

Type 2 Cytokines in the Pathogenesis of Sustained Airway Dysfunction and Airway Remodeling in Mice

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The mechanisms underlying airway hyperresponsiveness remain unclear, although airway inflammation and remodeling likely play important roles. We have observed sustained airway hyperreactivity and airway remodeling occurring in mice after chronic allergen exposure and persisting beyond resolution of allergen-induced inflammation. The aim of this study was to delineate mechanisms involved in allergen-induced airway hyperreactivity and airway remodeling and to examine evidence for a causal association between airway remodeling and sustained airway hyperreactivity. Wild-type (WT) and interleukin (IL)-4^{-/-}, IL-5^{-/-}, and IL-13-deficient (-/-) mice were sensitized and studied 4 weeks after chronic allergen exposure. By measuring airway responsiveness and airway morphometry, we demonstrated that WT mice developed sustained airway hyperreactivity and aspects of airway remodeling after chronic allergen exposure. Both IL-4^{-/-} and IL-13^{-/-} mice were protected from developing sustained airway hyperreactivity and aspects of airway remodeling. In contrast, IL-5^{-/-} mice developed sustained airway hyperreactivity and aspects of airway remodeling similar to that seen in WT mice. Our results confirm that IL-4 and IL-13, but not IL-5, are critical for the development of sustained airway hyperreactivity and airway remodeling after allergen exposure.

Keywords: allergic disease; asthma; interleukin-4; interleukin-5; interleukin-13

Airway hyperresponsiveness (AHR) is a central feature of asthma (1) and is characterized by exaggerated airway narrowing after exposure to nonspecific stimuli such as methacholine (MCh), histamine, or exercise (2). The dysfunction underlying AHR includes hypersensitivity (shift to the left of bronchoconstrictor dose-response curves), hyperreactivity (increased slope of these curves), and a greater maximum degree of induced bronchoconstriction. However, the mechanisms underlying these pathophysiologic abnormalities remain unclear. Numerous experimental and clinical studies have established that CD4⁺ T cell-mediated inflammation of the airways is central to the pathogenesis of asthma (3–5), and the contributions of acute immune-mediated airway inflammation to the pathogenesis of AHR have been investigated in animal models of allergen-induced airway responses (6–10). These studies confirm that the T helper type 2 cytokines interleukin (IL)-4, IL-5, and IL-13 contribute either directly or indirectly to the mechanisms under-

lying transient allergen-induced AHR by promoting the differentiation, survival, and function of key allergic effector cells (7–9, 11–19). Although these models have increased our understanding of the mechanisms underlying transient responses to allergen, the observed airway dysfunction is transient and appears to be related only to acute increases in inflammatory mediators. Thus, models of brief allergen exposure do not necessarily provide an accurate experimental model of the sustained AHR present in asthma.

Although some studies report that AHR in asthma is related to the extent of T cell-mediated airway inflammation (20, 21), the observation that profound AHR is sustained in asthma despite prolonged treatment with antiinflammatory corticosteroids (22–25) suggests that other mechanisms likely account for a major component of AHR. Evidence suggests that chronic structural changes in the airway, often termed airway remodeling, may be at least partly responsible for sustained AHR (26–32). These changes include thickening of the airway wall, subepithelial fibrosis, hyperplasia and hypertrophy of smooth muscle cells, and hyperplasia of fibroblasts/myofibroblasts and goblet cells (33–41). We have recently described a model in which airway dysfunction and aspects of airway remodeling develop in mice after chronic exposure to allergen (42). These abnormalities persist for at least 8 weeks after final allergen exposure, well beyond the resolution of acute inflammatory events, and suggest that airway remodeling occurs as a consequence of allergic airway inflammation and that aspects of airway remodeling contribute independently to the ongoing, sustained airway hyperreactivity. There have been other reports describing animal models in which structural airway changes occurred after chronic exposure to allergen (32, 43–46). In these studies, airway dysfunction was observed but was either only reported for the period immediately after the final exposure to allergen (43–46) or was measured at the time of ongoing cellular inflammation (32). Our recent observations are fundamentally different in that we have observed that sustained airway hyperreactivity persists for at least 8 weeks after final exposure to allergen (42).

