

Is Interleukin-13 Critical in Maintaining Airway Hyperresponsiveness in Allergen-challenged Mice?

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Interleukin (IL)-13 is regarded as being a central effector in the pathophysiology of airway hyperresponsiveness. We have described a mouse model in which chronic allergen exposure results in sustained airway hyperresponsiveness and aspects of airway remodeling, and here sought to demonstrate that this component of airway hyperresponsiveness is independent of biologically active IL-13. Sensitized mice were subjected to either brief or chronic periods of allergen exposure and studied 24 hours after brief or 4 weeks after chronic allergen inhalation. A soluble murine anti-IL-13 receptor fusion protein that specifically binds to and neutralizes IL-13 was given daily during the 4 days before the day of outcome measurements in both protocols. Outcome measurements included airway responses to intravenous methacholine, bronchoalveolar lavage fluid cell counts, and airway morphometry. Compared with the saline control, brief allergen challenge resulted in airway hyperresponsiveness, which was prevented by anti-IL-13 treatment. Chronic allergen challenge resulted in sustained airway hyperresponsiveness and indices of airway remodeling; IL-13 blockade failed to reverse this sustained airway hyperresponsiveness. These results confirm that IL-13 is critical for the development of airway hyperresponsiveness associated with brief allergen exposure, but is not necessary to maintain the sustained airway hyperresponsiveness associated with airway remodeling.

Keywords: airway inflammation; allergic disease; asthma; bronchial hyperreactivity

Experimental animal models point to an essential role for interleukin (IL)-13 in the induction of airway hyperresponsiveness (AHR) (1–9). The administration of recombinant IL-13 to the airways of naive mice, in the absence of allergen immunization, induces airway inflammation and AHR (1, 2). Furthermore, selective blockade of IL-13, performed by the systemic administration of a soluble IL-13 receptor fusion protein, is effective in abrogating allergen-induced AHR in mouse models that involve brief periods of allergen exposure (1, 2). Transgenic models, in which mice constitutively overexpress IL-13 in their airways, have also provided convincing evidence for the effector role of IL-13 in the pathogenesis of AHR. These mice develop acute and chronic airway inflammation, which is associated with spontaneous increases in airway resistance and airway hyperreactivity to nebulized methacholine (MCh) (3). Based largely on these preclinical observations, IL-13 is now widely regarded as being a central mediator in the pathophysiology of AHR (10–13).

However, the mechanisms underlying AHR in asthma are complex and likely to be multifactorial (14). We postulate that there are times when patients with stable asthma, but persistent AHR, will not have major ongoing immune events. The observation that profound AHR is sustained in asthma despite effective treatment with antiinflammatory corticosteroids (15–18) suggests that mechanisms other than acute T-helper type 2 (Th2) cell-mediated inflammation likely account for a major component of AHR. An increasingly large body of literature supports the paradigm that chronic structural changes in the airway, often termed *airway remodeling*, may be at least partly responsible for sustained AHR (19–27). These changes include airway wall thickening, subepithelial fibrosis, goblet cell metaplasia, and hypertrophy and hyperplasia of myocytes, fibroblasts, and myofibroblasts (28–33). Although modeling systems based on short-term exposure of sensitized animals to allergen have greatly increased our understanding of the mechanisms underlying Th2-mediated inflammatory responses, a relative limitation of these models is that they do not adequately account for the chronic structural changes present in asthma. Thus, the associated AHR is transient, disappearing 14 to 21 days after allergen inhalation, and appears to be related only to Th2-mediated airway inflammation and the activation of Th2 cytokine effector pathways (34). This is not equivalent to the sustained AHR present in individuals with established asthma and, thus, whereas these brief challenge models have provided valuable information, they are unlikely to provide a complete description of the mechanisms underlying AHR.

