

Interleukin-13-dependent bronchial hyper-responsiveness following isolated upper-airway allergen challenge in a murine model of allergic rhinitis and asthma

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Summary

Background We have previously shown that isolated allergic sensitization and challenge of the upper airway results in lower-airway inflammation, which supports the concept of the united airways.

Objective This study investigates the hypothesis that isolated upper-airway allergic sensitization is sufficient to induce bronchial hyper-responsiveness (BHR), characteristic of asthma, and that IL-13 is an essential mediator in both the upper and lower airways.

Methods BALB/c mice were sensitized and challenged by intranasal instillation of allergen ovalbumin (OVA) using our standard protocol. BHR to methacholine was determined and inflammation in nares and lung was assessed.

Results Isolated intranasal application of allergen in awake animals resulted in almost exclusive deposition in the upper airways while in anaesthetized mice there was almost equal distribution in the upper and lower airways. We have demonstrated significant BHR to methacholine challenge in animals receiving OVA only in the upper airway. Also noted was concomitant increase in eosinophilic infiltrates in lung and nares as well as increased granulocytes and IL-13 levels in bronchoalveolar lavage (BAL) fluid. Using a polyclonal anti-IL-13 antibody we have shown inhibition of airways inflammation, both in nares and in lung with significant reduction of granulocytes in BAL from anti-IL-13 treated mice ($P < 0.0001$). Anti-IL-13 treatment also abrogates allergen-induced BHR ($P < 0.01$).

Conclusion These data suggest that isolated upper-airway allergen deposition initiates allergic responses along the entire airway. IL-13 mediates both airway inflammation and BHR and may play a role in the communication between the upper and lower airways.

Keywords allergic rhinitis, animal models, asthma, methacholine challenge, united airways

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Introduction

Allergic sensitization of the airways is associated with the development of allergic rhinitis (AR) and asthma in humans and in animal models [1–10]. AR, a disease of the upper airway, is an independent risk factor for asthma in epidemiologic studies [1, 5, 11–14]. The development of allergic airways and interaction between the upper and lower airway in the provocation of asthma is an area of considerable interest [2–17]. We have previously studied the effects of isolated upper-airway allergen sensitization and challenge on the development of allergic inflammation in both the upper and lower airways [4]. Using a murine model, we have shown that nasal allergen deposition results in synthesis of allergen-

specific IgE as well as eosinophilic inflammatory changes in both nares and lungs of challenged animals. These data suggest a direct association between allergic signals in the upper airway and inflammatory events in the lower airway.

Allergic inflammation results from allergen-induced activation of T-helper type 2 (Th2) cells, release of cytokines such as IL-4 and IL-13 and their effects on smooth muscle cells, epithelia as well as B cells and other inflammatory cells [16, 18]. Several lines of evidence support a central role for IL-13 in the development of allergic inflammation and asthma [19]. IL-13 has been shown to have a direct effect on bronchial epithelial and smooth muscle cells [20–23]. Introduction of exogenous IL-13 into murine airways results in lymphocytic and eosinophilic inflammation, airway remodelling and airway hyper-responsiveness (AHR) [24, 25]. Finally, IL-13 knock-out mice are resistant to allergen-induced AHR [26, 27].

The purpose of this study was to determine if isolated upper-airway allergen challenge was sufficient to induce AHR, the central feature of asthma. We hypothesized that upper-airway allergen stimulus can provoke inflammatory

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and smooth muscle responses characteristic of asthma in the lower airways. We further postulated that, consistent with other asthma models, allergic inflammation as well as AHR are mediated in part by IL-13 in our model system.

Methods

Animals

BALB/c male mice were obtained from Harlan–Sprague Dawley (Indianapolis, IN, USA). Animals were between 6 and 8 weeks of age at the start of each experiment and were housed in a conventional animal facility at the Meakins-Christie Laboratories. All studies were evaluated and performed in accordance with the Animal Ethics Committee at McGill University.

