

Sustained Suppression of IL-13 by a Vaccine Attenuates Airway Inflammation and Remodeling in Mice

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We previously reported that a recombinant IL-13 peptide-based virus-like particle vaccine significantly suppressed murine acute airway allergic inflammatory responses. The impact of this strategy on the development of chronic airway inflammation and remodeling has not been investigated. We evaluated whether the vaccine-mediated sustained suppression of IL-13 attenuates features of chronic airway inflammation and remodeling in mice repeatedly challenged with allergen. BALB/c mice received two intraperitoneal sensitizing injections of ovalbumin (OVA) and alum, followed by six consecutive 2-day intranasal OVA challenges at 12-day intervals and then a 4-week recovery period. Anti-IL-13 antibodies were induced with a vaccine before (preventive experiments) or after (interventional experiments) the OVA challenge commenced. Respiratory mechanics were assessed using low-frequency forced oscillation with a small animal ventilator. Cytokine concentrations in bronchoalveolar lavage fluid (BALF) and lung histology were also assessed. In the preventive experiments, vaccination significantly suppressed IL-13 concentrations, the accumulation of inflammatory cells in BALF, lung mucus production, and collagen deposition. Furthermore, vaccination significantly attenuated OVA challenge-induced increases in airway resistance, tissue resistance, and tissue elastance, both acutely and after a 4-week recovery from allergen challenges. In the interventional experiments, vaccination decreased IL-13, TGF- β 1, and IL-12p40 concentrations in BALF, as well as mucus production and collagen deposition. Chronic inflammation and sustained airway hyperresponsiveness were not significantly reversed. The persistent suppression of IL-13 with a vaccine inhibits chronic airway inflammation and the development of several key components of airway remodeling, and this intervention is more effective at early stages than later during chronic inflammation.

CLINICAL RELEVANCE

The effects of the vaccine-mediated, sustained suppression of IL-13 on the development of airway remodeling have not yet been investigated. The present study reveals that this approach can reduce airway inflammation, remodeling, and hyperresponsiveness, thus enhancing our understanding of the role of IL-13 in chronic airway inflammation.

Keywords: asthma; IL-13; vaccine; airway remodeling; inflammation

Asthma is a chronic disease of the airways with features that include airway inflammation, airway hyperresponsiveness (AHR), and airway remodeling. Remodeling is characterized as structural changes that include epithelial injury, subepithelial thickening/fibrosis, airway smooth muscle tissue hypertrophy, increased goblet-cell number and mucous gland size, and angiogenesis (1). Airway remodeling is postulated to be a determinant of sustained AHR and of the accelerated loss of lung function linked to fixed airway obstruction in patients with long-term asthma (2).

IL-13 is a recognized central modulator in asthma pathology, and is associated with almost all the features of both acute and chronic asthmatic responses (3–6). The direct airway administration (7, 8) or transgenic overexpression of IL-13 in the lungs (9) induces mucus production, eosinophilia, IgE generation, subepithelial fibrosis, and AHR. On the other hand, IL-13 gene deficiency or interference with IL-13 activity significantly reduces or abolishes almost all features of allergen-induced acute airway inflammation and hyperresponsiveness (10–12). In mice that receive chronic allergen exposure, prolonged IL-13 blockade or the deletion of IL-13 gene expression suppresses inflammation, subepithelial collagen deposition, and the development of AHR, and can prevent or reverse mucus hypersecretion (13–16). Other studies in mice show IL-13 blockade to be of limited effect if initiated after the start of allergen challenge (16–18). For instance, after airway structural changes and AHR are established, IL-13 blockade does little to reverse these features. However, it is important to note that previous studies used an approach wherein multiple doses of antagonists, such as antibodies or soluble receptors, were applied with an interval of 4 to 7 days between each administration (14, 16, 17), or were administered continuously for short periods (e.g., 4 d) (18). Whether an interventional approach that provides the persistent suppression of IL-13 function is more effective in determining the role of IL-13 in airway remodeling remains to be determined.

Recently, vaccine strategies have emerged as a novel approach for blocking overexpressed endogenous molecules that play critical roles in the pathogenesis of airway disease (12, 19–23). In comparison with monoclonal antibodies and soluble receptors that need repeated administration with large doses to maintain efficacy, the vaccine strategy has the advantage of

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potentially providing long-lasting effects by inducing autoantibodies after only a few administrations. Because asthma is a chronic disease requiring long-lasting intervention, a vaccine strategy may offer a more powerful tool than the passive administration of monoclonal antibodies to achieve clinically significant improvements.

Our previous work has shown that an IL-13 vaccine prepared by inserting a murine IL-13 peptide into carrier protein, hepatitis B virus core antigen (HBcAg), induces sustained and intense IL-13-specific neutralizing IgG responses. This result was linked to the significant suppression of all features of ovalbumin (OVA)-induced acute airway allergic inflammatory responses (12). In the present study, using a vaccine strategy to achieve the persistent suppression of IL-13 *in vivo*, we aim to clarify the role of IL-13 in the development and maintenance of sustained AHR and remodeling in a chronic allergen exposure murine model of asthma.

MATERIALS AND METHODS

Animals

Female BALB/c mice (7–8 wk old) were purchased from Charles River Laboratories (Saint-Constant, PQ, Canada) and maintained at the Central Animal Care Services of the University of Manitoba. All protocols were approved by the Animal Ethics Committee of the University of Manitoba.

Vaccine

Recombinant IL-13 peptide-based virus-like particle vaccine was prepared as previously described (12). The vaccine and vaccine carrier hepatitis B core antigen (HBcAg) were expressed by *Escherichia coli* DH5 α cells and purified by ammonium sulfate precipitation and then chromatography on sepharose CL-4B (Sigma-Aldrich, St. Louis, MO), ceramic hydroxyapatite (Bio-Rad), and polymyxin (Bio-Rad, Hercules, CA) columns.

Protocols of Immunization, Sensitization, and Challenge

The protocols are summarized in Figure 1a (preventive experiments) and Figure 4a (interventional experiments). Chronic airway inflammation was induced by two intraperitoneal sensitizing injections of OVA (50 μ g, grade V; Sigma-Aldrich) and alum (2 mg), followed by six consecutive 2-day intranasal OVA challenges (100 μ g) at 12-day intervals and then a 4-week recovery, as described previously (13, 18, 24). Vaccine immunization was performed by four subcutaneous injections of 25 μ g of vaccine, as indicated in the protocols (Figures 1a and 4a). Mice receiving 25 μ g of carrier (HBcAg) or PBS (0.02 mol/L sodium phosphate buffer and 0.15 mol/L NaCl, pH 7.2) and normal mice served as controls.

Analysis of Airway and Lung-Tissue Mechanics

Airway reactivity was measured using a *flexiVent* small animal ventilator (Scireq Respiratory Equipment Inc., Montreal, PQ, Canada). As described previously (25), mice were anesthetized and received serial dilutions of methacholine, nebulized through a cannulae inserted in the trachea. Respiratory mechanics were assessed using a preset *flexiVent* Prime-8 low-frequency forced oscillation protocol.

Bronchoalveolar Lavage Fluid and Differential Cell Counts

After the cytospin of bronchoalveolar lavage fluid (BALF), the slides were stained with HEMA-3 (Fisher Diagnostics, Pittsburg, PA). Differential cell counts were performed according to standard hematological procedures. At least 400 cells were counted from each preparation.

