



Limonene-induced activation of A_{2A} adenosine receptors reduces airway inflammation and reactivity in a mouse model of asthma

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Received: 20 August 2019 / Accepted: 19 March 2020 / Published online: 13 August 2020
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Abstract

Animal models of asthma have shown that limonene, a naturally occurring terpene in citrus fruits, can reduce inflammation and airway reactivity. However, the mechanism of these effects is unknown. We first performed computational and molecular docking analyses that showed limonene could bind to both A_{2A} and A_{2B} receptors. The pharmacological studies were carried out with A_{2A} adenosine receptor knock-out (A_{2A}KO) and wild-type (WT) mice using ovalbumin (OVA) to generate the asthma phenotype. We investigated the effects of limonene on lung inflammation and airway responsiveness to methacholine (MCh) and NECA (nonselective adenosine analog) by administering limonene as an inhalation prior to OVA aerosol challenges in one group of allergic mice for both WT and KO. In whole-body plethysmography studies, we observed that airway responsiveness to MCh in WT SEN group was significantly lowered upon limonene treatment but no effect was observed in A_{2A}KO. Limonene also attenuated NECA-induced airway responsiveness in WT allergic mice with no effect being observed in A_{2A}KO groups. Differential BAL analysis showed that limonene reduced levels of eosinophils in allergic WT mice but not in A_{2A}KO. However, limonene reduced neutrophils in sensitized A_{2A}KO mice, suggesting that it may activate A_{2B} receptors as well. These data indicate that limonene-induced reduction in airway inflammation and airway reactivity occurs mainly via activation of A_{2A}AR but A_{2B} receptors may also play a supporting role.

Keywords A_{2A} adenosine receptors · Asthma · Airway inflammation · Airway responsiveness · Limonene

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11302-020-09697-z>) contains supplementary material, which is available to authorized users.

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Introduction

There are various drugs available for the management of asthma and its symptoms, such as corticosteroids, leukotriene modifiers, and β_2 agonists. Research is also ongoing with respect to the use of natural products with minimal side effects [1]. Flavonoids have emerged as the major naturally derived chemicals for the treatment of asthma. These compounds have anti-inflammatory properties, reduce levels of reactive oxygen species (ROS), and inhibit the release of various cytokines and cytotoxic materials [2].

The flavonoid of our interest is limonene, which is commercially available as D-(+)-limonene. Limonene has been used for the treatment of asthma, colic and heart problems in Chinese medicine [3]. It is the major component of essential oils obtained from the skin (peel) of citrus fruits like lemon, orange, yuzu, and grapefruit among others. These essential oils are used in aromatherapy for treating various disorders such as heartburn, stress relief, gastroesophageal reflux disease (GERD), and asthma [4]. Chemically, it is a monocyclic monoterpene with a characteristic lemon-like odor. Earlier studies have shown that limonene helps in reducing the levels of ROS, platelet aggregation and exhibits anti-tubercular properties [5, 6]. It has also been shown to inhibit the inflammatory response by inhibiting the IF- κ B inflammatory pathway [7].

Asthma is chronic lung disease characterized by increased inflammation and bronchoconstriction [8]. Various systemic and airway inflammatory conditions like eosinophilia and neutrophilia are observed in the asthma as a result of chemotactic movement [9, 10]. Though the detailed pathway of the asthma development is not fully known, several studies have shown a role for adenosine as an activator of inflammation and bronchoconstriction in asthma [11]. Asthmatic patients have increased levels of exhaled adenosine and higher bronchoresponsiveness to inhaled adenosine compared to healthy individuals, suggesting adenosine can act as a bronchoprovocant [12]. Studies also observed elevated level of adenosine in chronic asthmatic lungs [11, 13]. Adenosine signals through four main G protein coupled receptor subtypes namely A_1 , A_{2A} , A_{2B} , and A_3 . These receptors play different roles in asthma. A_1 and A_3 adenosine receptors (AR) are known to be involved in bronchoconstriction [14, 15] whereas A_{2A} AR and A_{2B} AR may mediate bronchodilation [16, 17]. Comparative studies have revealed that limonene may be a ligand for different adenosine receptor subtypes and could act like an agonist at the A_{2A} AR which plays a major role in anti-inflammatory processes [7, 18]. Selective A_{2A} agonist CGS 21680 and limonene show competitive binding to the A_{2A} AR with limonene specifically binding to the same site as that of CGS 21680, thereby increasing the levels of unbound CGS 21680 by 91.2% over that of controls [18]. This study confirmed that limonene has a high affinity towards A_{2A} AR and can act as agonistic ligand. Recent studies in mice also

show that inhaled limonene attenuated the development of airway hyperresponsiveness and reduced the levels of Th2 cytokines involved in the pathophysiology of airway inflammation [4]. Thus, our study focused on assessing the effects of limonene in asthma-induced and healthy wild-type (WT) as well as A_{2A} receptor deficient (A_{2A} KO) mice to identify the mechanism by which limonene may induce changes in asthma.

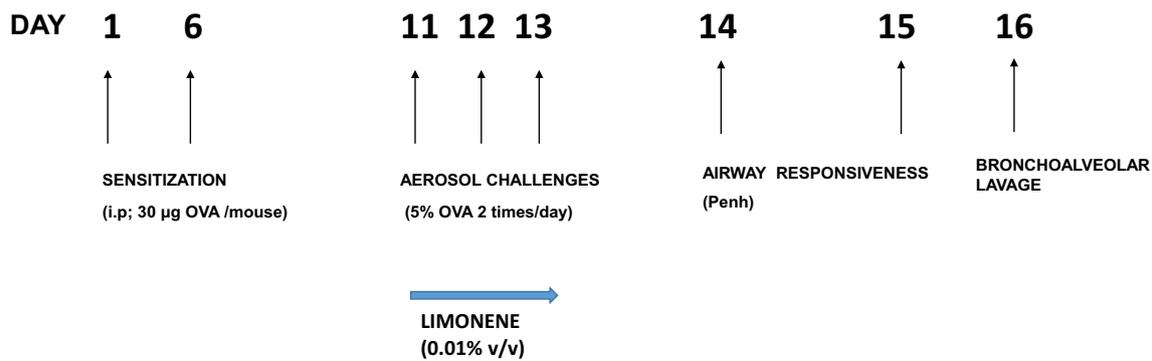
Materials and methods

Animals

Experiments were conducted with 6-week-old C57BL/6 wild-type (WT) and A_{2A} KO mice. Each group consisted of equal number of males and females. All the animals were inbred. A_{2A} KO mice (originally from C. Ledent, Universite Libre de Bruxelles, Brussels, Belgium) were obtained from Dr. Stephen Tilley, University of North Carolina Chapel Hill, on C57BL/6J background. C57BL/6J (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). A_{2A} KO mice were backcrossed 12 generations to the C57BL/6J background. A_{2A} KO mice were generated and genotyped by polymerase chain reaction. All the animals were housed under conventional animal room conditions at Long Island University wet lab facility in individually ventilated cages (IVCs) containing corn cob bedding, temperature of $23\text{ }^\circ\text{C} \pm 5\text{ }^\circ\text{C}$, 50–60% relative humidity, 12-h light/dark cycle and received standard rodent chow and water ad libitum. Animals were maintained at above conditions until they were 6 weeks old. The entire study adhered to the animal facility guidelines of Arnold and Marie Schwartz College of Pharmacy, Long Island University, Brooklyn, NY 11201.

