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Bryan G. Belikoff, Louis J. Vaickus, Michail Sitkovsky and
Daniel G. Remick

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A_{2B} Adenosine Receptor Expression by Myeloid Cells Is Proinflammatory in Murine Allergic-Airway Inflammation

Bryan G. Belikoff,^{*,1} Louis J. Vaickus,^{*,1} Michail Sitkovsky,[†] and Daniel G. Remick^{*}

Asthma is a chronic condition with high morbidity and healthcare costs, and cockroach allergens are an established cause of urban pediatric asthma. A better understanding of cell types involved in promoting lung inflammation could provide new targets for the treatment of chronic pulmonary disease. Because of its role in regulating myeloid cell-dependent inflammatory processes, we examined A_{2B} R expression by myeloid cells in a cockroach allergen model of murine asthma-like pulmonary inflammation. Both systemic and myeloid tissue-specific A_{2B} R deletion significantly decreased pulmonary inflammatory cell recruitment, airway mucin production, and proinflammatory cytokine secretion after final allergen challenge in sensitized mice. A_{2B} R deficiency resulted in a dramatic reduction on Th2-type airways responses with decreased pulmonary eosinophilia without augmenting neutrophilia, and decreased lung IL-4, IL-5, and IL-13 production. Chemokine analysis demonstrated that eotaxin 1 and 2 secretion in response to repeated allergen challenge is myeloid cell A_{2B} R dependent. In contrast, there were no differences in the levels of the CXC chemokines keratinocyte-derived chemokine and MIP-2 in the myeloid cell A_{2B} R-deficient mice, strengthening A_{2B} R involvement in the development of Th2-type airways inflammation. Proinflammatory TNF- α , IFN- γ , and IL-17 secretion were also reduced in systemic and myeloid tissue-specific A_{2B} R deletion mouse lines. Our results demonstrate Th2-type predominance for A_{2B} R expression by myeloid cells as a mechanism of development of asthma-like pulmonary inflammation. *The Journal of Immunology*, 2012, 189: 3707–3713.

Over the last several years, adenosine has gained attention as an important mediator of inflammatory processes. Adenosine levels increase locally and systemically in response to cell stress, infection, trauma, and chronic inflammation in humans and in animal models of disease. Adenosine drives proinflammatory and anti-inflammatory processes dependent on adenosine A₁, A_{2A} adenosine receptor (A_{2A} R), A_{2B} adenosine receptor (A_{2B} R), and A₃ adenosine receptor subtype interactions. A_{2A} Rs function as potent anti-inflammatory receptors in response to endogenously generated adenosine. The A_{2A} R provides a negative regulatory feedback loop to limit inflammatory processes. The anti-inflammatory effects of A_{2A} R engagement by adenosine are mediated by increases in intracellular cAMP levels (1). The chronic inflammatory mediator IL-13 is a central player in the pathogenesis of asthma and is shown to act synergistically with adenosine (2, 3). Adenosine is a known mediator of eosinophil airway influx secondary to AMP inhalation in patients with asthma (4, 5). In addition to the role of the A_{2A} R, past studies show a central role for A_{2B} R in the development of chronic pulmonary inflammation.

Systemic deletion of the A_{2B} R in mice attenuates chronic pulmonary inflammation, together with pulmonary levels of inflammatory mediators IL-4, IL-5, and IL-13 (6). Furthermore, adenosine deaminase-deficient mice develop severe pulmonary fibrosis that is prevented with exogenous administration of adenosine deaminase or an A_{2B} R antagonist, indicating a role for adenosine signaling through A_{2B} R in the development of this phenotype (7). The ability of the low-affinity A_{2B} R to mediate proinflammatory pathways may be attributed to their action as a Gq protein-coupled receptor. Importantly, a recent study using mice that lack the A_{2B} R in an allergy-induced model of chronic lung inflammation showed a deleterious role for A_{2B} R signaling in airway remodeling and inflammation (6). Mice that lack A_{2B} R also had decreased levels of circulating IgE and airway eosinophilia, providing further evidence of A_{2B} R involvement in allergic inflammation (6).

A number of immune and nonimmune cell types have been implicated in the pathogenesis of chronic inflammation in asthma. Specifically, the wide receptor distribution of the A_{2B} R on numerous cell types, not surprisingly, makes it difficult to delineate the cell types responsible for A_{2B} R involvement in the pathogenesis of asthma. The generation of A_{2B} R chimeric mice furthered our understanding of A_{2B} R distribution patterns showing high A_{2B} Rs expression on monocytes, endothelial cells, and vascular smooth muscle cells (8). Indeed, the discovery that A_{2B} R activation on mast cells promotes degranulation and proinflammatory cytokine secretion increased interest in the development of A_{2B} R antagonist for the treatment of asthma and other inflammatory airway diseases (2, 3, 9, 10). Furthermore, A_{2B} Rs function to promote differentiation of lung fibroblasts to a myofibroblast phenotype implicating their involvement with nonimmune-mediated airway remodeling associated with chronic pulmonary inflammation such as asthma (11).