Cytokines and Antibodies Measured by ELISA

The concentrations of cytokines (IL-13, IL-5, TGF- β , and IL-12p40) in BALF supernatants, serum OVA-specific IgE, and serum IL-13-specific IgG were assayed by ELISA, as described in previous reports (12) or according to the manufacturer's instructions (PharMingen, San Diego, CA).

Histological Assessment

Left lungs were fixed in formalin, and then embedded in paraffin and serially sectioned (5 μ m) sagittally at the anterior–posterior midline. Specimens were stained with hematoxylin and eosin to examine inflammation, with periodic acid–Schiff (PAS) to detect mucus production, or with Masson trichrome solution to detect collagen deposition. Semiquantitative analyses for peribronchiolar inflammation and airway goblet-cell hyperplasia were performed in a double-blinded fashion (10). The details of scoring systems are provided in the online supplement.

Soluble Collagen Assay

The total soluble collagen content of right lungs was determined using a Sircol Collagen Assay Kit (Accurate Chemical and Scientific–Biocolor, Westbury, NY), following the manufacturer's instructions.

Statistical Analysis

Differences between groups were assessed by unpaired Student *t* test. One-way ANOVA and then a Newman-Keuls multiple-comparisons test were performed when indicated (GraphPad Software, Inc., San Diego, CA). Values are reported as means $\pm$ standard errors. One symbol represents $P < 0.05$, two symbols represent $P < 0.01$, and three symbols represent $P < 0.001$. A pound sign represents an unpaired Student *t* test, and an asterisk represents one-way ANOVA in all figures, except for Figure 3a.

RESULTS

Preventive Experiments: IL-13 Vaccine Reduces Immune Cell Infiltration and Cytokine Accumulation in Airways, but Not IgE Concentrations

As in our previous reports, the results for mice receiving carrier and OVA and for mice receiving PBS and OVA were similar (12, 23). In the present study, emphasis was placed on analyzing the differences between mice receiving IL-13 vaccine and mice receiving carrier alone.

Vaccine immunization induced sustained, high-titer IL-13-specific IgG responses (Figure 1b). OVA-specific IgE responses were unaffected by vaccination at any of the time points assessed (Figure 1b). Differentiation cell counts in BALF were also performed. At time point B, vaccine immunization significantly suppressed both total cells and eosinophil accumulation, compared with carrier immunization (Figure 1c). At time point C, the total cell numbers in BALF in both groups reached a similar level, which was close to that in normal control mice, and eosinophils were rare in BALF cells, with the majority being monocytes. The results indicate that vaccine suppressed inflammatory cell infiltration of the airways, and after a 4-week recovery from the final challenge of OVA, inflammation naturally subsided, and thus no difference was evident between the vaccine and carrier groups.

The effects of preventative vaccine delivery on proinflammatory and profibrotic cytokines in BALF were also measured (Figure 1d). At time point A (early), OVA-induced IL-13 and IL-12p40 concentrations were significantly decreased, whereas TGF- β 1 was unaffected and, although numerically lower, the IL-5 concentration was not statistically different in vaccine-treated mice compared with the carrier group. Interestingly, at both later time points B and C, no significant differences were evident in cytokine concentrations between the two treatment groups, except that IL-13 remained significantly reduced in vaccinated mice.

Preventive Experiments: IL-13 Vaccine Diminishes Goblet-Cell Hyperplasia and Collagen Deposition

In formalin-fixed lungs, hematoxylin-and-eosin staining was performed to assess tissue inflammation (Figure 2a). In the carrier

