Adenosine Influences Myeloid Cells to Inhibit Aeroallergen Sensitization

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Running Head: Adenosine inhibits aeroallergen sensitization

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Abstract

Agonists of adenosine A2A receptors (A2AR) suppress the activation of most immune cells and reduce acute inflammatory responses. Asthma is characterized by sensitization in response to initial allergen exposure and by airway hyperreactivity in response to allergen rechallenge. We sought to determine if A2AR activation with CGS-21680 (CGS) is more effective when administered during sensitization or rechallenge. C57BL/6 wild type mice and Adora2a^−/−-LysMCre^+/− mice that lack A2ARs on myeloid cells were sensitized with intranasal OVA/LPS. Airway sensitization was characterized by a rapid increase in numbers of IL-6^+ and IL-12^+ macrophages and DCs in lungs. A2AR activation with subcutaneous CGS (0.1 μg/kg/min) only during sensitization reduced numbers of IL-6^+ and IL-12^+ myeloid cells in the lungs and reversed the effects of later OVA rechallenge to increase airway hyperresponsiveness to methacholine. CGS treatment during sensitization also reduced the expansion of lung Th1 and Th17 cells and increased expansion of Treg cells in response to OVA rechallenge. Most of the effects of CGS administered during sensitization were eliminated by myeloid-selective A2AR deletion. Administering CGS just during OVA rechallenge failed to reduce airway hyperresponsiveness. We conclude that myeloid cells are key targets of adenosine during sensitization and indirectly modify T cell polarization. The results suggest that a clinically useful strategy might be to use A2AR agonists to inhibit sensitization to new aeroallergens. We speculate that adenosine production by macrophages engulfing bacteria contributes to the curious suppression of sensitization in response to early-life infections.

Keywords: Adenosine, adenosine A2A receptor; asthma, allergen, macrophages
Introduction

Lung inflammation and airway hyperresponsiveness are hallmarks of asthma, which can be treated with anti-inflammatory agents such as corticosteroids and leukotriene D4 antagonists. Either Th1/Th17 or Th2-dominated immune responses to aeroallergens mediate asthma pathogenesis in different individuals, depending on the degree of co-exposure to LPS encountered from infection or inhalation of dust mites (23). LPS enhances Th1 priming with the production of IFN-γ and IL-1 (14) whereas the absence of LPS favors Th2 priming with the production of IL-4 and IL-13 (26). Hence, atopic asthma is now recognized to be heterogeneous disease with different inflammatory profiles (15). Zhu et al (48) found in mice that low-levels of LPS exposure along with allergen induces the production of TNF-α by dendritic cells, which enhances IL-4 and IL-13 production by mast cells and NKT cells. These cytokines favor naïve T cell differentiation into Th2 cells and subsequent eosinophilic inflammation. In the presence of high LPS, allergens stimulate macrophages and DCs to produce IL-6 and IL-12 that promote differentiation of naïve T cells into Th1 and Th17 cells that respond to allergens by producing cytokines such as IFN-γ and IL-17 to promote airway inflammation with neutrophils and macrophages, but not eosinophils (48).

Adenosine activates four G protein coupled receptors: A1, A2A, A2B and A3 (29). Activation of the A2AR that is expressed on most cells of the immune system generally acts to suppress Th1 inflammatory processes through Gs-coupled receptors that elevate cAMP in neutrophils (39, 40), macrophages (35), T cells (28) and NKT cells (27). By reducing inflammation, A2AR
activation indirectly influences airway responsiveness (13) in a way that is fundamentally different from direct airway bronchodilation in response to β2-adrenergic agonists (17). In the current study we examined the effects of A2AR activation in a high LPS mouse model of asthma that is associated with Th1/Th17 priming. The results indicate that A2AR activation effectively prevents airway sensitization, but marginally reduces airway inflammation when added during allergen rechallenge. By using Adora2a^{f/f}-LysMCre^{-/-} mice we demonstrate that suppression by CGS of aeroallergen-induced sensitization: 1) principally depends on myeloid A2ARs; 2) is associated with reduced myeloid production of IL-6 and IL-12, enhanced production of TGF-β; 3) is associated with airway expansion of Treg cells; and 4) is associated with reduced production of Th1 and Th17 cells. These findings suggest that it may be therapeutically effective to treat allergic individuals with A2AR agonists during their initial exposures to antigens in a new workplace or habitat.
Materials and Methods

