

ORIGINAL PAPER

Pulmonary eosinophilia correlates with allergen deposition to the lower respiratory tract in a mouse model of asthma

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Clinical and Experimental Allergy

Summary

Background Eosinophilic infiltration into the airways is frequently associated with allergic asthma; however, the role of antigen deposition in mediating this phenomenon has not been studied in detail.

Objective Using a murine model of ovalbumin (OVA) allergy, we examined how differential deposition of OVA during antigen challenge affects pulmonary eosinophilia, immune response and airway hyper-reactivity (AHR).

Methods Differential allergen deposition to the upper respiratory tract (URT) alone or combined upper and lower respiratory tract (ULRT) was accomplished by administering OVA intranasally to either anaesthetized or unanaesthetized mice, respectively. BALB/c mice (6–7 weeks old) were sensitized with OVA–alum via the intraperitoneal route, and then challenged intranasally using OVA, with or without anaesthesia. AHR, enumeration of inflammatory cells and quantitative measurement of inflammatory cytokines and chemokines in bronchoalveolar lavage fluid (BALF), lung histopathology and immune responses were subsequently assessed.

Results In sensitized animals challenged via the ULRT route, a profound eosinophilia and goblet cell hyperplasia was observed in lung tissue. Conversely, sensitized mice receiving an identical challenge dose via the URT route alone exhibited only negligible levels of inflammation. Interestingly, AHR and OVA-specific IgG₁ and IgE systemic responses were comparable between the two groups.

Conclusion This study indicates that direct exposure of allergen in the deep lung is highly correlated with airway eosinophilia and lung inflammation, but does not correlate with AHR or immune response.

Keywords airway hyper-reactivity, allergen deposition, allergy, anaesthesia, asthma, eosinophilia, lower respiratory tract, upper respiratory tract

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Introduction

Allergic asthma, which constitutes up to 70% of all asthma, is a chronic inflammatory disease of airways characterized by reversible airflow obstruction, airway inflammation and airway hyper-reactivity (AHR). These characteristics are thought to give rise to the clinical signs associated with asthma, which include recurrent, episodic bouts of coughing, shortness of breath, chest tightness and wheezing [1–3]. The prevalence of asthma has almost doubled in the last 20 years, especially in developed countries, and is the leading cause of hospitalizations among young children [4].

Currently, our understanding of asthma is incomplete due to its multifactorial aetiology and the complexity of the disease. The ovalbumin (OVA) mouse model of allergic asthma has been instrumental in advancing our understanding of the pathogenesis and aetiology of allergic airway diseases [5, 6]. Although it is widely acknowledged that this model has its limitations, it does recapitulate several immunological and pathophysiological hallmarks of the human disease [7] including pulmonary eosinophilia and AHR [2, 8]. Blood and/or tissue eosinophilia is a clinical feature common to a wide variety of diseases including allergic rhinitis (AR), asthma, urticaria, food

allergy, parasitic infections, Churg–Strauss syndrome, eosinophilic pneumonia and idiopathic hyper-eosinophilic syndrome [9]. Eosinophils are thought to play an important pathological role in these diseases by releasing a spectrum of cytotoxic, chemotactic and inflammatory mediators [8–10].

AR is often associated with asthma, lending support to the notion that the upper and lower respiratory tracts (ULRT) are integrated as one airway system [11–13]. Most studies that have examined this issue are based on epidemiological data showing an increased risk of asthma in patients with AR, but did not assess the role of differential antigen deposition in the upper vs. lower respiratory tract (LRT). It is largely unknown whether infiltration of eosinophils in the lungs and airways requires the presence of allergen in the LRT or whether they can infiltrate the lungs even when allergen deposition is restricted to the upper respiratory tract (URT). Studying allergen deposition and its effect on lung inflammation in human asthmatic patients is very difficult; therefore, a mouse model of asthma was used to test the hypothesis that lung inflammation and airway hyper-responsiveness were dependent on allergen deposition to the deep lung, irrespective of systemic immunity to OVA. Antigen deposition was altered by administering OVA intranasally in the presence or absence of anaesthesia during the challenge phase of allergen exposure. Luciferase tracking studies confirmed that intranasally administered protein did not reach the LRT in significant quantities when administered to unanaesthetized animals, but did in anaesthetized mice. The results of this study indicate that lung inflammation, especially eosinophilia and mucus production, is critically dependent on allergen deposition to the LRT.

Materials and methods

Mice

Specific pathogen-free, female, 6–7-week-old BALB/c mice were obtained from the Charles River Laboratories (Wilmington, MA, USA). Animals were age matched according to their date of birth within each experiment and housed in a temperature- and light-controlled animal facility. All animals were acclimated for at least 1 week before starting experimental procedures. For all experiments, five to six animals were used per group. The University of Connecticut's IACUC committee approved all procedures in advance and National Institute of Health guidelines were strictly followed.