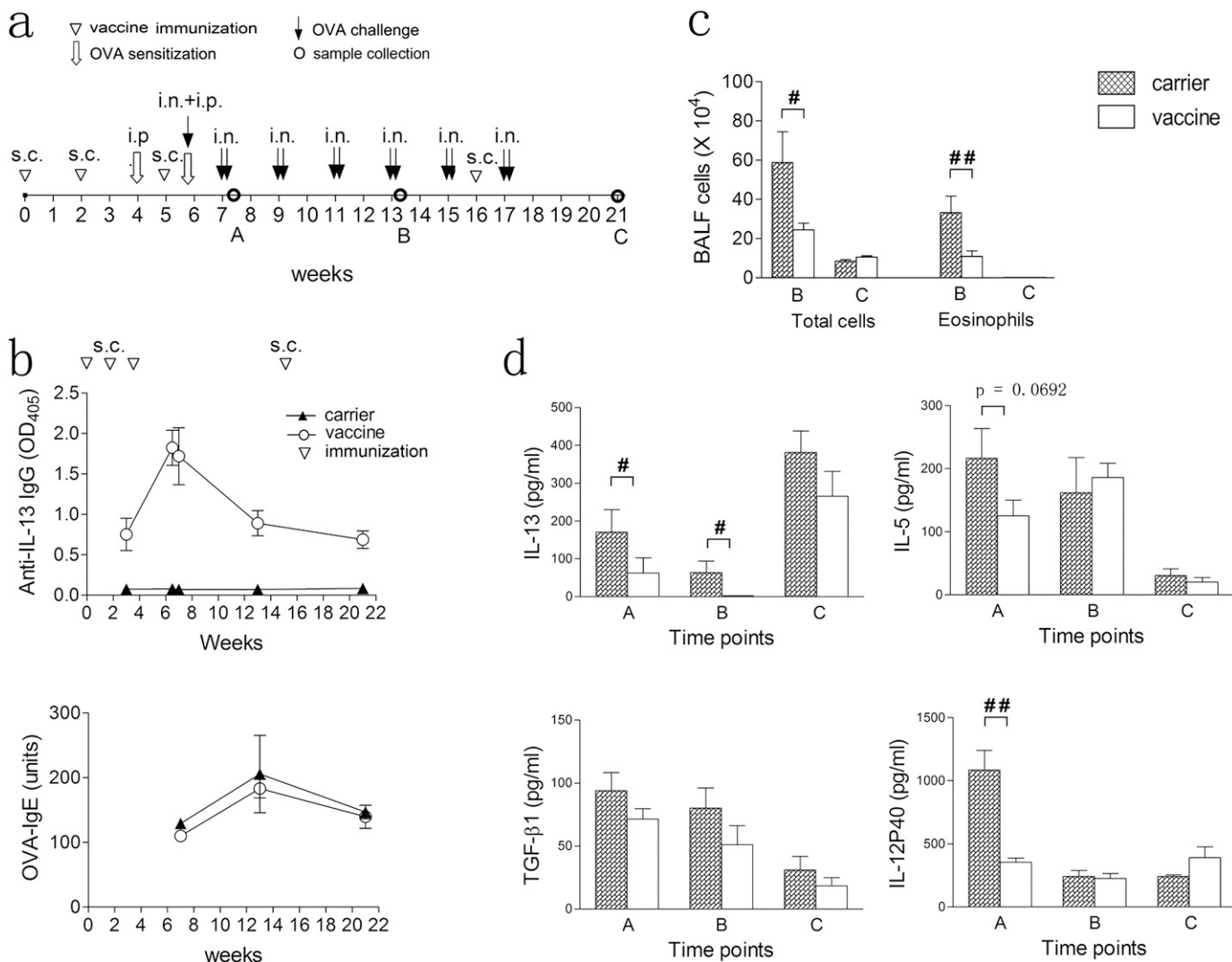


Figure 1. Preventive experiments. (a) Protocol of vaccine immunization and ovalbumin (OVA) sensitization and challenge. Mice were immunized with vaccine or carrier, and then sensitized/challenged with OVA ($n = 4$ /each group at time point A and B; $n = 8$ /each group at time point C). Subcutaneous (s.c.) vaccine immunizations were performed before OVA intraperitoneal (i.p.) sensitization/intranasal (i.n.) challenge commenced, as indicated by arrows. Mice were killed at time points A, B, and C. Another group of mice receiving PBS and OVA was included at time point A for the measurement of respiratory mechanics ($n = 4$). (b) Vaccine immunization elicits high concentrations of serum anti-IL-13 IgG, with no apparent difference in OVA-specific IgE concentrations between vaccine and carrier groups. (c) Vaccination suppresses the accumulation of total cells and eosinophils in bronchoalveolar lavage fluid (BALF) at time point B. (d) Vaccination markedly reduces the concentrations of IL-13, IL-12p40, and IL-5 in BALF at time point A. Unpaired Student *t* tests: * $P < 0.05$; ** $P < 0.01$.

group, repeated OVA challenges led to marked inflammatory cell infiltration (6 weeks after initiating repeated OVA challenges [time point B]). The majority of infiltrating cells were eosinophils and neutrophils (based on discernible morphological characteristics). Consistent with cell counts for BALF, after a 4-week recovery from OVA challenge (time point C), peribronchial and perivascular cell infiltration was significantly reduced. The majority of cells were mononuclear, whereas eosinophils and neutrophils were scarce. Compared with the carrier control mice, the inflammatory cell infiltration in the lung tissue of IL-13 vaccine-treated mice was slightly reduced at both time points studied (Figures 2a and 2b).

Airway goblet-cell abundance was analyzed on PAS-stained slides. Representative images and semiquantitative scoring analyses (Figures 2c and 2d) correlated well with lung tissue immune cell infiltration. In mice that received only carrier, repeated OVA challenges led to a marked increase in goblet cells (time

point B). After a 4-week recovery from OVA challenge (time point C), goblet-cell abundance returned to a low level. Hyperplasia persisted only in the main airways of the proximal lobar regions. Mice that received IL-13 vaccine showed markedly fewer goblet cells after allergen sensitization and repeated challenges at all time points, especially at time point B (Figures 2c and 2d).

Collagen deposition is an important feature of airway remodeling. Masson trichrome staining was used to visualize collagen distribution in the lung tissue, where collagen stained blue, and nuclei stained black with a red background. Trichrome staining revealed that significant collagen deposition was found in the airway subepithelial extracellular matrix in the carrier group (Figure 2e). By estimating the percentage of collagen-covered area, the IL-13 vaccine-treated group showed a reduced trend of this profibrotic response at all time points (data not shown). To make these observations more convincing, quantitative measurement was performed to detect the content of soluble collagen

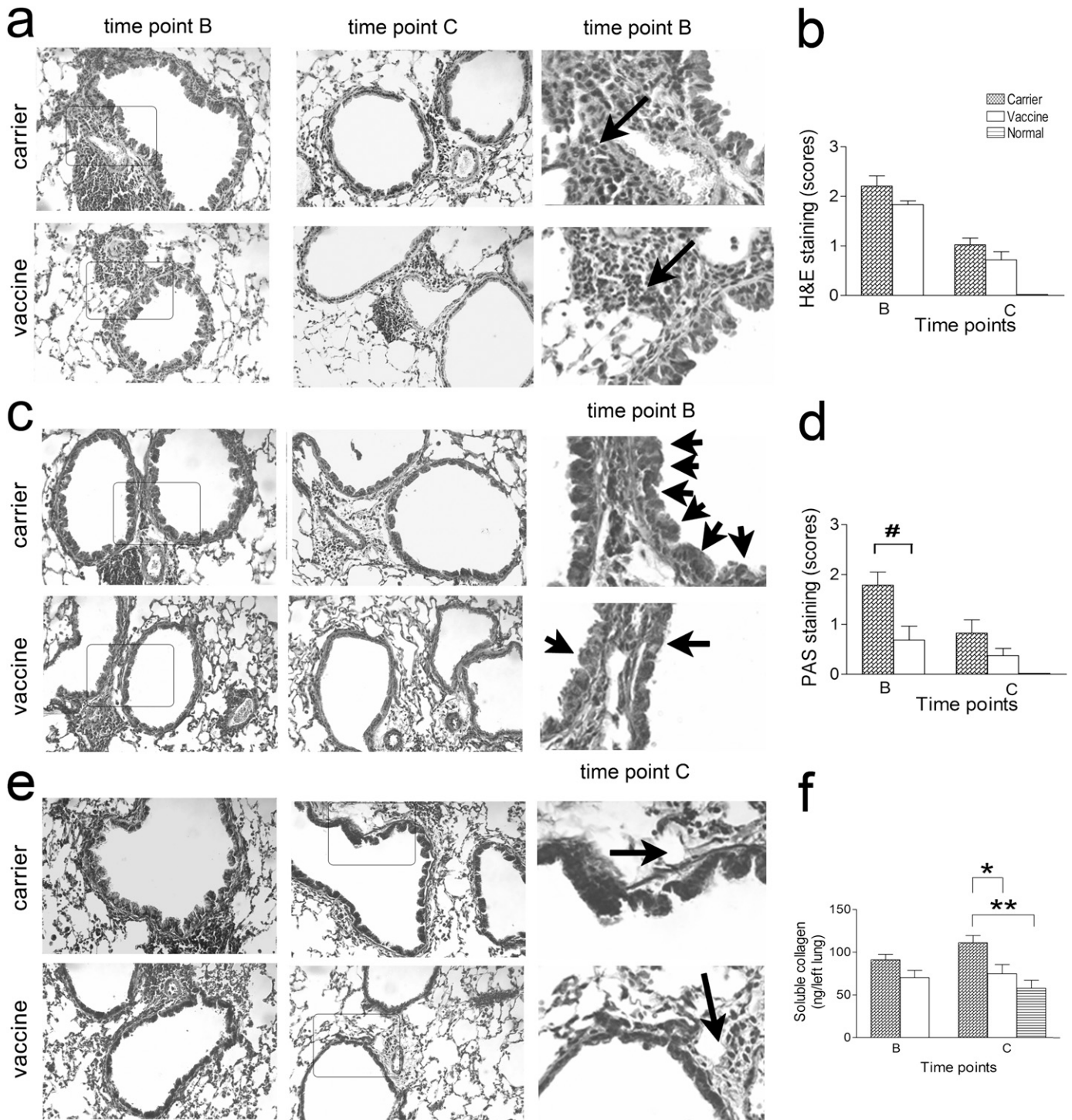


Figure 2. Histological analysis of the lung (preventive experiments). (a and b) Vaccine slightly suppresses lung inflammation. (a) Representative images stained with hematoxylin and eosin (H&E). The areas in frames were magnified and are shown at right. Arrowheads point to accumulated inflammatory cells that consist of infiltrated eosinophils and mononuclear cells. (b) Semiquantitative analysis. Peribronchiolar accumulation of inflammatory cells was assessed using an indexed scale. Scoring was examined in a double-blind fashion. (c and d) Vaccine significantly reduces goblet-cell hyperplasia. (c) Representative images stained with periodic acid-Schiff (PAS). The areas in frames were magnified and are shown at right. Arrowheads point to goblet cells within the respiratory epithelium. (d) Semiquantitative analysis. Goblet-cell abundance was measured as the percentage of PAS-positive cells in the total airway epithelia of medium-sized airways (90–150 μm in diameter). (e and f) Vaccine ameliorates fibrosis. (e) Representative images stained with Masson trichrome. The areas in frames were magnified and are shown at right. Arrowheads point to collagen fibers. (f) Soluble collagen assay. Unpaired Student *t* test, $^{\#}P < 0.05$. One-way ANOVA: $^*P < 0.05$; $^{**}P < 0.01$. Original magnification, $\times 200$.