We have described a model in which sustained AHR and indices of airway remodeling develop in mice after chronic exposure to allergen (34). We believe that both the AHR and much of the remodeling evident in this model are IL-13 dependent, as both are prevented when IL-13 gene-deficient mice are chronically exposed to allergen in this protocol (35). However, the fact that these abnormalities persist in wild-type mice for at least 8 weeks after final allergen exposure, beyond the resolution of acute inflammatory events, suggests that aspects of airway remodeling contribute independently to the ongoing, sustained airway hyperreactivity (34). We now hypothesize that the sustained airway dysfunction present in our model is no longer dependent on IL-13 effector mechanisms, but is instead associated with chronic structural airway remodeling. The purpose of the present study was to demonstrate that there is a component of AHR in mouse allergy models that is not responsive to anti-IL-13 treatment. To do this, we performed a series of experiments in which we initially sought to confirm that blockade of IL-13 with a soluble IL-13 receptor fusion protein prevented the development of the acute, inflammatory-associated transient AHR in mice after brief allergen exposure. These experiments served as a positive control to confirm the neutralizing effects of the anti-IL-13 fusion protein on allergen-induced AHR. In the second series of experiments, mice were exposed to our chronic allergen challenge protocol, and then, at a later point, several weeks after chronic challenge, treated with soluble IL-13 receptor fusion protein to block the effect of biologically active

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IL-13. Consistent with our hypothesis, we expected that IL-13 neutralization at this time was unlikely to attenuate the sustained AHR present in our model. Some of the results of these studies have been previously reported in the form of an abstract (36).

METHODS

Animals

Female BALB/c mice, aged 10 to 12 weeks, were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). Mice were housed in environmentally controlled specific pathogen-free conditions for 1 week before the study and for the duration of the experiments. All procedures were reviewed and approved by the Animal Research Ethics Board at McMaster University, and conformed to NIH guidelines for the experimental use of animals.

Sensitization

Mice were sensitized with intraperitoneal ovalbumin conjugated to aluminum potassium sulfate, as described by us previously (37).

Challenge and Treatment Intervention

Sensitized mice were subjected to either brief or chronic periods of allergen exposure, as described by us previously (34) (Figure 1). Control mice were subjected to the same sensitization protocol but received saline challenges. Allergen- and saline-challenged mice were studied 24 hours after the final exposure (Day 21) in the brief protocol, and 4 weeks after the final exposure in the chronic protocol. Blockade of IL-13 was performed by intraperitoneal administration of a soluble murine IL-13 α 2-humanIgG fusion protein (sIL-13 α 2.Fc) provided by Wyeth Pharmaceuticals Inc. (Cambridge, MA), which specifically binds to and neutralizes murine IL-13 (38). Control mice were treated with human IgG control protein. Mice were treated with 200 μ g/dose/mouse of sIL-13 α 2.Fc on Days -3, -2, and -1 before the outcome measurement day in each protocol, with a further 200 μ g dose given 1 hour before outcome measurements (Figure 1). In the brief model, this treatment was given on Days 18, 19, 20 and 21 (i.e., on the day before challenge, on 2 days of challenge, and on the day of outcome measurement). In the chronic model, treatment was given in the last week of the 4-week recovery period after the final allergen challenge on Day 90. The following outcome measurements were made: (1) *in vivo* airway responsiveness to intravenous MCh; (2) total and differential cell counts in bronchoalveolar lavage (BAL) fluid; and (3) airway morphometry using a computer-based image analysis system. Separate groups of 10 mice were studied in each treatment arm of each protocol.

Airway Responsiveness

The underlying contributors to airway responsiveness, namely airway reactivity (slope of increase in total respiratory system resistance [R_{RS}]

for a given increase in MCh dose), airway sensitivity (lowest dose of MCh to produce bronchoconstriction), and the maximum inducible bronchoconstriction (maximum R_{RS}) were measured after brief or chronic allergen challenge (Figure 2). These components of airway responsiveness were measured based on the response of R_{RS} to saline and increasing intravenous doses of MCh (Figure 2). R_{RS} was measured using the flow interrupter technique, modified for use in mice, and described in detail elsewhere (34, 37, 39).

BAL

After airway physiology measurements, BAL was performed as described by us previously (37). Differential cell counts were performed on 400 cells, counted by one investigator blind to the experimental conditions. IL-13 protein levels in BAL fluid were assessed by enzyme-linked immunosorbent assay (Quantikine; R&D Systems, Minneapolis, MN).

Lung Histology and Morphometry

The lungs were dissected and processed as described by us in detail previously (34). Transverse sections (3 μ m thick) were cut and assessed with the following stains: hematoxylin and eosin to demonstrate the presence of eosinophils, picosirius red to demonstrate the presence of collagen, and periodic acid Schiff to demonstrate the presence of mucin within goblet cells. Additional sections were prepared for immunohistochemistry using a monoclonal antibody (clone α sm-1; Novacastra Laboratories Ltd, Newcastle upon Tyne, UK) against α -smooth muscle actin to identify contractile elements. (We use the term 'contractile elements' rather than contractile smooth muscle, as this immuno-stain identifies the α -smooth muscle actin contractile protein present in both contractile and secretory smooth muscle phenotypes and in myofibroblasts). Morphometric quantification of the stained lung sections was performed using a customized digital image analysis system (Northern Eclipse; Empix Imaging Inc., Mississauga, ON, Canada), as described by us in detail previously (34, 40).