Luciferase deposition assay

A luciferase tracking assay was used to determine the distribution of intranasally administered luciferase to

different organs. Briefly, 2 μ L containing 30 μ g of Quantilum[®] recombinant luciferase (Promega Co., Madison, WI, USA) was mixed with 25 μ L of phosphate-buffered saline (PBS) and instilled into each nare of anaesthetized or conscious mice. Mice were anaesthetized by maintaining 2.5% isoflurane (Aerrane[®], Baxter Healthcare Co., Deerfield, IL, USA) in a closed chamber using a VetEquip Inhalation Anesthesia System (Pleasanton, CA, USA). Mice were killed 1–2 min after intranasal instillation of luciferase, upon intraperitoneal (i.p.) administration of a lethal dose (1.3 g/kg) of urethane (Sigma-Aldrich, St Louis, MO, USA). The nasal tissue (URT), lungs and trachea (LRT), stomach and oesophagus (upper gastro-intestinal tract) were immediately removed as described elsewhere [14] and homogenized in 3 mL PBS buffer (2 mL for nasal tissue). The supernatants were decanted following centrifugation at 10 000 g and luciferase activity was measured according to the manufacturer's instructions (Promega). The assay was performed using 96-well white opaque luminometer microtitre plates (Nunc, Rochester, NY, USA), and luminescence was measured using a FLUOSTAR Optima plate reader (BMG Labtech, Durham, NC, USA). The luminescence of each sample was used to calculate the percentage recovery of luciferase in different organs.

Sensitization and challenge

Animal sensitization to OVA (Grade V, Sigma-Aldrich) was performed by administering three-weekly i.p. injections. Briefly, 20 μ g OVA was adsorbed to alum by mixing for 1 h at room temperature in 2.25 mg of aluminium hydroxide (AlumImject; Pierce Biotechnology, Rockford, IL, USA) in a total volume of 200 μ L endotoxin-free PBS (Sigma-Aldrich). One week after the last sensitization, the challenge was initiated by instilling 25 μ g OVA in 25 μ L endotoxin-free PBS into each nostril (Fig. 1). OVA was depleted of LPS using Affinity-Pak[™] Detoxi-Gel[™] endotoxin-removing columns (Pierce Biotechnology) according to the manufacturer's instructions to avoid unintended immunomodulatory responses associated with this contaminant [15].

Whole body plethysmography

AHR to methacholine was measured 1 day after administration of the last challenge dose using a double chamber plethysmograph (Buxco Electronics, Sharon, CT, USA). Specific airway resistance (sRaw) was determined continuously via the non-invasive mechanics software of Buxco Electronics as described previously [16], with some modifications. Briefly, each unanaesthetized mouse was restrained in a two-chamber plethysmograph tube (separating the head from the body) to monitor pressure differences in both chambers. This approach allows a

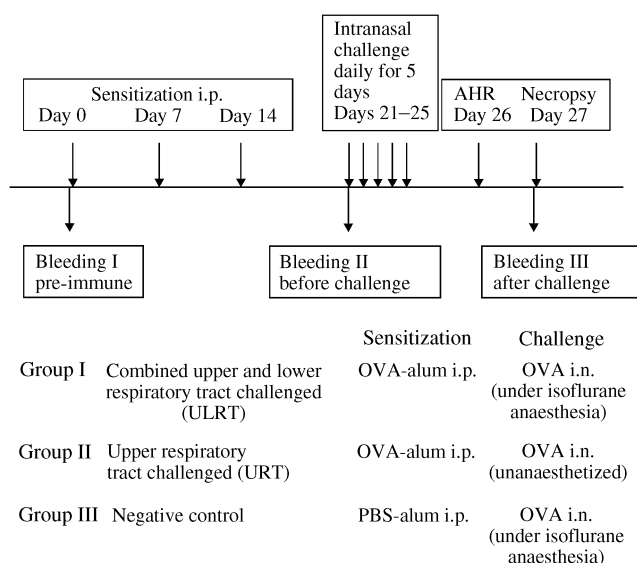


Fig. 1. Experimental design. Sensitization with OVA-alum was done weekly over 3 weeks to isoflurane-anaesthetized female BALB/c mice ($n = 6$) for groups I and II while the negative control group received only PBS-alum. Sensitization was followed by intranasal challenges daily for 5 days with OVA to all groups including the negative control, beginning 1 week after the last sensitization dose. The ULRT and negative control groups (groups I and III) were challenged intranasally while under isoflurane anaesthesia; the URT-challenged animals (group II) received OVA intranasally without anaesthesia. OVA, ovalbumin; PBS, phosphate-buffered saline.

direct estimation of sRaw, which is generally accepted as providing a more accurate measurement of AHR than Penh values [17]. Each mouse was acclimated in the chamber for 16 min, followed by baseline sRaw determination upon administration of aerosolized saline over 2 min to the nose chamber only, followed by 4 min of data recording. This was followed by 2-min exposures to incremental doses of aerosolized methacholine (3, 10, 30 and 100 mg/mL; Sigma-Aldrich), each followed by 4 min of data collection. The sRaw value was calculated for each 10-s interval of the 4-min recording period and averaged over each minute, allowing a final calculation of the sRaw at each methacholine dose that is reported as a fraction of the baseline sRaw. The methacholine dose that resulted in a 250% (PC 250) increase in baseline values for sRaw levels was calculated by log-linear regression for each animal and used for statistical analysis.