(Figure 2f), which was significantly lower in vaccine-treated mice than in untreated mice ($P < 0.05$). Indeed, the collagen concentration in the vaccine group was close to that found in

normal mice at time point C (Figure 2f), indicating an almost complete prevention of collagen deposition after IL-13 vaccine delivery. According to our preliminary data (not shown), the

amount of soluble collagen in normal lungs was similar between time points B and C.

Preventive Experiments: IL-13 Vaccine Inhibits the Development of AHR

The primary parameters of respiratory mechanics that were measured included airway resistance (R_{aw}), tissue resistance (G), and tissue elastance (H). After the initial OVA challenge, the IL-13 vaccine group showed a significant inhibition in allergen-augmented tissue G and H (time point A, Figure 3a).

In the carrier group, after recovery from OVA challenge (time point C), all parameters were reduced compared with the earlier acute responses (time point A). In particular, tissue G and H were reduced (Figure 3b). Responses at time point C were reduced compared with time point A, but remained higher than in unchallenged mice, indicating sustained AHR, perhaps tied to airway remodeling because inflammation had already subsided. Vaccination caused reduced airway resistance, tissue G , and tissue H , in comparison with the carrier group (Figure 3b).

In summary, as shown in Figures 2 and 3, upon chronic exposure to allergen, obvious collagen deposition, goblet-cell hyperplasia, and increased airway and lung tissue resistance were found in mice receiving carrier. These characteristics were significantly reduced in mice treated with vaccine. These results clearly indicate that IL-13 vaccine is effective in attenuating the development of airway remodeling.

Interventional Experiments: IL-13 Vaccine Reverses Collagen Deposition and Mucus Production, but Not AHR

To investigate whether IL-13-specific IgG responses (induced after the establishment of acute allergic responses) can reverse chronic features of AHR and remodeling, we performed an interventional experiments, as outlined in Figure 4a. Specific

antibodies against IL-13 were induced and maintained at a high concentration throughout the experiments (Figure 4b). Total cell and eosinophil accumulation in BALF was reduced significantly at time point C, compared with that at time point B (Figure 4c). Compared with the carrier group, the total inflammatory cell number and the number of eosinophils in the vaccine group were slightly decreased (Figure 4c).

To assess the cytokine response and determine the impact of vaccine treatment, cytokine concentrations in BALF were measured at time points B and C. In chronic airway inflammation, TGF- β 1 and IL-5 concentrations were dramatically reduced at time point C, 4 weeks after OVA challenges were discontinued, compared with time point B, whereas IL-13 remained at a relatively high concentration (Figure 4d). The concentrations of IL-13, TGF- β 1, IL-12p40, and IL-5 in the vaccine-treated group were significantly lower than those in the carrier group.

Lung-tissue inflammation in both the carrier and vaccine groups, as assessed by hematoxylin-and-eosin staining, showed significant inflammatory cell infiltration around the bronchial and vascular trees at time point B. This infiltration was dramatically reduced but still present at time point C (Figure 5a). Semiquantitative analysis showed that no significant difference was evident (Figure 5b) between the vaccine and carrier groups, indicating that the vaccine failed to suppress inflammatory cell influx in the interventional experiments. Increased goblet-cell abundance, a feature of airway remodeling, was reversed in the vaccine group compared with the carrier group (Figures 5c and 5d). Extracellular matrix deposition, another feature of airway remodeling, was examined by trichrome staining and a soluble collagen assay. As shown in Figure 5e, repeated OVA challenges induced significant extracellular matrix deposition around the bronchial and vascular tracts at time point B. This was also seen at time point C, 4 weeks after discontinuing the OVA challenges. The vaccine intervention significantly decreased collagen deposition in comparison to

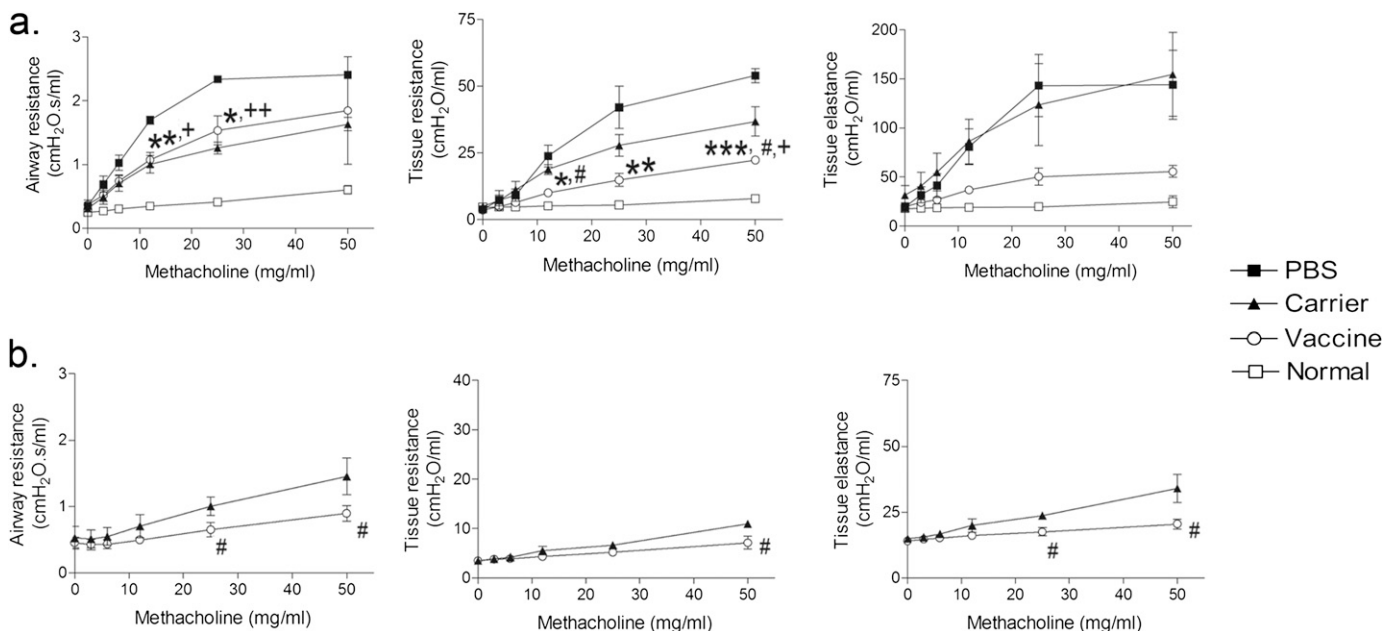


Figure 3. Respiratory mechanic responses to inhaled methacholine, analyzed by using low-frequency forced oscillation with a small animal ventilator (preventive experiments). (a) Vaccine significantly suppresses tissue resistance and elastance of the lung during the acute inflammatory phase (time point A). One-way ANOVA, followed by the Newman-Keuls multiple-comparison tests, was performed. *Between vaccine and PBS. #Between vaccine and carrier. +Between vaccine and normal. (b) Immunization with vaccine significantly suppresses sustained airway hyperreactivity at 4 weeks after the final OVA challenge (time point C). Respiratory mechanics were assessed by calculating airway resistance, peripheral tissue and airway resistance, and tissue elastance or stiffness, allowing the lung mechanics to be divided into central and peripheral components.