Animal sensitization

The sensitization protocol used to develop the asthma phenotype is shown in Fig. 1, based on an established protocol [19, 20]. Forty-eight animals (24 each of WT and A_{2A} KO) were randomly chosen and were separated into 4 groups ($n=6$). Albumin from chicken egg white (ovalbumin; OVA) was obtained from Sigma-Aldrich (St. Louis, MO). A suspension containing 250 $\mu\text{g/ml}$ of OVA was prepared in Imject® Alum (Thermo Scientific) solution for intraperitoneal (i.p.) sensitization. Five percent OVA solution was prepared in normal saline (0.85% w/v sodium chloride) for aerosol challenge. (R)-(+)-Limonene was obtained from Sigma-Aldrich. It was first dissolved in DMSO (1:9) and then diluted with normal saline (0.9 w/v sodium chloride) to make the desired concentration of 0.01% (v/v) limonene solution [4]. The mice were sensitized on days 1 and 6 by i.p. injection of 25–30 μg of OVA suspended in 200 μl of Imject alum solution. Control mice were



➤ **Control animals received 0.2ml vehicle (alum) for sensitization and aerosolized saline for challenges**

Fig. 1 Protocol used to develop allergic model and treatment groups. Mice were divided into control (CON) and allergen sensitized (SEN) groups. Mice were sensitized with ovalbumin (OVA) on day 1 and 6 (25–30 µg; i.p.), followed by 5% OVA aerosol challenge (days 11–13)

to obtain the SEN group. For limonene-treated groups, the mice were first exposed to aerosolized 0.01% (v/v) limonene solution (7.5 ml) for 20 min followed by OVA aerosol challenges

injected with a matching volume of Imject alum solution alone on the same days. On days 11, 12, and 13, the sensitized mice were exposed to aerosolized 5% OVA (allergen) in 0.9% saline (vehicle) while the controls were exposed to vehicle only for 20 min twice a day and 6 h apart (morning and evening) using an ultrasonic nebulizer (De Vilbiss Healthcare, Somerset, PA). For limonene-treated groups, the mice were first exposed to aerosolized 0.01% (v/v) limonene solution (7.5 ml) for 20 min and then the OVA challenges were performed 90 min later. The flow rate of the aerosolized allergen was performed at 2 ml/min and the median aerodynamic diameter of the aerosol particles < 4 µm (De Vilbiss Healthcare). In addition, based on some of our initial data, we tested the effects of A_{2B}AR activation in A_{2A}KO mice. For this purpose, one group of sensitized A_{2A}KO received selective A_{2B} agonist BAY 60-6583 (Millipore Sigma; 10 mg/kg) on days 11–13 prior to the OVA challenges.

Study groups

Wild-type and A_{2A}KO mice were divided into the following study groups, respectively:

- Non-asthmatic mice: control (CON).
- Non-asthmatic mice receiving limonene: CON+LIM.
- Asthmatic mice: allergen sensitized-challenged (SEN).
- Asthmatic mice receiving limonene: allergen sensitized-challenged + LIM (SEN + LIM).

Computational analysis

Model selection

A previously published crystal structure for the human A_{2A}AR in complex with agonist UK-432097 was available

in the Protein Data Bank (PDB; Code: 3QAK) [21]. We selected this structure for molecular docking based on the resolution (2.71 Å), geometric, and refinement statistics. In addition, this structure has the fewest mutations (for crystal formation) compared to other higher resolution structures.

Homology modeling

Currently, there is no available crystal structure for the A_{2B}AR for computational analysis. A homology model for A_{2B}AR was constructed using the A_{2A}AR structure (3QAK) as a starting template. The program used for homology modeling was Prime (Schrödinger) [22]. The amino acid sequence for the A_{2B}AR was obtained using protein search in NCBI. This sequence (AAA51598) was used to build the homology model. A BLAST search in Prime identified models with high sequence identity and homology. The 3QAK structure was predicted to have high homology and 61.3% sequence identity in the BLAST search. Using 3QAK as the template, PRIME (Schrodinger) was used to construct the homology model for A_{2B}AR. Model validity was assessed based on the following parameters: score; % positives (match) % gaps and (mismatch).

Molecular docking

A docking model for A_{2A}AR (PDB: 3QAK) and limonene was prepared using the molecular docking program Glide [23]. The A_{2A}AR structure was prepared using the Protein Preparation tool; this program added hydrogens to the structure and assigned proper ionization states. Using Grid Generation, we generated a docking grid that occupied all residues interacting within the ligand UK-432097 binding site (~6 Å radius around UK-432097 and interacting residues) [21]. The UK-432097 molecule in the structure was deleted,

and limonene was docked into the receptor model. Three poses for limonene were generated and the best pose was selected for further analysis. To check the accuracy of our docking method, we “docked in place” UK-432097 and CGS21680 (Supporting Information Table 1) into the receptor grid and found that the best pose matched with original crystal structures.

Airway responsiveness to MCh and NECA

The airway hyperresponsiveness was assessed by using unrestrained whole body plethysmography (WBP) assembly manufactured by Buxco Electronics Inc. (Sharon, CT). This system uses a dimensionless parameter known as enhanced pause (Penh) to estimate the total pulmonary airflow. Animals were placed in individual Plexiglas chambers and allowed to adjust to their surroundings for 10 min. Baseline and vehicle readings were recorded for 5 min each, and the animals were then exposed to methacholine (MCh) via the Buxco aerosol delivery system (version 1.5; Buxco, Sharon, CT). Increasing concentrations of MCh (1.5, 3, 6, 12, 24, and 48 mg/ml) dissolved in normal saline was aerosolized for 2 min to establish a dose-response relationship. Each consecutive dose was administered only after the mice returned to baseline Penh levels. Penh values were normalized to the vehicle values and the increase in response was calculated as the percentage difference between the dose and vehicle values of Penh. On day 15, the entire procedure was repeated to determine the airway hyperresponsiveness to NECA (nonselective adenosine agonist).

