Article Type: Original Article: Experimental Allergy and Immunology

Pollen-derived adenosine is a necessary co-factor for ragweed allergy

Maria Wimmer, PhD[a,b,j]*, Francesca Alessandrini, PhD[b,j]*, Stefanie Gilles, PhD[a,j] , Ulrike Frank, PhD[c,j], Sebastian Oeder, PhD[b,j], Michael Hauser, PhD[d,j], Johannes Ring, MD[e,j], Fatima Ferreira[d], PhD, Dieter Ernst, PhD[c], Jana Barbro Winkler, PhD[f], Philippe Schmitt-Kopplin, PhD[g,h], Caspar Ohnmacht, PhD[b], Heidrun Behrendt, MD[b,j], Carsten Schmidt-Weber, PhD[b], Claudia Traidl-Hoffmann, MD[a,e,i#], Jan Gutermuth, MD[b,e,i#]

[a]Institute of Environmental Medicine, UNIKA-T, Technische Universität München, Germany
[b]Center of Allergy and Environment (ZAUM), Technische Universität and Helmholtz Zentrum München, Member of the German Center for Lung research (DZL) , Germany
[c]Institute of Biochemical Plant Pathology, Helmholtz Zentrum München, Germany
[d]Christian Doppler Laboratory for Allergy Diagnosis and Therapy, Department of Molecular Biology University of Salzburg, Austria
[e]Department of Dermatology and Allergy Biederstein, TU Munich, Germany
[f]Research Unit Environmental Simulation at the Institute of Biochemical Plant Pathology, Helmholtz Zentrum München, Germany
[g]Research Unit Analytical BioGeoChemistry, Helmholtz Zentrum München, Germany
[h]Analytical Food Chemistry, Technische Universität München, Germany
[i]Department of Dermatology, Universitair Ziekenhuis Brussel, Vrije Universiteit Brussel, Belgium
[j]Christine Kühne – Center for Allergy Research and Education, Switzerland

*These authors contributed equally to this work.

[#]These authors contributed equally to this work.

Corresponding author:

PD Dr. Francesca Alessandrini
Center of Allergy and Environment (ZAUM),
Technische Universität and Helmholtz Zentrum München

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.12642

Ingolstaedter Landstr. 1
85764 Neuherberg, Germany
Tel. +49 +89 3187-2524
Fax. +49 +89 3187-2540
email: franci@helmholtz-muenchen.de

Short title: Pollen-derived adenosine as cofactor in ragweed allergy

Supported by

Christine Kühne – Center for Allergy Research and Education (CK-CARE), Davos (J.R., H.B.)

KKF C grant 8761150, Faculty of Medicine, Technische Universität München (J.G.)

Grant 3/09 CK-CARE Individual project of Kühne Foundation (U.F.)

OZR/BOF 2497, Vrije Universiteit Brussel (VUB), (JG)

Author contributions: MK and FA designed, performed and analyzed the in-vivo studies; SG performed and analyzed the in vitro studies; UF, MH, FF, DE, JBW, PS-K were responsible for ragweed controlled growth and analysis; CS-W, CT-H, JG supported, made substantial contribution in the interpretation of the data, in writing the manuscript and, together with SO, JR, CO, HB, revised the manuscript to its final form. All authors read and approved the final manuscript.

Conflicts of interest: The authors declare that they have no conflicts of interest.

ABSTRACT

Background: Ragweed (*Ambrosia artemisiifolia*) is a strong elicitor of allergic airway inflammation with worldwide increasing prevalence. Various components of ragweed pollen are thought to play a role in the development of allergic responses. Aim of the study was to identify critical factors for allergenicity of ragweed pollen in a physiologic model of allergic airway inflammation.

Methods: Aqueous ragweed pollen extract, the low molecular weight fraction or the major allergen Amb a 1 were instilled intranasally on 1 - 11 consecutive days and allergic airway inflammation was evaluated by bronchoalveolar lavage, lung histology, serology, gene-expression in lung tissue and measurement of lung function. Pollen-derived adenosine was removed from the extract enzymatically in order to analyze its role in ragweed-induced allergy. Migration of human neutrophils and eosinophils towards supernatants of ragweed-stimulated bronchial epithelial cells was analyzed.

Results: Instillation of ragweed pollen extract, but not of the major allergen or the low molecular weight fraction, induced specific IgG_1, pulmonary infiltration with inflammatory cells, a Th2-associated cytokine signature in pulmonary tissue and impaired lung function. Adenosine aggravated ragweed-induced allergic lung inflammation. *In vitro*, human neutrophils and eosinophils migrated towards supernatants of bronchial epithelial cells stimulated with ragweed extract only if adenosine was present.

Conclusions: Pollen-derived adenosine is a critical factor in ragweed-pollen induced allergic airway inflammation. Future studies aim at therapeutic strategies to control these allergen-independent pathways.

Key words

Adenosine, adjuvant, allergic inflammation, ragweed, sensitization

Abbreviations

ADO	adenosine
BAL	bronchoalveolar lavage

BALF	bronchoalveolar lavage fluid
i.n.	intranasal
i.p.	intraperitoneal
NHBE cells	normal human bronchial epithelial cells
OVA	chicken ovalbumin
RWE	ragweed pollen extract
<3kDa	low molecular weight fraction of ragweed pollen extract
>3kDa	high molecular weight fraction of ragweed pollen extract

INTRODUCTION

Ragweed (*Ambrosia artemisiifolia*) pollen is a major cause for hay fever and allergic asthma in Northern America and currently spreads as a neo-allergen in Europe, becoming one of the main causes for allergic reactions in late summer or autumn (1). Amb a 1 has been identified as the major ragweed allergen and belongs to the pectate lyase family of proteins (2-4). The status as neo-allergen in European populations and its high allergenicity makes ragweed an ideal allergen to study the course and adjuvant factors of allergic sensitization towards pollen.

The sensitization process is initially driven by an epithelial response to an allergen, which results in a pathogenic Th2-response in atopic eczema, allergic rhinitis, and allergic asthma

(5-7). It is still incompletely understood how these initial steps are elicited and which pollen-intrinsic co-factors play a role in triggering the induction of allergen-induced airway inflammation.

In the context of sensitization by pollen grains, it has been shown that pollen is not only a carrier of allergen, but also contains bioactive pollen-associated lipid mediators (PALMs) (8). These small molecular biogenic cofactors lead to the breaking of immunological tolerance by recruiting inflammatory cells like eosinophils and neutrophils to the site of allergic sensitization (9-11), or generating a Th2-favouring micromilieu in pollen-exposed tissues (12-14). Adenosine, which has been increasingly implicated in the pathophysiology of asthma (15, 16), has been recently found to be contained in pollen grains of different plant species (17), giving rise to speculations of its possible role in pollen-induced allergic reactions. In a murine model it was shown that elevated endogenous adenosine levels in adenosine deaminase deficient mice induce pulmonary inflammation with infiltration of eosinophils, mucus hypersecretion and airway obstruction (18) and that exogenous adenosine challenge in sensitized mice leads to enhanced influx of inflammatory cells into the lung (19).