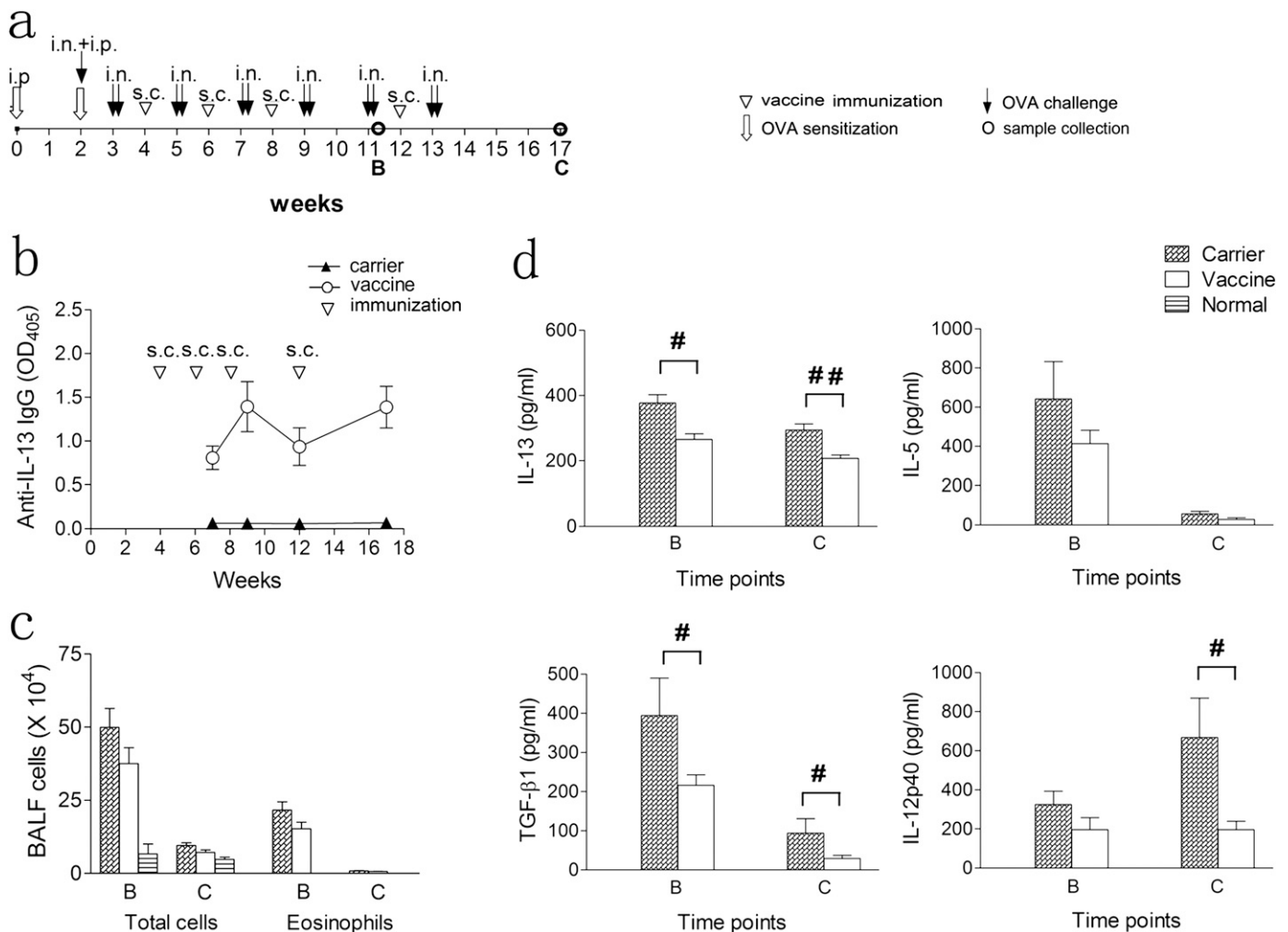


Figure 4. Interventional experiments. (a) Protocol of vaccine immunization and OVA sensitization/challenge. Mice receiving OVA were subsequently injected with vaccine or carrier ($n = 4/\text{group}$ at time point B; $n = 8/\text{group}$ at time point C). Three groups of mice ($n = 8\text{--}12/\text{group}$) were included and killed at time points B and C. OVA sensitization began during Week 0, and vaccinations were administered during Weeks 4, 6, 8, and 12. (b) Vaccine immunization elicits high concentrations of serum anti-IL-13 IgG. (c) Vaccination leads to a slight reduction in total cell and eosinophil accumulations in BALF at time point B. (d) Vaccination significantly decreases IL-13, TGF- β 1, and IL-12p40 concentrations in BALF, and shows a reduced trend for IL-5 concentrations. * $P < 0.05$, ** $P < 0.01$, unpaired Student t test.

the carrier group when analyzed by quantitative soluble collagen assay (Figure 5f).

To assess the functional impact of these biomarkers of airway remodeling, airway and lung tissue mechanics were measured at time point C, when acute inflammatory responses had declined (Figure 6). We observed significantly lower R_{aw} , with especially noticeable decreases for tissue G and H , in both groups when compared with those measured during the acute phase (see Figure 3a). Unlike the data obtained in the preventive experiments, no difference was evident between the vaccine and carrier groups in the interventional experiments.

DISCUSSION

In studies where IL-13 has been neutralized using monoclonal antibodies (mAbs) in murine models of chronic airway inflammation, outcomes have generally been examined immediately after the OVA challenge, when acute inflammation is most severe (14, 16–18). IL-13 neutralization by mAbs can reduce inflammation and ameliorate AHR. However, because such data are obtained during the early stages of inflammation, the results do not reflect the possible contributions of IL-13 to airway

remodeling or the impact of airway remodeling on airway physiology. Furthermore, in these studies, the mAb-mediated inhibition of IL-13 is temporary, and thus the effects may be limited. In the present study, a murine model with chronic exposure to allergen was used, in which sustained AHR and airway remodeling developed. In addition, a novel strategy involving a vaccine to generate long-lasting anti-IL-13 antibodies to block IL-13 activity was used. Thus, this study more directly assessed the role of IL-13 in the development and maintenance of features associated with chronic asthma. In our experiments, the only difference between the carrier and vaccine groups involved the inserted IL-13 epitope in the vaccine. Thus, comparisons between these two groups reflect the specificity of anti-IL-13 vaccine effectiveness. In the prevention study, vaccinated mice exhibited significant improvement in most features of chronic airway inflammation, including inflammatory cell accumulation and cytokine concentrations in BALF, goblet-cell hyperplasia, collagen deposition, and AHR when compared with the carrier group. In the interventional experiments, vaccine treatment appeared less effective than in the preventive experiments. In the interventional study, vaccination decreased IL-13, TGF- β 1, and IL-12p40 concentrations in BALF, as well as mucus