The purpose of this study was to further delineate the mechanisms underlying allergen-induced sustained airway hyperreactivity and airway remodeling and to examine evidence for a causal association between the presence of ongoing sustained airway hyperreactivity and airway remodeling. Although the requirements for IL-4, IL-5, and IL-13 in initiating IgE-mediated eosinophilic airway inflammation and airway dysfunction are established, their respective roles in initiating sustained AHR are not known. By studying mice deficient for IL-4, IL-5, and IL-13, we aimed to determine whether sustained airway dysfunction and aspects of airway remodeling share common critical mechanistic pathways. Some of the results of these studies have been reported previously in the form of an abstract (47).

METHODS

Animals

Female BALB/c wild-type (WT) mice, aged 10 to 12 weeks, were purchased from The Jackson Laboratory (Bar Harbor, ME). Female mice of a BALB/c background of similar age, deficient for the IL-4

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gene (IL-4^{-/-}), IL-5 gene (IL-5^{-/-}) (12), or IL-13 gene (IL-13^{-/-}) (48), were obtained from The Jackson Laboratory, the Australian National University in Canberra, Australia, and from Dr. Andrew McKenzie at the Medical Research Council Laboratory of Molecular Biology, Cambridge, UK, respectively. All gene-deficient mice were developed on either a BALB/c or a C57/BL6 background (in which case they were backcrossed at least eight generations onto a BALB/c background). Mice were housed in environmentally controlled specific pathogen-free conditions for the duration of the experiments. All procedures were approved by the Animal Research Ethics Board at McMaster University and conformed to the National Institutes of Health guidelines for the experimental use of animals.

Sensitization

Mice were sensitized with intraperitoneal ovalbumin conjugated to aluminum potassium sulfate injected on Days 1 and 11 and intranasal ovalbumin on Day 11, as described by us previously (49).

Challenge

Sensitized mice were subjected to either brief or chronic periods of exposure to allergen, as described by us previously (42) (Figure 1). Mice were studied 24 hours after brief exposure protocols or 4 weeks after the chronic protocols. Separate groups of 10 sensitized mice were subjected to saline or ovalbumin exposure in each protocol, and 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.

Airway Responsiveness

The underlying contributors to airway responsiveness were measured on the basis of the response of respiratory system resistance to saline and increasing (10, 33, 100, and 330 $\mu\text{g/kg}$) intravenous doses of MCh, as described elsewhere in detail by us and others (42, 49, 50).

Bronchoalveolar Lavage

After airway physiology measurements, BAL was performed, as described previously (49).

Lung Morphometry

Lung tissue was prepared for morphometric analysis, as described by us in detail previously (42, 51). Three-micrometer-thick transverse sections were cut and assessed with the following stains: Congo red for the presence of eosinophils; picrosirius red to demonstrate the presence of collagen; and periodic acid-Schiff to demonstrate the presence of mucin within goblet cells. Furthermore, 3- μm sections were prepared for immunohistochemistry using a monoclonal antibody (Novacastra Laboratories Ltd., Newcastle upon Tyne, UK) against α -smooth muscle actin (α -SMA) to identify contractile elements. Morphometric quantification of the stained lung sections was performed using a customized digital image analysis system (Northern Eclipse; Empix Imaging Inc., Missis-

sauga, ON, Canada), as described by us previously (42, 51). Analysis of the Congo red-stained tissue involved identifying the first generation airway under a microscope at $\times 20$ magnification and capturing images into the computer by an investigator blinded to the tissue codes for the study groups. Analysis involved drawing a line along the basal border of the epithelium; then, using that line as a reference point, the software identified a 50- μm band of tissue in a basal direction. The numbers of eosinophils within this region were counted manually and the results expressed as cells per square millimeter of airway tissue.

Analysis of picrosirius red-stained (viewed using polarized light microscopy) and α -SMA-stained tissue involved identifying the same first generation airway and capturing images of airway wall that were not adjacent to neighboring vessels. As before, a line was drawn along the basal border of the airway epithelium from which a 20- μm band of tissue was projected in a basal direction. The software then calculated the percentage of each band that was positively stained for the individual stains on the basis of previously determined color plane settings. The final score for each stained section was expressed as a weighted mean, with each result weighted in proportion to the total area examined. Goblet cells were identified as periodic acid-Schiff-positive cells in the epithelium and counted manually and expressed as cells per millimeter length of airway wall.