Statistical Analysis

Reported values are expressed as mean and standard error of the mean (SEM). Comparisons with respect to airway reactivity (slope of the R_{RS} -log transformed MCh dose-response curve), maximal bronchoconstriction (maximal MCh induced R_{RS}), cell counts, and indices of airway remodeling between saline control mice and mice receiving either brief or chronic allergen exposure, treated with either sIL-13 α 2.Fc or IgG control protein, were made using analysis of variance. Post hoc multiple comparison testing was performed using Duncan's test to assess for significant effects. All comparisons were two-tailed, and *p* values less than 0.05 were considered to be significant.

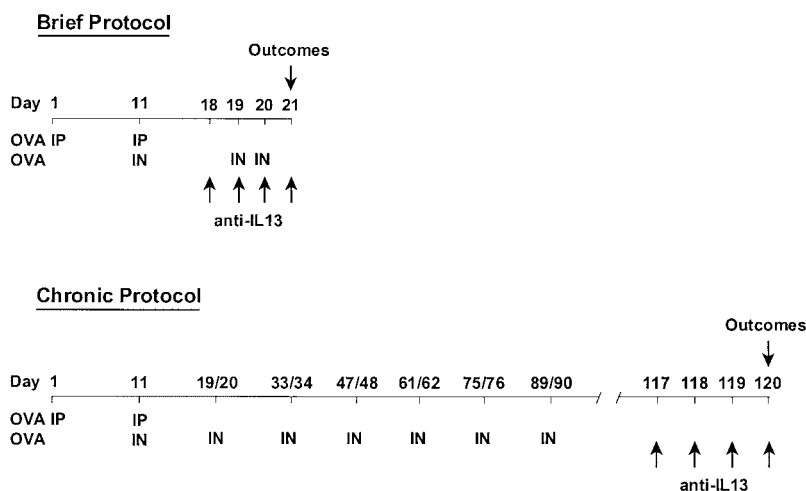


Figure 1. Study protocols. Sensitization and challenge protocols used in brief (upper panel) and chronic (lower panel) challenge models. Note that anti-IL-13 (sIL-13 α 2.Fc) and IgG control treatments were given 4 days before the day of outcome measurements in each protocol.

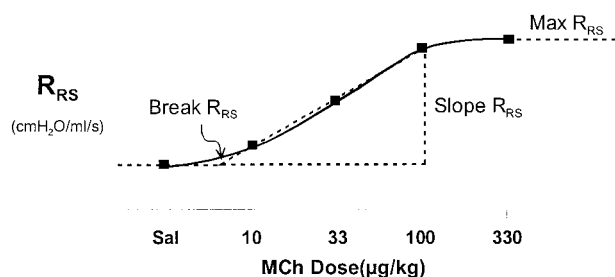


Figure 2. Airway responsiveness methods. Total respiratory system resistance (R_{RS}) was measured in response to increasing doses of intravenous methacholine (MCh). Using the resulting R_{RS} -MCh dose-response curve, indices of airway reactivity (Slope R_{RS}), airway sensitivity, or the lowest dose to produce bronchoconstriction (Break R_{RS}) and maximal degree of bronchoconstriction (Max R_{RS}) were measured.

RESULTS

Treatment with sIL-13R α 2.Fc During Brief Allergen Exposure Prevents the Development of Th2-Mediated AHR

After a period of brief allergen challenge, mice treated with IgG control protein exhibited a significant increase in total cell counts and eosinophils in BAL fluid compared with mice exposed to saline and treated with IgG control protein ($p < 0.001$) (Figure 3). BAL fluid IL-13 levels increased from 14.22 (SEM 0.48) pg/ml to 24.45 (SEM 2.73) pg/ml in saline versus allergen-challenged mice ($p < 0.05$). Treatment with sIL-13R α 2.Fc during the period of brief allergen challenge (Figure 1) resulted in significant attenuation of the allergen-induced increase in total cell counts ($p = 0.02$), but had no significant effect on the number of eosinophils in BAL fluid (Figure 3). The magnitude of BAL fluid eosinophilia in response to brief allergen challenge, and the subsequent lack of attenuation by sIL-13R α 2.Fc treatment were similar to that seen in the airway tissue (data not shown).