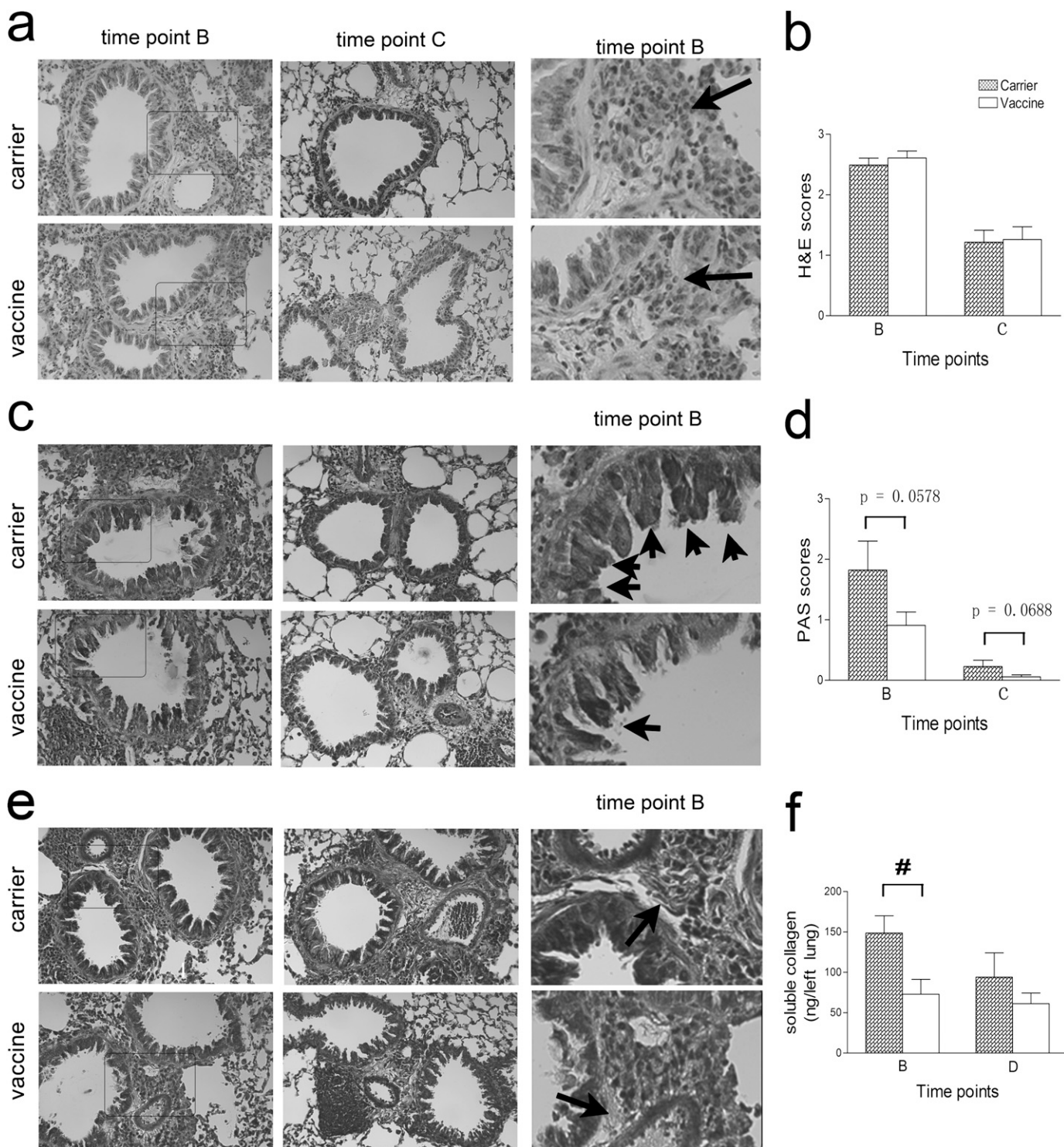


Figure 5. Histological analysis of the lung (interventional experiments). (a and b) No change is apparent in the vaccine group when compared with the carrier control group in inflammation analyses. (a) Representative images stained with hematoxylin and eosin for inflammation. (b) Semiquantitative analysis. (c and d) The vaccine group shows a trend toward reduced goblet-cell hyperplasia. (c) Representative images stained with PAS for mucus production. (d) Semiquantitative analysis. (e and f) Vaccination significantly decreases collagen deposition at time point B. (e) Representative images stained with Masson trichrome for collagen deposition. (f) Semiquantitative analysis. $^{\#}P < 0.05$ in an unpaired Student *t* test. Original magnification, $\times 200$.

production and collagen deposition, but chronic inflammation and IL-5 in BALF and sustained AHR were not changed significantly.

Our results from both the preventive and interventional experiments showed that although the vaccine significantly

suppressed the accumulation of goblet cells, it only slightly reduced the influx of inflammatory cells to the lung, indicating that neutralizing IL-13 was more efficient on the control of mucus overproduction than on cell influx. This result may be explained by the finding that IL-13 is more critical in the development of

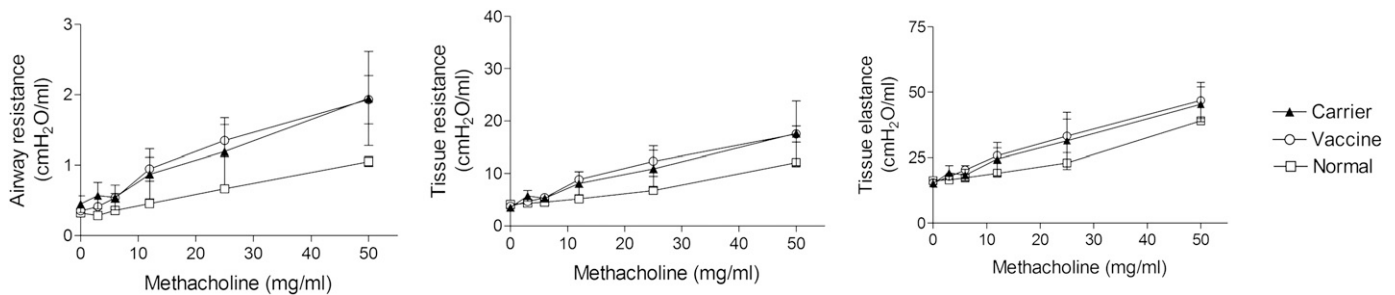


Figure 6. Respiratory mechanic responses to inhaled methacholine, analyzed by using low-frequency forced oscillation with a small animal ventilator (interventional experiments). Four weeks after the final OVA challenge (time point C), mice in both the carrier and vaccine groups showed clearly elevated airway resistance, tissue resistance, and tissue elastance compared with the normal group. However, no difference was evident between the vaccine and carrier groups.

goblet-cell hyperplasia. IL-13, but not IL-4 and IL-5, was reported to be indispensable for this response (26, 27), whereas cytokines other than IL-13 also significantly contributed to other aspects involved in control of the development and maintenance of inflammation. Our previous study reported significantly reduced OVA-specific IgE and IL-5 concentrations after IL-13 vaccination (12). The unchanged IgE and IL-5 concentrations in the present study may have been caused by changes in the protocol. In the present study, OVA doses were higher, the interval between OVA intraperitoneal sensitization and intranasal challenge was shorter, vaccine doses were lower, and OVA sensitization/challenge commenced earlier than in the previous study (12). These changes may have induced more severe airway inflammation, resulting in less IgE and IL-5 inhibition.