Aim of this study was to analyze the sensitizing and proinflammatory properties of ragweed pollen extract (RWE) in an *in vivo* model that mimics the physiologic route of pollen exposure to the airways.

We demonstrated that intranasal (i.n.) instillations with RWE lead to a rapid Th2-biased sensitization and inflammatory airway infiltration, initiated by influx of neutrophils and followed by eosinophils and lymphocytes. These subsequent innate and adaptive immune

responses were modulated by adenosine contained naturally in ragweed pollen, which resulted in a significant augmentation of the elicitation phase.

METHODS

Animals

Female, 6-10 weeks old BALB/c mice were obtained from Charles River (Sulzfeld/Germany), housed under specific pathogen free conditions in individually ventilated cages (VentiRack; Biozone, Margate/UK) and fed by standard diet. The study was conducted under federal guidelines for the use and care of laboratory animals and was approved by the Government of the District of Upper Bavaria and the Animal Care and Use Committee of the Helmholtz Center Munich.

Allergen sensitization protocol

Mice were sensitized by bilateral i.n. instillations of RWE pollen extract (10mg/ml; 10µl/nostril) or its fractions once a day for up to 11 days. Control animals received the same amount of PBS. For i.p. sensitization, mice were injected with 1.5 µl RWE absorbed to 2 mg alum (ImjectAlum, Thermo Fischer Scientific, Rockford, IL) in 200 µl PBS.

Analysis of bronchoalveolar lavage, lung histology, and allergen specific serology

Measurement of IgE, bronchoalveolar lavage and lung histology were performed as described previously (20). Mucus hypersecretion and inflammatory cell infiltration were

graded in a blinded fashion on a scale from 0 to 4 (0=none, 1=mild, 2=moderate, 3=marked, 4=severe), reflecting the degree of the pathological alteration (20). For measurement of Amb a 1-specific IgG_1, 96-well plates were coated with Amb a 1 and mouse plasma was added. Subsequently, a biotinylated detection antibody (BD Biosciences, Heidelberg), streptavidin-horseradish peroxidase (Calbiochem, Bad Soden/Germany) and tetramethylbenzidine (Fluka, Neu-Ulm/Germany) were used according to manufacturer instructions.

RNA extraction and real-time PCR

RNA extraction and real-time PCR were performed as described previously (21).

Lung function analysis

Lung function analysis was performed 24h after the last intranasal exposure in intubated, mechanically ventilated animals (n=6-10/group; Buxco® Research Systems, Wilmington, NC, USA) (22).

Neutrophil and eosinophil migration assay

Normal human bronchial epithelial (NHBE) cells were incubated for 24h with indicated stimulants in basal medium. The chemotactic activity of NHBE supernatants was evaluated by measuring neutrophil and eosinophil migration, as described previously (10, 23). The

granulocyte migration index was calculated in relation to the migration towards supernatants of unstimulated NHBE cells (Migration index=number of migrated stimulated cells/number of migrated control cells).

Additional details on pollen cultivation and preparation of extracts and methods utilized in this study are provided in the Supporting Information (Data S1).

Data analysis

Results are shown as boxplots indicating minimum, 25% percentile, median, 75% percentile, and maximum, or as mean ± SD. Statistical significance was determined by Mann-Whitney U test and by two-way ANOVA with post-hoc Bonferroni test for lung function analysis. Results were considered significant as $p \leq 0.05$.

RESULTS

Eleven i.n. instillations induce lung inflammation, IgG_1 secretion and airway hyperresponsiveness

In order to assess the kinetic of sensitization and elicitation of allergic airway inflammation on cellular and serologic level, BALB/c mice were instilled once, three, eight, or 11 times i.n. with RWE. Control mice were either instilled i.n. 11 times with PBS or left untreated (Fig. 1A). In RWE-instilled mice, total BAL cells increased with increasing numbers of instillations. Numbers of macrophages did not change, but the number of neutrophils in BALF started to increase after three instillations, peaked after 8 instillations and decreased again after 11

instillations. Lymphocytes and eosinophils started to rise after 8 instillations, but increased significantly after 11 instillations. Eleven PBS instillations led to a minor increase of total cells, neutrophils and lymphocytes, which did not reach statistical significance (Fig. 1B). In plasma, Amb a 1 specific IgG_1 was initially detectable after 8 i.n. instillations, but showed significantly higher titers after 11 instillations (Fig. 1C). The level of Amb a 1 specific IgE was under detection limit (data not shown). Eleven RWE instillations significantly increased airway resistance and decreased dynamic compliance following increasing methacholine concentrations compared to PBS (Fig. 1D). To assess the kinetic of the serologic allergic response, mice were treated 11 times i.n. with RWE and boosted on days 21-23 (Fig. 1A). Total IgE was slightly, but significantly elevated on day 12 and more pronounced on day 24 after the booster instillations. Likewise, levels of Amb a 1 specific IgG_1 were elevated significantly on day 12, but also reached much higher levels on day 24 (Fig. 1E).

Eleven days of RWE instillation induce a systemic ragweed-specific Th2-response

In order to judge the potential of intranasally administered RWE to induce systemic Th2-responses, intranasal RWE exposure was compared to classical i.p./alum sensitization protocol. Non-i.p.-sensitized (NS) mice were instilled on 11 consecutive days with RWE (NS/RWE) or with PBS (NS/PBS). Classically sensitized mice were i.p. injected with RWE (S) in combination with alum and instilled with PBS (S/PBS), (Fig. 2A).

On day 12, splenocytes of mice of all three groups were restimulated ex vivo with culture media, OVA (protein-control), Amb a 1, or RWE. After six days of restimulation, splenocytes

of i.p.-sensitized mice as well as mice that had received RWE only intranasally, secreted significantly increased levels of IL-5 and IL-13 compared to non-sensitized animals (Fig. 2B). In contrast, levels of IFN-γ showed no significant changes. Medium and protein-controls did not affect cytokine secretion.