The wide cellular distribution and multiple inflammatory properties of the A_{2B} R add to the complexity of this receptor involvement in airway disease. Early studies showed the A_{2B} R ability to limit inflammation in models of acute vascular injury that describes A_{2B} R's anti-inflammatory property. Conversely, a recent study by

^{*}Department of Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, MA 02118; and [†]New England Inflammation and Tissue Protection Institute, Northeastern University, Boston, MA 02115

¹B.G.B. and L.J.V. are co-first authors who contributed equally to this article.

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D.G.R. contributed the conceptual design of the experiments in this paper.

Address correspondence and reprint requests to Dr. Daniel G. Remick, Boston University School of Medicine, 670 Albany Street, Room 405, Boston, MA 02118. E-mail address: remickd@bu.edu

Abbreviations used in this article: A_{2A} R, A_{2A} adenosine receptor; A_{2B} R, A_{2B} adenosine receptor; BAL, bronchoalveolar lavage; CRA, cockroach allergen; EPO, eosinophil peroxidase; KC, keratinocyte-derived chemokine; KO, knockout; LH, lung homogenate; LysM, lysozyme M promoter-driven Cre recombinase; MPO, myeloperoxidase; PAS, periodic acid-Schiff; WT, wild type.

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Zaynagetdinov et al. (6) determined a proinflammatory role for A_{2B} Rs during chronic pulmonary inflammation using an allergen-induced model. This study was limited by the use of systemic A_{2B} R knockout (KO) mice, thereby leaving the cell types responsible for this phenotype to be determined. More recent studies demonstrate high levels of A_{2B} R expression on hypoxia-treated dendritic cells inducing the cells toward a Th2-polarizing phenotype, further strengthening our interest in the role of A_{2B} Rs and asthma (12). Our study sought to determine whether A_{2B} R expression by myeloid cells is involved in the pathogenesis of chronic airway inflammation. We hypothesized that A_{2B} R expression on immune cell types of the myeloid lineage, which include neutrophils, monocytes, eosinophils, and basophils, play a central role in the pathogenesis of chronic pulmonary inflammation. These findings may have implications for the mode of adenosine receptor modulator drug delivery. Therefore, we made use of mice lacking A_{2B} Rs on the myeloid cells lineage (lysozyme M promoter-driven Cre recombinase [LysM] KO) using A_{2B} R-floxed/floxed LysM cre^{+/+} transgenic mice (13), and established a mouse model of airway inflammation using German cockroach extract to induce airway inflammation via direct inhalation (14–17). Our results support the hypothesis that A_{2B} R expression on myeloid cells is partially responsible for the pathogenesis of chronic airway inflammation in asthma.

Materials and Methods

Experimental mice

Female mice 8–12 wk old were used for all experiments. C57BL/6 mice were used as age-matched controls and were given 1 wk to acclimate to the pathogen-free housing facility at Northeastern University (Boston, MA). Ozgene developed the A_{2B} R-deficient mice on a C57BL/6 background as described previously (13). Myeloid tissue-specific A_{2B} R KO mice were used to determine the myeloid cell A_{2B} R-dependent contributions to allergic-type inflammation. Myeloid tissue-specific A_{2B} R KO were generated and validated using the Cre/loxP system as described previously (13). All animal experiments were conducted in accordance with Institutional Animal Care and Use Committee guidelines of Northeastern University.

Allergen challenge and asthma induction

On days 0, 14, and 21, mice received direct hypopharyngeal challenges of 4, 2, and 2 µg lyophilized whole-body German cockroach allergen (CRA; Greer, Lenoir, NC) in 50 µl sterile PBS using our previously described protocol (17, 18). In brief, mice were lightly anesthetized with isoflurane and suspended by the front incisors from an inclined board. Using forceps, the tongue was gently extended and held against the lower mandible. The allergen solution was delivered to the hypopharynx in two separate 25 µl aliquots. Inhalation of the solution was confirmed by inspiratory sounds and previous radiological studies (data not shown). In these studies, mice were lightly anesthetized and given a radio-opaque dye hypopharyngeally either with the tongue extended (epiglottis held shut) or with the tongue in place (epiglottis unencumbered), and real-time x-ray videos were taken showing the progression and final location of the dye, allowing distinction between esophageal/gastrointestinal and tracheal/bronchial progression. Within 5 min of dosing, all mice resumed normal activity. Mice received a total of three pulmonary CRA challenges at days 0, 14, and 21, and then were examined and sacrificed at 24 h after final allergen challenge for analysis.