Mice

Male C57Bl/6 mice 8-10 weeks of age were obtained from Jackson Laboratories (Bar Harbor, ME). Adora2a/C-LysMCre\(^{+/-}\) and Adora2a/C-LysMCre\(^{-/-}\) littermate controls were generated by crossing C57BL/6 LysMCre\(^{+}\) mice with congenic Adora2a/C mice produced as described (8). The Animal Care and Use Committee of the La Jolla Institute approved experiments in accordance with National Institutes of Health Guidelines.

Mouse sensitization, rechallenge and treatment with CGS

Mice were sensitized by intranasal administration of 100 µg OVA and 15 µg LPS in a total volume of 20 µl on days 0, 1, 2 and 7. Mice were rechallenged with three exposures for 30 min to aerosolized 1% OVA in PBS on days 14, 15 and 21. This regimen is associated with production of Th1/Th17 cells and neutrophil recruitment into alveoli (24). To evaluate the in \textit{vivo} effects of treatment with an A\(_{2A}\)R agonist, mice were treated with CGS (21) (100 ng/kg/min) administered via 3-day Alzet-minipumps implanted subcutaneously 5 hours before day 0 sensitization, and with a 1µg/kg i.p. bolus 30 min before day 7 sensitization. Other mice were treated with CGS only during OVA rechallenge (\textbf{Figure 1}). CGS treatment via Alzet minipumps was started 24 hours before day 14 rechallenge and with an i.p. bolus of 1µg/kg 30 min before day 21 rechallenge.

Vascular permeability

Pulmonary vascular leak was determined by measuring the extravasation of Evans blue dye (EBD) from the circulation to the lungs. EBD (30 mg/kg body weight in 200 µl) was injected intravenously in mice anesthetized with ketamine/xylazine and allowed to circulate for 30 minutes. The chest was opened, the inferior vena cava transected, and the pulmonary
vasculature flushed with 10 ml saline via the right ventricle to remove intravascular dye. The lung was homogenized and incubated in 100% formamide at 37°C for 24 hours to extract EBD. The concentration of dye was determined by spectrophotometry with correction for heme pigments as previously described using the equation: $E_{620 \text{ (corrected)}} = E_{620} - (1.426 \times E_{740} - 0.03)$ (45). Data are expressed as micrograms EBD per gram lung.

**Airway response to methacholine challenge**

Following sensitization with intranasal OVA/LPS and rechallenge with aerosolized OVA, mice were treated with aerosolized methacholine at doses of 0, 6.25, 12.5 and 25 mg/ml in PBS. Methacholine-induced changes in Penh were determined using noninvasive whole body plethysmography (Buxco Electronic) according to the manufacture’s instructions.

**Pulmonary immunohistochemistry and histopathologic grading**

Lung inflammation was scored on a scale of 0 to 4 as described (2). After perfusion with PBS, lungs were fixed with 4% paraformaldehyde overnight. Paraffin sections (5 μm) were stained with hematoxylin and eosin (H&E).