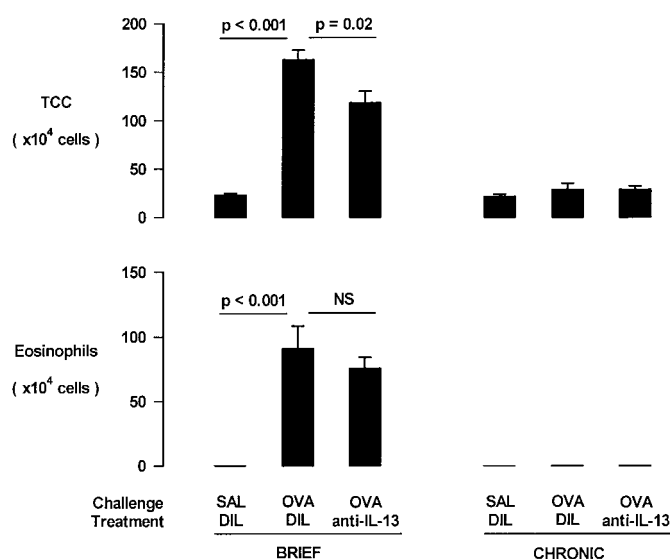


Figure 3. Bronchoalveolar lavage (BAL) fluid cell counts after brief or chronic exposure to saline or allergen. Total cell counts (TCC) (upper panel) and eosinophil counts (lower panel) measured in BAL fluid after brief challenge with either saline or ovalbumin and treatment with either IgG control antibody or anti-IL-13. DIL = IgG control antibody.

After brief exposure to allergen, mice treated with IgG control protein exhibited significant increases in airway reactivity ($p < 0.001$) and maximal bronchoconstriction ($p < 0.001$) compared with the saline control groups (Figure 4). We were also able to confirm, as others have shown before (1, 2), that sIL-13R α 2.Fc treatment during the period of brief allergen challenge completely abrogated the development of airway hyperreactivity and the increased maximal bronchoconstriction present in allergen challenged/IgG-treated control mice (Figure 4).

Chronic Allergen Exposure Results in Aspects of Airway Wall Remodeling and Sustained AHR that is not Attenuated by sIL-13R α 2.Fc Treatment

As in the brief challenge protocol, sIL-13R α 2.Fc or IgG control treatment was given during the 4 days before the outcome measurements were made, but the timing of this intervention in the chronic protocol meant that treatment with sIL-13R α 2.Fc was only given some 3 weeks after the final allergen challenge (Figure 1), at a time when airway remodeling was established (34). BAL fluid IL-13 measured at this time was not different between saline-challenged (13.29; SEM 1.36 pg/ml) and allergen-challenged (13.20; SEM 1.04 pg/ml) mice ($p > 0.05$). After chronic allergen exposure, IgG control protein-treated mice exhibited a significant and sustained increase in airway reactivity ($p < 0.01$) and maximal bronchoconstriction ($p < 0.01$) (Figure 5) compared with saline control mice. In contrast to our observations in the brief exposure model, treatment with sIL-13R α 2.Fc after chronic allergen exposure had no attenuating effect on either this sustained airway hyperreactivity or the sustained increase in maximal bronchoconstriction (Figure 5). There was a significant increase in the amount of mucin-containing, periodic acid Schiff-positive goblet cells (Figures 6, 7A, and 7B), subepithelial collagen deposition (Figures 6, 7D, and 7E), and α -smooth muscle actin staining (Figures 6, 7G, and 7H) in the airways of the IgG-treated control mice 4 weeks after chronic allergen exposure, compared with saline control mice ($p < 0.01$). As we had anticipated *a priori*, neutralization of IL-13 had no effect on any of these indices of airway remodeling (Figures 6, 7C, 7F, and 7I). Consistent with our previous studies (34), minimal numbers of eosinophils were observed in the BAL fluid of IgG-treated control and sIL-13R α 2.Fc-treated mice 4 weeks after chronic allergen exposure, and these values were not significantly different from baseline values observed in saline control mice (Figure 3).