Sacrifice and tissue collection

Mice were anesthetized with ketamine/xylazine and sacrificed by exsanguination followed by cervical dislocation. The lungs were lavaged with 2 ml warm HBSS in 250 µl aliquots. This bronchoalveolar lavage (BAL) fluid was centrifuged to pellet the cells. The cell pellets were combined, counted, placed on slides, and a differential was performed. The right lung of each mouse was homogenized and used to assess eosinophil peroxidase (EPO) and neutrophil-specific peroxidase activity and cytokine analysis. In brief, lung homogenates (LHs) were centrifuged to separate tissue components from supernatant. Supernatants were reserved for cytokine/chemokine analysis. LH cell pellets were treated with cetyltrimethylammonium chloride or hexadecyltrimethylammonium bromide and sonicated to release eosinophil and neutrophil granules, respectively. Activity was measured as described later. The left lung was fixed in 70% ethanol for histological analysis.

Assessment of LH cytokines and chemokines

Cytokines and chemokines in LH samples were assessed by sandwich ELISA using biotin-linked detection Abs and streptavidin HRP development as previously described (19).

Assessment of EPO and myeloperoxidase

LH were treated with cetyltrimethylammonium chloride or hexadecyltrimethylammonium bromide and treated as described earlier. Activity was measured as previously described (18). Measurements were taken without the addition of degranulating agents to measure release representing ongoing allergen-induced eosinophil/neutrophil activation in the lung tissue.

Histological analysis

Fixed lungs were sent to the Boston University School of Medicine, Department of Pathology and Laboratory Medicine Core Services for histology sectioning and staining. Image analysis for the periodic acid–Schiff (PAS)-stained area was performed using ImageJ freeware (<http://rsbweb.nih.gov/ij/>). In brief, whole-lung images were captured at ×2 magnification. These images were subjected to background subtraction and color deconvolution. The magenta-separated image was converted to a black-and-white image, and the total PAS (black)-stained area was calculated.

Statistical analysis

All data are presented as the mean ± SEM, with the numbers of animals provided in the figure legend. Groups of data were analyzed by ANOVA with a Dunnett's or Bonferroni post hoc analysis to compare groups if appropriate. Student *t* test was used to compare data between two groups when appropriate. The groups used for comparison are listed in the figure legends. All statistical analyses were performed using GraphPad Prism 4.0 software.

Results

To determine the degree of allergic pulmonary inflammation in the wild type (WT) mice, systemic A_{2B} R and A_{2B} R LysM KO mice were used to examine multiple mediators and metrics of asthma-like pulmonary inflammation. First, we assessed pulmonary mucous production because airways mucous hypersecretion is a characteristic feature of asthma-associated inflammation in humans. Histological sections of fixed whole left lungs were stained with PAS for mucus, which stains the mucus within the epithelial cells dark magenta. The A_{2B} R KO and A_{2B} R LysM KO mice showed markedly diminished mucous staining 24 h after the final allergen challenge in representative images (Fig. 1A). The total mucous-stained area in the entire section of the lung was then quantitated using

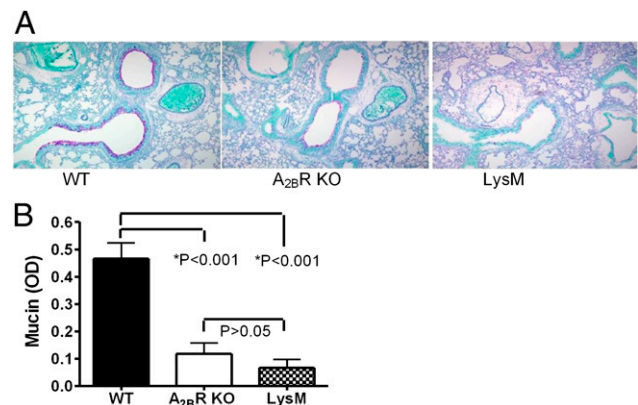


FIGURE 1. Airways mucous production. **(A)** PAS stain in lung histological sections of WT, A_{2B}R KO, and LysM KO groups of mice. Each panel is a representative image; the mucin is stained magenta within the airway epithelial cells. All images are the same magnification. **(B)** Quantization of the total area of PAS-stained mucus using ImageJ photoanalysis software. A_{2B}R mice showed significant decreases in airways mucous production as compared with WT. Values are mean ± SEM of 2 independent experiments for WT (*n* = 5), A_{2B}R KO (*n* = 6), and LysM KO (*n* = 6). The *p* values are indicated in the figure.

image analysis software that showed that there was significantly less mucous-stained area in the A_{2B} R KO or the A_{2B} R LysM KO animals as compared with their WT mice (Fig. 1B).