Only total ragweed is capable to induce allergic lung inflammation

Because pollen release allergens and a multitude of low molecular weight substances (8), we analyzed if either the major allergen Amb a 1 or the low molecular weight fraction of RWE (<3kDa RWE) are dominant in induction of airway inflammation. Mice were instilled with PBS, Amb a 1, <3kDa RWE, Amb a 1 in combination with <3kDa RWE, or total RWE (Fig. 3A). Total cell number and macrophages were increased significantly after instillation with Amb a 1, Amb a 1 plus <3kDa, and total RWE compared to controls (PBS) (Fig. S2B). Neutrophils were increased significantly in all groups compared to PBS, including the group instilled with the <3kDa. Lymphocyte and eosinophil counts were increased significantly only in animals treated with total RWE when compared to PBS-treated animals (Fig. 3B). Only total RWE, but neither the low molecular weight fraction nor Amb a 1 induced Amb a 1 specific IgG_1 (Fig. 3C). Importantly, instillation of Amb a 1 or <3kDa RWE alone had only a minor effect on lung histopathology, whereas the instillation of Amb a 1 in combination with <3kDa RWE led to mild cell infiltration (score >1, Fig. S2C; Fig. S2D) as well as a minor increase in Amb a 1 specific IgG_1 (Fig. 3C), suggesting an adjuvant effect of low molecular weight substances. A marked to severe inflammatory infiltrate and mucus hyper-secretion were observed only after intranasal instillation with total RWE (Fig. S2C, Fig. S2D).

Only total RWE instillation promotes a proinflammatory micromilieu in pulmonary tissue

To decipher the pulmonary micromilieu of intranasally sensitized mice, quantitative real time PCR (qPCR) of lung tissue was performed after 11 intranasal instillations (Fig. 3D, Fig. S2E). Instillation of total RWE induced a significant increase of mRNA expression of typical Th2-associated cytokines (IL-4, IL-5 and IL-13) as well as IL-10. As expected, RWE instillation did not affect expression of the Th1 cytokine IFN-γ or TNF-α. The markers for alternative activation of macrophages Arg1, as well as mucin genes (Muc 2 and Muc 5ac) were upregulated by total RWE. Furthermore, instillation of total RWE led to a higher expression of IL-22 and GM-CSF, but had no effect on IL-33. Lastly, total RWE increased the expression of CCL24 and, although not significantly, of CCL11, crucial chemokines in eosinophil chemotaxis. Importantly, gene expression of the before mentioned genes remained unaffected by instillation with all investigated fractions of pollen extract including Amb a 1.

Adenosine depletion abrogates RWE-induced local and systemic Th2 responses

Adenosine was identified to be a potent immunoregulatory substance in pollen (17). In order to evaluate the effect of pollen-derived adenosine, adenosine was digested from total RWE by adenosine deaminase treatment (Fig. S1A-C). Mice were i.n. instilled on 11 consecutive days with RWE, with RWE depleted of adenosine (RWE w/o ADO) or with adenosine (ADO) alone (Fig. 4A). Depletion of adenosine from RWE prevented the increase of total cells in BALF significantly when compared to total RWE. This prevention could be

attributed mainly to a significant reduction of lymphocyte and eosinophil infiltration. Adenosine instillation alone did not affect cell numbers in BALF (Fig. 4B). After 11 instillations of RWE w/o ADO, plasma levels of Amb a 1 specific IgG_1 were significantly lower when compared to mice instilled with total RWE (Fig. 4C). In order to evaluate the systemic effects of adenosine in RWE, splenocytes were isolated and restimulated ex vivo with medium, control protein (OVA), Amb a 1, or RWE. In supernatants of Amb a 1- or RWE-restimulated splenocytes from mice instilled with RWE w/o ADO, the production of IL-5, IL-13 (and even IFN-γ) was significantly lower when compared to mice instilled with total RWE (grey shaded bars compared to dark grey bars) (Fig. 4D). No regulation was detected upon mock-stimulation with OVA.

Adenosine-reconstitution reestablishes lung inflammation and hyperresponsiveness

To verify whether adenosine removal abrogates airway inflammation, adenosine was added in the original concentration to the adenosine-depleted RWE. Mice were instilled i.n. either with RWE w/o ADO or with RWE w/o ADO supplemented with adenosine (RWE w/o ADO +ADO, Fig. 5A). Reconstitution of adenosine significantly increased total cell counts in BALF, mainly by increasing the number of eosinophils. No significant effect was observed for other BAL cells (Fig. 5B). Lung function tests showed that RWE w/o ADO failed to induce the RWE-dependent increased airway hyperresponsiveness. Importantly, reconstitution of adenosine in RWE w/o ADO+ADO significantly increased airway resistance compared to RWE w/o ADO, demonstrating an important role of adenosine in pollen-induced airway inflammation (Fig 5C).

Ragweed pollen-derived adenosine augments the elicitation phase of ragweed allergy

To clearly delineate the impact of adenosine during the sensitization and the elicitation phase of allergic airway inflammation, two different approaches were used (Fig. 6A).

A limited number of instillations of total RWE did not lead to a detectable cellular infiltration in BALF or to type 2 associated cytokine production from restimulated splenocytes (Fig. 6B, C left). In contrast, instillation with RWE w/o ADO resulted in enhanced antigen-specific secretion of IL-5 and IL-13 from restimulated splenocytes (Fig. 6C, left), but had no effect on BALF cellular influx (Fig. 6B, left). To address the impact of pollen-derived adenosine uniquely during the elicitation phase, mice were i.p. treated with RWE/alum to guarantee efficient sensitization to RWE (Fig. 2B) and then challenged with RWE or RWE w/o ADO (Fig. 6A, right). A strong influx of neutrophils, eosinophils and lymphocytes into the BALF was observed upon challenge with RWE (Fig. 6B, right). Interestingly, instillation with adenosine-depleted RWE almost completely prevented cellular influx and reduced production of IL-5/IL-13 effector cytokines by splenocytes (Fig. 6B, C right).

Taken together, pollen-derived adenosine was necessary for exacerbation of allergic airway inflammation during the elicitation phase in sensitized animals.

RWE induces secretion of neutrophil and eosinophil chemoattractants by human bronchial epithelial cells

Intranasal RWE induced a significant granulocyte infiltration into the lung in the *in vivo* model (Fig. 1B). To evaluate this effect also in a human *in vitro* setting, we analyzed the

effect of the supernatants of RWE, RWE w/o ADO, or ADO-stimulated normal human bronchial epithelial (NHBE) cells on neutrophil and eosinophil migration. We observed an increased IL-8 secretion induced by RWE (Fig. 7A) and an enhanced neutrophil migration towards the supernatants of RWE-stimulated NHBE cells (Fig. 7B). Stimulation of NHBE cells with RWE w/o ADO significantly reduced neutrophil migration towards NHBE supernatants. This effect was mirrored by reduced levels of IL-8 secretion by NHBE-cells in the absence of ADO. Stimulation of NHBE cells by adenosine alone neither led to neutrophil migration by NHBE supernatants, nor to an induction of IL-8 secretion (Fig. 7A, B). Similarly to neutrophils, eosinophil migration was increased towards supernatants of NHBE cells stimulated with total RWE. This increase was significantly reduced by removal of adenosine form the ragweed extract (Fig. 7C).