Bronchoalveolar lavage

On day 27 (the second day after administration of the final OVA challenge dose), animals were euthanized upon placement in an isoflurane-saturated inhalation chamber. Immediately thereafter, the lungs were lavaged by cannulating the trachea and gently injecting/recovering ($4 \times$) 0.8 mL of a pre-warmed (37°C) lavage fluid (Mg^{2+} - Ca^{2+}

free PBS). The collected bronchoalveolar lavage fluid (BALF) was centrifuged at $300g$ at 4°C for 10 min and the supernatant was stored at -20°C for OVA-specific antibody and cytokine/chemokine measurement. The cell pellet was re-suspended in 0.5 mL of RPMI 1640+10% FBS (Invitrogen Corp., Carlsbad, CA, USA) medium and an aliquot of cells was counted using a hemocytometer. The remaining cells were cytocentrifuged onto microscope slides using a Shandon Cytospin 4 (Thermo Electron, Waltham, MA, USA) and stained with Hema 3[®] Stat Pak (Fisher Scientific, Middletown, VA, USA) for differential counting based on cell morphology and staining patterns; at least 400 cells were counted in a random field of view. The mean of four independent counts of 100 cells was used to calculate the percentage of each cell population.

Lung histopathology

Following bronchoalveolar lavage (BAL), the lungs were gently filled with 1 mL of 10% neutral-buffered formalin (Fisher Scientific), and then immersed in the formalin solution for fixation. The lungs were subsequently embedded in paraffin, sectioned at a thickness of $5\mu\text{m}$ and stained with haematoxylin and eosin (H&E) for light microscopy. A board-certified veterinary pathologist (S. W.), blinded to the experimental design, scored the lung tissues for inflammatory lesions. Inflammatory lesion scores in peribronchiolar and perivascular regions of lung tissue were scored as follows: 5-marked, 4-moderate, 3-medium, 2-mild, 1-minimal and 0-no inflammation. To detect mucus-producing goblet cells in the lung tissues, paraffin-embedded lung sections were subjected to periodic acid-schiff (PAS) staining using the PAS staining system (Sigma-Aldrich) according to the manufacturer's instructions. PAS staining was quantified as described elsewhere [18]; briefly, at least three medium-sized bronchioles were evaluated on each slide for PAS⁺ cells. The results are expressed as the percentage of PAS⁺ cells per bronchiole, which was determined from the number of PAS⁺ cells divided by the total number of epithelial cells in each bronchiole.

Quantitative analysis of chemokine and cytokines in bronchoalveolar lavage fluid

The level of Eotaxin-2 in BALF was determined using a capture ELISA kit (R&D Systems, Minneapolis, MN, USA) as per the instructions provided with the kit. To determine the level of other inflammatory cytokines (IL-1 β , IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17, IFN- γ and TNF- α) in undiluted BALF, a custom-made Bioplex array system (Bio-Rad, Hercules, CA, USA) was used as per the protocols of the company. Samples were measured using a Luminex-200 system (Luminex, Austin, TX, USA), and

cytokine levels were determined by interpolating sample values from standard curves generated from murine recombinant cytokines and analysed using Luminex bead array software (Luminex).

Measurement of ovalbumin-specific antibodies

Mice were bled at day 0 (pre-immune), day 21 (after sensitization) and day 27 (after challenge) using a retro-orbital puncture; serum was collected and stored at -20°C after centrifugation. OVA-specific IgG₁, IgG_{2a} and IgE were measured by ELISA using isotype-specific secondary antibodies (anti-mouse IgG₁, IgG_{2a} and IgE conjugated to HRP, Bethyl Laboratories, Montgomery, TX, USA), both in BALF and in serum samples as described previously [19]. For IgE estimation, serum and BALF samples were pre-incubated for 1 h at room temperature with Protein G (Amersham Biosciences, Uppsala, Sweden) beads to deplete interfering OVA-specific IgG₁ antibodies, before assay. Results were expressed as net OD₄₅₀ at a specific serum or BALF dilution after background subtraction.

Statistics

Results are expressed as means $\pm$ SEMs. Statistically significant differences between groups were determined by Student's unpaired *t*-test (two groups) or one-way ANOVA (three or more groups), followed by the Newman-Keuls post-hoc test, with $P \leq 0.05$ being considered to be statistically significant.

Results

Luciferase tracking assay

Luciferase was administered to anaesthetized or unanaesthetized mice to determine its distribution in the respiratory tract [14]. In anaesthetized mice, most of the luciferase activity (~ 50 – 60%) was deposited in the LRT, in stark contrast to that observed in unanaesthetized animals (Fig. 2; ~ 0.5 – 1.0% ; $P \leq 0.0002$). There was no significant difference in luciferase activity measured in the URT (~ 3.0 – 5.0%) between the two groups. This deposition pattern was identical, regardless of whether an injectable or an inhalation anaesthesia was used (data not shown). This differential protein deposition pattern was subsequently used to examine the effects of differential allergen (OVA) deposition on lung inflammation, AHR and immune responses.

Airway hyper-reactivity

To investigate the role of differential deposition of allergen during challenge on AHR, whole-body plethysmo-