Statistical Analysis

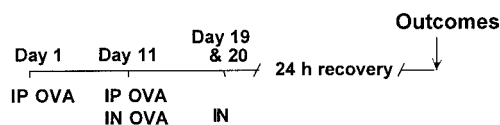
Reported values are expressed as mean and SEM. Comparisons between saline control mice and mice receiving either brief or chronic allergen exposure with respect to airway reactivity (slope of the respiratory system resistance [R_{RS}] – log-transformed MCh dose–response curve), maximal bronchoconstriction (maximal MCh-induced R_{RS}), cell counts, and indices of airway remodeling were made using analysis of variance. Similar between-group comparisons were made in mice receiving either brief or chronic allergen exposure. *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.

RESULTS

MCh Airway Responses after Brief or Chronic Exposure to Allergen

After brief exposure to allergen, WT mice exhibited significant increases in airway reactivity and maximal bronchoconstriction, compared with the saline control groups ($p < 0.01$) (Figures 2A, 3, and 4). Similarly, chronic allergen exposure resulted in a significant and sustained increase in airway reactivity and maximal bronchoconstriction in WT mice at 4 weeks, compared with mice given chronic saline exposure ($p < 0.01$) (Figures 2B, 3, and 4). It should be noted that the magnitude of allergen-induced changes in airway function differed between the groups of WT mice used as controls for each gene-deficient group. Although

Brief Protocol



Chronic Protocol

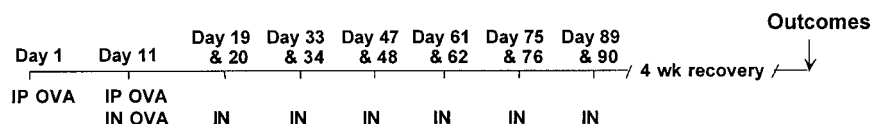


Figure 1. Study protocols. Sensitization and challenge protocols used in brief- and chronic-challenge models. Note that all outcome measurements were made after the final challenge in each protocol.

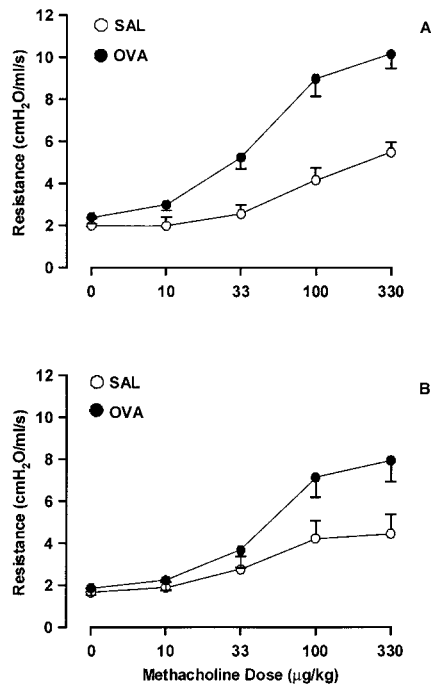


Figure 2. Airway responsiveness in wild-type (WT) mice after brief or chronic exposure to saline or allergen. Total respiratory system resistance (R_{RS}) was measured in response to increasing doses of intravenous methacholine (MCh) in WT mice either 24 hours after brief exposure to saline or allergen (A) or 4 weeks after chronic exposure to saline or allergen (B). Using the resulting R_{RS} – MCh dose-response curve, indices of airway reactivity (slope R_{RS}) and maximal degree of bronchoconstriction (max R_{RS}) were calculated.

we cannot be certain of the reason for this, it likely reflects the fact that, for logistic reasons, there were differences in the time points at which different groups of mice were studied. All gene-deficient mice were studied at the same time as the corresponding WT mice, and as such, statistical comparisons have only been made between each gene-deficient group and their own WT control group.

After brief exposure to allergen, IL-4^{-/-} mice demonstrated significant airway hyperreactivity ($p < 0.01$) and increased maximum bronchoconstriction ($p < 0.05$), compared with IL-4^{-/-} mice exposed to saline (Figures 3 and 4). However, after chronic allergen exposure, IL-4^{-/-} mice demonstrated normal airway reactivity and normal maximal bronchoconstriction, similar to that present in IL-4^{-/-} mice that had been chronically exposed to saline (Figures 3 and 4). In contrast to IL-4^{-/-} mice, IL-13^{-/-} mice exposed to brief allergen challenge demonstrated normal airway reactivity and normal maximal bronchoconstriction, similar to that present in both WT and IL-13^{-/-} mice given brief saline challenge (Figures 3 and 4). Like the IL-4^{-/-} mice chronically exposed to allergen, IL-13^{-/-} mice also demonstrated normal airway reactivity and normal maximal bronchoconstriction after chronic allergen exposure, similar in magnitude to that present in WT and IL-13^{-/-} mice chronically exposed to saline (Figures 3 and 4).