DISCUSSION

In this study shows that i.n. exposure with an aqueous ragweed pollen extract rapidly induced a Th2-biased sensitization and allergic airway inflammation. Neutrophils most likely played a crucial role, as they were the first cells to infiltrate the lung upon *in vivo* sensitization. Subsequent eosinophil and lymphocyte accumulation in the lung indicated the development of an allergic adaptive immune response that was strongly dependent on adenosine. Furthermore, human neutrophils and eosinophils migrated towards the supernatants of human bronchial epithelial cells stimulated by total ragweed extract. Currently, there is no *in vivo* model of allergic airway responses available allowing the examination of the pro-allergic properties of ragweed via the physiological mucosal route. Instead, most models of allergic airway inflammation use intraperitoneal chicken

ovalbumin (OVA) in combination with alum as adjuvant. Although there are some asthma and conjunctivitis models which use ragweed for sensitization, all of these models need alum as adjuvant (24, 25). Only few models exist that elicit sensitization against natural allergens in wild type mice, such as house dust mite or Cupressaceae pollen, without using adjuvant substances (26, 27). Our model is characterized by rapid sensitization and induction of allergic airway inflammation upon nasal exposure to ragweed pollen as an environmentally relevant allergen without use of any additional adjuvants.

Human migrant studies revealed that manifestation of atopic diseases in most cases requires more than two years, with repeated periods of pollen exposure (28, 29). However, here ragweed-specific T- and B cell response were detected after 11 days of intranasal instillation of RWE, which corresponds to about half of the duration of a typical birch pollen season, or about one fourth of a ragweed pollen season (30). This observation in allergy-prone BALB/c mice, which serve as a surrogate for susceptible human individuals, provides experimental support for the clinical observation that even adult migrants or local residents, which are exposed to potent (neo-)allergens, can rapidly develop sensitization upon allergen exposure. Therefore, even after short-term exposure during a period of high pollen counts, early testing for newly developed sensitizations should be considered, when clinical symptoms and allergen exposure are indicative for a neo-sensitization.

Analysis of the differential impact of total RWE, Amb a 1 and the low molecular weight fraction revealed that only instillation with total RWE induced a complete allergic airway inflammation. However, the low molecular weight fraction induced isolated proinflammatory influx of neutrophils into the lung, confirming a physiological *in vivo*

relevance of human *in vitro* studies (10, 11). In contrast, cultured human bronchial epithelial cells secreted large amounts of IL-8 and induced recruitment of neutrophils only upon stimulation with total RWE. Additionally, only total RWE, but not Amb a 1 alone nor in combination with the low-molecular weight fraction, was able to induce ragweed-specific IgG_1. Thus, additional components present in total RWE were crucial for the development of allergic lung inflammation.

Besides a typical Th2 expression pattern in lung tissue, an elevated expression of Arg1 suggested alternative activation of macrophages (31). This macrophage population can promote disease progression and therefore enforce a chronic allergic airway inflammation (32). Further, mucins (Muc2 and Muc5ac) were up regulated after i.n. instillation of RWE correlating well with secretory cell hyperplasia in histological lung sections of RWE-treated mice (33).

Adenosine is an important mediator, is known for its role in inflammation, but it also exerts immune regulatory and suppressive effects and acts on a wide range of cells (34). Both, endogenous and exogenous adenosine sources have been implicated in the asthma pathogenesis with increased adenosine levels in BALF that correspond with airway inflammation and tissue damage (35). Furthermore, exogenous adenosine causes potently bronchoconstriction in asthmatic patients but not in healthy subjects (15). In a murine asthma model adenosine challenge significantly enhanced the influx of inflammatory cells into the lung (19). Adenosine has been shown to mediate asthma features through its receptors in experimental models, especially through A2A and A2B receptor signaling (36, 37). Furthermore, an adenosine A3 receptor knockout mouse showed decreased

neutrophils recruitment into the lung (38). Adenosine also may promote type 2 immunity as recent studies suggest, that A2B adenosine receptor signaling attenuates chronic pulmonary inflammation (39) and induces protective antihelminth type 2 immune responses (40). In the present study, we showed an important effect of pollen-derived adenosine for neutrophil and eosinophil migration in a human *in vitro* setting as well as for the reduction of symptoms in a novel allergic airway inflammation model. More precisely, adenosine had an effect as a cofactor in combination with the proteins contained in RWE, as there was no effect of adenosine instillation alone or the adenosine rich <3kDa RWE fraction.

Moreover, in a previous human *in vitro* study, pollen-derived adenosine was shown to drive dendritic cell primed T cell responses towards a regulatory response, and the effect was less pronounced if dendritic cells were derived from pollen-sensitized donors. This suggested a protective role of adenosine during sensitization, whereas it might act aggravating in already sensitized individuals (17). This principle was confirmed by our i*n vivo* study exploring the effect of adenosine in the early sensitization and elicitation phase of ragweed-specific airway inflammation.

Taken together, we propose a physiological *in vivo* model that mimics mucosal exposure of ragweed pollen to the airways. We demonstrated that as early as 11 days after intranasal allergen exposure, clinically relevant sensitization can be induced and allergic airway inflammation elicited. Thus, even short-term exposure to potent allergens sources can induce clinically relevant de-novo sensitizations. As pollen-derived adenosine seems to play an important adjuvant role during sensitization and elicitation of disease, these

findings potentially define adenosine receptors as drug targets for prevention and treatment of IgE-mediated allergies.

Acknowledgments

We thank Alexandra Seisenberger, Johanna Grosch, Benjamin Schnautz, Katja Haslauer, and Brigitte Look for excellent technical assistance. We thank Stefan Haak and Jenny Westphal for fruitful discussions. Ragweed seeds were kindly provided by Dr. Beate Alberternst (Friedberg).

Figure Legends

Figure 1 Intranasal ragweed extract induces allergic airway inflammation, **A**, Experimental setup. **B,** BAL cell analysis. **C,** Amb a 1 specific IgG_1 levels. n=6 mice/group; *p≤0.05; **p≤0.01 vs untreated. **D,** Lung function analysis performed 24h after 11 intranasal exposures. n=10 mice/group *p≤0.05, **p≤0.01 vs PBS at same methacholine concentrations. **E**, Total IgE and Amb a 1 specific IgG_1 levels of extended protocol. n=6 mice/group; *p≤0.05, **p≤0.01 vs PBS. Representative data of two independent experiments.

Figure 2 Intranasal ragweed instillation induces systemic Th2-response. **A,** Experimental setup. Mice were non-sensitized (NS), i.n. treated with PBS or RWE, or i.p.-sensitized with RWE/alum (S) plus i.n. PBS. **B**, Splenocytes were restimulated with medium, OVA [10 µg/ml], Amb a 1 [10 µg/ml] or RWE [1,25 mg/ml]. Supernatants were analyzed for indicated cytokines. Data:

Mean+SD. n=6 mice/group. *p≤0.05, **p≤0.01 vs NS/PBS. #p<0.05 versus OVA-restimulated cells. Representative data of two independent experiments.