Lung eosinophilia is a widely accepted characteristic of the asthmatic lung (20). These inflammatory cells can typically be recovered in the BAL and also observed in the lung parenchyma of asthmatic patients. In mice, eosinophils are recruited to the lung via eotaxins 1 and 2, as well as a number of other cytokines and chemokines. In our experiments, CRAs produced significantly more eotaxin 1 and 2 in the lung parenchyma in WT mice compared with either of the A_{2B} R KO groups of mice (Fig. 2A, 2B). The number of peribronchial eosinophils was also decreased in the A_{2B} R KO and A_{2B} R LysM KO mice by routine light microscopy (Fig. 2C). Total lung eosinophils were quantified by measuring lung EPO to confirm the histologic findings. EPO levels were significantly reduced in both groups of A_{2B} R KO mice compared with the WT mice (Fig. 2D). Notably, EPO levels were measured after BAL had been performed. We also evaluated the number of eosinophils in the BAL fluid recovered 24 h after the last allergen challenge. Cytospin preparations showed reduced influx of eosinophils (representative photomicrographs in Fig. 2E). Quantification of the slides

demonstrated a significant reduction of eosinophil recruitment to the bronchoalveolar spaces in both the A_{2B} R KO and A_{2B} R LysM KO mice compared with WT mice (Fig. 2F).

In addition to pulmonary eosinophilia, infiltration by neutrophils is often observed in asthmatic patients, typically in the most therapeutically recalcitrant cases. In mice, neutrophils are recruited by a number of cytokines, chemokines, and other mediators including complement fragments and leukotriene metabolites. BAL neutrophils and LH neutrophil-specific myeloperoxidase (MPO) activity did not differ significantly among the experimental groups (Fig. 3A, 3B). We measured the lung levels of two important CXC chemokines, keratinocyte-derived chemokine (KC) and MIP-2, murine analogs of IL-8, and found no significant differences between WT and A_{2B} R LysM KO mice. We did find a significant reduction in KC and MIP-2 levels in systemic A_{2B} R KO mice compared with WT mice, albeit a modest reduction (Fig. 3C, 3D). No significant differences in RANTES levels were found between all experimental groups (Fig. 3E).

Macrophages are the only inflammatory cells present in normal lungs in the form of resident alveolar cells. During inflammatory insults, these resident macrophages perform a number of immune