Ovalbumin sensitization and challenge

Ovalbumin (OVA) (grade V; Sigma Chemical Co., St Louis, MO, USA) was used in all experiments. Animals were sensitized and challenged as described previously [4]. Briefly, awake 6-week-old animals were sensitized daily for 10 days by local instillation of 5 µL in each nare of 1% OVA in phosphate-buffered saline (PBS). Following 2 weeks rest, animals received intranasal challenge daily for 5 days with 1% OVA. Experiments were performed 24 h following fifth challenge dose as detailed below. For the following experiments, the positive control group consisted of animals sensitized using a standard protocol. These mice received 250 µg/mL OVA mixed with 4 mg/mL Al(OH)₃ via the intraperitoneal (i.p.) route weekly for 3 weeks. These animals were then challenged intranasally with 5 µL in each nare of 1% OVA once daily for 5 days. Animals sensitized and challenged with intranasal application of normal saline represented the negative control group. In some experiments, animals received a one time dose of 7 µg of monoclonal anti-mouse IL-13 neutralizing antibody (R&D Systems, Minneapolis, MN, USA) or isotype control normal goat IgG (R&D Systems) via both the intranasal and intravenous routes prior to the first of 5 days IN challenge.

Distribution of intranasal particles

Ten microlitres of a 5 mg/mL solution of Evan's Blue dye in PBS was instilled into each nostril of awake and anaesthetic mice, which were then killed by means of anaesthetic overdose. The nares, lungs and trachea, stomach and spleen of each animal were removed, and Evan's Blue was extracted in 2 mL formamide in a 60 °C water bath for 24 h. The optical density of the supernatant was assessed at 620 nm by using a spectrophotometer. The extracted Evan's Blue was quantified by interpolation on a standard curve of Evan's Blue dye concentrations in the range of 0–100 µg/mL.

Measurement of ovalbumin-specific immunoglobulins G and E

Serial serum samples were obtained by saphenous vein puncture and OVA-specific IgG and IgE were assessed by ELISA as described previously [4]. Briefly, ELISA plates were

coated with 20 mg/mL OVA and blocked with PBS-Tween 20. For assessment of OVA-specific IgG standard mouse purified mouse IgG (Zymed Laboratories, San Francisco, CA, USA) was used, serially diluted. Appropriate dilutions of serum samples were loaded into each well and incubated for 2 h at 37 °C. Plates were washed and biotin-labelled rabbit anti-mouse IgG plus horseradish peroxidase–streptavidin (Zymed Laboratories) was added and plate incubated for 60 min at room temperature. Following washing, TMB substrate (Zymed Laboratories) was added. The reaction was stopped with 1 M H₃PO₄ and read at 450 nm. OVA-specific IgE was performed using an ELISA kit (Pharmingen OPTEIA IgE Kit, San Diego, CA, USA) with the following modifications: serum was incubated overnight at 4 °C with protein G sepharose beads prior to loading onto ELISA plates. Plates were coated with OVA and serum added as described above. Standards were plated as per OPTEIA protocol. Samples were incubated overnight at 4 °C and ELISA performed as per protocol (Pharmingen OPTEIA mouse IgE set).

Bronchoalveolar lavage

Under deep anaesthesia immediately post mortem, the trachea was isolated and a 22-gauge stainless steel catheter was inserted into the proximal trachea and secured with 5-0 silk suture. The lungs were lavaged using 0.5 mL of ice-cold 0.9% NaCl. The fluid obtained was frozen at –70 °C for analysis later. IL-13 was assayed in bronchoalveolar lavage fluid (BALF) using an IL-13-ELISA kit using protocol provided by the manufacturer (Biosource, Camarillo, CA, USA). Cells were spun onto glass slides and stained using the Diffquick method. Differential cell counts were obtained manually under light microscopy. 2–3 × 100 cells were counted per slide and means obtained.

Histological analysis

Histology was performed as described previously. Briefly, lungs were slowly inflated with 1 mL of formalin and then placed entirely in formalin. The skull was also isolated and placed in formalin. The specimens were embedded in paraffin and 5.0 µm sections cut. Slides were stained using Giemsa staining and examined under standard light microscopy.

Assessment of lung hyper-responsiveness by methacholine challenge

Bronchial responsiveness was measured 24 h after OVA challenge. Mice were deeply anaesthetized with xylazine and sodium pentobarbital and paralysed with pancuronium bromide as per animal protocol specifications. The tail vein was cannulated for drug administration. The trachea was exposed and a cannula was inserted into the trachea and secured with silk ties. The tracheal cannula was attached to a computer-controlled small-animal ventilator (FlexiVent, SCIREQ, Montreal, QC, Canada). Mice were ventilated quasi-sinusoidally with a tidal volume of 8 mL/kg and respiratory rate of 150 breaths/min. Respiratory system resistance was measured. Methacholine was injected through the tail vein at a dose of 10, 20, 40, 80, 160 and 320 µg/kg and the maximal resistance and elastance measurement were recorded.