**Staining of immune cells for flow cytometry**

After BAL to remove alveolar cells, lungs were removed, minced and incubated in digestion buffer containing 1mg/ml collagenase type Ia, 60U/ml hyluronidase type I-s, and 60 U/ml DNase 1 for 45 minutes at 37°C. Single cells were prepared by passing digested tissue through a 40 μm cell strainer. Cells were resuspended at $2 \times 10^6$ cells/mL in RPMI media and incubated in 48 well plates with PMA (50ng/ml) and Ionomycin (500ng/ml) for 4 hours, with the addition of GolgiStop (BD Bioscience) added during the last 2 hours. Fcγ receptors were blocked and live cells (fixable live/dead yellow, Invitrogen) were stained to detect
surface markers. Some cells were fixed and permeabilized for intracellular staining of IL-17A, IFN-γ, Foxp3, IL-6 and IL-12p40. Stained cells were analyzed using a LSR II flow cytometer (BD Bioscience) and data analyzed using FlowJo version 9.6.4 software (Tree Star, Inc).

Cell numbers and cytokines in BAL fluid

Mice were euthanized using CO₂ and tracheae were cannulated. The lungs were flushed 3 times with one ml of PBS. The recovered fluid from the first ml was used for cytokine measurements. Total cells in the pooled BALs were counted using a Viab cell counter. To determine the composition of cells in the BAL approximately $2 \times 10^3$ cells were spun onto glass slides using a Cytospin cytocentrifuge and stained with Protocol Hema 3 stain set (Fisher Scientific). At least 300 cells were counted and identified as macrophages, lymphocytes, neutrophils, or eosinophils, according to standard morphological criteria.

Cytokines in BAL were measured by ELISA (eBioscience), according to the manufacturer’s instructions.

Analysis of A₂A R Responses in Spleen-derived Th1 and Th17 Cells

Splenocytes were isolated from 8-week-old C57Bl/6J mice. CD4⁺/CD62L⁺ cells were prepared by using T Cell Isolation Kit II (Miltenyi Biotech, Auburn, CA, USA). One x $10^5$ cells/well were cultured in 200 µl RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 mM HEPES in 96 well plate. Th1-polarizing conditions were produced by adding 3 µg/ml anti-CD3 Ab (BD Pharmingen, San Diego, CA, USA), 2 µg/ml anti-CD28 Ab (BD Pharmingen), 20 ng/ml IL-2 (R&D System, Minneapolis, MN, USA), 20 ng/ml IL-12 (R&D System) and 10 µg/ml anti-IL-4 (R&D System) to T cell cultures. Th17-polarizing conditions
were produced by adding 3 μg/ml anti-CD3 Ab, 2 μg/ml anti-CD28, 5 ng/ml TGF-β  
(PeproTech, Rocky Hill, NJ, USA), 100 ng/ml IL-6 (R&D System), 10 μg/ml anti-IL-4  
(R&D System) and 10 μg/ml anti-IFN-γ (R&D System). After 5 days in culture, cells were  
washed and CGS or vehicle was added to the cells 30 minutes before restimulation with anti-  
CD3 (3 μg/ml) and anti-CD28 (2 μg/ml). After restimulation for 24 h, supernatants were  
collected for cytokine measurements.

Statistical analysis

Prism version 6 software (GraphPad) was used for statistical analysis. Data are presented as  
the mean ± standard error of the mean. Unpaired Student’s t-test, one-way ANOVA with  
Tukey posttesting, or two-way ANOVA with Bonferroni posttesting were used as appropriate  
to compare experimental groups. P values of less than 0.05 were considered to be significant.

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Results

Airway disease was produced by sensitizing mice with intranasal OVA (100 µg) in combination with a high dose of LPS (15 µg) administered four times over 7 days (24). This was followed by rechallenge with aerosolized OVA (10 mg/ml for 30 min) on days 14, 15 and 21 (Figure 1). In prior studies similar mouse models of asthma provoked the rapid accumulation of pulmonary macrophages and DCs. Antigen rechallenge in such models causes an expansion of Th1 and Th17 cells and an increase in airway responsiveness to methacholine challenge (24, 25). Our strategy for studying the effects of CGS on the allergic disease process is illustrated in Figure 1. Since innate immune responses occur rapidly, we measured the effects of CGS on lung myeloid cells and myeloid cytokine accumulation in the BAL acutely, on day 3 following OVA/LPS sensitization on days 0, 1, and 2. Adaptive immune responses are manifest more slowly. For that reason, T cell accumulation in the lung and lymphokine accumulation in the BAL were sampled on day 21, following OVA rechallenge on days 14, 15 and 21. Airway hyperresponsiveness and lung inflammation were measured on day 23 following OVA rechallenge on days 14, 15 and 21, based on prior reports suggest that this corresponds to the time of peak responses.