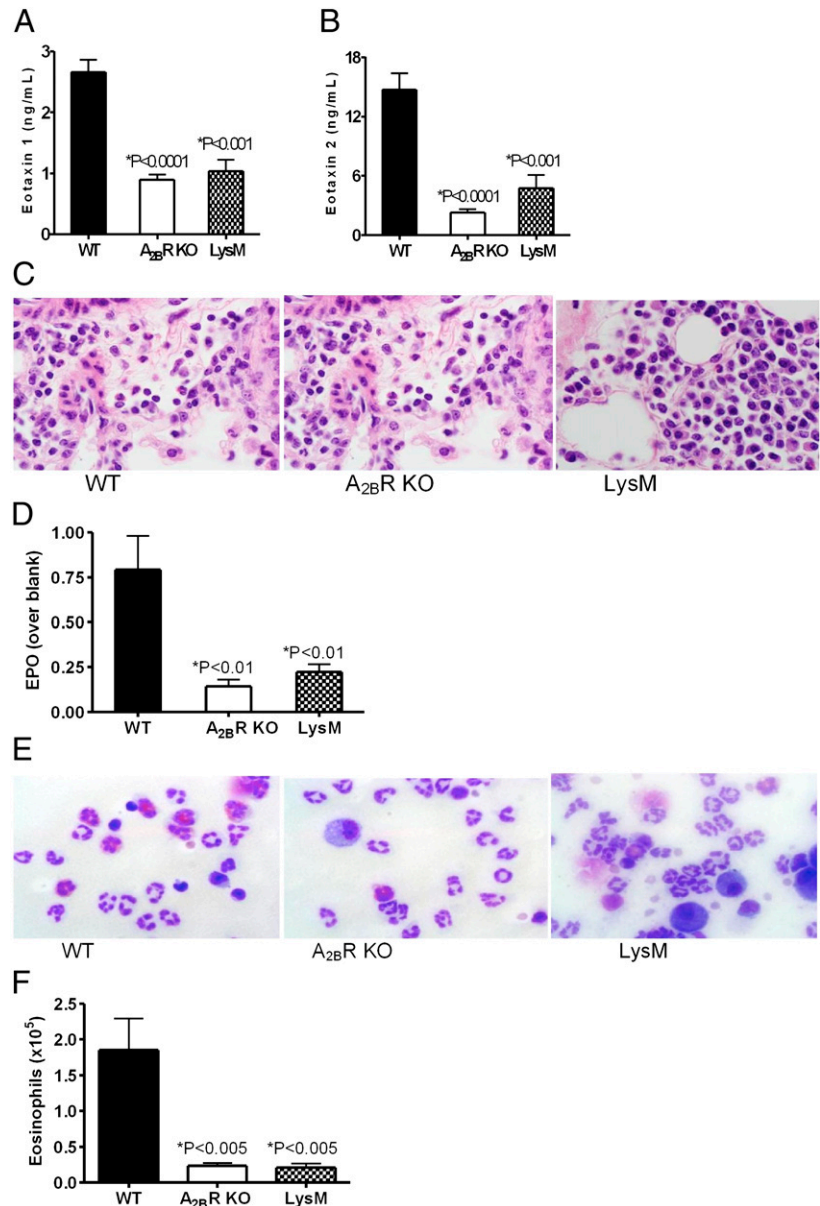


FIGURE 2. Pulmonary eosinophil recruitment and infiltration. LH concentrations of eotaxins 1 (A) and 2 (B) in WT ($n = 5$), A_{2B} R KO ($n = 6$), and LysM KO ($n = 6$) mice. (C) Representative images of parenchymal eosinophils in H&E sections of whole left lung. All images are the same magnification. (D) LH supernatant EPO activity in WT, A_{2B} R KO, and LysM KO mice. (E) Representative cytopsin photomicrographs of BAL cells including eosinophils from the indicated mice. All images are the same magnification. (F) Absolute eosinophil counts in the BAL fluid. The A_{2B} R-deficient groups of mice demonstrated markedly reduced eosinophil infiltration into the BAL and lung parenchyma, and concurrent diminished production of eosinophil chemoattractants. (A, B, D, and F) Values are the mean \pm SEM of two independent experiments. (D and F) $n = 9$ for WT, $n = 10$ for A_{2B} R KO, and $n = 10$ for LysM. The p values are indicated in the figure.

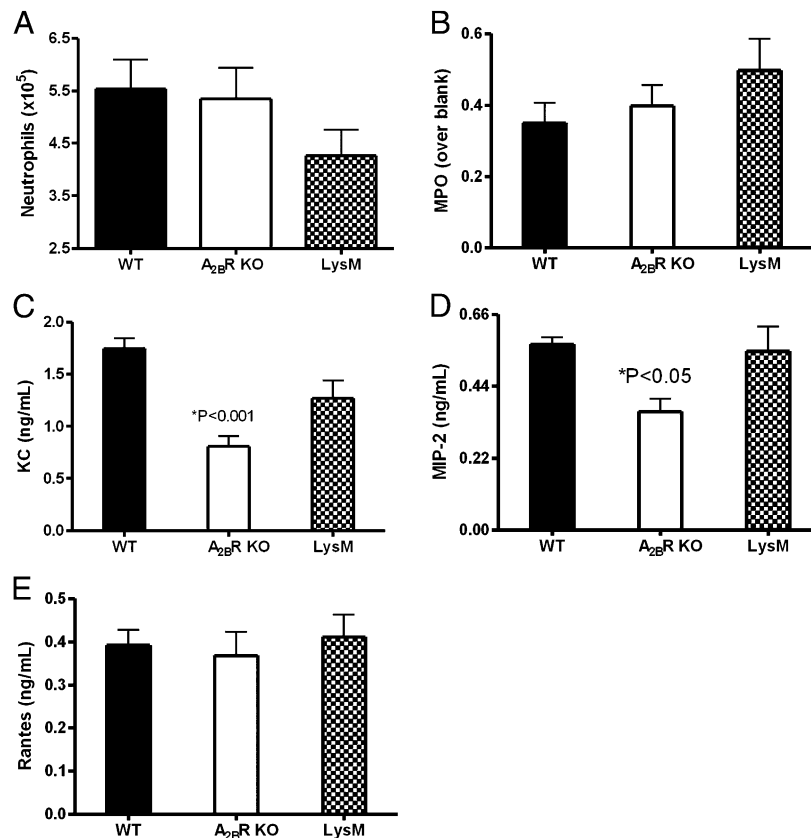


FIGURE 3. Pulmonary neutrophil infiltration. Absolute counts of neutrophils recovered from the BAL fluid (A). LH supernatant MPO activity (B). No significant differences were observed in the infiltration of neutrophils into the airspaces or lung parenchyma between the experimental groups. Values are the mean ± SEM of two independent experiments with $n = 9$ for WT, $n = 10$ for A_{2B}R KO, and $n = 10$ for LysM KO. LH concentrations of KC (C), MIP-2 (D), and RANTES (E) in WT, A_{2B}R KO, and LysM KO groups of mice. (C–E) Values are mean ± SEM of WT ($n = 5$), A_{2B}R KO ($n = 6$), and LysM KO ($n = 6$). The p values are indicated in the figure.

functions including Ag processing and presentation and phagocytosis. As the inflammatory response continues, the alveolar macrophages are augmented by and replaced with fresh circulating monocytes recruited by various inflammatory mediators. In our experiments, there was a trend toward decreased alveolar macrophages in the BAL of A_{2B}R KO mice and significantly reduced macrophage levels in the lung of the A_{2B}R LysM KO mice as compared with WT mice (Fig. 4A). Lymphocytes, similar to eosinophils and neutrophils, are typically not present in the healthy lung. Although granulocytes tend to arrive early in the inflammatory timeline, lymphocytes typically arrive many hours to days after the initial insult and orchestrate the Ag-specific response (21, 22). These cells are attracted by a number of mediators and themselves produce a complex array of Th1, Th2, and other cytokines and chemokines. In our model, the A_{2B}R-deficient groups of mice

exhibited significantly fewer BAL lymphocytes as compared with the WT mice after final allergen challenge (Fig. 4B).