Statistical analysis

The results are expressed as mean $\pm$ standard error of the mean. Statistical significance was measured by Student's *t*-test or one-way ANOVA followed by Tukey's *post hoc* tests for individual group comparisons using SPSS software.

Results

Distribution of particles following intranasal challenge in awake and anaesthetized mice

In our model of airways allergy, awake, restrained animals are sensitized and challenged via the intranasal route while in contrast most other models using intranasal allergen use anaesthetized mice. We have previously demonstrated that intranasal application of Evan's Blue dye solution in awake mice resulted in almost exclusive deposition of particles in the upper airways while there was no significant deposition in lungs [4]. In contrast, other studies have shown significant deposition of solution in the lungs following intranasal challenge of anaesthetized mice [17, 28, 29]. We therefore compared deposition of Evans Blue dye in animals challenged while awake vs. anaesthetized. As shown in Fig. 1, intranasal application of solution in awake animals resulted in the almost exclusive deposition in the upper airway as we have shown previously [4]. The amount of dye detected in the lungs of these mice was insignificant. In contrast, there was almost equal distribution of dye particles in the upper and lower airways of anaesthetized animals. These results show that in our model, and in contrast to others, the site of greatest allergen deposition and therefore stimulation is concentrated in the upper airway. These results accord with those of Southam et al. [29] who used intranasal instillation of 25 μ L of radiolabelled ^{99m}Tc -sulphide colloid tracer at a fivefold increase in the volume used in our method. They demonstrated that greater than 60% of tracer was detectable in the heads of animals, instilled when awake, while detectable tracer levels in the lungs were equivalent to the background carcass. In contrast, when these animals were anaesthetized almost 40% of the tracer was detected in the lungs. Taken

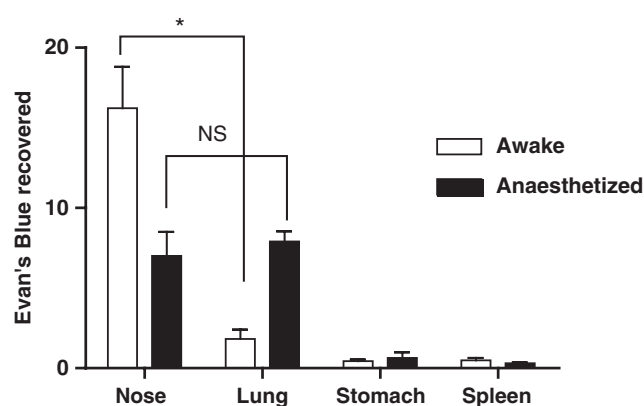


Fig. 1. Distribution of Evan's Blue dye in awake and anaesthetized mice. Evan's Blue dye was instilled into the nares of awake and anaesthetized mice and distribution in nares, lungs, spleen and stomach was assessed as described in the Methods section. * $P = 0.003$.

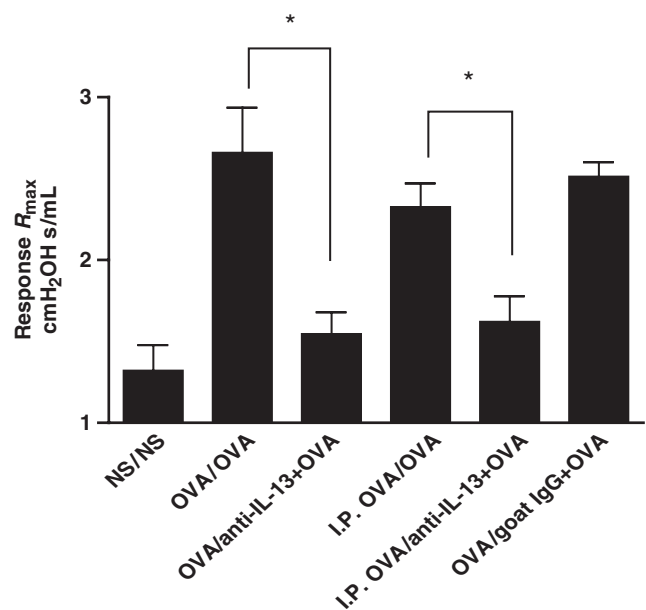


Fig. 2. Bronchial hyper-responsiveness following intranasal challenge. Mice were sensitized and challenged with ovalbumin (OVA) or normal saline (NS) with or without anti-IL-13 antibody pretreatment and airway responsiveness to 10 μ g/kg methacholine measured as described in the Methods section. Six to eight mice were used per group. * $P < 0.01$.

together with our results (Fig. 1), these data show that intranasal instillation of small volumes in awake animals results in restricted upper-airway distribution with statistically insignificant deposition in the lower airways.