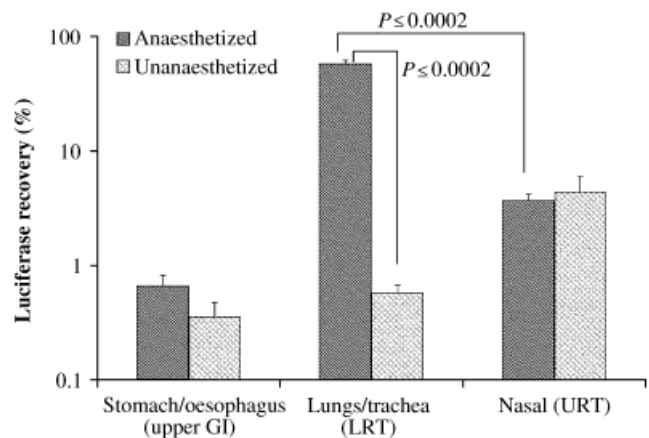


Fig. 2. Luciferase deposition assay. Two groups of female BALB/c mice (6–7 weeks old, $n = 5$) were either conscious or isoflurane-anaesthetized during intranasal administration of luciferase. Immediately after luciferase administration (1–2 min) each mouse was euthanized and selected tissues/organs were harvested and pooled as part of the Upper-GI, LRT or URT, then homogenized in PBS. Luciferase activity was measured in the supernatant after centrifugation of the homogenate. Data is represented as percentage of luciferase recovered (means $\pm$ SEM) for five animals per group. GI, gastro-intestinal tract; LRT, lower respiratory tract; PBS, phosphate-buffered saline; URT, upper respiratory tract.

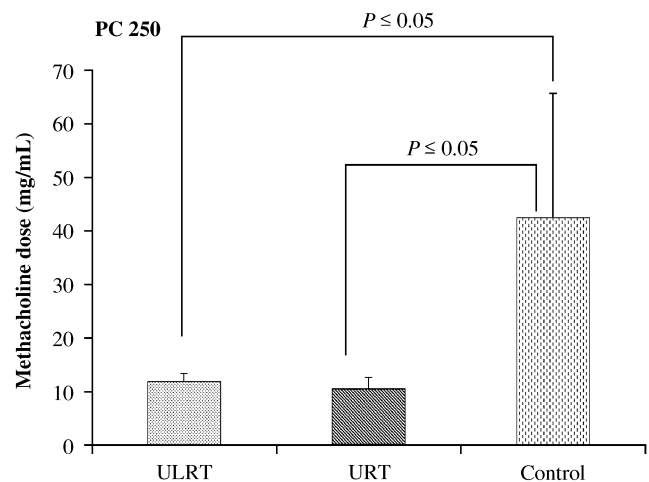


Fig. 3. Airway hyper-reactivity (AHR). One day after the last intranasal challenge, airway response to methacholine was measured by whole-body plethysmography for each animal. The methacholine dose increasing the maximum sRaw over baseline by 250% (PC 250) was calculated for each animal. Both ULRT and URT groups reacted to lower doses of methacholine in comparison with the negative control. sRaw, specific airway resistance; ULRT, upper and lower respiratory tract; URT, upper respiratory tract.

graphy was performed. In anaesthetized animals (designated as ULRT based on antigen deposition patterns), the OVA sensitization and challenge resulted in increased airway hyper-responsiveness, as judged by a lower PC250 methacholine dose, in comparison with control animals (Fig. 3; $P \leq 0.05$). A similar yet unanticipated sensitivity to methacholine was also observed in the

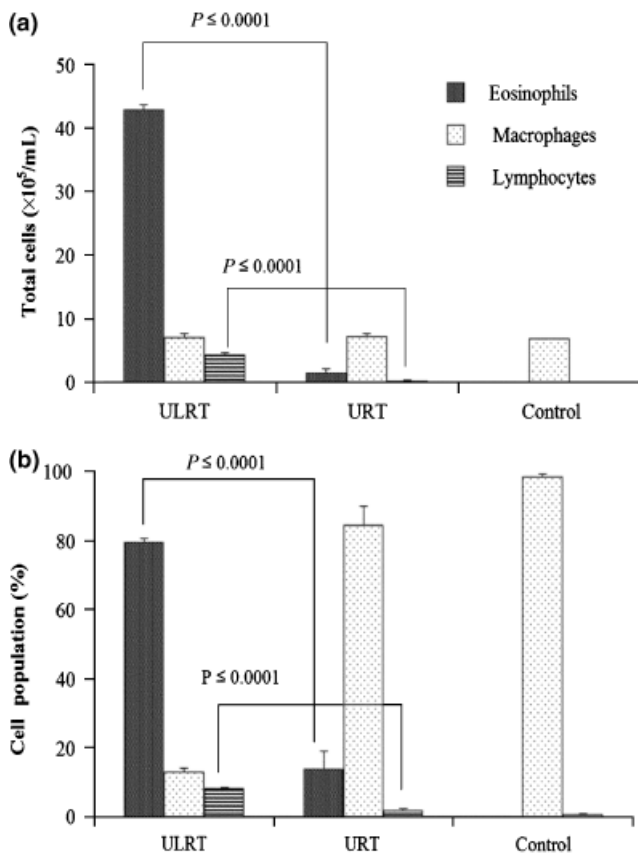


Fig. 4. Differential leucocyte count on BALF cells. Immediately following sacrifice, each animal's lungs were lavaged and harvested cells were cytocentrifuged, stained and enumerated by differential counting. Total number (a) and percentage (b) of lymphocytes and eosinophils in ULRT group was significantly higher than in URT group. ULRT, upper and lower respiratory tract; URT, upper respiratory tract.

URT-only group when compared with control animals ($P \leq 0.05$).

Bronchoalveolar lavage cells analysis

Allergen deposition to the LRT during challenge resulted in a robust inflammatory response, with significantly increased numbers of eosinophils ($P \leq 0.0001$) and lymphocytes ($P \leq 0.0002$) as shown in Figs 4a and b in the ULRT-challenged group in comparison with the URT-only challenged group. The PBS-sensitized negative control group had negligible numbers of inflammatory cells in BALF, but did have the anticipated population of alveolar macrophages, which were comparable in number to those of the experimental groups. The significant decrease in macrophage percentages observed in the ULRT group reflects the lower proportion of these cells in the presence of large numbers of infiltrating eosinophils, not a decrease in their raw numbers. Statistical analysis was not performed on neutrophils due to their negligible numbers, nor on the negative control group

due to the virtual absence of eosinophils and lymphocytes in this group.