A2ARs are expressed on multiple cells of the immune system, including macrophages, DCs, neutrophils and T cells. Since macrophages and DCs present antigens to T cells and produce cytokines that influence T cell polarization, we reasoned that A2AR signaling in myeloid cells might influence OVA sensitization. In order to test this hypothesis, myeloid-selective A2AR deletion was produced in Adora2a^f/f-LysCre mice as described (7).

Effects of CGS on Pulmonary Myeloid Cells During Sensitization.

Airway sensitization following three exposures to intranasal OVA/LPS triggered a marked increase in inflammatory cells in the BAL on day 3 (Figure 2A). CGS significantly inhibited
the accumulation of total cells, neutrophils, lymphocytes and eosinophils in the BAL. The

202 total number of macrophages in the BAL was not significantly affected by CGS. In a similar

203 mouse model, Bonneau et al. (9) also found that among cells that accumulate in the BAL in

204 response to OVA, only macrophage numbers are not affected by intranasal CGS. These

205 findings suggest that factors produced in acutely inflamed alveoli that are chemotactic to

206 circulating monocytes or lung macrophages are not strongly influenced by CGS. In

207 Adora2a^{fl}LysMCre^{+} mice in which A2A Rs are deleted from macrophages and neutrophils

208 (7), the effect of CGS on total cells and neutrophil accumulation into the BAL were abolished

209 (Figure 2A), suggesting that neutrophil chemotaxis is controlled by A2A receptors on

210 myeloid cells, and perhaps in part by cell intrinsic A2A Rs (12). BAL lymphocyte

211 accumulation in response to CGS was attenuated by myeloid-selective A2A R deletion (Figure

212 2A), suggesting that lymphocyte chemotaxis is partly controlled by myeloid A2A Rs and partly

213 by T cell-intrinsic receptors. Only very small numbers of eosinophils accumulated in the

214 BAL as expected with this asthma model. The chemotaxis of eosinophils was inhibited by

215 CGS and this inhibition was not affected by myeloid-selective A2A R deletion (Figure 2A),

216 suggesting that eosinophil chemotaxis may be controlled by eosinophil-intrinsic A2A R

217 signaling or chemotactic cytokine production by non-myeloid cells (1).

218 We also examined the effects of CGS on the acute accumulation cytokines in the

219 BAL in response to OVA/LPS (Figure 2B). CGS treatment during sensitization significantly

220 blunted the accumulation of IL-6 and IL-12 and enhanced the production of TGF-β. The

221 effects of CGS were reversed completely in Adora2a^{fl}LysMCre^{+} mice with myeloid-

222 selective A2A R deletion, suggesting that the source of these cytokines is macrophages that are

223 regulated by A2A R signaling.

224 We next examined populations of myeloid cells derived from enzymatically dispersed

225 lungs following BAL to remove most immune cells from alveoli. As shown in Figure 2C,D
CGS administered during OVA/LPS sensitization significantly reduced the rapid (day 3) accumulation of F4/80⁺/CD11c⁺ cells (composed of activated macrophages), and F4/80⁻ CD11c⁺ (composed of DCs). Increases in numbers of these myeloid cells were significantly reduced by A₂A-R activation with CGS administered during sensitization. Among the myeloid cells that accumulated in the lungs in response to OVA sensitization were IL-6⁺ and IL-12⁺ cells. CGS treatment reduced the numbers of lung myeloid cells expressing these cytokines. The effects of CGS were completely eliminated in Adora2a⁺LysMCre⁺ mice lacking myeloid A₂A-Rs.