Lung responsiveness to methacholine following upper-airway allergen challenge

Using this model of local, intranasal, allergen sensitization and challenge, we have previously shown evidence of lower-airway inflammation [4]. We were interested in determining if in this model these animals also developed AHR, characteristic of asthma. As shown in Fig. 2, animals sensitized locally as well as animals sensitized systemically by intraperitoneal injection demonstrate significant bronchial hyper-responsiveness (BHR) to low-dose methacholine, 24 h following intranasal challenge. These data suggest that allergen stimulation of the upper airway can induce physiologic effects in lower airways in the absence of significant allergen deposition in the lungs. During experiments animal heart rates were continually monitored as an indication of systemic parasympathomimetic effects of methacholine. Significant changes in airways resistance were noted with very low doses of methacholine, while significant changes in heart rate occurred at higher doses (data not shown) suggesting that the airways allergen sensitized and challenged animals were very sensitive to low-dose methacholine while control animals were not.

Interleukin-13 is an important effector cytokine mediating airway hyper-responsiveness

We hypothesized that in our model system of allergen-induced AHR IL-13 was also an important mediator. To support this, we investigated the effect of blocking IL-13

using an anti-IL-13 antibody delivered before intranasal allergen challenge. Animals were treated with anti-IL-13 antibody, both via the intraperitoneal and intranasal routes 30 min prior to the first of 5 challenge days. As shown in Fig. 3, allergen sensitized and challenged animals showed significantly elevated levels of IL-13 in BALF. Treatment with anti-IL-13 antibody resulted in a decrease of IL-13 to baseline levels. BHR to methacholine was measured as described above, 24 h following the 5th day of challenge. As shown in

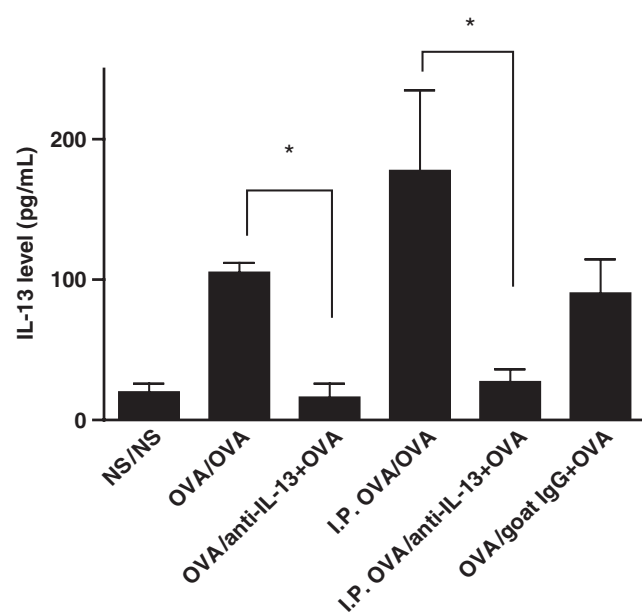


Fig. 3. IL-13 levels in bronchoalveolar lavage (BAL) following intranasal challenge. Mice were sensitized and challenged with ovalbumin (OVA) or normal saline (NS) with or without anti-IL-13 antibody pretreatment and IL-13 levels were determined in BAL fluid by ELISA as described in the Methods section. * $P \leq 0.02$.

Fig. 2, anti-IL-13 significantly inhibited AHR in OVA-allergic animals, suggesting that, in this model, IL-13 plays an important role in mediating asthma. These data show that allergen deposition primarily in the upper airway of sensitized animals can cause lower-airway hyper-responsiveness in an IL-13-dependent manner.

Anti-interleukin-13 inhibits airway inflammation

As shown in Figs 4 and 5, histological sections of nares and lung tissue obtained following local allergen challenge show inflammatory changes. In Fig. 4, the nares of allergic mice (panels b and c) show evidence of mucous cell hyperplasia, eosinophilic as well as lymphocytic infiltration and disruption of the brush border compared with the saline-negative control group (panel a). In contrast, nares from animals treated with anti-IL-13 (panels d and e) do not demonstrate these inflammatory changes and resemble the negative control group (panel a). Panel f animals, treated with the isotype (sham) control antibody, demonstrated inflammatory changes similar to those seen in panels b and c.