Bronchoalveolar lavage (BAL)

On day 16, the animals were sacrificed by i.p. injecting 65 mg/kg of sodium pentobarbital. Bronchoalveolar lavage (BAL) was performed according to an established protocol [19, 20, 24]. BAL involved tracheotomy wherein the trachea was exposed and a small incision was made in the upper region of the trachea and was cannulated. This was followed with 3 lung lavages with normal saline (0.8 ml saline/lavage) and the fluid was withdrawn for cell collection. The collected BAL fluid (BALF) was stored on ice during collection and was centrifuged at 1800 rpm for 7 min at 4 °C (model GS-6R centrifuge; Beckman, Fullerton, CA). The cell pellet was suspended in 1 ml normal saline.

Total cell count

The total cell count analysis was performed using 400 µl of 0.4% Trypan Blue added to 100 µl of cell suspension. Using a pipette, 100 µl of this suspension was applied to the hemocytometer and the cells were counted under 10× magnification.

Differential cell analysis

The remaining cell suspension was spun on glass slides. Briefly, the glass slides were labeled with respective animal IDs. Cytotunnel was placed on the slide and firmly assembled with the cytoclip. Cell suspension (450 µl) was aliquoted into each well of the cytotunnel and slides were placed in the Cytospin 3® (Shandon Scientific Ltd., MA) and spun at room temperature for 5 min at 800 rpm. After cytospin and air drying for 24–36 h, cells were stained with Kwik-Diff staining solutions (Shandon, Fisher Scientific) as shown in the protocol provided by the manufacturer and were finally washed gently with distilled water. The slides were air dried and different types of cells were counted under 40X magnification. A total of 300 cells were counted on each slide. ELISA analysis was performed on the BAL supernatant using kits for Th2 cytokine IL-5 (Invitrogen, Carlsbad CA catalog # BMS610), OVA-specific IgE (Cayman Chemicals, Ann Arbor MI catalog # 500840) and OVA-specific IgG (Chondrex Inc., Redwood WA catalog #3011). The plates were prepared as per the manufacturers' instructions and absorbances were measured in a Synergy microplate reader at 450 nm.

Drugs and chemicals

Unless stated otherwise, all chemicals were of the highest grade available and were purchased from Sigma Chemicals (St. Louis, MO).

Statistical analysis

The data are expressed as mean ±SEM. Comparison between different groups of WT and A_{2A}KO were performed using two-way ANOVA followed by Tukey's multiple comparison test. For IgG analysis, 3-way ANOVA followed by Tukey's multiple analysis was used. A “*p*” value less than 0.05 (*p* < 0.05) was considered as significant. All statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA; Software Version 8.2.1).

Results

Computational analysis of docking models for limonene and A_{2A}AR/A_{2B}AR

Our selected A_{2B}AR homology model has the following parameters: highest model score; 74% positives, and 12% gaps. The docking scores and receptor interactions suggest that limonene can bind in a similar region of A_{2A}AR as CGS21680 (Supporting Information, Table 1). Based on structural

analysis, limonene appears to occupy a region at the top of the receptor. Receptor interactions captured in the docking model include van der Waals contacts with S67, V84, L85, F168, E169, W246, H278, and I274 (Fig. 2a). In comparing interactions with the receptor, it appears that these same residues (Phe168, Leu249, Asn253, and Ile 274) interact with the diphenyl group of UK-432097 [21]. Based on the molecular analysis, we hypothesize that limonene has affinity for A_{2A} AR and may act as an agonist or partial agonist. We also looked at A_{2B} AR-Limonene interactions, as functional data suggested another adenosine receptor may be involved. We found that limonene may also have similar binding interactions with the A_{2B} AR based on the docking analysis with the homology model. Several residues at the top of the binding site of A_{2B} AR are homologous to A_{2A} AR. Thus, limonene interacts with similar residues S68, V85, L86, F173, W247, H280, and I 276 of A_{2B} AR (Fig. 2b).

Airway responsiveness to MCh in WT and A_{2A} KO

WT There was no significant difference in airway responsiveness to MCh (48 mg/ml) in control (CON; $87.2 \pm 40.7\%$) and control mice treated with limonene (CON+LIM; $63.1 \pm 26.7\%$). Allergen sensitized and challenged mice or allergic mice (SEN) showed the highest response to MCh ($444.5 \pm 87.5\%$; $p < 0.05$ compared to CON). However, treatment with limonene (SEN + LIM) significantly reduced the airway

hyperresponsiveness in allergic mice (SEN $444.5 \pm 87.5\%$ vs. SEN + LIM $69.1 \pm 34.2\%$, $p < 0.05$) compared to the untreated sensitized groups (SEN). This showed that limonene effectively reduced the airway hyperresponsiveness in the asthmatic mice (Fig. 3a).

A_{2A} KO There was significant difference in the airway responsiveness to MCh (48 mg/ml) between control and sensitized groups ($104.8 \pm 15.698\%$ in CON vs $553 \pm 85\%$ in SEN, $p < 0.05$). However, the limonene-treated sensitized mice did not show any significant reduction as compared to the untreated sensitized mice ($588 \pm 34\%$ in SEN + LIM). This confirmed that the absence of A_{2A} AR renders the treatment with limonene ineffective (Fig. 3b).

Airway responsiveness to NECA in WT and A_{2A} KO mice

WT Similar to the MCh responses, exposure to NECA (750 μ g/ml) showed significant increase in the airway responsiveness in the sensitized group compared to the control group ($90.6 \pm 57.7\%$ in CON vs. $1002.8 \pm 161.7\%$ in SEN, $p < 0.05$). On the other hand, sensitized animals treated with limonene showed significant reduction in the airway hyperresponsiveness as compared to the sensitized untreated mice ($201.1 \pm 89.1\%$ in SEN + LIM vs. $664.1 \pm 327.9\%$ in SEN, $p < 0.05$). NECA being a nonselective adenosine agonist and limonene being an agonist of the A_{2A} AR suggest that