Lung histopathology

Significant pulmonary inflammation was observed in the ULRT allergen deposition groups, characterized by an infiltration of eosinophils and lymphocytes in perivascular and peribronchiolar spaces (Figs 5a and b). Interestingly, we did not observe any inflammation in the LRT of URT-only-challenged mice as shown in Fig. 5c, which was comparable to the negative control group (Fig. 5d). These observations were further substantiated by comparing the histopathologic lesion scores, which showed a large and statistically significant difference between the ULRT and the URT-only group (Fig. 5f; $P \leq 0.001$). In contrast, there were no differences between the control and the URT-only challenged mice. In a separate experiment in which OVA-sensitized mice were challenged with PBS while under anaesthesia, no inflammation was observed, ruling out a direct effect of the anaesthetizing agent (Fig. 5e).

PAS staining also indicated a similar trend with regard to goblet cell hyperplasia. Examination of PAS⁺ cells in the bronchial epithelium of the ULRT-challenged group revealed a significant increase ($P \leq 0.0002$) in mucus production compared with the URT-only challenged group (Figs 6a–d).

Cytokine and chemokine measurement

Eotaxin-2, an important eosinophil-specific chemokine, was also significantly higher in BALF collected from the ULRT group than in the URT-only group (Fig. 7c; $P \leq 0.0047$). Bioplex array-based cytokine measurements showed significantly higher responses for IL-4 ($P \leq 0.001$), IL-5 ($P \leq 0.001$) and IFN- γ ($P \leq 0.03$) (Figs 7a–d) in the ULRT group than in the URT-only group. In negative control animals, cytokine responses were either equivalent to the URT-only group (IL-4 and IL-5) or below the limits of detection (eotaxin-2 and IFN- γ). Other assayed cytokines (IL-1 β , IL-6, IL-10, IL-12p70, IL-13, IL-17 and TNF- α) were either absent or were below the limits of detection in all groups (data not shown).

Antibody response to ovalbumin in serum samples and bronchoalveolar lavage fluid

As anticipated, OVA sensitization resulted in a considerable OVA-specific serum IgG₁ response, which increased three-to-four fold after an intranasal challenge (Fig. 8a). Similar responses were observed for OVA-specific IgE (Fig. 8c). Interestingly, we did not find any difference in OVA-specific serum IgG₁ or IgE between the ULRT and the URT-only challenged groups. However, local OVA-specific IgG₁ and IgE in BALF were significantly different ($P \leq 0.05$)