Figure 5 shows histological sections from lungs of allergen challenged and control mice and following treatment with anti-IL-13 antibody. As seen in the upper airway (Fig. 4) there is evidence of eosinophilic and lymphocytic peribronchial inflammation in allergic animals (panels c and d) as well as bronchial wall thickening compared with negative controls (panel a). In contrast, allergic animals treated with anti-IL-13 antibody prior to allergen challenge (panels d and e) show airway histology similar to negative control (panel a) with no allergic inflammation seen. Isotype (sham antibody) control-treated allergic mice (panel f) demonstrate peribronchial eosinophilic and lymphocytic changes similar to allergic animals (panels b and c). Of note, the degree of inflammation seen in our allergic animals is somewhat reduced when compared with other asthma models [6, 7]. This may be a direct effect of antigen deposition as in our model there is no

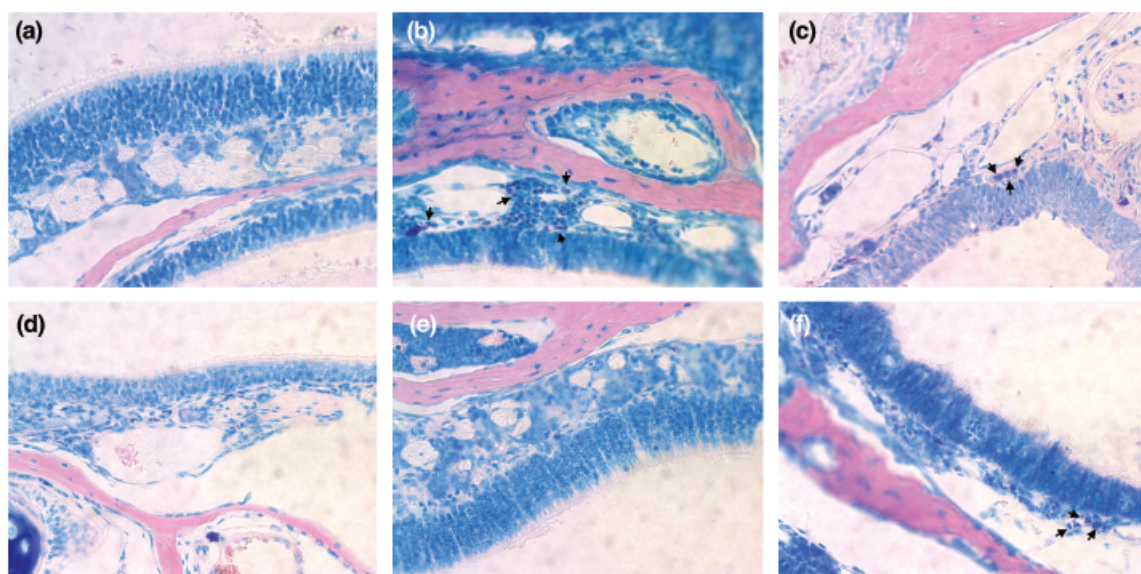


Fig. 4. Histology of BALB/c nares following challenge. (a) Sham-negative control NS/NS; (b) i.n. OVA/OVA; (c) i.p. OVA/OVA; (d) i.n. OVA/anti-IL-13 + OVA; (e) i.p. OVA/anti-IL-13 + OVA; (f) i.n. OVA/goat IgG + OVA. Sections were stained with Giemsa stain. Eosinophils highlighted with arrows. Magnification $\times 400$. NS, non-saline; i.n., intranasal; i.p., intraperitoneal; OVA, ovalbumin.