We expected that the vaccine targeting IL-13 would suppress the lung fibrosis induced by chronic OVA exposure. The impact of IL-13 on fibrotic responses was well described in a previous review (28). IL-13 induces the activation of fibroblasts into myofibroblasts and their proliferation (29, 30), stimulates the gene expression of extracellular matrix (such as collagen), and increases the expression of TGF- β 1, which is a key fibrotic mediator (31, 32). Notably, Kaviratne and colleagues (33) demonstrated that an intraperitoneal injection of recombinant IL-13 in TGF- β 1-deficient mice leads to the induction of several repair-associated genes, including extracellular matrix proteins, tissue inhibitor of metalloproteinase 1, and various matrix metalloproteinases to concentrations equivalent to those observed in IL-13-injected wild-type mice. They also showed that, in an animal model of *Schistosoma*-induced hepatic fibrosis, IL-13 can activate a mechanism of tissue fibrosis that is independent of TGF- β 1 to drive Th2-associated reparative/fibrotic responses (33). The anti-IL-13 neutralizing antibody treatment of IL-13-deficient mice in chronic OVA challenge models is protective against the increase in subepithelial collagen deposition (13–15, 34). On the other hand, constitutive IL-13 overexpression in the lungs of naive mice leads to the development of subepithelial fibrosis (35). To support these findings, IL-13 and TGF- β 1 concentrations in the present study were suppressed in both preventive and interventional experiments, and accordingly collagen deposition was also reduced, although the suppression of TGF- β 1 production in the preventive experiments was not statistically significant. The different reductions of TGF- β 1 production in the two sets of experiments may be attributable to the relatively low concentrations of anti-IL-13 antibodies in the preventive experiments at time points B and C (measured at 8 and 16 weeks, respectively, after the third immunization) than those in the interventional experiments (measured 3 and 9 weeks, respectively, after the third immunization). Because IL-13 stimulates TGF- β 1 expression (33), IL-13 suppression may directly

contribute to reduced TGF- β 1 concentrations. Other parameters such as eosinophils, lung inflammation, and AHR may also be significantly affected by other inflammatory factors such as IL-5. This may explain why the significant suppression of TGF- β 1, but less inhibition of inflammation and BALF cells, was observed in the interventional experiments.

We used the forced oscillation technique with a *flexiVent* small animal ventilator to measure lung mechanic responses to inhaled methacholine challenges. The forced oscillation technique partitions “Zrs” (respiratory impedance) into airway and tissue mechanics, allowing for the detection of more subtle changes than total lung or respiratory system impedance. “R_{aw}” reflects the central component of lung mechanics. “G” reflects the parenchymal distortion that occurs when the airways constrict, and as with “H,” “G” increases with an elevated degree of regional airflow heterogeneity throughout the lung. “H” increases because of airway closure and increased intrinsic tissue stiffness, which can result from the distortion of the parenchyma as the airways narrow, and from the development of regional heterogeneities in lung tissue (36). In the present report, in the preventive experiments, the vaccine significantly suppressed tissue resistance and tissue elastance (Figure 3a) at the acute inflammation checkpoint (time point A), which reflects effects on peripheral lung components. This finding is consistent with data reported in previous studies (36–38), which showed that AHR in OVA-inflamed mice is entirely attributable to the exaggerated closure of peripheral and small airways. The significantly reduced lung mechanic responses to inhaled methacholine challenges at time point A correspond to other results obtained in the study, such as the decrease in inflammation, goblet-cell hyperplasia, and airway remodeling present mainly around the major airways. Four weeks after the discontinuation of OVA challenge (time point C), mice showed a decreased yet relatively high level of airway resistance, along with dramatically decreased tissue resistance and elastance, compared with those measured during the acute phase. At this time point, vaccinated mice showed a clear suppression of airway resistance, tissue resistance, and tissue elastance (Figure 3b). On the other hand, in the interventional experiments, vaccination failed to induce a decrease of AHR (as assessed by airway resistance, tissue resistance, and tissue elastance) compared with carrier group (Figure 6). These results indicate that IL-13 suppression during the early phase is more effective than suppression during the late phase of chronic inflammation.

A recent clinical study has shown that the administration of lebrikizumab (mAb against human IL-13) improves lung function in patients with uncontrolled asthma, and that the effect is most significant in patients with increased concentrations of circulating periostin and in patients exhibiting a high Th2

phenotype (39). The data suggest that further studies, to be more informative, may need to establish stronger Th2-type responses during the chronic phases of this model. As in the present study, repeated OVA challenges in mice induced some degree of immune tolerance and dramatically reduced inflammatory responses to allergen stimulation, which may have dimmed the effects of the IL-13 vaccine in the interventional experiments. Moreover, based on the efficacy of anti-IL-13 antibody therapy in adults, the vaccinated mice may be fully or partly protected when IL-13 again becomes more important for the induction of inflammation. Thus, in our forthcoming study, after the Week 13 challenge before another round of challenges, a longer break of up to 5 to 6 weeks will be implemented to allow for the restoration of inflammation and reconstitution of an IL-13 effect, as seen in seasonal asthma.

We have explored the effectiveness of the sustained suppression of IL-13 by using a vaccine in a murine model that exhibits features of AHR and remodeling, in which airway and lung-tissue mechanics were appropriately measured by using a *flexiVent* small animal ventilator. Our results indicate that, based on a preventive strategy, IL-13 vaccination could suppress the development of chronic OVA exposure-induced airway remodeling features such as mucus overproduction, subepithelial collagen deposition, and AHR. However, this strategy was less effective after sustained AHR and remodeling were established, as shown in the interventional experiments. This indicates the critical need for such studies in model systems of chronic inflammation, before considering human interventional studies, because the majority of asthma cases begin in childhood, with subbasement membrane collagen deposition completed by age 10 years (40).