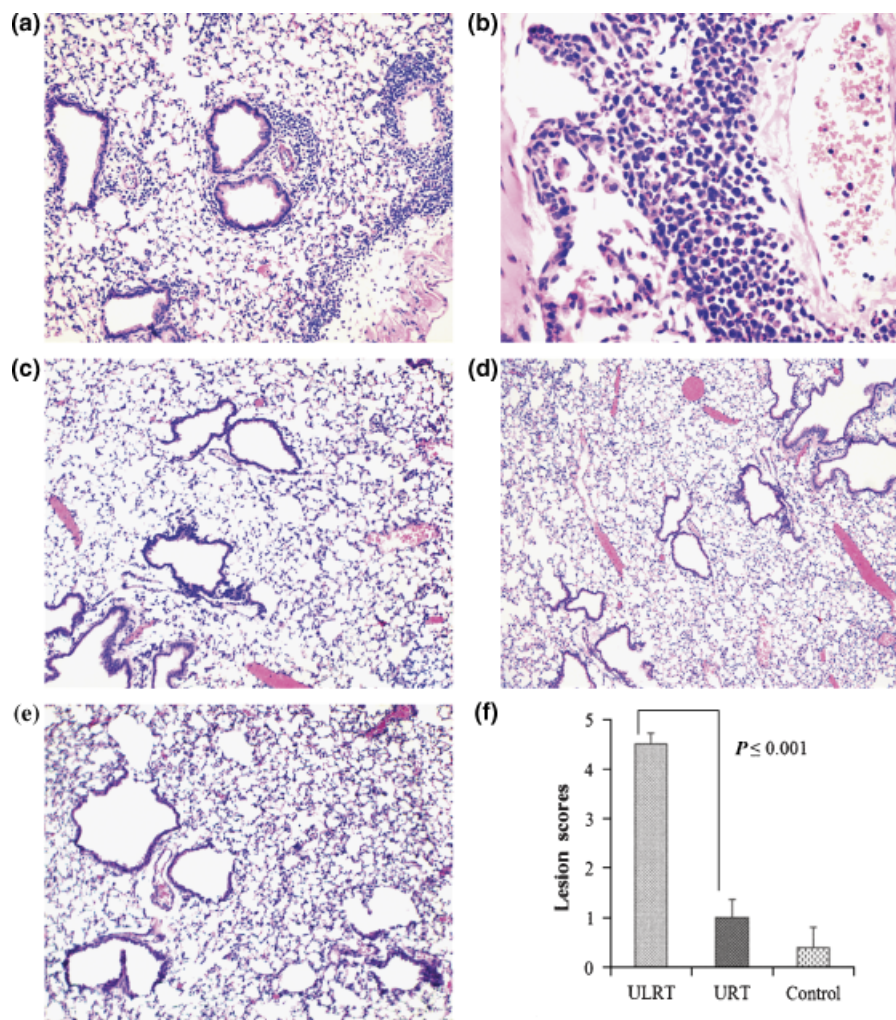


Fig. 5. Lung histopathology. Formalin fixed, H&E-stained lung tissues were evaluated and scored for inflammatory lesions. Marked infiltration of inflammatory cells in peribronchiolar space (a) and perivascular space (b) were observed in the ULRT group. There were no significant inflammatory lesions present in the representative lung sections belonging to the groups of URT (c), PBS–alum-sensitized/OVA-challenged control (d) or OVA–alum-sensitized/PBS challenged control (e). Lesions scores (f) were graded on the basis of severity of inflammation as described in ‘Materials and methods’. The ULRT group had significantly higher lesion scores than the URT-only group. H&E, haematoxylin and eosin; OVA, ovalbumin; PBS, phosphate-buffered saline; ULRT, upper and lower respiratory tract; URT, upper respiratory tract.

between these two groups (Figs 8b and d). We did not find any measurable IgG₁ or IgE in PBS–alum-sensitized control mice, even though they received the five daily OVA doses intranasally. No OVA-specific IgG_{2a} was observed in any group (data not shown).

Discussion

Previous studies using the OVA mouse model of allergic asthma have examined the role of antigen deposition in the isolated URT and subsequent lung inflammation and systemic humoral immune responses [20–22]. However, the linkage between allergen deposition in the upper and/or LRT, to pulmonary inflammation, AHR and immune response has not been well characterized. The current study addresses this question by examining several para-

meters of lung inflammation, cytokine/chemokine secretion, airway hyper-responsiveness and immune response in allergen-sensitized mice challenged in the presence or absence of anaesthesia, which was used as a method for accomplishing differential allergen deposition.

Previous studies investigating respiratory allergen deposition following an intranasal challenge have used either radioactive colloidal particles [14] or coloured dye [20, 22] as tracking markers. In the current study, the enzyme luciferase, which is similar in molecular weight to OVA, was used to study deposition of intranasally delivered antigen in the respiratory tract. Our results indicate that intranasally delivered protein was effectively aspirated to the LRT in anaesthetized mice, in contrast to negligible amounts measured in unanaesthetized mice. Although the current study was conducted in mice, it is in

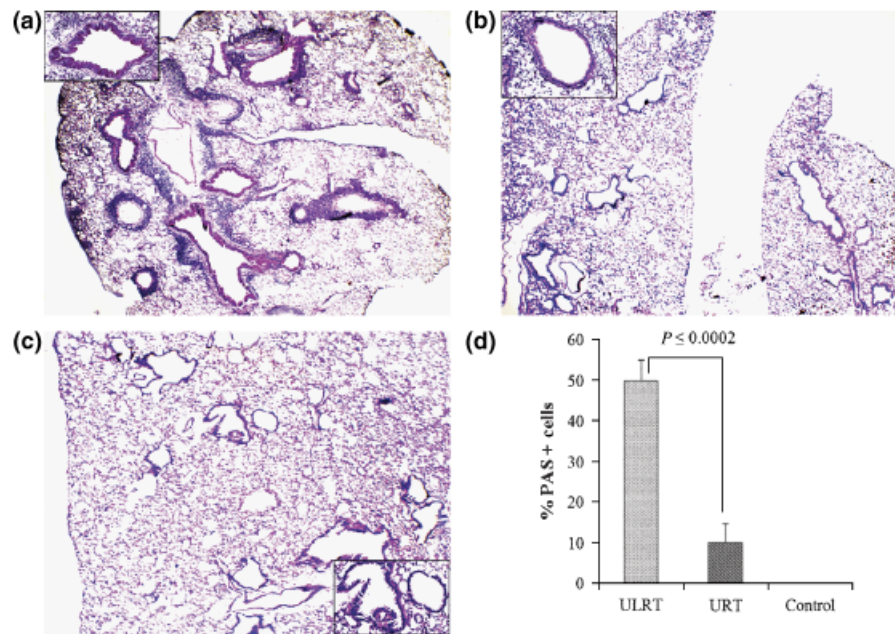


Fig. 6. Lung PAS Staining. Formalin-fixed lung tissues were stained with the PAS staining kit to detect goblet cells in the bronchial epithelium. Profound goblet cells hyperplasia was observed in the ULRT-challenged group (a) in comparison to the URT-only challenged group (b) or negative control (c). Quantification of goblet cells, which was done as per detail described in 'Materials and methods', shows significantly higher %PAS⁺ cells in the ULRT-challenged group than in the URT-only group (d). PAS, periodic acid-schiff; ULRT, upper and lower respiratory tract; URT, upper respiratory tract.