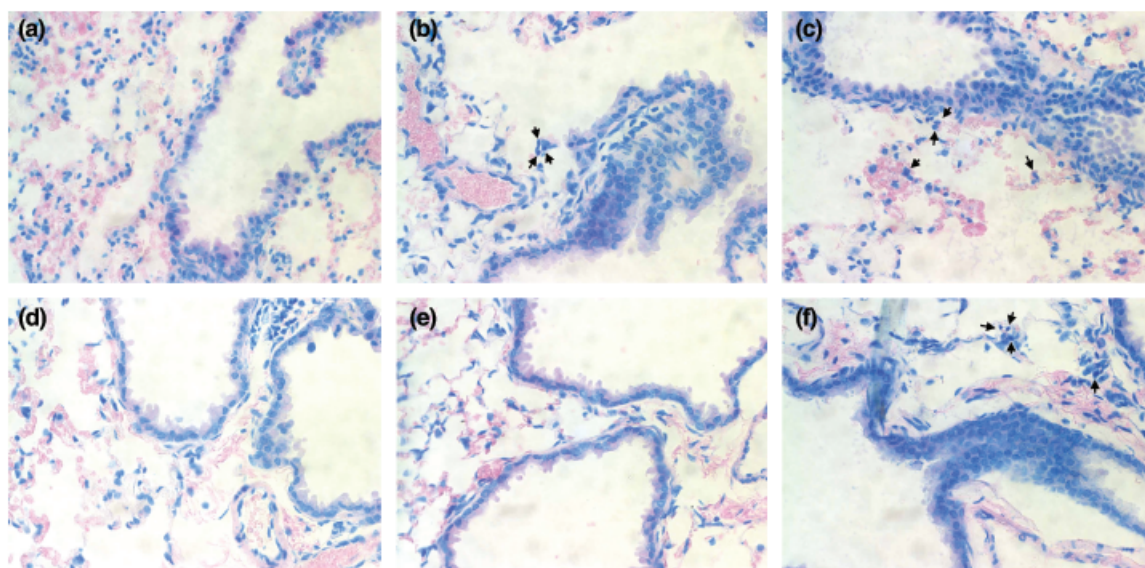


Fig. 5. Histology of BALB/c lungs following challenge. (a) Sham-negative control NS/NS; (b) i.n. OVA/OVA; (c) i.p. OVA/OVA; (d) i.n. OVA/anti-IL-13 + OVA; (e) i.p. OVA/anti-IL-13 + OVA; (f) i.n. OVA/goat IgG + OVA. Sections were stained with Giemsa stain. Eosinophils highlighted with arrows. Magnification $\times 200$. NS, non-saline; i.n., intranasal; i.p., intraperitoneal; OVA, ovalbumin.

significant antigen deposition in the lower airways while in most other asthma model animals are challenged while anaesthetized to maximize antigen delivery to the lower airways [6, 7]. Interestingly, we thus demonstrate that significant AHR can be induced in allergic animals without large amounts of antigen in the lower airways and with only moderate airway inflammation (Figs 1, 2 and 4).

We also performed differential leucocyte counts from BALF from these animals. As seen in Fig. 6, treatment of allergic animals with anti-IL-13 antibody significantly reduces the number of inflammatory cells in the BALF, although not to control levels. The degree of inflammatory response to allergen challenge varies depending upon the route of sensitization as greater numbers of granulocytes are detected in BAL from systemically vs. locally sensitized animals. However, allergic responses generated by either route appear to be IL-13 dependent.

Taken together, these data show that allergic inflammation in both the upper and lower airways of allergen-challenged mice is inhibited by anti-IL-13 and suggest that IL-13 is an important mediator of lower-airway inflammation following upper-airway challenge.

Anti-interleukin-13-antibody-mediated inhibition is independent of allergen-specific immunoglobulin E

We have previously demonstrated elaboration of allergen (OVA)-specific IgE from animals [4]. OVA-specific IgE levels were assayed in serum from animals before and after antigen challenge. There were no differences noted in IgE levels from animals treated with anti-IL-13 antibody prior to challenge compared with untreated animals (Fig. 7). Similarly, OVA-specific IgG levels were also not different between these groups.

This suggests that while IgE likely plays a role in inducing the allergic response, IL-13 plays an essential role in inflammation and AHR in both the upper and lower airways.