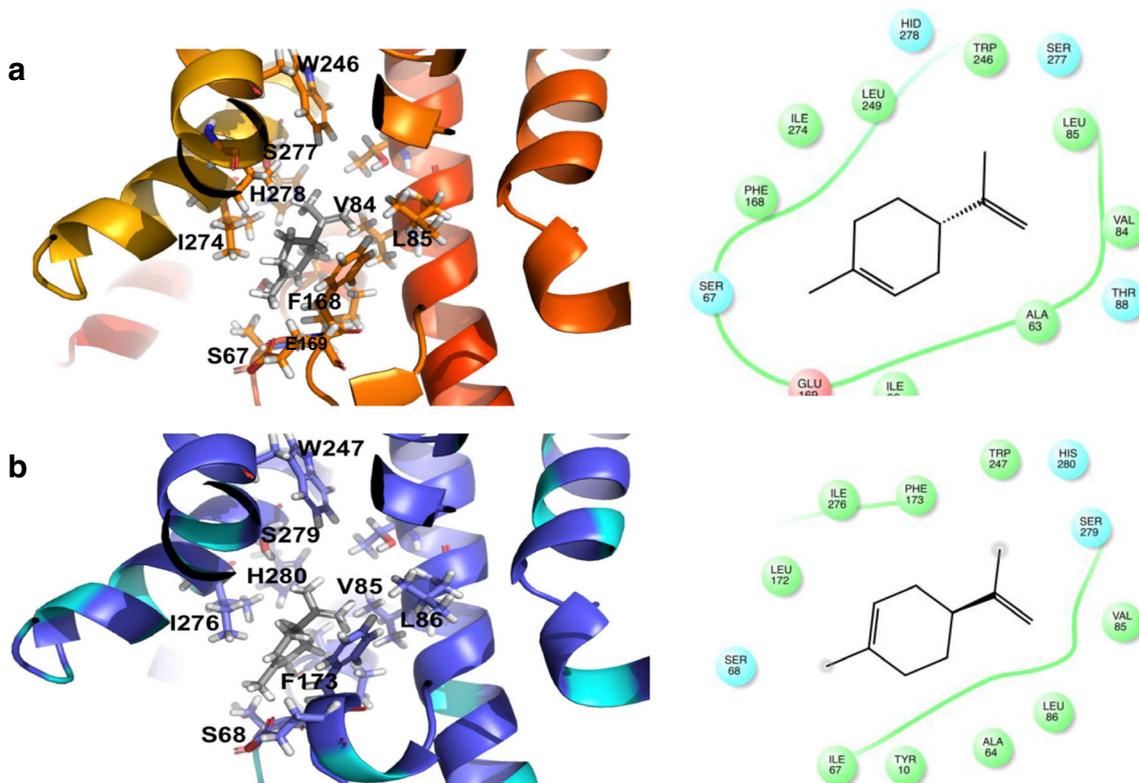


Fig. 2 Docking model for limonene binding to A_{2A} (Fig. 2a) and A_{2B} (Fig. 2b) receptors

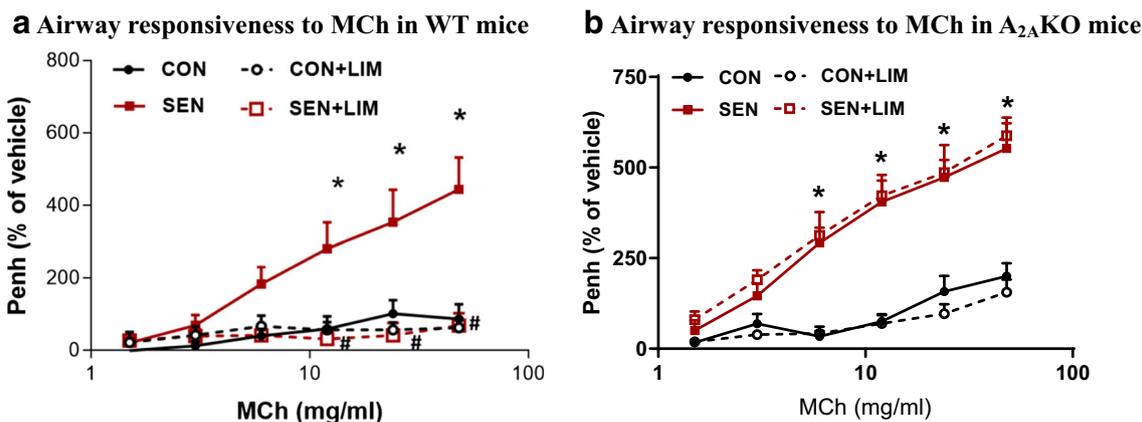


Fig. 3 Methacholine-induced airway hypersensitivity (enhanced pause, i.e., Penh) in **a**) wild-type study groups. Values for Fig. 3a are expressed as mean \pm SEM, * p < 0.05 compared to WT CON; # p < 0.05 compared to

WT SEN, n = 6 mice per group, and **b**) A_{2A} KO study groups. Values for Fig. 3b are expressed as mean \pm SEM, * p < 0.05 compared to A_{2A} KO CON, n = 6 mice per group

limonene acts through the A_{2A} AR to reduce the airway hyperresponsiveness (Fig. 4a).

KO Airway responsiveness to NECA (750 μ g/ml) was significantly higher in the sensitized group compared to the control group ($104 \pm 52\%$ in CON vs. $541 \pm 44\%$ in SEN, p < 0.05). Treatment with limonene in the SEN + LIM group had no significant effect in reducing the airway responsiveness of KO SEN mice ($528 \pm 147\%$ in SEN + LIM; Fig. 4b).

Total cell count of BAL fluid in WT and A_{2A} KO

WT Significant increase was observed in the total cell counts of sensitized WT mice compared to controls ($6 \times 10^4 \pm 1$ in CON vs. $23.6 \times 10^4 \pm 1.9$ in SEN, p < 0.05). Limonene treatment effectively reduced the number of cells significantly in the limonene treated sensitized mice ($14.1 \times 10^4 \pm 1.5$; Fig. 5a).

A_{2A} KO Similarly, we observed that sensitized groups of A_{2A} KO mice had significantly elevated total cell count compared to control ($6.6 \times 10^4 \pm 1.2$ in CON vs. $26.8 \times 10^4 \pm 2.5$ in SEN, p < 0.05). However, limonene treatment did not lower the cell count in sensitized KO mice ($25.5 \times 10^4 \pm 1.6$ in SEN + LIM; Fig. 5b).