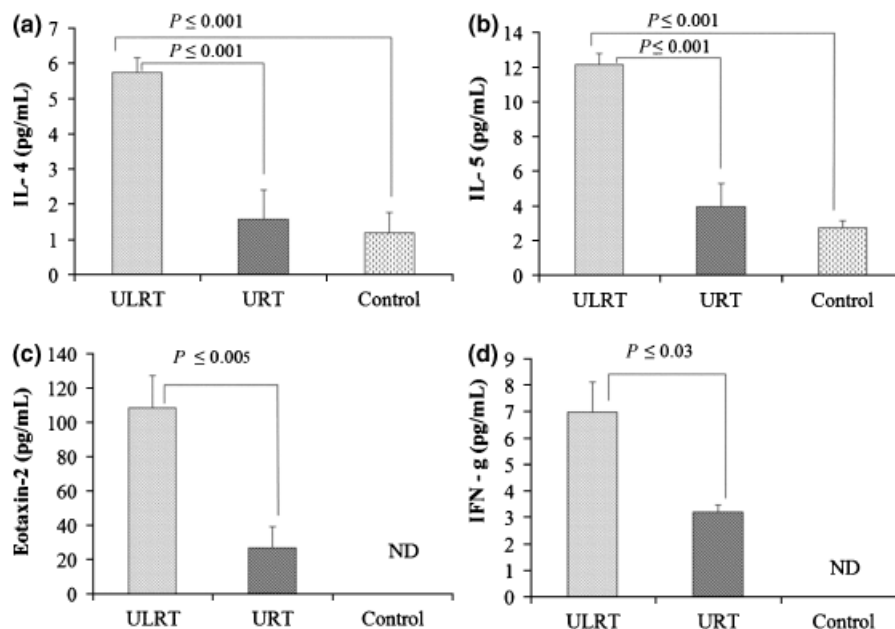


Fig. 7. Quantification of cytokines and chemokine. A capture ELISA kit was used to determine the concentration of eotaxin-2 in the undiluted BALF which clearly shows (a) significantly more eotaxin-2 in the ULRT group than the URT-only group. A Bioplex cytokine array system was used to quantify IL-4 (b), IL-5 (c) and IFN-γ (d) in undiluted BALF which were significantly higher in the ULRT group than the URT-only group. IL-4 and IL-5 levels in the URT alone group vs. the negative control group were not statistically different. Eotaxin-2 and IFN-γ levels in the negative control animals could not be determined because their level was below the detection limit. BALF, bronchoalveolar lavage fluid; ULRT, upper and lower respiratory tract; URT, upper respiratory tract.

keeping with reports in humans indicating that nasal aspiration occurs during sleep and depressed consciousness [23–25]. Thus, allergen exposure while sleeping may be of considerable importance with regard to antigen

deposition in the lung. Moreover, these results are also in general agreement with previous reports of antigen deposition studies using the mouse model [14, 20, 22]. Thus, intranasal challenge of anaesthetized mice represents an

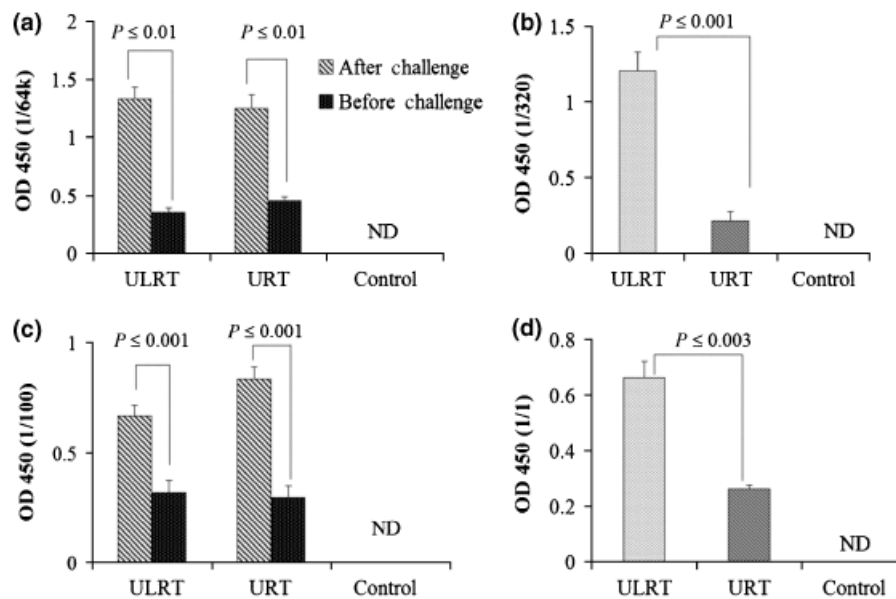


Fig. 8. OVA-specific immune response. (a) OVA-specific IgG₁ was measured by ELISA in serum samples collected before and after challenge. Data is presented as OD₄₅₀ values at 1/64 k dilution of serum. (b) OVA-specific IgG₁ in BALF at 1/320 dilution. (c) OVA-specific IgE immune responses in serum samples collected before and after challenge at 1/100 dilution; and (d) OVA-specific IgE in BALF without dilution. BALF, bronchoalveolar lavage fluid; OVA, ovalbumin.

alternate method to reproducibly deliver antigen to the LRT [26, 27]. Moreover, this differential antigen administration pattern provided a convenient model to examine the potential impact of URT vs. ULRTS antigen deposition on lung histopathology, AHR, eosinophilia and immune response.