Figure 3 Intranasal instillation of RWE induces airway inflammation. **A**, Experimental setup. **B,** BAL cell analysis. **C**, Amb a 1 specific IgG_1 levels. **D**, Gene expression in pulmonary tissue. Pulmonary tissue was analyzed for mRNA-expression of indicated genes. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$method. GAPDH: housekeeping gene Data are displayed as boxplots. n=6 mice/group. *p≤0.05; **p≤0.01 vs PBS. Data are representative of two independent experiments.

Figure 4 Depletion of adenosine decreases Th2 response. **A**, Experimental setup. **B,** BALF analysis. Data: Boxplot-analysis. **C**, Amb a 1 specific IgG_1 levels. **D,** Splenocytes restimulation with medium, OVA, Amb a 1 or RWE. Supernatants were analyzed for indicated cytokines. Data are expressed as mean+SD. n=6 mice/group. *p≤0.05, **p≤0.01 vs PBS; $^{+}$p≤ 0.05, $^{++}$p≤0.01 vs RWE. Representative data of two independent experiments.

Figure 5 Adenosine supplementation reestablished allergic airway inflammation. **A**, Experimental setup. **B,** BAL cell analysis. Data are displayed as boxplots. n=6 mice/group. *p≤0.05, **p≤0.01 vs RWE w/o ADO. **C,** Lung function analysis was performed 24h after the last of 11 intranasal exposures. n=6-10 mice/group. *p≤0.05 vs PBS; $^{+++}$p≤0.001 vs RWE at same methacholine concentrations.

Figure 6 Pollen-derived adenosine exacerbates the elicitation phase. **A,** Experimental setup. **B,** BAL cell analysis. n=6 mice/group. **p≤0.01. Data are displayed as boxplots. n=6 mice/group. **C,**

Splenocytes were restimulated with medium, OVA, Amb a 1 or RWE. Supernatants were analyzed for indicated cytokines. Data are expressed as mean+SD. n=6 mice/group. *p≤0.05.

Figure 7 Neutrophils and eosinophils migrate towards supernatants of RWE-stimulated NHBE cells. **A,** IL-8 production by NHBE-cells (stimulus indicated) **B,** Neutrophil migration and **C,** eosinophil migration towards supernatants of NHBE-cells stimulated with RWE, adenosine-depleted RWE, or adenosine alone. n=4 experiments, *p≤0.05, **p≤0.01 vs PBS; $^{+}$p≤0.05, $^{++}$p≤0.01 vs RWE.

Literature

1. D'Amato G, Cecchi L, Bonini S, Nunes C, Annesi-Maesano I, Behrendt H, et al. Allergenic pollen and pollen allergy in Europe. *Allergy* 2007;**62**(9):976-990.
2. Wopfner N, Gadermaier G, Egger M, Asero R, Ebner C, Jahn-Schmid B, et al. The spectrum of allergens in ragweed and mugwort pollen. *International archives of allergy and immunology* 2005;**138**(4):337-346.
3. Wopfner N, Jahn-Schmid B, Schmidt G, Christ T, Hubinger G, Briza P, et al. The alpha and beta subchain of Amb a 1, the major ragweed-pollen allergen show divergent reactivity at the IgE and T-cell level. *Molecular immunology* 2009;**46**(10):2090-2097.
4. Leonard R, Wopfner N, Pabst M, Stadlmann J, Petersen BO, Duus JO, et al. A new allergen from ragweed (Ambrosia artemisiifolia) with homology to art v 1 from mugwort. *The Journal of biological chemistry* 2010;**285**(35):27192-27200.
5. Paul WE, Zhu J. How are T(H)2-type immune responses initiated and amplified? *Nature reviews. Immunology* 2010;**10**(4):225-235.
6. Barlow JL, Bellosi A, Hardman CS, Drynan LF, Wong SH, Cruickshank JP, et al. Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. *The Journal of allergy and clinical immunology* 2011.
7. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 2010;**464**(7293):1367-1370.
8. Behrendt H, Kasche A, Ebner von Eschenbach C, Risse U, Huss-Marp J, Ring J. Secretion of proinflammatory eicosanoid-like substances precedes allergen release from pollen grains in the initiation of allergic sensitization. *International archives of allergy and immunology* 2001;**124**(1-3):121-125.
9. Miyagawa F, Gutermuth J, Zhang H, Katz SI. The use of mouse models to better understand mechanisms of autoimmunity and tolerance. *Journal of autoimmunity* 2010;**35**(3):192-198.