Like WT and IL-4^{-/-} mice, but in contrast to IL-13^{-/-} mice, IL-5^{-/-} mice demonstrated significant airway hyperreactivity ($p < 0.05$) and increased maximum bronchoconstriction ($p < 0.01$) after brief exposure to allergen, compared with IL-5^{-/-} mice exposed to saline (Figures 3 and 4). After chronic allergen exposure, and in contrast to both IL-4^{-/-} and IL-13^{-/-} mice, IL-5^{-/-} mice demonstrated significant airway hyperreactivity ($p <$

0.05) and increased maximum bronchoconstriction ($p < 0.01$) compared with IL-5^{-/-} mice chronically exposed to saline (Figures 3 and 4).

Eosinophilic Airway Response after Brief or Chronic Allergen Exposure

After brief allergen exposure, there was a significant increase in the number of peribronchial eosinophils in WT mice compared with the saline control group ($p < 0.01$) (Figure 5). Both IL-4^{-/-} IL-13^{-/-} mice had an attenuated eosinophil response in comparison with WT mice but still demonstrated a significantly increased eosinophil response in comparison with saline control mice ($p < 0.01$) (Figure 5). In contrast, the allergen-induced eosinophil response was completely abrogated in IL-5^{-/-} mice after brief allergen exposure, with levels being similar to those in the saline control mice (Figure 5). There was no evidence of significant peribronchial eosinophilia in any of the WT or the IL-4^{-/-}, IL-13^{-/-}, or IL-5^{-/-} mice when examined 4 weeks after chronic allergen challenge (Figure 5). The magnitude of BAL eosinophilia in response to brief allergen challenge was similar to that seen in the airway tissue (data not shown). Compared with saline control mice, WT as well as IL-4^{-/-} and IL-13^{-/-} mice developed a robust BAL eosinophilia after brief allergen exposure ($p < 0.01$). In contrast, IL-5^{-/-} mice had a negligible BAL eosinophil response after brief allergen exposure. No BAL eosinophils were detected in any of the study groups 4 weeks after chronic allergen exposure.

Subepithelial Airway Collagen Deposition after Brief or Chronic Allergen Exposure

There was a significant increase in the amount of collagen deposition present in the airways of WT mice 4 weeks after chronic allergen exposure, compared with saline control mice ($p < 0.05$) (Figures 6, 7A, and 7B). These changes were not present in IL-4^{-/-} and IL-13^{-/-} mice (Figures 6 and 7C–7F). In contrast, IL-5^{-/-} mice exhibited a significant increase in airway collagen deposition 4 weeks after chronic allergen challenge compared with the saline control mice ($p < 0.05$); indeed, these changes were of a similar magnitude to those seen in WT mice after chronic exposure to allergen (Figures 6, 7A, 7B, 7G, and 7H).

Airway Epithelium Mucin Changes after Brief or Chronic Allergen Exposure

The number of goblet cells staining positively with periodic acid-Schiff for mucin was significantly increased in the airway epithelium of WT mice at 4 weeks after chronic exposure to allergen, compared with saline control mice ($p < 0.01$) (Figures 6, 8A, and 8B). These changes were completely abrogated in IL-4^{-/-} and IL-13^{-/-} mice after chronic allergen exposure (Figures 6 and 8C–8F). In contrast, IL-5^{-/-} mice exhibited a significant increase in airway epithelium mucin content 4 weeks after chronic allergen challenge compared with saline-exposed mice at the same time point ($p < 0.01$). This response was however attenuated in comparison with the increase in the amount of mucin present in the airway epithelium of WT mice after chronic allergen exposure ($p < 0.05$) (Figures 6, 8G, and 8H).