ELISA analysis of BALF supernatant for IL-5

The supernatant of the BALF was analyzed for IL-5 in WT (Fig. 6a) and A_{2A} KO (Fig. 6b). IL-5 was significantly elevated in WT SEN (286.2 ± 45.2 pg/ml compared to 74.2 ± 18.6 pg/ml in CON; p < 0.05). Limonene significantly reduced IL-5 in treated WT asthmatic mice (81.4 ± 25.4 pg/ml in SEN + LIM; p < 0.05) compared to the nontreated SEN mice. A_{2A} KO SEN also had elevated IL-5 compared to the KO control (231.2 ± 30.5 pg/ml vs. 78.5 ± 6.8 pg/ml in CON; p < 0.05), but limonene treatment had no effect on IL-5 levels in asthmatic KO

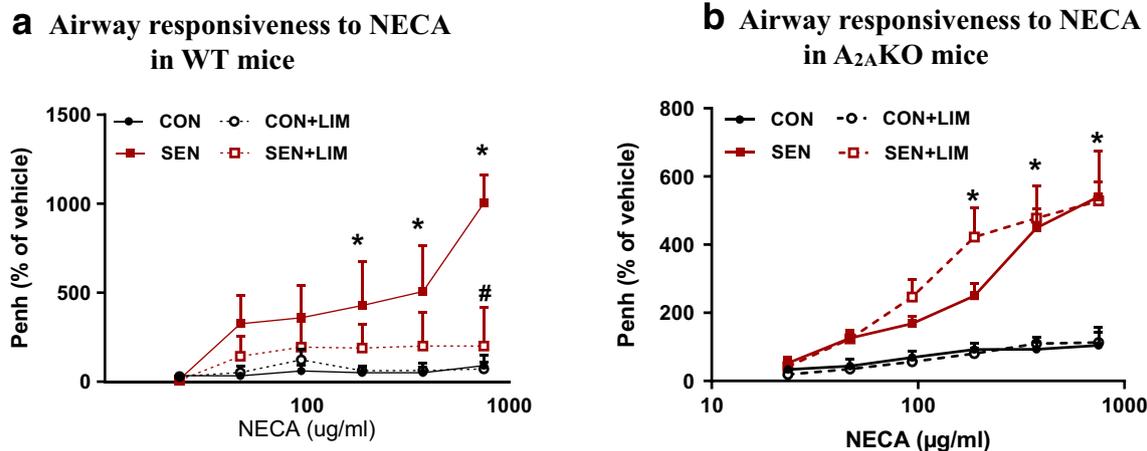
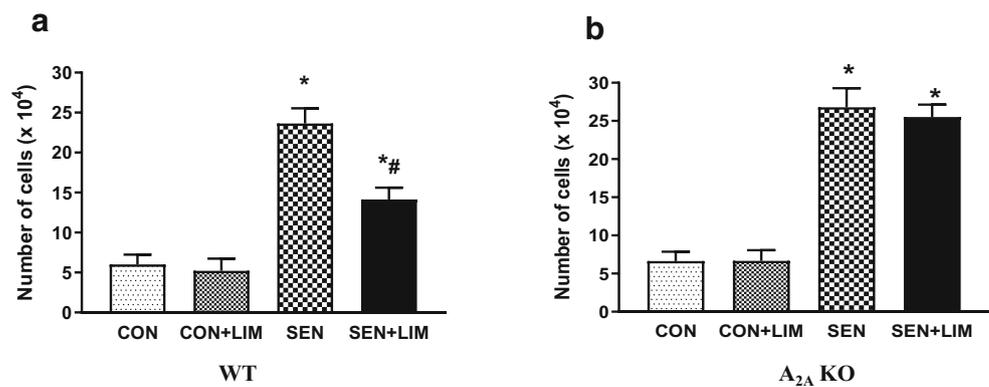


Fig. 4 NECA-induced airway responsiveness in **a**) wild-type study groups. Values for Fig. 4a are expressed as mean \pm SEM, * p < 0.05 compared to WT CON; # p < 0.05 compared to WT SEN, n = 6 mice

per group, and **b**) A_{2A} KO study groups. Values for Fig. 4b are expressed as mean \pm SEM, * p < 0.05 compared to A_{2A} KO CON, n = 6 mice per group

Fig. 5 Total cell count in BAL from **a**) wild-type study groups. Values for Fig. 5a are expressed as mean \pm SEM, $*p < 0.05$ compared to WT CON; $\#p < 0.05$ compared to WT SEN, $n = 6$ mice per group, and **b**) A_{2A} KO study groups. Values for Fig. 5b are expressed as mean \pm SEM, $*p < 0.05$ compared to A_{2A} KO CON, $n = 6$ mice per group



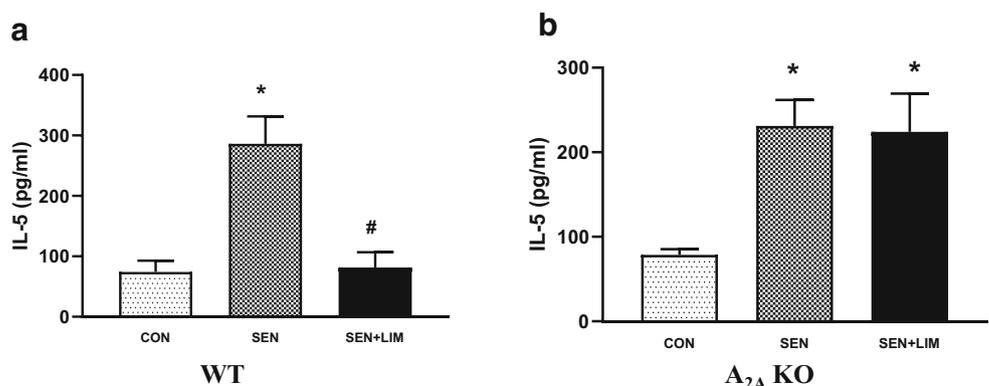
mice (224 ± 45.3 pg/ml). These data suggest that limonene reduced IL-5 only in the presence of A_{2A} AR.

ELISA analysis of BALF supernatant for OVA-specific IgE and OVA-specific IgG

Analyses for OVA-specific IgE (Fig. 7a) and IgG (Fig. 7b) were performed using the BALF supernatant in all groups. IgE was significantly elevated in both sensitized WT (18.3 ± 6.4 pg/ml in SEN vs. undetected in CON; $p < 0.001$) and A_{2A} KO (19.9 ± 3.9 pg/ml vs. undetected in CON; $p < 0.001$) groups compared to respective controls. Limonene significantly reduced IgE levels in SEN WT (0.7 ± 0.4 pg/ml in SEN + LIM; $p < 0.0001$) and A_{2A} KO (2.4 ± 0.8 pg/ml in SEN + LIM; $p < 0.001$). There was no difference between WT and A_{2A} KO in the BALF levels of OVA-specific IgE.

Elevated levels of OVA-specific IgG were observed in SEN WT (231.7 ± 43.4 pg/ml vs. 12.2 ± 8.2 pg/ml in CON; $p < 0.0001$) and SEN A_{2A} KO (96 ± 1.7 pg/ml in SEN vs. 44.2 ± 15.8 in CON; $p < 0.05$). The level of IgG in WT SEN was significantly higher than sensitized A_{2A} KO mice ($p < 0.01$). While limonene reduced the IgG levels in SEN WT (74.8 ± 11.4 , $p < 0.001$), it did not reduce the IgG level in treated SEN A_{2A} KO (100.1 ± 4.2 pg/ml; $p > 0.05$).