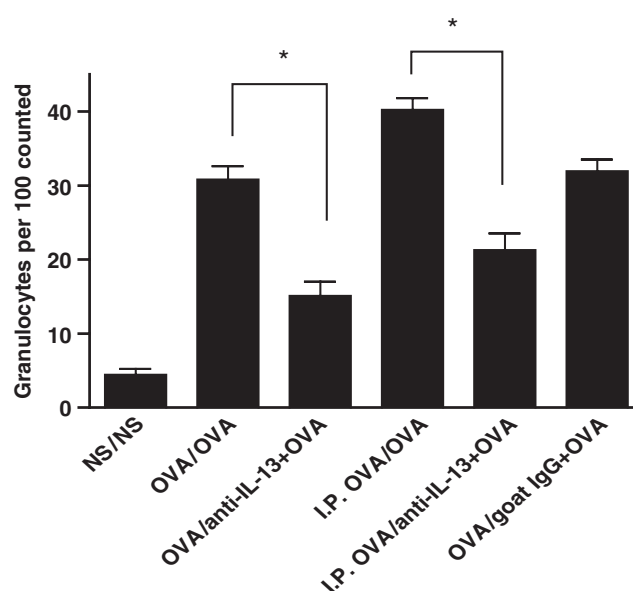


Fig. 6. Differential cell counts from bronchoalveolar lavage (BAL) fluid of mice 24 h after final challenge. Lymphocyte and granulocytes from BAL were enumerated by light microscopy as described in the Methods section. Six mice were used per group. * $P = 0.0001$.

Discussion

In the present study, we examined the effects of isolated upper-airway allergen challenge on the development of asthma and rhinitis. We have demonstrated that lower airways BHR is IL-13-dependent and that inhibition of IL-13 abrogates both upper and lower airways allergic inflammation, supporting the concept of commonality in the induction and regulation of immune responses in the airway mucosa as a whole.

Epidemiologic studies continue to show the strong link between AR and asthma development [1, 12, 14]. Mucosal