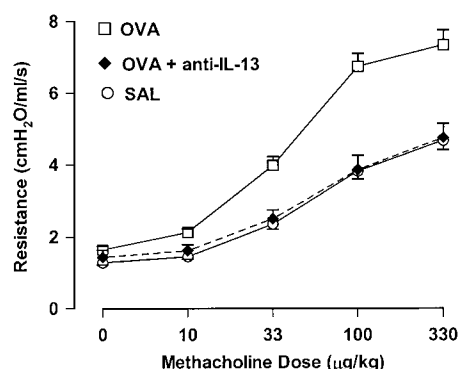


Figure 4. Airway responses after brief exposure to saline or allergen. Maximum airway bronchoconstriction and airway reactivity, calculated as the dose response slope to intravenous MCh, measured 24 hours after brief exposure to saline (circles), ovalbumin (squares), or ovalbumin and anti-IL-13 (diamonds).

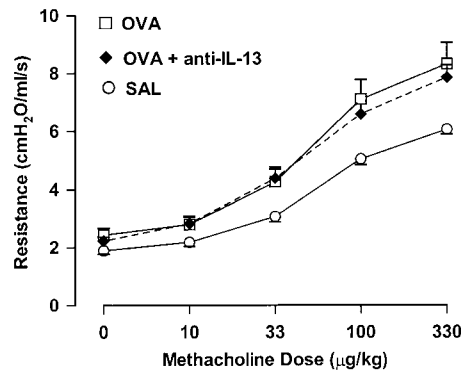


Figure 5. Airway responses after chronic exposure to saline or allergen. Maximum airway bronchoconstriction and airway reactivity, calculated as the dose response slope to intravenous MCh, measured 4 weeks after brief exposure to saline (circles), ovalbumin (squares), or ovalbumin and anti-IL-13 (diamonds).

DISCUSSION

This is the first study to directly examine the role of IL-13 in a model of established, sustained AHR. Our main purpose with this study was to demonstrate an anti-IL-13-insensitive component of allergen-induced AHR. We therefore waited until a time point after chronic allergen challenge when IL-13 levels had decreased, and blocked any residual biological activity of IL-13 using the sIL-13Rα2.Fc fusion protein. The results of our study clearly illustrate that blocking IL-13 activity at this time is not capable of reversing the sustained AHR.

Although there have been other reports of animal models in which chronic allergen or fungal exposure has resulted in structural airway changes and AHR (41–45), our model is fundamentally different in that the sustained AHR we observe is present for at least 8 weeks after the final allergen challenge, at

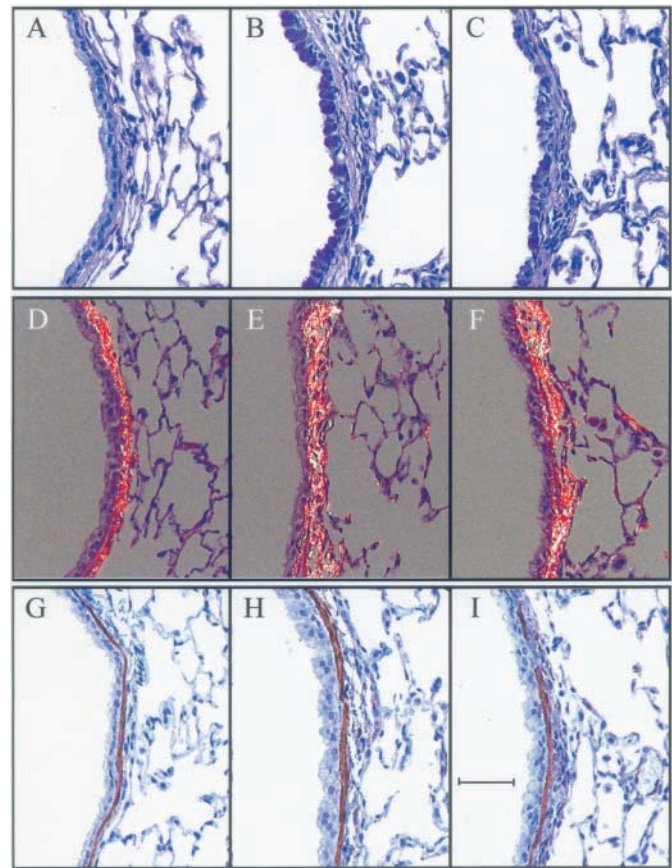


Figure 7. Histological sections of airway wall from chronically challenged mice. Staining for PAS-positive goblet cells (A–C), collagen deposition (PSR, viewed using polarized light microscopy) (D–F), and contractile elements (α-SMA) (G–I) in the airways of mice after chronic exposure to saline with IgG control antibody treatment (A, D, G), allergen with IgG control antibody treatment (B, E, H), or allergen with anti-IL-13 (sIL-13Rα2.Fc) treatment (C, F, I). Bar (I) indicates 50 μm.