Effects of CGS on Pulmonary T Cell Polarization.

We next sought to determine if CGS added just during OVA sensitization or just during OVA rechallenge produced any modification of pulmonary T cells that appear in the lung during OVA rechallenge. Numbers of T cell subsets in enzymatically-dispersed lungs (after washout of BAL cells) were measured on day 21 (refer to Figure 1). Intracellular staining for IFN-γ, IL-17A and Foxp3 was used to define Th1, Th17 and Treg cells, respectively. As shown in Figure 3A, OVA sensitization and rechallenge resulted in substantial increases in numbers of Th1, Th17 and Tregs in the lung. Addition of CGS only during OVA/LPS sensitization reduced Th1 and Th17 cell numbers, and significantly increased numbers of Treg cells. Addition of CGS just during OVA rechallenge modestly reduced Th1 cell numbers, but failed to influence numbers of Th17 cells or Treg cells. BAL cytokines paralleled these responses; CGS treatment during sensitization reduced BAL IFN-γ and IL-17 (measured on day 21) whereas CGS added only during rechallenge reduced BAL IFN-γ but not IL-17 (Figure 3B). These findings suggest that A₂A-R activation reduces the polarization of naïve T cells to Th17 cells that occurs during sensitization, but does not influence IL-17 release from effector Th17 cells during OVA rechallenge. To investigate this further we measured the acute effects of
CGS directly on spleen-derived Th1 and Th17 cells \textit{in vitro}. As illustrated in Figure 3C Th1
but not Th17 cells are inhibited by A$_{2A}$R activation.

Effects of CGS on airway responsiveness to methacholine, vascular leak, and
inflammation.

We next sought to determine how exposure to CGS during OVA/LPS sensitization affects
pulmonary hyperresponsiveness to methacholine and inflammatory responses during
subsequent OVA rechallenge. As shown in Figure 4A, exposure to CGS just during
OVA/LPS sensitization nearly completely prevented airway hyperresponsiveness to
methacholine following later rechallenge with aerosolized OVA. In contrast, exposure to
CGS only during rechallenge with OVA produced only a small statistically insignificant
reduction in responsiveness to methacholine. Airway inflammation in response to OVA/LPS
sensitization and rechallenge were also inhibited much more effectively by CGS added only
during sensitization than only during rechallenge. These inflammatory responses include
pulmonary vascular leak (Figure 4B) neutrophil accumulation in the BAL (Figure 2C) and
lung inflammation (Figure 4 D,E).

Blockade of airway hyperresponsiveness by CGS is mediated by A$_{2A}$Rs on myeloid cells.
As shown in Figure 5, the effects of CGS administered during OVA/LPS sensitization to
reduce lung inflammation, vascular leak, and respiratory hyperresponsiveness to
methacholine were absent in mice with myeloid-selective A$_{2A}$R deletion. These findings
suggest that A$_{2A}$Rs on myeloid cells are primarily responsible for inhibition of aeroallergen
sensitization by A$_{2A}$R activation.
Discussion

Anti-inflammatory effects of $A_{2A}$R activation are mediated by suppression of the activation of most immune cells, including NKT cells, T cells, macrophages, dendritic cells and neutrophils (10, 19, 27, 28, 37). $A_{2A}$R activation reduces LPS-induced lung inflammation and injury by activating receptors on myeloid cells (37). Although adenosine $A_{2A}$ agonists can inhibit inflammatory responses in lung as well as other tissues, clinical trials with inhaled $A_{2A}$ agonists such as GW328267X (31) or UK-432097 (ClinicalTrials.gov NCT00430300) to treat asthma have not been successful. Moreover, $A_{2A}$R activation with CGS administered intranasally to sensitized mice was found to reduce allergen-driven cell influx into the airway, but not to affect airway hyperreactivity (5). The current study demonstrates that the $A_{2A}$R agonist CGS activates $A_{2A}$Rs on myeloid cells to inhibit airway hyperresponsiveness much more effectively when given during aeroallergen sensitization than when given during allergen rechallenge. The results indicate that myeloid cells play a central role in controlling airway inflammation and hyperresponsiveness. These findings provide an explanation for the lack of clinical response in established asthmatics to $A_{2A}$R agonist therapy and suggest that therapy with an $A_{2A}$R agonist might be useful to blunt airway disease if administered during the initial exposure of an allergic individual to a new antigen, as might occur as a result of moving to a new habitat or changing workplaces.