Airway α -SMA Changes after Brief or Chronic Allergen Exposure

There was a significant increase in the amount of α -SMA staining present in WT mice at 4 weeks after chronic exposure to allergen, compared with saline control mice ($p < 0.01$) (Figures 6, 9A, and 9B). There were no significant differences in α -SMA staining at 4 weeks in any of the IL-4^{-/-}, IL-13^{-/-}, or IL-5^{-/-} mice after chronic exposure to allergen, compared with their corresponding saline control groups (Figures 6 and 9C–9H). However, com-

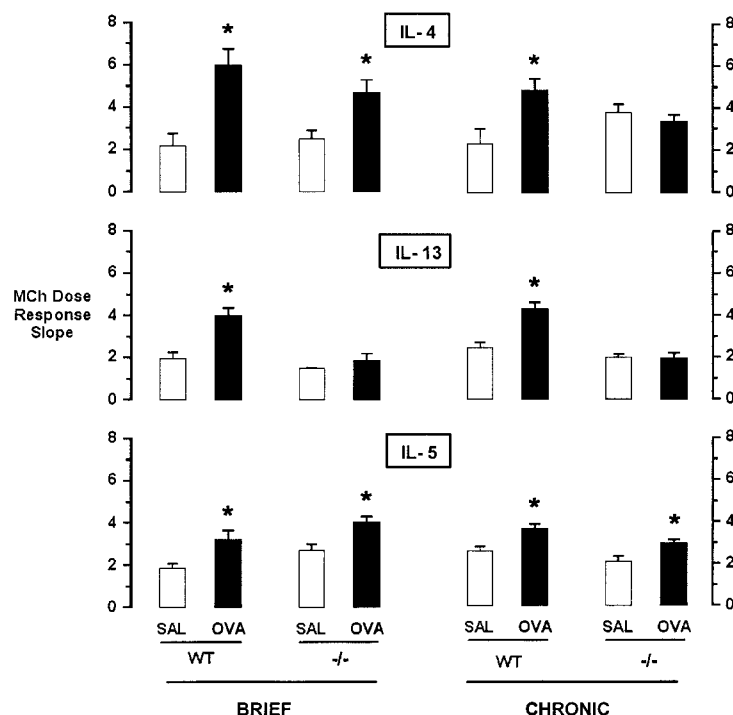


Figure 3. Airway reactivity in interleukin (IL)-4 $^{-/-}$, IL-13 $^{-/-}$, and IL-5 $^{-/-}$ mice and their corresponding WT control mice after brief or chronic exposure to saline or allergen. Airway reactivity, calculated as the dose-response slope to intravenous MCh, measured 24 hours after brief exposure to saline or allergen and 4 weeks after chronic exposure to saline or allergen in IL-4 $^{-/-}$, IL-13 $^{-/-}$, and IL-5 $^{-/-}$ mice and the corresponding WT control mice. *Indicates p value less than 0.05 compared with saline-challenged mice.

pared with saline-challenged WT mice, all the gene-deficient groups had significantly increased amounts of α -SMA staining present, regardless of whether they were chronically exposed to saline or allergen ($p < 0.01$) (Figures 6, 9A, 9C, 9E, and 9G).

DISCUSSION

In this study, we have confirmed that WT mice develop sustained airway hyperreactivity and aspects of airway remodeling in

response to chronic allergen exposure. However, both IL-4 $^{-/-}$ and IL-13 $^{-/-}$ mice were protected from developing sustained airway hyperreactivity and the associated aspects of airway remodeling, whereas IL-5 $^{-/-}$ mice were not completely protected from either of these outcomes. These results are consistent with our underlying hypothesis that sustained hyperreactivity is not observed when aspects of remodeling are prevented.