10. Plotz SG, Traidl-Hoffmann C, Feussner I, Kasche A, Feser A, Ring J, et al. Chemotaxis and activation of human peripheral blood eosinophils induced by pollen-associated lipid mediators. *The Journal of allergy and clinical immunology* 2004;**113**(6):1152-1160.
11. Traidl-Hoffmann C, Kasche A, Jakob T, Huger M, Plötz S, Feussner I, et al. Lipid mediators from pollen act as chemoattractants and activators of polymorphonuclear granulocytes. *The Journal of allergy and clinical immunology* 2002;**109**(5):831-838.
12. Mariani V, Gilles S, Jakob T, Thiel M, Mueller MJ, Ring J, et al. Immunomodulatory mediators from pollen enhance the migratory capacity of dendritic cells and license them for Th2 attraction. *Journal of immunology* 2007;**178**(12):7623-7631.
13. Gilles S, Mariani V, Bryce M, Mueller MJ, Ring J, Behrendt H, et al. Pollen allergens do not come alone: pollen associated lipid mediators (PALMS) shift the human immune systems towards a T(H)2-dominated response. *Allergy, asthma, and clinical immunology : official journal of the Canadian Society of Allergy and Clinical Immunology* 2009;**5**(1):3.
14. Gutermuth J, Bewersdorff M, Traidl-Hoffmann C, Ring J, Mueller MJ, Behrendt H, et al. Immunomodulatory effects of aqueous birch pollen extracts and phytoprostanes on primary immune responses in vivo. *The Journal of allergy and clinical immunology* 2007;**120**(2):293-299.
15. Cushley MJ, Tattersfield AE, Holgate ST. Inhaled adenosine and guanosine on airway resistance in normal and asthmatic subjects. *Br J Clin Pharmacol* 1983;**15**(2):161-165.
16. Alfieri A, Parisi A, Maione F, Grassia G, Morello S, Ialenti A, et al. Hyperresponsiveness to adenosine in sensitized Wistar rats over-expressing A1 receptor. *Eur J Pharmacol* 2012;**695**(1-3):120-125.
17. Gilles S, Fekete A, Zhang X, Beck I, Blume C, Ring J, et al. Pollen metabolome analysis reveals adenosine as a major regulator of dendritic cell-primed T(H) cell responses. *The Journal of allergy and clinical immunology* 2011;**127**(2):454-461 e451-459.
18. Blackburn MR, Volmer JB, Thrasher JL, Zhong H, Crosby JR, Lee JJ, et al. Metabolic consequences of adenosine deaminase deficiency in mice are associated with defects in alveogenesis, pulmonary inflammation, and airway obstruction. *The Journal of experimental medicine* 2000;**192**(2):159-170.
19. Fan M, Jamal Mustafa S. Role of adenosine in airway inflammation in an allergic mouse model of asthma. *Int Immunopharmacol* 2006;**6**(1):36-45.
20. Alessandrini F, Schulz H, Takenaka S, Lentner B, Karg E, Behrendt H, et al. Effects of ultrafine carbon particle inhalation on allergic inflammation of the lung. *The Journal of allergy and clinical immunology* 2006;**117**(4):824-830.
21. Alessandrini F, Weichenmeier I, van Miert E, Takenaka S, Karg E, Blume C, et al. Effects of ultrafine particles-induced oxidative stress on Clara cells in allergic lung inflammation. *Particle and fibre toxicology* 2010;**7**:11.
22. Marzaioli V, Aguilar-Pimentel JA, Weichenmeier I, Luxenhofer G, Wiemann M, Landsiedel R, et al. Surface modifications of silica nanoparticles are crucial for their inert versus proinflammatory and immunomodulatory properties. *International Journal of Nanomedicine* 2014;**9**:2815-2832.
23. Beck I, Jochner S, Gilles S, McIntyre M, Buters JT, Schmidt-Weber C, et al. High environmental ozone levels lead to enhanced allergenicity of birch pollen. *PloS one* 2013;**8**(11):e80147.
24. Dharajiya N, Vaidya SV, Murai H, Cardenas V, Kurosky A, Boldogh I, et al. FcgammaRIIb inhibits allergic lung inflammation in a murine model of allergic asthma. *PloS one* 2010;**5**(2):e9337.
25. Stern ME, Siemasko K, Gao J, Duong A, Beauregard C, Calder V, et al. Role of interferon-gamma in a mouse model of allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 2005;**46**(9):3239-3246.
26. Cates EC, Fattouh R, Wattie J, Inman MD, Goncharova S, Coyle AJ, et al. Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. *Journal of immunology* 2004;**173**(10):6384-6392.

27. Kamijo S, Takai T, Kuhara T, Tokura T, Ushio H, Ota M, et al. Cupressaceae pollen grains modulate dendritic cell response and exhibit IgE-inducing adjuvant activity in vivo. *Journal of immunology* 2009;**183**(10):6087-6094.
28. Lombardi C, Canonica GW, Passalacqua G. The possible influence of the environment on respiratory allergy: a survey on immigrants to Italy. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* 2011;**106**(5):407-411.
29. Gibson PG, Henry RL, Shah S, Powell H, Wang H. Migration to a western country increases asthma symptoms but not eosinophilic airway inflammation. *Pediatric pulmonology* 2003;**36**(3):209-215.
30. Buters JT, Weichenmeier I, Ochs S, Pusch G, Kreyling W, Boere AJ, et al. The allergen Bet v 1 in fractions of ambient air deviates from birch pollen counts. *Allergy* 2010;**65**(7):850-858.
31. Barron L, Smith AM, El Kasmi KC, Qualls JE, Huang X, Cheever A, et al. Role of arginase 1 from myeloid cells in th2-dominated lung inflammation. *PloS one* 2013;**8**(4):e61961.
32. Moreira AP, Cavassani KA, Hullinger R, Rosada RS, Fong DJ, Murray L, et al. Serum amyloid P attenuates M2 macrophage activation and protects against fungal spore-induced allergic airway disease. *The Journal of allergy and clinical immunology* 2010;**126**(4):712-721 e717.
33. Voynow JA. What does mucin have to do with lung disease? *Paediatr Respir Rev* 2002;**3**(2):98-103.
34. Antonioli L, Blandizzi C, Pacher P, Hasko G. Immunity, inflammation and cancer: a leading role for adenosine. *Nat Rev Cancer* 2013;**13**(12):842-857.
35. Driver AG, Kukoly CA, Ali S, Mustafa SJ. Adenosine in bronchoalveolar lavage fluid in asthma. *Am Rev Respir Dis* 1993;**148**(1):91-97.
36. Csoka B, Selmeczy Z, Koscso B, Nemeth ZH, Pacher P, Murray PJ, et al. Adenosine promotes alternative macrophage activation via A2A and A2B receptors. *FASEB J* 2012;**26**(1):376-386.
37. Nadeem A, Ponnoth DS, Ansari HR, Batchelor TP, Dey RD, Ledent C, et al. A2A adenosine receptor deficiency leads to impaired tracheal relaxation via NADPH oxidase pathway in allergic mice. *J Pharmacol Exp Ther* 2009;**330**(1):99-108.
38. Inoue Y, Chen Y, Hirsh MI, Yip L, Junger WG. A3 and P2Y2 receptors control the recruitment of neutrophils to the lungs in a mouse model of sepsis. *Shock* 2008;**30**(2):173-177.
39. Zaynagetdinov R, Ryzhov S, Goldstein AE, Yin H, Novitskiy SV, Goleniewska K, et al. Attenuation of chronic pulmonary inflammation in A2B adenosine receptor knockout mice. *Am J Respir Cell Mol Biol* 2010;**42**(5):564-571.
40. Patel N, Wu W, Mishra PK, Chen F, Millman A, Csoka B, et al. A2B adenosine receptor induces protective antihelminth type 2 immune responses. *Cell Host Microbe* 2014;**15**(3):339-350.