Fig. 6 Analysis of IL-5 levels in BAL from **a**) wild-type study groups. Values for Fig. 6a are expressed as mean \pm SEM, $*p < 0.05$ compared to WT CON; $\#p < 0.05$ compared to WT SEN, $n = 6$ mice per group, and **b**) A_{2A} KO study groups. Values for Fig. 6b are expressed as mean \pm SEM, $*p < 0.05$ compared to A_{2A} KO CON, $n = 6$ mice per group



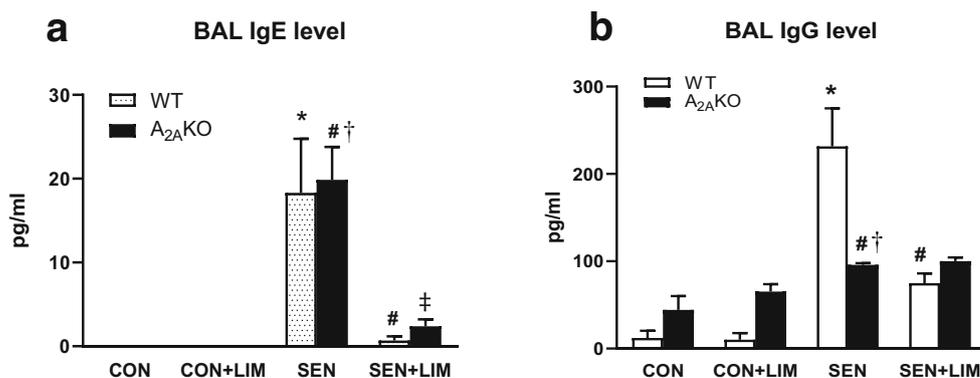
Differential cell studies of bronchoalveolar lavage

The differential cell count for eosinophils, macrophages, neutrophils, and lymphocytes was obtained for all study groups.

Effects of limonene on eosinophils, neutrophils, macrophages, and lymphocytes in WT

Sensitized WT mice (nontreated asthmatic group) had significantly higher number of eosinophils compared to control group ($0 \pm 0\%$ in CON vs. $70.7 \pm 2.6\%$ in SEN; $p < 0.0001$; Fig. 8a). Limonene significantly reduced number of eosinophils in asthmatic mice ($48.7 \pm 1.4\%$ in SEN + LIM, $p < 0.05$) compared to nontreated SEN groups (Fig. 7a). Neutrophils were significantly increased in SEN group compared to CON ($5 \pm 2.5\%$ in CON vs. $15.9 \pm 1.1\%$ of cells in SEN; $p < 0.05$; Fig. 8b). However, limonene-treatment had no effect on the increase in neutrophils ($16.8 \pm 1\%$ in SEN + LIM). In control groups, macrophages had the highest contribution to the cellular infiltrate while in SEN group, macrophages were reduced significantly ($90.6 \pm 1.8\%$ in CON vs. $9.2 \pm 2.8\%$ in SEN; Fig. 8c). In the limonene-treated sensitized group, the decrease in macrophages was significantly attenuated compared to the SEN group ($27.2 \pm 4.4\%$; $p < 0.05$). Lymphocytes were significantly elevated in asthmatic WT mice ($7 \pm 1\%$ in

Fig. 7 Analysis of IgE (a) and IgG (b) levels in BAL from WT and A_{2A} KO mice. Values are expressed as mean \pm SEM, * p < 0.05 compared to WT CON; # p < 0.05 compared to WT SEN; † p < 0.05 compared to A_{2A} KO CON; ‡ p < 0.05 compared to A_{2A} KO SEN; n = 4 mice per group



SEN vs. $1 \pm 1\%$ in CON; p < 0.05; Fig. 8d) and limonene reduced the elevated lymphocytes observed ($2 \pm 0.04\%$ in SEN + LIM; p < 0.05).

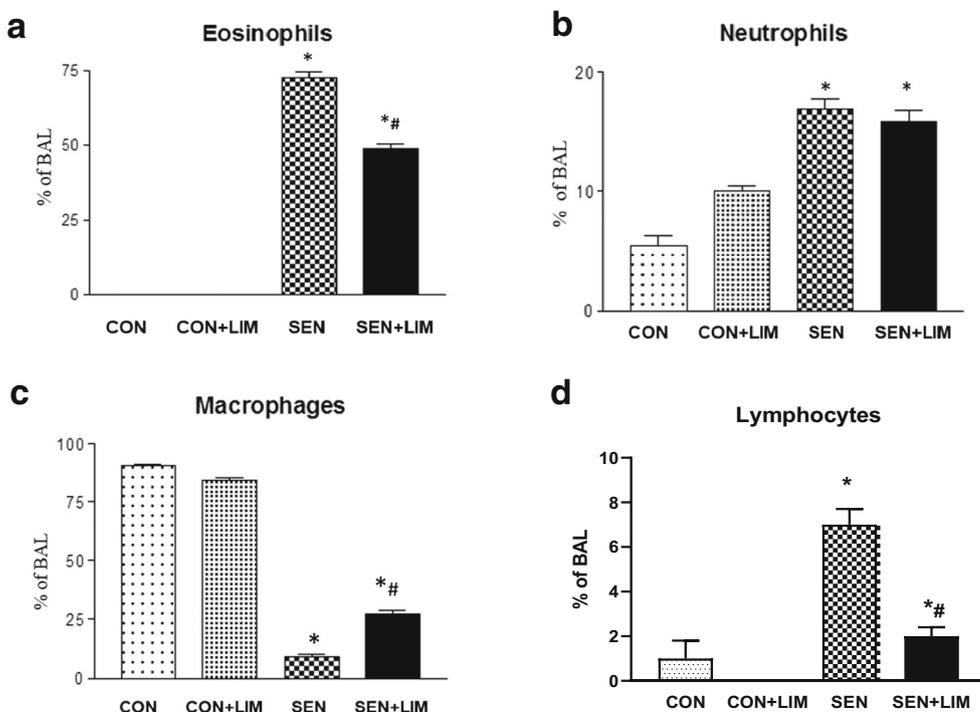
Effects of limonene on eosinophils, neutrophils, macrophages, and lymphocytes in A_{2A} KO

Sensitized A_{2A} KO mice (nontreated asthmatic group) had significantly higher number of eosinophils compared to control group ($0 \pm 0\%$ in CON vs. $76.3 \pm 1.4\%$ in SEN; p < 0.0001; Fig. 9a). Limonene failed to reduce the eosinophils in treated KO asthmatic mice ($79 \pm 0.69\%$ in SEN + LIM). In A_{2A} KO mice, macrophages were significantly reduced in SEN mice with limonene treatment having no effect on the macrophage count (Fig. 9b). Lymphocytes were also significantly elevated in asthmatic A_{2A} KO mice ($7.5 \pm 0.6\%$ in SEN vs. $1.6 \pm 0.5\%$

in CON; p < 0.05; Fig. 9c) and limonene reduced the elevated lymphocytes observed ($3.4 \pm 0.4\%$ in SEN + LIM; p < 0.05).