This is the first study to directly examine the role of IL-4, IL-5,

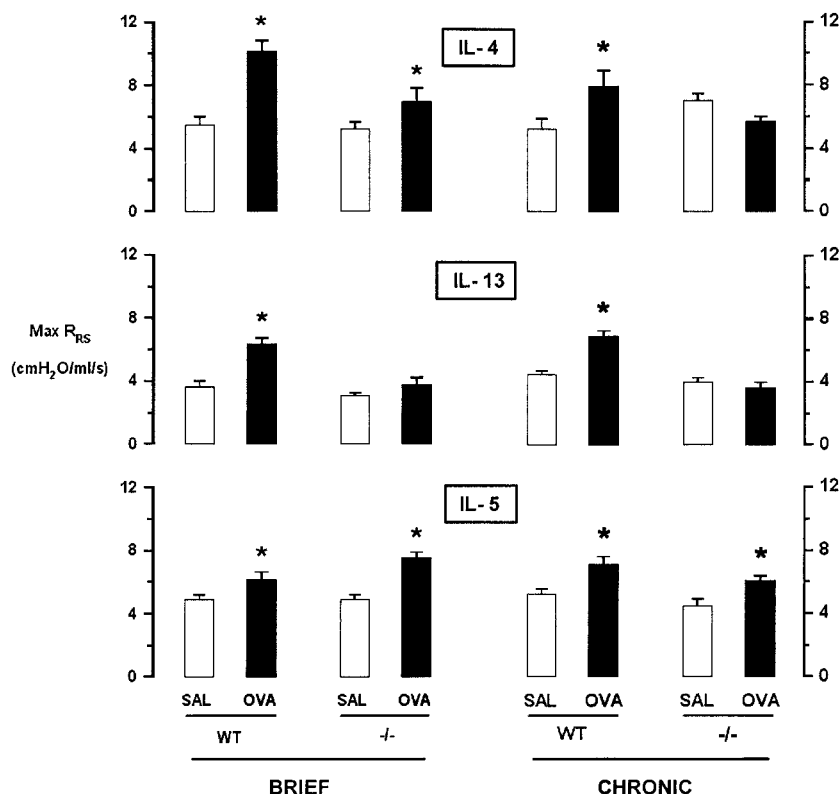


Figure 4. Maximum bronchoconstriction in IL-4 $^{-/-}$, IL-13 $^{-/-}$, and IL-5 $^{-/-}$ mice and their corresponding WT control mice after brief or chronic exposure to saline or allergen. Maximum airway bronchoconstriction in response to intravenous MCh, measured 24 hours after brief exposure to saline or allergen and 4 weeks after chronic exposure to saline or allergen in IL-4 $^{-/-}$, IL-13 $^{-/-}$, and IL-5 $^{-/-}$ mice and the corresponding WT control mice. *Indicates p value less than 0.05 compared with saline-challenged mice.

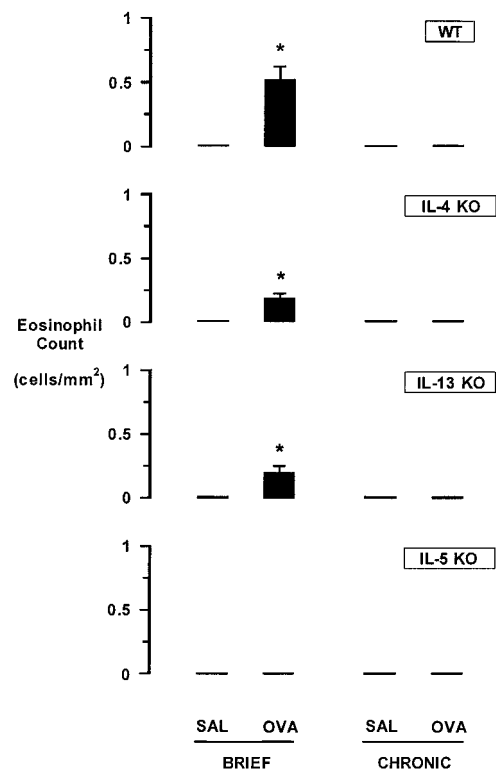


Figure 5. Airway tissue eosinophil counts in WT, IL-4^{-/-}, IL-13^{-/-}, and IL-5^{-/-} mice after brief or chronic exposure to saline or allergen. Airway tissue eosinophil counts (expressed as cells per square millimeter) in the 50-μm region beneath the epithelium in the Congo red-stained sections. *Indicates p value less than 0.05 compared with saline-challenged mice.

and IL-13 in an animal model in which both airway remodeling and sustained airway dysfunction are present. The ongoing sustained airway dysfunction that we have observed in our model is fundamentally different from the airway dysfunction described in reports of other models of chronic allergen or fungal exposure, where airway dysfunction is observed at times when cellular airway inflammation is still marked (32, 43, 45, 46). In our current model,