To complete the evaluation of the impact of differential A_{2B}R expression, we examined the production of a number of classical Th2 and proinflammatory/immunomodulatory cytokines. IL-4, IL-5, and IL-13 are produced principally by activated lymphocytes and are traditionally considered to be part of the Th2 classification. These cytokines have a number of effects including the promotion of an allergic-type, eosinophil predominant inflammatory response (23–25). In our experiments, the Th2 cytokines were significantly diminished in the A_{2B}R-deficient mice as compared with the WT group (Fig. 5A–C) after final allergen challenge. In addition, we examined the production of proinflammatory mediators that are considered essential in the development of asthma. IL-17, the signature cytokine produced by Th17 cells, plays a critical role in the development of the asthmatic response. IL-17 was significantly reduced in both the A_{2B}R KO and the A_{2B}R LysM KO mice after allergen challenge (Fig. 5D). TNF- α has been shown to be a potent mediator of several parameters in CRA-induced asthma (26). Interestingly, lack of A_{2B}R that resulted in decreased inflammation also resulted in decreased levels of TNF- α (Fig. 5E). IFN- γ is a classically defined Th1 mediator. To determine whether the adenosine receptors drove the model toward a Th1 inflammatory response, we measured IFN- γ in the LHs, where both the systemic A_{2B}R and A_{2B}R LysM KO mice had reduced levels (Fig. 5F).

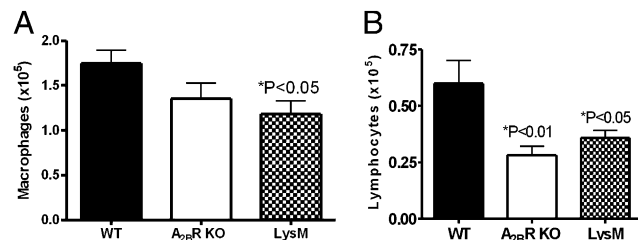


FIGURE 4. Pulmonary macrophage and lymphocyte infiltration. Absolute counts of macrophages (A) and lymphocytes (B) recovered in the BAL fluid of WT, A_{2B}R KO, and LysM KO groups of mice. Macrophages recovered in BAL trended toward decreased but were only significantly depressed in the LysM KO mice. In contrast, lymphocyte levels were markedly reduced in the BAL fluid of A_{2B}R-deficient groups of mice. Values are the mean ± SEM of two independent experiments with $n = 9$ for WT, $n = 10$ for A_{2B}R KO, and $n = 10$ for LysM KO. The p values are indicated in the figure.

Discussion

The outcome of adenosine activation of inflammatory pathways is complex and depends on the stimulus for inflammation, as well as the adenosine receptor subtype activated. Adenosine–A_{2B}R interactions promote immune-mediator release such as histamine and cytokines to direct proinflammatory and anti-inflammatory outcomes. Despite the overall immunomodulatory role of A_{2B}Rs in inflammation, A_{2B}R-mediated mechanisms that control inflammatory

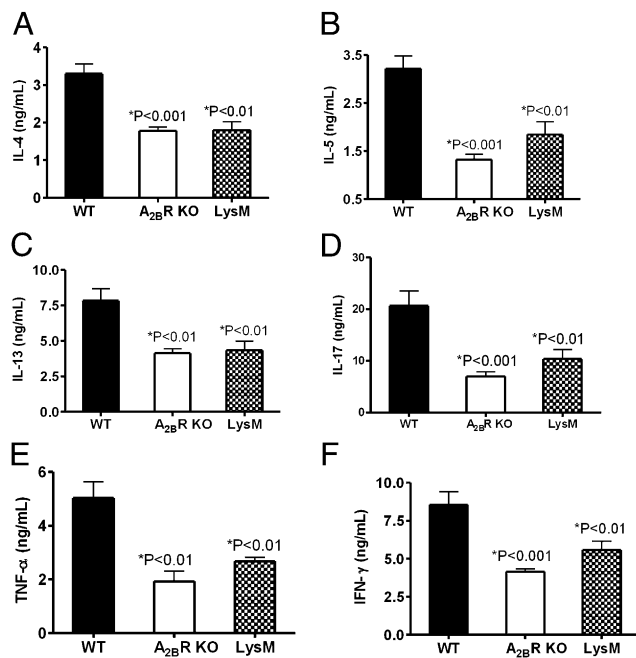


FIGURE 5. Th2 and proinflammatory/immunomodulatory cytokines. LH concentrations of the Th2 cytokines IL-4 (A), IL-5 (B), and IL-13 (C) all demonstrated a significant decrease in the Th2 cytokines. In addition, the proinflammatory cytokines IL-17 (D) and TNF- α (E) were decreased in the A_{2B}R and LysM KO mice. The classic Th1 cytokine, IFN- γ , was also significantly decreased in the KO groups (F). Values are the mean \pm SEM of two independent experiments with WT ($n = 5$), A_{2B}R KO ($n = 6$), and LysM KO ($n = 6$). The p values are indicated in the figure.