The most striking observation of the current study was the absence of pulmonary inflammation in the airways and lung tissues of URT-only challenged animals, as assessed by BALF cell counts, cytokine/chemokine secretion patterns and lung histopathology. In contrast, the ULRT-challenged group had significant eosinophilia (~80%) in BALF cell counts with a concomitant but lower percentage of lymphocytes. Inflammatory cell infiltration in airways, as represented by BALF cells, paralleled lung histopathology, further suggesting that pulmonary inflammation occurs only if antigen is delivered to the LRT of sensitized mice during challenge. PAS staining also showed robust goblet cell hyperplasia among epithelial cells of bronchioles in the ULRT-challenged group while URT-only challenged and control groups showed only minimal goblet cell staining. To rule out the direct effect of the inhalant gas (Isoflurane) on inflammation, separate experiments were performed using Avertin, an injectable anaesthetic agent, during intranasal OVA challenge. These experiments yielded results comparable to those observed with isoflurane (data not shown).

Inflammation in the ULRT group was also accompanied by increased levels of both T-helper (Th)-2 and Th-1 cytokines IL-4, IL-5 and IFN- γ in BALF, and the level of eotaxin-2 was also significantly higher in the BALF of this group. Eotaxin-2 is known to be an important eosinophil-specific chemokine that is associated with the infiltration

of eosinophils into the airways [28]. IL-4 and IL-5 are well-known cytokines produced by Th-2 cells that play critical roles in allergic asthma pathophysiology by class switching B cells to produce IgE (IL-4) and eosinophil development and activation (IL-5) [1]. Interestingly, IFN- γ , which is produced by Th-1 and other cells, was also elevated, in keeping with previous reports of allergic inflammation in the lung and the exacerbation of asthma [2, 29, 30]. The URT-only challenged group, which had insignificant numbers of inflammatory cells in lung tissue and BALF, also had much lower levels of the aforementioned cytokines and chemokine in BALF. Taken together, these data support the notion that inflammation in the ULRT group is due to increased level of cytokines/chemokines in the lung by leukocytes and possibly other cell populations in response to allergen deposition in the LRT.

The OVA model of allergic asthma is known to invoke a vigorous, Th-2-biased immune response due to repeated i.p. injections of allergen complexed to alum [6, 7]. The current study confirmed this observation as evidenced by robust systemic OVA-specific IgG₁ and IgE responses before challenge, and the absence of OVA-specific IgG_{2a}. After intranasal challenge, the systemic antigen-specific immune response was boosted by approximately three to four-fold in both the ULRT and the URT groups of mice, indicating that antigen deposition to the URT-only vs. ULRT plays little or no role in boosting systemic responses. A moderate local booster effect was observed in ULRT-challenged animals as evidenced by increased levels of OVA-specific IgG₁ and IgE levels in BALF, indicating that LRT allergen deposition did augment local

immune responses. This booster effect could be in part due to the leakage of serum antibodies as a consequence of pulmonary inflammation and oedema.

The current study provides evidence that suggests allergen deposition to the LRT is necessary for pulmonary inflammation to occur in allergen-sensitized mice. A study involving isolated URT intranasal challenge reported [20] inflammation in the LRT and concluded that eosinophilic infiltration occurred in the lungs, despite a lack of direct allergen deposition in the lower airways, in contrast to our findings. However, that study did not include a ULRT-challenged group, which, in the current study, demonstrated significant inflammation in comparison with the URT-only challenged group. Another report investigating isolated URT challenge [21] in the mouse model also found a lack of inflammation in the LRT, in agreement with the current study. Similarly, this study did not perform a comparison involving a ULRT challenge to show potential differences in pulmonary inflammation. Moreover, that study was more focused on changes in the haematopoietic system in response to nose-only challenge than to studying the effects of allergen deposition on pulmonary inflammation.

Airway hyper-responsiveness to a variety of allergens and non-specific stimuli is known to be a hallmark of asthma. To assess potential differences in AHR in the mouse model, whole-body plethysmography was performed to measure sRaw upon exposure to incremental doses of methacholine. Surprisingly, both the ULRT and the URT-only challenged groups showed comparable levels of AHR, suggesting that AHR may be independent of pulmonary inflammation/eosinophilia. It is well known that eosinophils play an important role in asthma pathophysiology, especially in AHR [8, 9], but our data suggest otherwise in that the URT-only challenged group had equivalent levels of AHR, but without pulmonary eosinophilia. This apparent lack of association between AHR and lung eosinophilia has also been reported by others [31, 32]. However, AHR measurement by non-invasive whole-body plethysmography in the current study does not differentiate between URT and LRT responsiveness to methacholine. Therefore, it is acknowledged that the AHR data in the URT-only challenged mice in the current study may reflect methacholine-induced nasal obstruction, whereas the data from the ULRT-challenged mice are indicative of nasal and/or lung responsiveness to methacholine. It has been shown in other studies of AR that increased response to methacholine in the nose reflects nasal rather than lung inflammation [21, 33, 34]. Alternatively, it is also possible that as yet unknown URT processes are necessary for the development of LRT hyper-responsiveness as suggested by a classic study [35] showing increased lower airway responsiveness associated with sinusitis in a complement factor-induced inflammation model. This responsiveness was prevented

when the link between URT and LRT was blocked by intubation of the trachea. Future studies are needed to resolve these possibilities.

In conclusion, the current study indicates that pulmonary inflammation appears to be highly correlated with allergen deposition to the LRT during the challenge phase in sensitized mice. Moreover, allergen deposition to the LRT does not influence AHR and systemic immune response. The current study also suggests that allergen deposition to the URT alone does not cause lung inflammation. Taken together, the current study indicates that the upper and lower respiratory tract may not be as tightly integrated as others have suggested, at least as far as allergen deposition and lung inflammation are concerned.

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