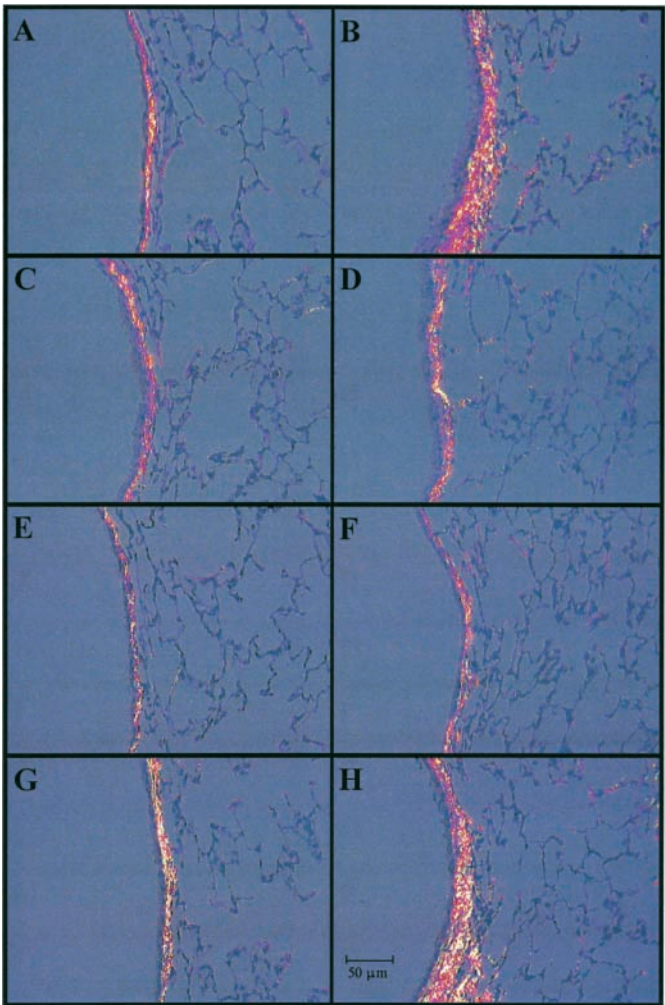
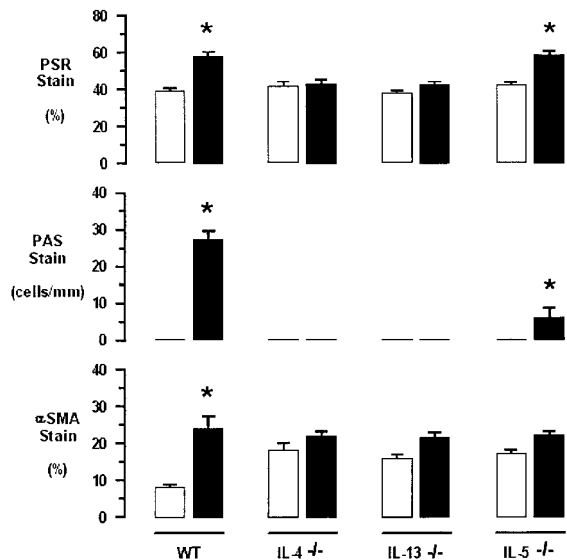


Figure 7. PSR-stained sections of airway wall from chronically challenged mice. Staining for PSR, viewed using polarized light microscopy, in the airways of WT mice after chronic exposure to saline (A) or allergen (B), in IL-4^{-/-} mice after chronic exposure to saline (C) or allergen (D), in IL-13^{-/-} mice after chronic exposure to saline (E) or allergen (F), and in IL-5^{-/-} mice after chronic exposure to saline (G) or allergen (H). Bars indicate 50 μm. These photomicrographs depict the upper limit of allergen-induced effects; the mean differences are illustrated in the bar graphs in Figure 6.

sustained airway dysfunction is present for at least 8 weeks after the final allergen challenge, at a time when acute, immune-mediated inflammatory response has resolved (42), and our current results further strengthen the concept that the sustained airway

Figure 6. Morphometric changes in the airway of WT, IL-4^{-/-}, IL-13^{-/-}, and IL-5^{-/-} mice after brief or chronic exposure to saline or allergen. Morphometric quantification of collagen (picrosirius red [PSR]), mucin (periodic acid-Schiff [PAS]), and contractile elements (α-smooth muscle actin [α-SMA]) in the airway wall of WT, IL-4^{-/-}, IL-13^{-/-}, and IL-5^{-/-} mice after chronic exposure to saline or allergen. Data are expressed as the percentage of positively stained tissue in the region of interest in the PSR- and α-SMA-stained sections and as the number of goblet cells staining for mucin (PAS positive), expressed per length (cells per millimeter) of airway wall. *Indicates p value less than 0.05 compared with saline-challenged mice.

associated airway dysfunction after chronic allergen exposure in this model. Our results confirm the observations of several reports, which have suggested that IL-5 (and by implication, eosinophilic inflammation) is not critical for the development of airway dysfunction or aspects of airway remodeling (52, 55). However, given that there may be differences in eosinophil activation in murine models of allergic airway inflammation and in asthma (56), we should not necessarily conclude that eosinophilic inflammation in asthma does not contribute to functionally important airway remodeling. Furthermore, although sustained airway hyperreactivity was still present in IL-5^{-/-} mice, it appeared to be reduced (although not significantly) in relation to the WT mice. We would therefore caution against concluding that IL-5, or by implication airway eosinophilia, is not involved in the development of sustained airway dysfunction.