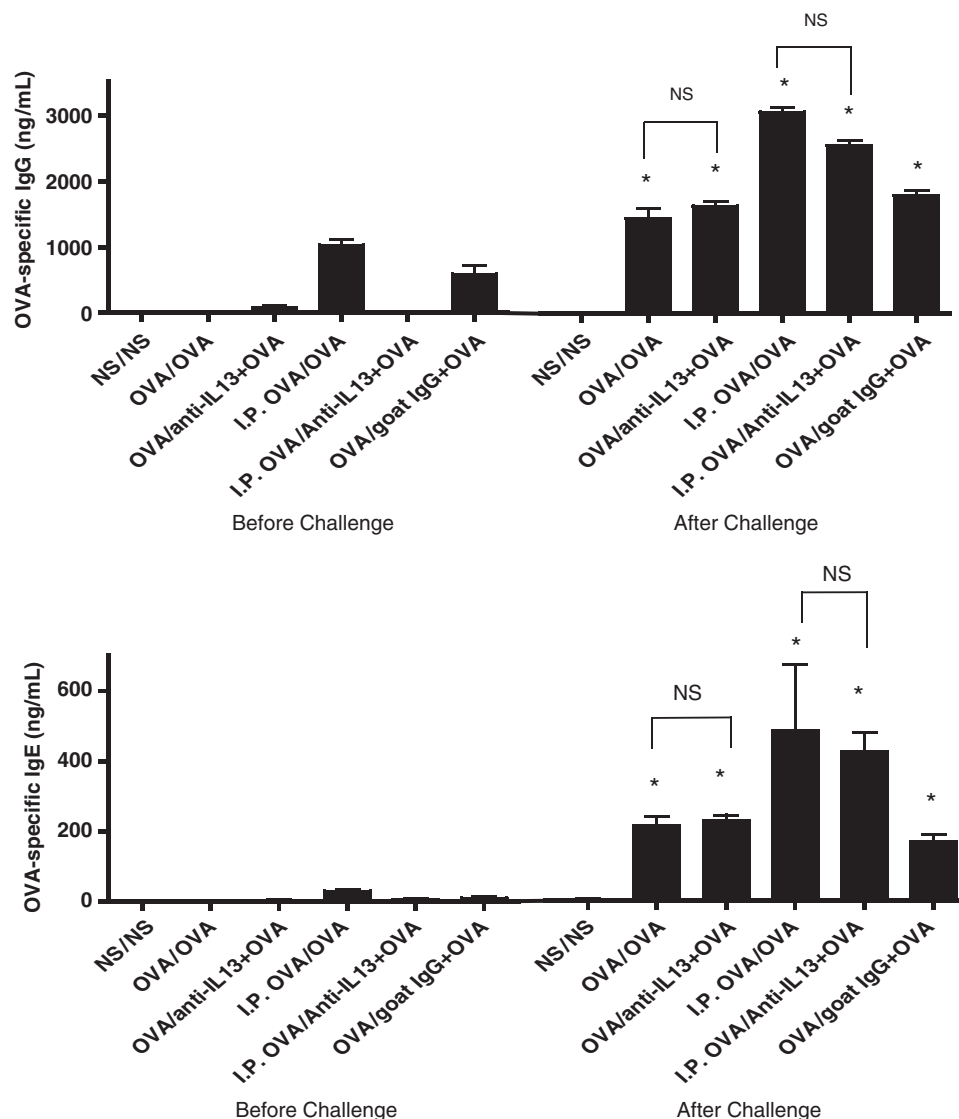


Fig. 7. Ovalbumin (OVA)-specific IgG and IgE measured before and after challenge. Specific anti-OVA antibodies were measured in mouse sera by ELISA. Six to eight mice were used per group. * $P < 0.001$.

contact between innocuous antigens and local immune system leads to the development of allergen-specific IgE and the allergic phenotype in genetically predisposed individuals. More than 75% of patients with asthma show signs and symptoms of AR [14]. Allergic sensitization early in life, i.e. before the age of 6 years, increases the risk of chronic asthma in later years [30]. However, while AR and asthma share characteristics of a common allergic inflammatory process, an aetiological role of the upper-airway mucosal immune response in asthma development has not been defined.

We have previously shown that allergen sensitization and challenge using small volume nasal instillation in awake mice results in almost exclusive deposition in the animals' upper airways [4]. In the present paper we have shown that nasal challenge in anaesthetized animals allows for the significant lower-airway deposition that has been demonstrated by others (Fig. 1) [17, 28, 29]. However, allergic inflammation can be demonstrated in both the upper and lower airways of awake mice, in the absence of significant lower-airway

mucosal-allergen interaction. We were interested in determining if this airways inflammation was also sufficient to induce BHR. Using methacholine challenge, we were able to demonstrate significant increases in airway resistance in allergen sensitized and challenged animals compared with controls. This increase in airway resistance was evident beginning at very low doses of methacholine (Fig. 2) prior to development of systemic parasympathomimetic side-effects such as bradycardia. This suggests that the allergen stimulation of the upper airway, causing moderate inflammation in both the upper and lower airways (Figs 4 and 5), is also sufficient for the development of asthma-like symptoms. This supports our hypothesis that mucosal immune responses in the nares can precipitate similar responses in distal airways.

Studies in humans with AR and/or asthma support this united airways concept [12, 18, 31]. In a recent study, patients with seasonal asthma and/or AR underwent segmental bronchoprovocation [15]. The investigators demonstrated a similar pattern of allergic inflammation in both groups

regardless of their usual disease phenotype. However, the question remains 'how do the upper and lower airways communicate?'

Several studies have highlighted the effects of IL-13 in mediating asthma such as direct effects on smooth muscle and airway epithelial cells as well as allergic inflammation [20–27]. IL-13-deficient mice do not develop AHR despite vigorous allergic stimulation [26, 27]. We therefore postulated that IL-13 was an important mediator in this model system. Allergen-sensitized animals were treated with anti-IL-13-antibody immediately prior to the first day of intranasal allergen challenge. Following challenge, anti-IL-13-treated mice resembled negative control mice and showed no evidence of allergic inflammation (Figs 4–6) or AHR (Fig. 2) in an IgE-independent manner (Fig. 7). This has led to the speculation that, although not exclusive, IL-13 may play a key role in the interaction between the upper and lower airways during allergic responses. Taken together, these data suggest that upper-airway mucosal sensitization and challenge is sufficient to induce allergic responses along the airway tree and that IL-13 is an important mediator in these responses.

The respiratory mucosa plays a critical role in determining the characteristics of immune responses to antigen challenge. It has been shown that intranasal application of allergen in anaesthetized animals followed by systemic (intraperitoneal) exposure to allergen results in the development of tolerance to subsequent intranasal challenge [17, 32]. This effect is mediated by the induction of IL-10-producing regulatory T cells that appear to inhibit expression of Th2 cells in the airways [31]. In contrast, we show that exclusive local allergen exposure induces allergic inflammation. Taken together, these studies show that route and timing of antigen exposure significantly affects the characteristics of subsequent immune responses in the airway mucosa. This suggests that understanding the regulation of the upper-airway mucosal immune response to allergen is critical in determining mechanisms to redirect allergic responses toward a more tolerant phenotype and ultimately abrogate allergic disease of the upper and lower airways.

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