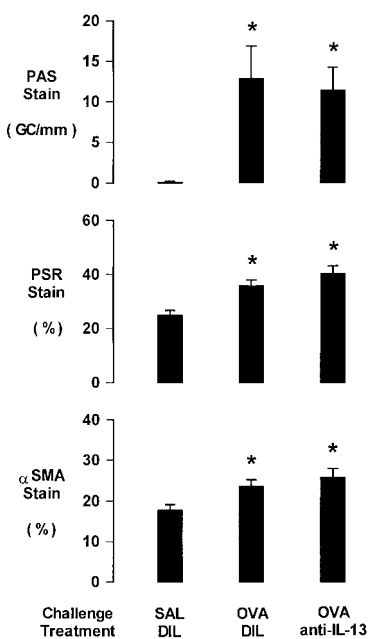


Figure 6. Morphometric changes in airways of mice after chronic exposure to saline or allergen. Morphometric quantification of mucin containing goblet cells (as demonstrated by periodic acid Schiff staining [PAS]), collagen (as demonstrated by picrosirius red [PSR] staining), and contractile elements (as demonstrated by staining with antibody to smooth muscle actin [α-SMA]) in the airways of mice after chronic challenge with saline or ovalbumin and treatment with IgG control antibody or sIL-13Rα2.Fc (anti-IL-13). Data are expressed as the number of goblet cells staining for mucin (PAS positive), expressed per length (cells/mm) of airway wall, and as the percentage of positively

stained tissue in the region of interest in the PSR and α-SMA stained sections. **p* < 0.05 compared with saline-challenged mice.

a time when acute Th2-mediated inflammatory responses have largely resolved (34). This is in contrast to the AHR described in the other models of chronic allergen or fungal exposure, where AHR is observed at times when cellular airway inflammation is still marked (41, 43–45). We have now shown that neutralizing IL-13, at a time when chronic allergen exposure has already resulted in the establishment of airway remodeling, did not attenuate the sustained AHR observed in our model. These observations are consistent with our underlying hypothesis, namely that IL-13-mediated effector pathways are not required to maintain sustained AHR; they also provide further evidence that sustained AHR is a consequence of airway remodeling, rather than of ongoing Th2 cytokine-mediated airway inflammation. We do however recognize that earlier Th2 immune-mediated inflammatory events are likely to be critical in the initial pathogenesis of functionally important airway remodeling processes, as evidenced by the fact that IL-13 gene-deficient mice are protected from developing aspects of airway remodeling or sustained AHR when subjected to this chronic allergen exposure protocol (35).

We have also shown that specific neutralization of IL-13 in mice during a period of brief allergen exposure, at a time when IL-13 protein levels were significantly increased in BAL fluid compared with saline control mice, prevented the development of transient, Th2 cytokine mediated-AHR. By demonstrating

that treatment with sIL-13R α 2.Fc was able to completely prevent this component of AHR after brief allergen challenge, we have confirmed that sIL-13R α 2.Fc is functionally effective at neutralizing the effects of endogenous IL-13. Our observations thus confirm the critical role of IL-13 in initiating the acute Th2-mediated inflammatory events that lead to transient AHR in this model, and are consistent with other published reports in which IL-13 blockade resulted in the abrogation of allergen-induced AHR (1, 2). However, whereas these experimental models of brief allergen exposure point to the fact that IL-13 may be an important therapeutic target in the treatment of AHR, they only allow evaluation of interventions on acute Th2-mediated responses, and do not take into account the chronic structural changes that are characteristic of established asthma and which have been implicated in the pathophysiology of AHR.

In agreement with previous observations by us, BAL fluid IL-13 levels were not elevated above control levels in chronically challenged mice (35). We felt, however, that this was not sufficient for concluding that anti-IL-13 treatment would not be effective at this time. Ongoing local production of IL-13 may well have been affecting smooth muscle function in these mice without elevating BAL fluid IL-13 levels. Here, however, we have shown that anti-IL-13 treatment at this time was ineffective, indicating that ongoing production of IL-13 was not playing a role in maintaining AHR.