On the basis of our findings, it is tempting to postulate that indices of airway remodeling, as well as sustained airway dysfunction, can be prevented using strategies that do not affect eosinophilic inflammation. Indeed, we have observed that both these outcome variables are prevented in the IL-4^{-/-} and the IL-13^{-/-} mice despite the fact that eosinophilic airway inflammation was not completely abrogated 24 hours after a brief allergen challenge. However, we do not have data confirming that eosinophilia in these mice persisted throughout the whole period of chronic allergen challenge; we also cannot state that eosinophilic activity was not affected in these mice. Thus, although we believe that we are justified in concluding that airway remodeling in this model is possible in the absence of eosinophilic inflammation, it is clear that further experiments are warranted to address whether controlling eosinophilia can modulate remodeling of the airway.

Thus, our findings are largely consistent with the published literature and strongly support the hypothesis that IL-4 and IL-13 are necessary for the development of aspects of airway remodeling. Our observations also provide a substantial novel finding in that we have observed a consistent relationship between the blocking effects of these cytokines on aspects of airway remodeling and the development of sustained airway dysfunction. We cannot however postulate any role for these cytokines in the remodeling of airway contractile elements, given that the α -SMA staining was increased in all three gene-deficient groups (IL-4^{-/-}, IL-13^{-/-}, and IL-5^{-/-}), compared with WT mice after chronic saline challenge. Although we have provided no direct evidence of a causal relationship between airway remodeling and sustained dysfunction, the consistent association of these variables suggests that such a relationship exists. Bradford-Hill has proposed a set of formal criteria for evaluating and assigning causality (57). Thus, features that would strengthen the argument for a cause and effect relationship include an appropriate temporal relationship, a strong association between purported cause and effect, the existence of a dose-response relationship, a fall in risk when the purported cause is removed, consistency amongst several studies, biological plausibility, and analogy to similar cause and effect relationships. In our experimental model, we have demonstrated both an appropriate temporal relationship as well as a strong association between purported cause and effect between the requirement of IL-4 and IL-13 for the development of airway remodeling after chronic allergen exposure and the associated development of sustained airway hyperreactivity. We have confirmed that sustained airway dysfunction and different aspects of airway remodeling occur in both WT and IL-5^{-/-} mice after chronic exposure to allergen. However, indices of airway remodeling were not seen in either IL-4^{-/-} or IL-13^{-/-} mice, and these mice were completely protected from developing sustained airway hyperreactivity. In our study, we have not demonstrated the existence of a dose-response relationship between IL-4 and IL-13, the development of aspects of airway remodeling,

and the subsequent presence of sustained airway dysfunction. However, indirect evidence for this comes from other studies in which the dose-dependent overexpression of IL-13 in transgenic mice results in collagen deposition and goblet cell hyperplasia and the development of airway dysfunction (17).

In summary, we have demonstrated that IL-4 and IL-13 gene-deficient mice were completely protected from developing aspects of airway remodeling and sustained airway hyperreactivity after chronic allergen exposure. In contrast, and despite the virtual absence of tissue eosinophilia, IL-5 gene-deficient mice developed aspects of airway remodeling and sustained airway hyperreactivity similar to that seen in WT mice after chronic allergen exposure. Our results strongly support the concept that airway remodeling and sustained airway dysfunction occur as a consequence of repeated T helper type 2 immune-mediated airway inflammation. Furthermore, they illustrate that IL-4 and IL-13, but not IL-5, are critical for the development of these phenomena.

Conflict of Interest Statement: R.L. has no declared conflict of interest; R.E. has no declared conflict of interest; J.N.W. has no declared conflict of interest; J.A.H. has no declared conflict of interest; K.I.M. has no declared conflict of interest; P.S.F. has no declared conflict of interest; P.M.O. has no declared conflict of interest; M.D.I. has no declared conflict of interest.

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