Myeloid cells as targets of $A_{2A}$R signaling.

Numerous studies have shown that $A_{2A}$R stimulation acutely inhibits airway inflammation (20, 34, 42, 44). However, it has not been clear which cells are most important for mediating anti-inflammatory pulmonary responses. During allergen sensitization, one effect of adenosine is to influence the polarization of naïve T cells. In models of autoimmunity, $A_{2A}$
receptor activation was shown to inhibit Th1 and Th17 effector cell generation (47). This
could occur because adenosine has a direct effect on T cells or because adenosine indirectly
influences cytokine production by macrophages and other antigen presenting cells to
indirectly influence T cell polarization. Both the accumulation of Th1/Th17 cells in the lungs
and the accumulation of IFN-γ and IL-17A in the BAL in response to antigen sensitization
and rechallenge were inhibited by CGS treatment during sensitization, while BAL TGF-β and
numbers of FoxP3+ Treg cells in the lungs were increased. These findings are consistent with
prior reports in other disease settings demonstrating that adenosine can increase numbers of
Tregs and promote their immunosuppressive activity (36). Changes in T cell polarization in
response to adenosine or CGS have been attributed to modified myeloid production of IL-6,
IFN-γ and TGF-β (46). TGF-β is thought to attenuate allergen-induced airway
hyperresponsiveness by increasing airway Treg cells (6). The data in the current study
indicate that the effects of CGS are mediated by A2ARs on myeloid cells, thus confirming that
the activation-state of myeloid cells plays a critical role in the regulation of effector and Treg
cell development. These findings are consistent with prior reports that suggest a role for
APCs in determining whether the response to allergens will be tolerogenic or inflammatory
(16) and further suggest that other agents that modify allergen presentation and cytokine
production by APCs may be effective in modifying T cell polarization.

**Weak cell-intrinsic effects of A2AR activation on Th17 cells.**

During allergen rechallenge adenosine can act directly on T effector cells to suppress
cytokine release. However, the results of the current study suggest that A2AR signaling is not
very effective at suppressing the activation of T effector cells enough to significantly inhibit
pulmonary inflammation or hyperresponsiveness to methacholine. CGS added during OVA
rechallenge did partially reduced IFN-γ release from Th1 cells, but CGS had no effect on
numbers of Th17 cells or IL-17 in the BAL. We also found that CGS applied to Th1 cells in vitro inhibited the release of IFN-γ. In contrast, Th17 cells in vitro were insensitive to CGS.

The results suggest that once Th17 cells are produced during sensitization, they cannot be directly inhibited by A2AR activation during aeroallergen rechallenge. A human population of Th17 cells that express CD39 also are resistant to the effects of adenosine as a consequence of low expression of A2ARs (30). Moreover, IL-17 may be particularly important for controlling airway hyperresponsiveness (43). The findings also demonstrate that CGS administered at a dose of 100 ng/kg/min does not have any significant direct bronchodilator effects. In this regard CGS differs from epinephrine and selective β2-adrenergic agonists (17).

Influence of environmental factors on allergic responses.