Neutrophils were significantly increased in SEN group compared to CON ($4 \pm 0.5\%$ in vs $18.2 \pm 0.7\%$ in SEN; p < 0.05; Fig. 9d). However, unlike the WT group, limonene-treatment significantly reduced neutrophils in treated KO mice ($11.2 \pm 1.1\%$ in SEN + LIM, p < 0.05). This suggested that in the absence of A_{2A} AR, limonene could activate another receptor that affected neutrophilic infiltration. Based on the docking model studies, it appeared A_{2B} AR could also be activated by limonene. To confirm the function of the A_{2B} AR, we treated sensitized A_{2A} KO mice with selective A_{2B} agonist BAY 60–6583 [25]. These data showed that BAY 60–6583 also significantly reduced the neutrophilic infiltration in sensitized A_{2A} KO mice ($18.2 \pm 0.68\%$ in SEN vs $10 \pm 2\%$ in SEN + BAY; p < 0.05 Fig. 9e).

Fig. 8 Differential cell count of a) eosinophils, b) neutrophils, c) macrophages, and d) lymphocytes in BAL from WT mice. Values are described as mean \pm SEM, * p < 0.05 compared to WT CON; # p < 0.05 compared to WT SEN; n = 6 mice per group



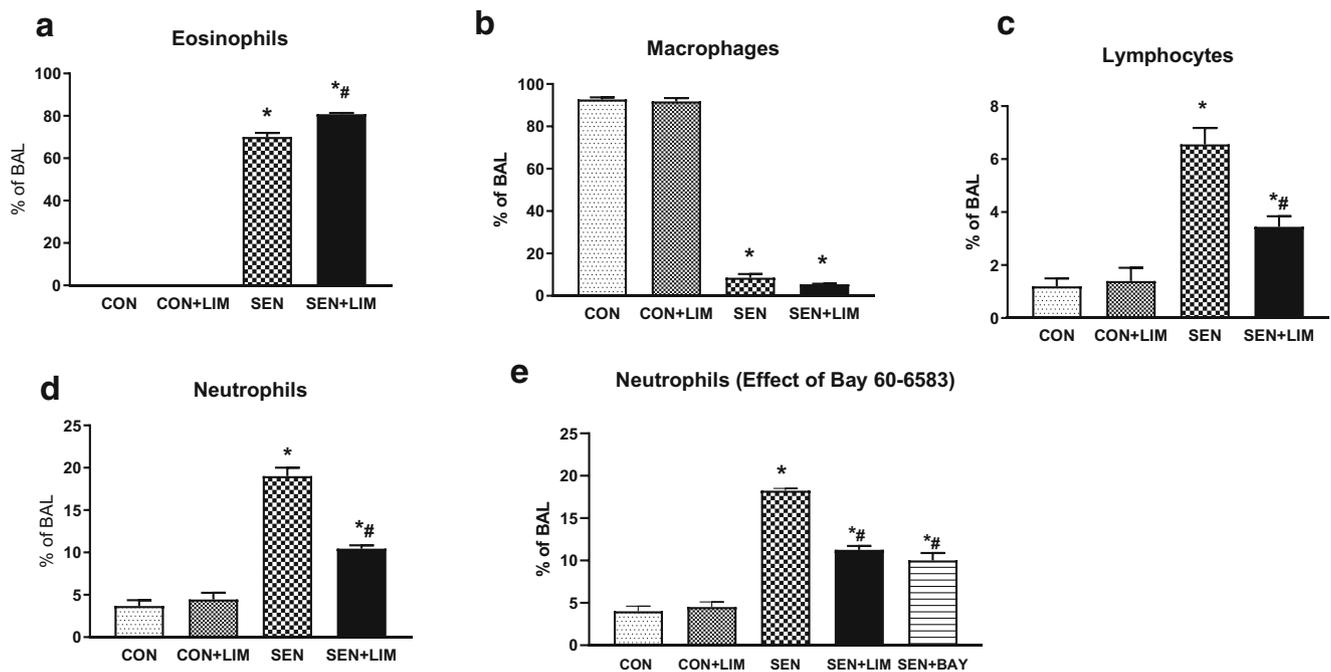


Fig. 9 Differential cell count of **a)** eosinophils, **b)** macrophages, **c)** lymphocytes, **d)** neutrophils, and **e)** neutrophils in groups treated with BAY 60-6583 from A_{2A} KO mice. Values are described as mean \pm

SEM, * $p < 0.05$ compared to A_{2A} KO CON; # $p < 0.05$ compared to A_{2A} KO SEN; $n = 6$ mice per group

Discussion

In the current study, we investigated the effects of limonene using A_{2A} KO and WT allergic mice to identify whether limonene attenuated inflammation and airway hyperresponsiveness by activating A_{2A} receptors. The primary finding of this study is that allergic WT mice had increased bronchoconstriction and eosinophilic inflammation, which were lowered by limonene. Allergic A_{2A} KO mice also had elevated bronchoconstriction and eosinophilic inflammation but treatment with limonene failed to lower these outcomes, suggesting that limonene mediated its effects partly through the A_{2A} AR. Limonene, however, can also bind to A_{2B} AR and may have an effect on neutrophil infiltration via activation of these receptors.

Our computational analysis and molecular docking data confirmed that limonene binds to the A_{2A} AR as an agonist and to A_{2B} AR as well. The effects of limonene on other adenosine receptors (A_1 , A_3) remain unclear, and more studies are required to characterize if limonene acts as an agonist, partial agonist or antagonist at these receptors. From our studies, it appears that limonene acts as a nonselective agonist at the A_{2A} and A_{2B} receptors.

Next, we measured airway responsiveness using whole-body plethysmography with methacholine as a bronchoprovocant as previously described [24, 26]. While the use of whole body plethysmography is controversial, it continues to be used to assess airway responsiveness [24, 26–29]. We measured effect of limonene on airway responsiveness as enhanced pause (Penh) using increasing doses of

methacholine and nonselective adenosine analog NECA in A_{2A} KO and WT mice. Significantly increased airway responsiveness to both MCh and NECA was observed in sensitized WT and A_{2A} KO mice compared to their respective controls. These responses were significantly lowered with limonene treatment in WT sensitized mice. However, limonene had no effect on increased airway responsiveness in A_{2A} KO group. This data suggested that limonene attenuated the airway responsiveness through activation of the A_{2A} AR and the absence of A_{2A} AR renders the treatment with limonene ineffective.