outcomes may differ within the same tissue compartment. For instance, the downstream effects of A_{2B} R activation tend to be proinflammatory during chronic pulmonary inflammation (in the OVA asthma model) and anti-inflammatory during acute pulmonary inflammation (after LPS exposure) (6, 7, 27, 28). Immune cell types that drive allergic-type inflammation such as T cells and myeloid cells secrete proinflammatory cytokines (IL-4, IL-5, IL-13) and chemokines (CC and CXC) in the lungs of sensitized mice when re-exposed to the same allergen and may be regulated by A_{2B} R stimulation. It is known that A_{2B} R-mediated pathology in allergic pulmonary inflammation includes airways eosinophilia and mucous production after OVA peptide inhalation in sensitized mice (6). In vitro studies show that A_{2B} R contributes to the proinflammatory adenosine signaling on mast cells as part of the allergic response (3). However, the relative contribution of A_{2B} Rs and proinflammatory adenosine signaling in immune cells of the myeloid cell lineage in response to the inhalation of an allergen is relatively unknown and is important for the development of novel pharmacological approaches to modulate A_{2B} Rs for the treatment of disease.

Recent studies confirm A_{2B} R function specificity by showing that A_{2B} Rs play cell-type-specific roles in regulating inflammation. Nakatsukasa et al. (29) showed a significant role for the A_{2B} R in regulating T cell differentiation, independent of TCR signaling. An independent study determined a mechanism of adenosine to stimulate dendritic cell IL-6 secretion via the A_{2B} R through the pathway of indirectly regulating T cell differentiation to the Th17 subtype (30). These studies suggest a dominant role for A_{2B} Rs to regulate myeloid cell proinflammatory pathways, as opposed to other immune cell types, adding cell specificity to A_{2B} R-mediated inflammatory outcomes. Furthermore, studies show a greater specificity of A_{2B} R regulatory functions within immune cells of the myeloid cell lineage, such as A_{2B} R regulation of neutrophil function

(13, 31). Taken together, the cellular specificity of the A_{2B} R immunoregulatory function along with the proinflammatory role of A_{2B} Rs in the development of chronic pulmonary inflammation led us to hypothesize that the proinflammatory effects of adenosine in allergic pulmonary disease are myeloid cell A_{2B} R dependent. Using myeloid tissue-specific A_{2B} R KO mice, we designed our study to determine the effects of myeloid-specific A_{2B} R gene ablation on allergen-induced pulmonary inflammation in a murine model of CRA-induced asthma.

Similar to previous studies, our investigation of cellular parameters in the BAL fluid recovered from allergic mice showed A_{2B} R ablation to have the most striking effect in reducing airway eosinophilia (6). These findings were supported by a dramatic reduction in EPO activity in LHs from A_{2B} R KO mice and A_{2B} R LysM KO mice compared with WT mice. Lymphocytes and macrophages recovered from BAL fluid were also decreased in the absence of A_{2B} Rs on myeloid cells when compared with control mice. From these data, we conclude that A_{2B} R engagement on myeloid cells is partially responsible for airways inflammatory cell recruitment. The significant reduction in pulmonary leukocyte airway recruitment was accompanied by decreases in Th2 cytokines (IL-4, IL-5, and IL-13) secreted in the lungs of A_{2B} R KO and A_{2B} R LysM KO mice compared with controls, suggesting that proinflammatory mediators secreted in response to adenosine–A_{2B} R interactions may be responsible.