We recognize that immunologic tolerance is likely to occur during our chronic challenge protocol. This may decrease the contribution of IL-13 to the sustained AHR present in our chronic model. Nonetheless, the major focus of the present study was to demonstrate that there is a component of AHR that is not reversible by anti-IL-13 treatment; this aspect of AHR should not be affected by the development of immunologic tolerance. We would interpret the results of our present study as demonstrating that whereas both tolerance and the blocking of IL-13 reduce the immune-mediated component of AHR, there is a substantial residual component of AHR that is independent of immune-mediated mechanisms and the effects of IL-13. This interpretation would be wholly consistent with our overall hypothesis that there is a component of AHR that is not responsive to anti-IL-13 treatment.

Given that transgenic overexpression of IL-13 results in tissue eosinophilia in mouse airways (3), we might have expected that sIL-13R α 2.Fc treatment would result in a more substantial attenuation of the allergen-induced airway eosinophilia than was noted in this study. However, our results are also consistent with a previously published report (1) in that IL-13 blockade before brief allergen exposure did not significantly attenuate allergen-induced airway eosinophilia. It is also consistent with our own previous work in which IL-13 gene-deficient mice had only a modest reduction in tissue eosinophilia compared with wild-type control mice after brief periods of allergen exposure (35). These observations suggest that eosinophil biology is likely influenced by a number of mediators and cytokines, in addition to IL-13. It is also likely that IL-13-dependent AHR occurs by mechanisms that are independent of airway eosinophilia.

In this study, we have not attempted to reverse structural changes, but rather to leave them intact and remove a specific immune mediator. For this reason we elected to treat mice with sIL-13R α 2.Fc for a relatively brief 4-day period. Although this was sufficient to neutralize IL-13 in the airways and to address our study hypothesis, we recognized *a priori* that this treatment regimen was likely to be too brief to have any effect on the indices of airway remodeling measured in the study. Thus, our observations that IL-13 blockade had no attenuating effect on indices of remodeling were entirely expected; they do, however,

point to the contribution that chronic structural changes play in the pathogenesis of sustained AHR.

Our study design also provides an opportunity to speculate on the potential therapeutic effects of extended sIL-13R α 2.Fc treatment during the period of chronic allergen exposure. Interventions that regulate Th2 cytokine effector pathways are attractive as potential therapeutic targets, and we assume that concomitant sIL-13R α 2.Fc treatment throughout the period of chronic allergen exposure might result in abrogation of Th2 immune-mediated airway inflammation, with the subsequent attenuation of aspects of airway remodeling and AHR. It is, however, perhaps more intriguing to speculate on whether prolonged sIL-13R α 2.Fc treatment, given after a period of chronic allergen exposure, might facilitate some resolution of the AHR and associated airway remodeling. Data from preclinical studies suggest a potential impact of IL-13 antagonism on airway remodeling, with evidence that sIL-13R α 2.Fc treatment significantly blocks collagen formation in a model of hepatic fibrosis (46), and also prevents collagen deposition in chronic allergic airway inflammation (47). Formal testing of this hypothesis has important implications for targeted anti-IL-13 as a potential therapy for asthma, and is clinically relevant in that the majority of patients with asthma already have aspects of airway remodeling present at the time of clinical presentation (48).

In summary, we have demonstrated that neutralization of IL-13 during a period of brief allergen challenge, at a time when acute Th2-mediated airway inflammation is associated with transient AHR, results in the prevention of this component of AHR. In contrast, neutralization of IL-13 after chronic repeated allergen exposure, at a time when airway remodeling has already become established, has no effect on the sustained AHR. Our results strongly support the paradigm that the transient airway hyperreactivity occurring after brief exposure to allergen is dependent on IL-13-mediated effector pathways. However, our observations extend that understanding by providing substantial novel evidence that there is a component of AHR that is associated with aspects of airway remodeling, and that blocking the effect of biologically active IL-13 has no effect on this component of AHR. Our observations also imply that anti-IL-13 strategies may be no more effective in the clinical management of established asthma than currently available inhaled corticosteroid therapies that target immune-mediated inflammation (49).

Conflict of Interest Statement: R.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; R.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; J.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; D.D.D. has been an employee of Wyeth Research since 1987, and holds stock options in Wyeth that are part of the compensation package consistent with the company policy and patents issued and developed during the course of employment at Wyeth; M.D.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this article.

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