Increased level of adenosine is seen in BAL fluid of asthma patients and studies show that adenosine contributes to increased inflammation in asthma [11]. We carried out total cell count of BAL fluid to assess the cellular infiltration in the lungs in each experimental group. Sensitized mice had the highest number of inflammatory cells compared to nonsensitized group in both WT and A_{2A} KO groups, suggesting infiltration of inflammatory cells in the lungs. Treatment with limonene significantly attenuated the number of cells in allergic WT mice whereas the elevated total cell count noted in sensitized A_{2A} KO mice remained unchanged with limonene treatment. These results indicate that limonene attenuated the inflammatory infiltration via the A_{2A} AR, as in the absence of the A_{2A} AR, there was no change in the increased cell counts in the allergic mice. Thus, limonene may have an anti-inflammatory role by acting as an agonist of A_{2A} AR. This supports previous findings that activation of A_{2A} AR has anti-inflammatory effects in a

mouse model of asthma [30]. Previous studies have examined the effects of limonene and ozone exposures on inflammation in asthma [31]. The findings of these studies suggest that while limonene has an effect on lowering inflammation, it did not affect cellular infiltration to a great extent. The differences in this study and ours are possibly due to the differences in exposure duration of limonene, the dose selected, and the model used.

Elevated levels of inflammatory cells such as eosinophils and neutrophils are typically observed in the BAL fluid in mouse models of asthma [19, 20, 30, 32]. We assessed the lung inflammation in all study groups by differential cell analysis of the BAL to identify the cell types. Presence of highly elevated eosinophil counts in the BAL indicates the presence of allergic inflammation and exacerbates airway hyper-reactivity [9, 33]. We found that both WT and A_{2A} KO sensitized mice had highest number of eosinophils compared to their respective nonsensitized controls. This eosinophilic infiltration was lowered significantly with the treatment with limonene in WT mice. However, treatment with limonene did not affect eosinophil number in A_{2A} KO sensitized mice, suggesting again that limonene was reducing the eosinophilia via A_{2A} receptor activation. The predominant cells in BAL in healthy individuals are macrophages [34]. In the present study, macrophages were highest in BAL of control groups of both WT and A_{2A} KO mice and were lowered in sensitized mice. Limonene attenuated the decrease in macrophage number in sensitized WT mice but did not have any effect in A_{2A} KO. Neutrophils are also elevated in asthma [10]. Differential cell analysis showed that neutrophils were increased in the sensitized mice in both WT and A_{2A} KO groups. Interestingly, limonene did not lower the neutrophilia in sensitized WT mice but significantly lowered the neutrophil count in sensitized A_{2A} KO mice. This led us to surmise that limonene could possibly activate other pathways as well to reduce inflammation. Based on our docking studies and previously published work that showed A_{2B} AR activation lowered neutrophil infiltration [35], it was possible that the A_{2B} AR was being activated by limonene in our model. In order to confirm the effect of A_{2B} activation on neutrophil infiltration, we treated sensitized A_{2A} KO mice with selective A_{2B} agonist BAY 60-6583. Our data showed that BAY 60-6583 also lowered the neutrophil count in the sensitized A_{2A} KO mice to the same degree as limonene. This led us to conclude that limonene lowered eosinophilia via A_{2A} AR activation and could lower neutrophilia by activation of the A_{2B} AR. However, we did not observe lowered neutrophils in the WT mice, even though the A_{2B} receptors were present in these mice. It has been established that among the adenosine receptors, the A_{2B} receptor is considered as the low affinity receptor [35]. Thus, high amounts of agonist are required before activation of A_{2B} AR can occur. It is possible that the dose of limonene given to WT mice was

not enough to activate both A_{2A} and A_{2B} receptors, with the likelihood that A_{2A} AR activation dominated in the WT. On the other hand, in the absence of the A_{2A} AR in the A_{2A} KO, the limonene administered could now solely target the A_{2B} AR. Taken together with the BAY 60-6583 compound effects, it appears A_{2B} AR activation may be responsible for the lowered neutrophil counts observed in A_{2A} KO. This is a novel finding where limonene may have differential effects in asthma by activating A_{2A} and A_{2B} receptors.

We measured OVA-specific IgE and IgG in the BAL and found elevations for both immunoglobulins in the sensitized WT and A_{2A} KO mice compared to controls. The role of IgE in allergic asthma is well-established [36] and there also appears to be a role for IgG in driving airway hyperreactivity and inflammation, as evidenced by IgE knockout mice still developing some of the features of allergic asthma [37, 38]. Limonene reduced IgE in both WT and A_{2A} KO sensitized mice. For IgG, we observed that sensitized WT had higher IgG levels compared to A_{2A} KO sensitized mice. While limonene reduced the level of IgG significantly in allergic WT mice, it did not affect the elevated IgG level in sensitized A_{2A} KO mice. We also found that IL-5 was significantly elevated in the BAL in sensitized mice from both WT and A_{2A} KO groups. Limonene reduced the IL-5 level in the WT sensitized group but not the A_{2A} KO sensitized mice. IL-5 is a key cytokine for the increased infiltration of eosinophils in allergic asthma [39]. Our finding on IL-5 corresponded with the exacerbated eosinophilia in sensitized A_{2A} KO mice that was unaffected by limonene. IL-5 is also known to increase airway responsiveness [35]. Our findings on IL-5 and eosinophils correlated with the Penh measurements of airway hyperresponsiveness in sensitized A_{2A} KO mice treated with limonene. Studies have also shown that neutrophil numbers may not be associated with higher responsiveness to methacholine [40]. Thus, even though there were lowered neutrophils in treated sensitized A_{2A} KO mice, the unabated eosinophilia and elevated IL-5 could possibly be responsible for the higher airway responsiveness in this group. IgE and IgG may both be elevated on account of initial allergen sensitization.

In summary, our data shows that limonene decreases airway inflammation and airway responsiveness in allergic mice by the activation of A_{2A} AR. In the absence of the A_{2A} receptor, limonene fails to have an effect on eosinophilia and airway reactivity. However, limonene can also lower the neutrophilia observed in asthma by activation of A_{2B} receptors. These novel findings support a role for limonene as an adenosine receptor agonist, targeting both A_{2A} and A_{2B} receptors for beneficial effects in asthma. To our knowledge, this is the first study to show that limonene may have therapeutic benefits in asthma by acting on adenosine-mediated pathways through both A_{2A} and A_{2B} receptor activation.

Acknowledgments This work was supported by LIU startup funds (DSP) and National Institutes of Health grant HL027339 (SJM).

Funding information Long Island University start-up funds (DSP), HL027339 (SJM).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional guidelines for the care and use of animals were followed. All procedures performed were in accordance with the ethical standards of Long Island University under an approved IACUC protocol DP-AA-2018.

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