Past studies have shown that the role for neutrophils in the development of A_{2B} R-mediated inflammatory disease is limited, whereas the A_{2A} Rs predominate in this respect (13, 31). Airway neutrophilia results when infused OVA-specific Th1 cells are challenged with aerosolized OVA in mice. Conversely, airway eosinophilia results when infused OVA-specific Th2 cells are challenged with aerosolized OVA in mice (32). In our studies, final challenge with cockroach Ags resulted in a mixed airway response with BAL fluid cells consisting of eosinophils and neutrophils. The mixture of allergens (cockroach proteins Blag1 and Blag2) and innate immune activators (LPS, chitin, proteases) combine to elicit an allergen-specific Th2 response in addition to an innate Th1 inflammatory response. OVA models use a simplified allergen mixture that contains low quantities of the LPS, chitin, and proteases than found in CRA, and thus elicit a cleaner and more isolated Th2 response. However, OVA is not a real-world allergen, whereas a significant percentage of urban asthmatic children are demonstrably bronchially allergic to CRA, which is encountered as a complex mix of allergens and innate immune activators as in our model (33, 34). Accordingly, we found a minimal contribution of A_{2B} R regulatory function on airways neutrophilia by measured lung MPO activity and absolute number of alveolar neutrophils recovered from allergen-challenged sensitized WT mice compared with systemic A_{2B} R KO, and A_{2B} R LysM KO mice. The limited response of A_{2B} R deletion on airways neutrophilia together with decreased airways eosinophilia and IL-4, IL-5, and IL-13, and in the lungs of myeloid-specific A_{2B} R KO mice suggests that downstream effects of A_{2B} R activation on myeloid cells regulate Th2-type-dependent airway inflammation. The effect of A_{2B} R ablation to decrease proinflammatory cytokines measured in the lungs of all groups of A_{2B} R KO mice likely reflects the overall decrease in pulmonary macrophages and lymphocytes that orchestrate Th2 responses. The data also show specificity to the lack of A_{2B} R because not all inflammatory parameters were reduced.

We determined chemokine levels in the lungs of allergic mice after allergen challenge. Eotaxins are CC chemokines that are potent activators and chemoattractants of both eosinophils and Th2 lymphocytes (35, 36). Whereas Th1-dependent airway neutrophilia is CXC chemokine dependent (32), we found significant

airway eosinophilia associated with dramatic increases in both eotaxins 1 and 2 in sensitized WT mice compared with A_{2B} R KO and A_{2B} R LysM KO mice challenged with allergen. We did not detect significant reductions in CXC chemokine KC and MIP-2 in mice lacking A_{2B} Rs on myeloid cells. The presence of neutrophils in the BAL fluid recovered in A_{2B} R-deficient mice suggest other unmeasured neutrophil chemotactic proteins are responsible for airway neutrophilia that are A_{2B} R independent in our model. Adenosine receptor redundancy may provide an explanation for neutrophil chemotaxis in A_{2B} R KO mice as adenosine acting via the A_{2A} R and A₃ increase neutrophils in lung tissue (31). Other neutrophil chemotactic factors such as leukotrienes may serve to recruit these cells.

Our results strongly suggest a mechanism that adenosine stimulates myeloid cells to secrete CC chemokines in the lung parenchyma by A_{2B} R engagement resulting in pulmonary eosinophil recruitment via T cell polarization to Th2-type lymphocytes. Additional effects of eotaxin to attract lymphocytes to the inflamed lung, as well as the modulation of Th2 cytokine responses (as measured IL-4, IL-5, IL-13), are relevant to eosinophil activation. However, the exact phenotype of the T cell population modulated by myeloid cell A_{2B} R expression at the site of inflammation remains to be determined. IL-4 production can be derived from a number of cell types including Th lymphocytes and cells of the myeloid cell lineage (eosinophils and basophils) to orchestrate Th2 allergic response, thereby enhancing eosinophil airway recruitment. Our studies using A_{2B} R LysM KO mice suggest a critical role for myeloid cells to result in IL-4 production in CRA-induced lung pathology. We expand our current knowledge of A_{2B} Rs to act upstream on myeloid cell types to regulate IL-4 production. Whether adenosine acts on the A_{2B} R directly or indirectly through myeloid cells to stimulate IL-4 production is uncertain. For example, non-myeloid cell types (CD4⁺ T cells and NKT cells) may be Th2 polarized in response to IL-10 production on macrophages subsequent to via A_{2B} Rs to produce IL-4 during allergic-type inflammation. Future studies to elucidate these pathways are warranted. Nevertheless, the decrease in measured Th2 cytokines in the lungs of systemic A_{2B} R KO and A_{2B} R LysM KO mice compared with WT mice suggest that A_{2B} R expression by myeloid cells play a role.

The cellular source of eotaxin after A_{2B} R stimulation is unknown but may include resident cells such as epithelial and endothelial as well as mononuclear cells recruited to the site of inflammation. Alternatively, decreased levels of IL-4 may be responsible for the decrease in eotaxins levels, because IL-4 is known to stimulate the secretion of eotaxin from type II epithelial cells (37).

Taken together, our results suggest that eotaxin regulation by A_{2B} Rs is a major mechanism for promoting tissue inflammation. Our studies better define A_{2B} R expression on myeloid cells as an independent contributor to the pathogenesis of allergen-induced pulmonary inflammation and indicate that blocking adenosine-A_{2B} R interactions on myeloid cells may be an effective immunomodulatory therapy to treat allergic-type inflammatory disease. Furthermore, our findings suggest that using nanoparticle delivery of adenosine immunomodulatory therapy to act on myeloid tissue may be a promising approach for the treatment of patients with asthma. Whether disruption of A_{2B} R activation on myeloid cells using nanoparticle technology can be achieved remains to be determined.

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Disclosures

The authors have no financial conflicts of interest.

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