Studies in children indicate that the inner-city environment can promote allergic disease and wheezing. Curiously, early-life exposure to certain allergens together with bacteria has been counter-intuitively associated with significant reductions in airway hyperresponsiveness (32). It has been observed that in high allergen environments, enhancing microbial exposure is more effective for preventing hypersensitivity reactions than allergen abatement (32). One factor produced in response to microbial infection that prevents airway hyperresponsiveness is adenosine. Moreover bacteria, or macrophages that have engulfed bacteria, were found to release adenosine, which influenced cytokine release from mast cells (33). We speculate that adenosine produced by bacteria or by macrophages engulfing bacteria also influences allergen sensitization by effects on APCs.

Since myeloid cells express A2BRs as well as A2ARs, the activation of A2BRs on myeloid cells may also influence allergen sensitization by adenosine. In fact, A2BR blockade has been found to enhance macrophage-mediated bacterial phagocytosis (4). A2BR activation
by adenosine is also used by *Leishmania* parasites within DCs to inhibit their function and evade immune responses (11). Nevertheless, A$_{2B}$R activation should probably be avoided as a strategy to treat asthma due to stimulation by A$_{2B}$R activation of the release of Th2 cytokines and degranulation of mast cells (3, 18, 38).

A$_{2A}$R agonists for immunotherapy?

Subcutaneous or sublingual allergen-specific immunotherapy is sometimes used effectively to treat allergic asthma (22). Immunotherapy induces desensitization and long-term allergen-specific immune tolerance, as well as the suppression of allergic inflammation in affected tissues. Since CGS was found in the current study to enhance immune tolerance, e.g. by increasing FoxP3$^+$ Treg cell polarization, it will be of interest to determine if co-administration of A$_{2A}$R agonists will improve the effectiveness of immunotherapies that appear to be of some use for the treatment of allergic asthma and rhinitis.

**Effects of changing environment in asthmatics.**

One strategy that asthmatics use to relieve symptoms of seasonal asthma or allergic rhinitis is to change habitat in order to avoid exposure of aeroallergens. However, the benefit of such relocation may be short-lived due to high sensitivity of such individuals to novel allergens present in the new environment. Also, certain occupational environments produce a high incidence of allergy (41). The data in the current study suggest that treatment of allergic individuals with A$_{2A}$R agonists during their initial exposure to a new allergen, such as highly allergenic pollen or workplace allergen, might be a useful strategy to produce tolerance.

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Disclosure: JL owns equity in Adenosine Therapeutics, LLC, and Lewis and Clark Pharmaceuticals. These companies manufacture drugs targeting adenosine receptors.
Figure Legends

Figure 1. Scheme for sensitizing mice and for treatment with the adenosine A2A R agonist CGS-21680 (CGS) during allergen sensitization (SEN) or rechallenge (RE). Mice were sensitized with four intranasal doses of ovalbumin OVA/LPS over 7 days and rechallenged with 3 doses of aerosolized OVA on days 14, 15 and 21. To evaluate the in vivo effects of CGS-21680 (CGS) in response to OVA, mice were treated with vehicle or 100 ng/kg/min CGS administered from 3-day Alzet-minipumps implanted subcutaneously 5 hours before day 0 sensitization and with an i.p. bolus injection of 1 μg/kg CGS, 30 min before day 7 sensitization. To evaluate the in vivo effects of CGS in response to OVA rechallenge, mice were treated with CGS by Alzet minipump plus a bolus injection during rechallenge instead of during sensitization. Mice were sacrificed on day 3, 21 or 23 as indicated.