Figure 1

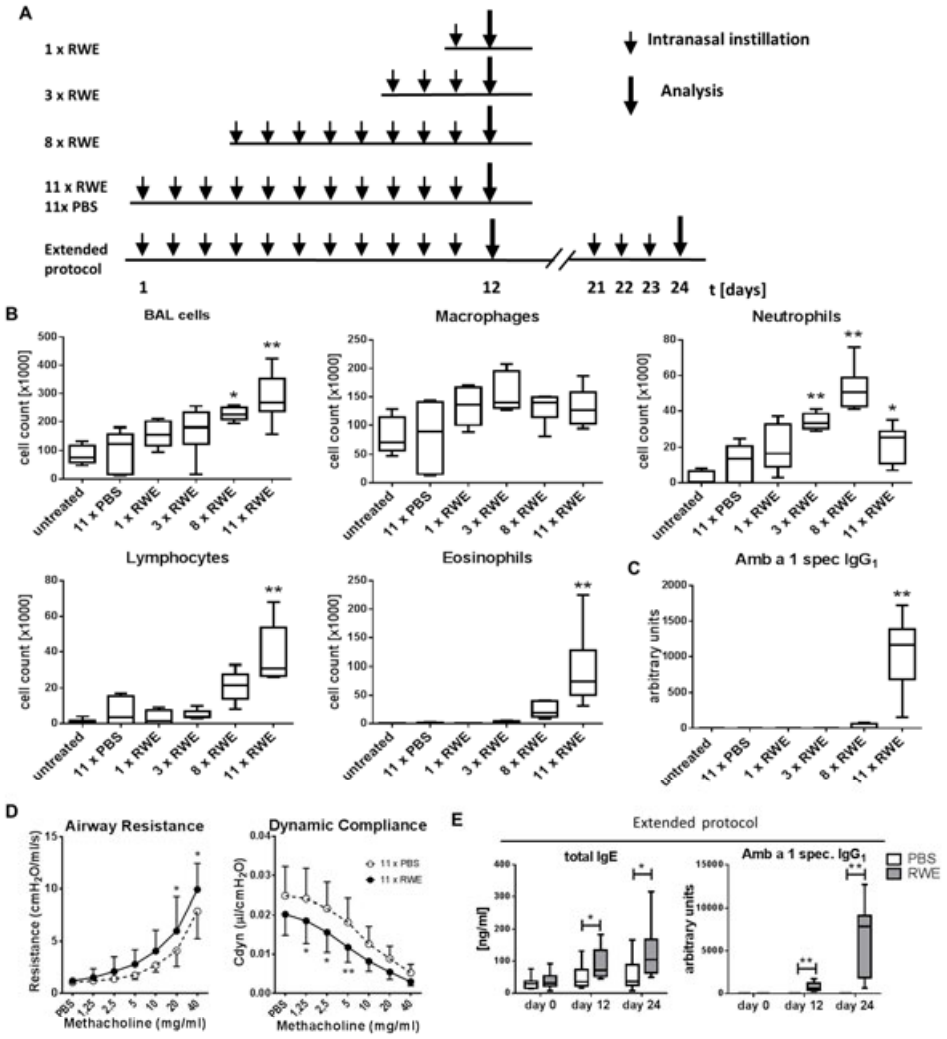

Figure 2

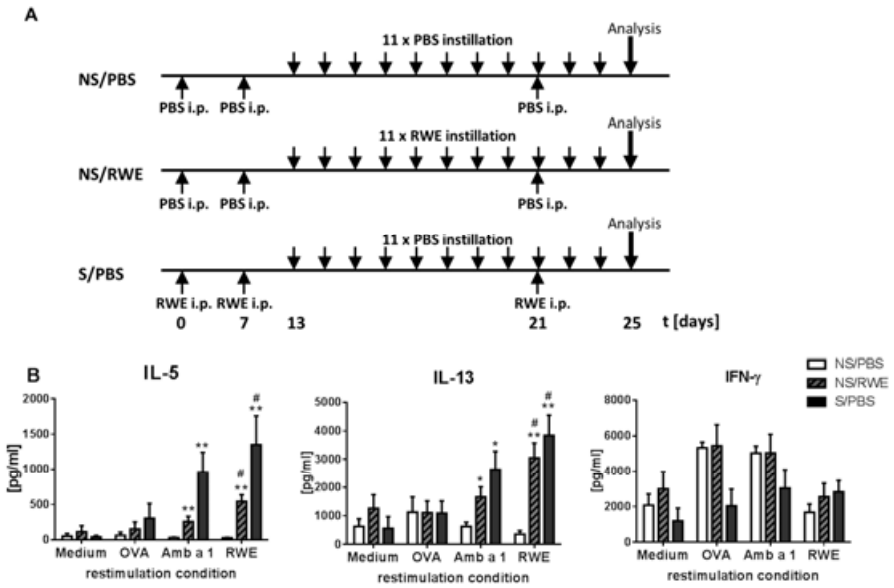

Figure 3

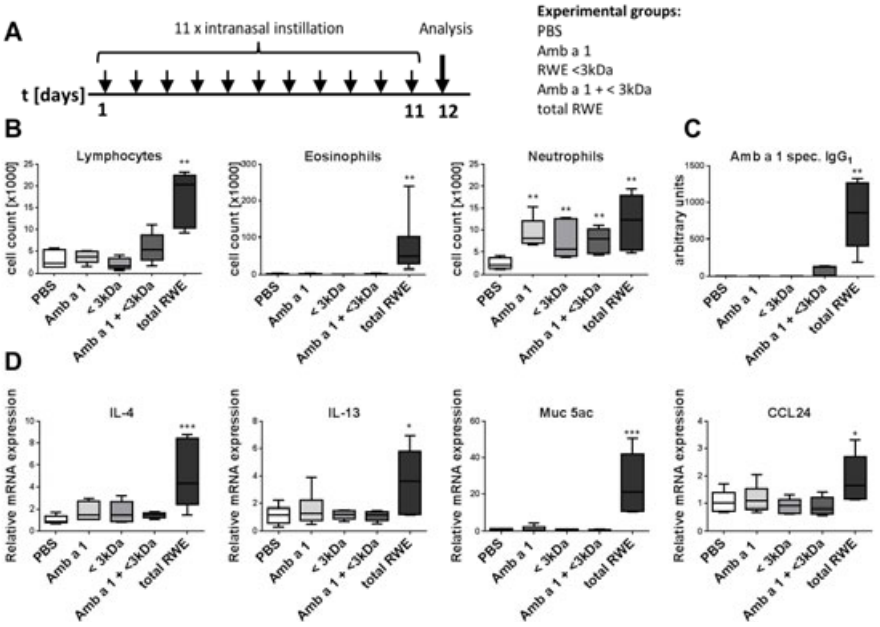