Figure 2. Effects of A2AR activation on pulmonary myeloid responses during OVA/LPS sensitization. C57BL/6J wild type mice (B6 Control) or mice with myeloid-selective A2AR deletion (A2a$^{flox/flox}$LysMCre) were treated with vehicle or CGS only during sensitization (SEN). BAL cells or cells harvested from digested lung after BAL (lung cells) were prepared on day 3 (see Figure 1). (A) Numbers of various cells in BAL as determined after cytospin by morphology and staining with the HENA 3 stain set. (B) BAL cytokine concentrations. (C) Representative FACS plots of lung cells stained for F4/80$^+$ and CD11c. (D) Effects of CGS treatment and myeloid A2AR deletion on numbers of lung myeloid cells. N=6, *P < 0.05 vs OVA/LPS, **P < 0.01, ***P < 0.001 by one way ANOVA and Tukey’s multiple comparison tests.
Figure 3. Effects of CGS treatment on lung and spleen T cells and BAL cytokines. Mice were treated with CGS only during OVA/LPS sensitization (SEN) or only during OVA rechallenge (RE). CD4+ T cells were prepared from lungs on day 21 (see Figure 1) and stained for intracellular cytokines or Foxp3 as indicated. (A) Representative FACS plots and average total numbers of lung IFN-γ+, IL-17A+ and Foxp3+ cells gated on CD4+ pulmonary lymphocytes derived from enzymatically-dispersed lungs after BAL. (B) Effects of GGS added only during OVA sensitization or only during OVA rechallenge on concentrations of IL-17A and IFN-γ in BAL measured by ELISA on day 21. (C) Naïve T cells purified from spleen were differentiated into Th1 or Th17 cells in vitro as described in Methods. The cells were then activated for 24 hours with various amounts of CGS and IFN-γ (Th1) or IL-17A (Th17) in the supernatants were measured by ELISA. CGS did not affect IL-17A production, but inhibited IFN-γ production with an IC50 of 24 nM. *P < 0.05 vs OVA/LPS, **P < 0.01, ***P < 0.001 by one way ANOVA and Tukey’s multiple comparison tests.

Figure 4. Effects of CGS treatment just during OVA/LPS sensitization (SEN), or just during OVA rechallenge (RE) on airway responsiveness to MCh and lung inflammation. Control mice were not exposed to OVA or LPS. Other mice were challenged with OVA and LPS as depicted in Figure 1. Data were collected from C57BL/6J mice (N = 6) on day 23. (A) Dose-dependent changes in Penh in response to methacholine, ***P< 0.001 by repeated measures ANOVA and Bonferroni’s multiple comparison test. (B) Pulmonary vascular leak assessed by accumulation of Evans Blue dye uptake into the lung. (C) Accumulation of various cells into the BAL as determined after cytospin by morphology and staining with the HENA 3 stain set. (D) Representative images of H&E-stained lungs (original magnification, x20. (E) Inflammation scores (see “Methods”) calculated by analysis
of H&E-stained mouse lungs. \*P < 0.05 vs OVA/LPS, \*\*P < 0.01, \*\*\*P < 0.001 by one way ANOVA and Tukey’s multiple comparison tests.

Figure 5. Effect of myeloid-selective deletion of A2ARs on the anti-inflammatory effects of CGS added only during OVA sensitization. Control mice were not treated with OVA or LPS. Adora2a\textsuperscript{fl}-LysMC\textsuperscript{+/−} mice and Adora2a\textsuperscript{fl}-LysMC\textsuperscript{−/−} littermate controls (N = 4) were sensitized with OVA/LPS and evaluated on day 23, after OVA rechallenge (refer to Figure 1). (A) Representative images of H&E-stained lungs (original magnification, x20). (B) Inflammation scores (see “Methods”). (C) Methacholine-induced Penh in mice after allergen rechallenge of A2ARf/fLysMC\textsuperscript{ Cre+} mice (Cre+) and Cre- littermate controls. ** P < 0.01, *** P < 0.001 vs OVA/LPS by one way ANOVA and Tukey’s multiple comparison tests.
References:


exposure levels of lipopolysaccharide determine type 1 versus type 2 experimental asthma. 


Mouse Th1/Th17 Asthma Model

Sensitization (SEN)
Intranasal OVA (100μg) + LPS (15μg)

Day 0 1 2 7
CGS infusion bolus

Rechallenge (RE)
Aerosolized OVA (10mg/ml for 30 min)

14 15 21 23
CGS infusion bolus

Figure 1
Figure 2A
Figure 2 B
Figure 2 C-D
Figure 3
Figure 4
Figure 5