Figure 4

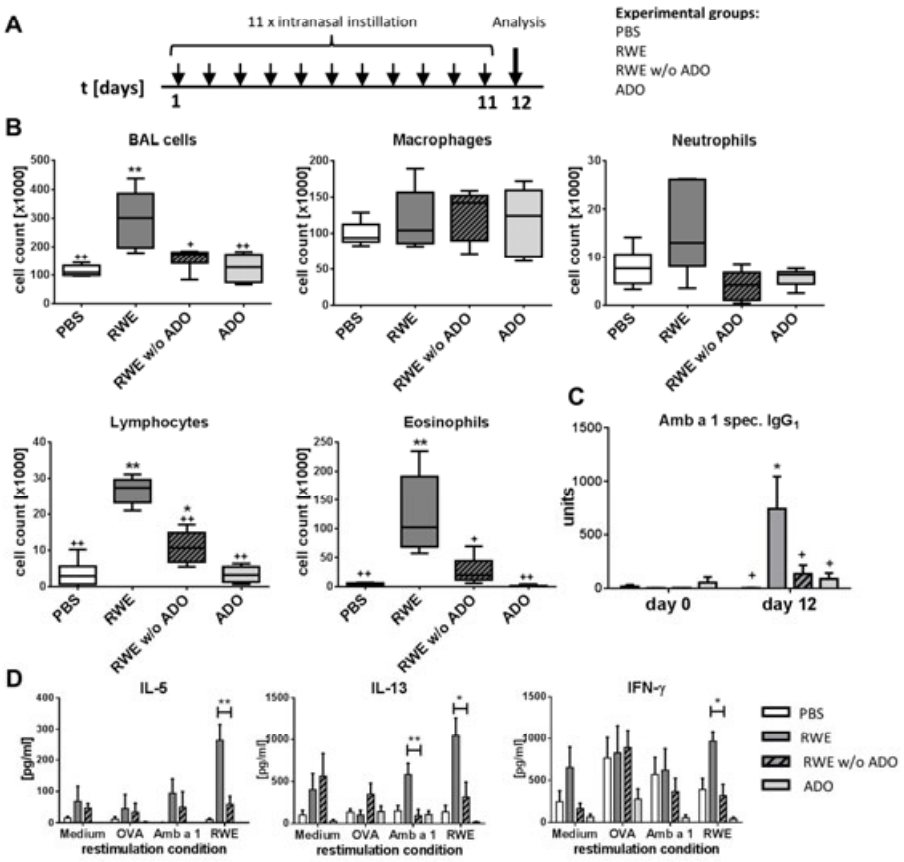

Figure 5

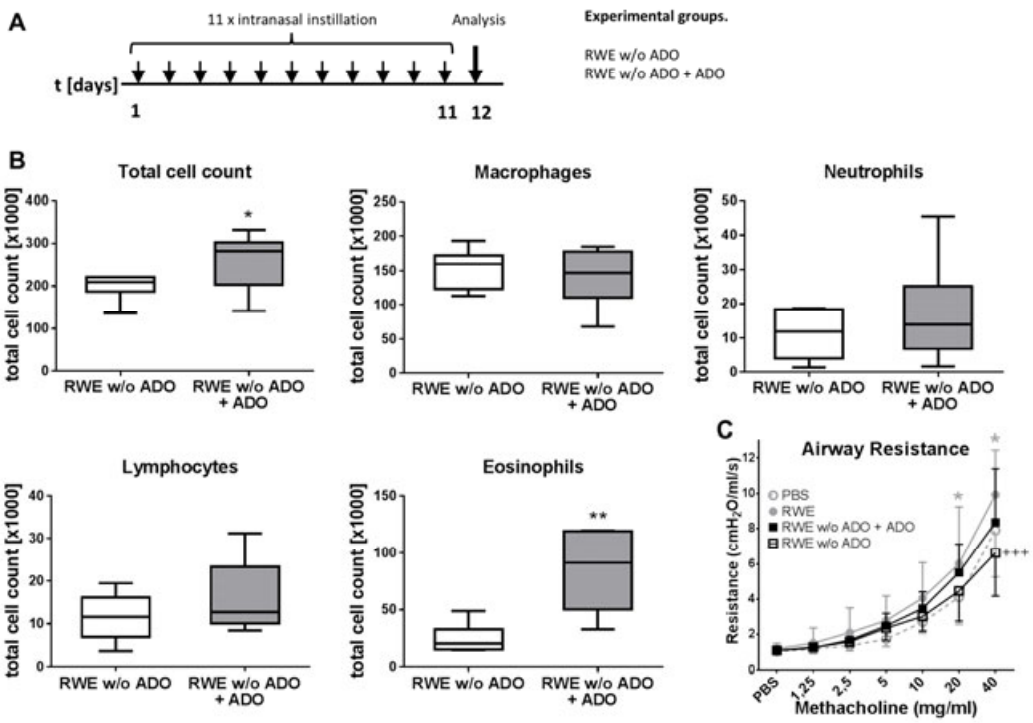

Figure 6

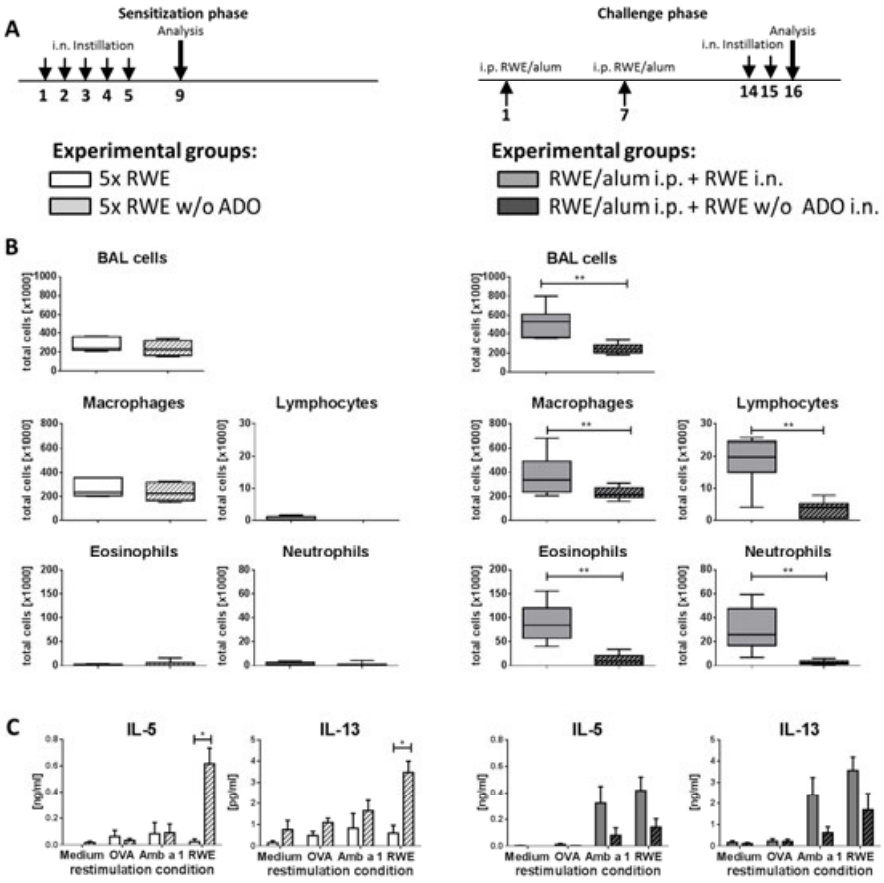

Figure 7