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References

- Durrani SR, Viswanathan RK, Busse WW. What effect does asthma treatment have on airway remodeling? Current perspectives. *J Allergy Clin Immunol* 2011;128:439–448.
- Lloyd CM, Robinson DS. Allergen-induced airway remodelling. *Eur Respir J* 2007;29:1020–1032.
- Wills-karp M. Interleukin-13 in asthma pathogenesis. *Immunol Rev* 2004;202:175–190.
- Oh CK, Geba GP, Molfino N. Investigational therapeutics targeting the IL-4/IL-13/STAT-6 pathway for the treatment of asthma. *Eur Respir Rev* 2010;19:46–54.
- Lewis CC, Aronow B, Hutton J, Santeliz J, Dienger K, Herman N, Finkelman FD, Wills-Karp M. Unique and overlapping gene expression patterns driven by IL-4 and IL-13 in the mouse lung. *J Allergy Clin Immunol* 2009;123:795–804.
- Nguyen TH, Casale TB. Immune modulation for treatment of allergic disease. *Immunol Rev* 2011;242:258–271.
- Nakano T, Inoue H, Fukuyama S, Matsumoto K, Matsumura M, Tsuda M, Matsumoto T, Aizawa H, Nakanishi Y. Niflumic acid suppresses interleukin-13-induced asthma phenotypes. *Am J Respir Crit Care Med* 2006;173:1216–1221.
- Kibe A, Inoue H, Fukuyama S, Machida K, Matsumoto K, Koto H, Ikegami T, Aizawa H, Hara N. Differential regulation by glucocorticoid of interleukin-13-induced eosinophilia, hyperresponsiveness, and goblet cell hyperplasia in mouse airways. *Am J Respir Crit Care Med* 2003;167:50–56.
- Fulkerson PC, Fischetti CA, Hassman LM, Nikolaidis NM, Rothenberg ME. Persistent effects induced by IL-13 in the lung. *Am J Respir Cell Mol Biol* 2006;35:337–346.
- Ford JG, Rennick D, Donaldson DD, Venkayya R, McArthur C, Hansell E, Kurup VP, Warnock M, Grunig G. IL-13 and IFN- γ : interactions in lung inflammation. *J Immunol* 2001;167:1769–1777.
- Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998;282:2261–2263.
- Ma Y, Hayglass KT, Becker AB, Fan Y, Yang X, Basu S, Srinivasan G, Simons FE, Halayko AJ, Peng Z. Novel recombinant interleukin-13 peptide-based vaccine reduces airway allergic inflammatory responses in mice. *Am J Respir Crit Care Med* 2007;176:439–445.
- Leigh R, Ellis R, Wattie JN, Hirota JA, Matthaie KI, Foster PS, O'Byrne PM, Inman MD. Type 2 cytokines in the pathogenesis of sustained airway dysfunction and airway remodeling in mice. *Am J Respir Crit Care Med* 2004;169:860–867.
- Yang G, Volk A, Petley T, Emmell E, Giles-Komar J, Shang X, Li J, Das AM, Shealy D, Griswold DE, et al. Anti-IL-13 monoclonal antibody inhibits airway hyperresponsiveness, inflammation and airway remodeling. *Cytokine* 2004;28:224–232.
- Kumar RK, Herbert C, Yang M, Koskinen AM, McKenzie AN, Foster PS. Role of interleukin-13 in eosinophil accumulation and airway remodelling in a mouse model of chronic asthma. *Clin Exp Allergy* 2002;32:1104–1111.
- Yang G, Li L, Volk A, Emmell E, Petley T, Giles-Komar J, Rafferty P, Lakshminarayanan M, Griswold DE, Bugelski PJ, et al. Therapeutic dosing with anti-interleukin-13 monoclonal antibody inhibits asthma progression in mice. *J Pharmacol Exp Ther* 2005;313:8–15.
- Kumar RK, Herbert C, Webb DC, Li L, Foster PS. Effects of anti-cytokine therapy in a mouse model of chronic asthma. *Am J Respir Crit Care Med* 2004;170:1043–1048.
- Leigh R, Ellis R, Wattie J, Donaldson DD, Inman MD. Is interleukin-13 critical in maintaining airway hyperresponsiveness in allergen-challenged mice? *Am J Respir Crit Care Med* 2004;170:851–856.
- Spohn G, Keller I, Beck M, Grest P, Jennings GT, Bachmann MF. Active immunization with IL-1 displayed on virus-like particles protects from autoimmune arthritis. *Eur J Immunol* 2008;38:877–887.
- Guan QD, Ma Y, Hillman C-L, Qing G, Ma AG, Weiss CR, Zhou G, Bai A, Warrington R, Bernstein CN, et al. Targeting IL-12/IL-23 by employing a p40 peptide-based vaccine ameliorates TNBS-induced acute and chronic murine colitis. *Mol Med* 2011;17:646–656.
- Guan Q, Ma Y, Aboud L, Weiss CR, Qing G, Warrington RJ, Peng Z. Targeting IL-23 by employing a p40 peptide-based vaccine ameliorates murine allergic skin and airway inflammation. *Clin Exp Allergy* 2012;42:1397–1405.
- Ma Y, Guan Q, Bai A, Weiss CR, Hillman C, Ma AG, Zhou G, Qing G, Peng Z. Targeting TGF- β 1 by employing a vaccine ameliorates fibrosis in a mouse model of chronic colitis. *Inflamm Bowel Dis* 2010;16:1040–1050.
- Ma Y, HayGlass KT, Becker AB, Halayko AJ, Basu S, Simons FER, Peng Z. Novel cytokine peptide-based vaccines: an interleukin-4 vaccine suppresses airway allergic responses in mice. *Allergy* 2007;62:675–682.
- Leigh R, Ellis R, Wattie J, Southam DS, De Hoogh M, Gaudie J, O'Byrne PM, Inman MD. Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. *Am J Respir Cell Mol Biol* 2002;27:526–535.
- Kadkhoda K, Wang S, Fan Y, Qiu H, Basu S, Halayko AJ, Yang X. ICOS ligand expression is essential for allergen-induced airway hyperresponsiveness. *Int Immunol* 2011;23:239–249.
- Webb DC, McKenzie AN, Koskinen AM, Yang M, Mattes J, Foster PS. Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. *J Immunol* 2000;165:108–113.
- Whittaker L, Niu N, Temann UA, Stoddard A, Flavell RA, Ray A, Homer RJ, Cohn L. Interleukin-13 mediates a fundamental pathway for airway epithelial mucus induced by CD4 T cells and interleukin-9. *Am J Respir Cell Mol Biol* 2002;27:593–602.
- Fattouh R, Jordana M. TGF- β , eosinophils and IL-13 in allergic airway remodeling: a critical appraisal with therapeutic considerations. *Inflamm Allergy Drug Targets* 2008;7:224–236.
- Saito A, Okazaki H, Sugawara I, Yamamoto K, Takizawa H. Potential action of IL-4 and IL-13 as fibrogenic factors on lung fibroblasts *in vitro*. *Int Arch Allergy Immunol* 2003;132:168–176.
- Ingram JL, Rice A, Geisenhoffer K, Madtes DK, Bonner JC. Interleukin-13 stimulates the proliferation of lung myofibroblasts via a signal transducer and activator of transcription-6-dependent mechanism: a possible mechanism for the development of airway fibrosis in asthma. *Chest* 2003;123:422S–424S.

31. Doucet C, Brouty-Boye D, Pottin-Clemenceau C, Jasmin C, Canonica GW, Azzarone B. IL-4 and IL-13 specifically increase adhesion molecule and inflammatory cytokine expression in human lung fibroblasts. *Int Immunol* 1998;10:1421–1433.
32. Lindemann D, Racke K. Glucocorticoid inhibition of interleukin-4 (IL-4) and interleukin-13 (IL-13) induced up-regulation of arginase in rat airway fibroblasts. *Naunyn Schmiedeberg Arch Pharmacol* 2003;368:546–550.
33. Kaviratne M, Hesse M, Leusink M, Cheever AW, Davies SJ, McKerrow JH, Wakefield LM, Letterio JJ, Wynn TA. IL-13 activates a mechanism of tissue fibrosis that is completely TGF-beta independent. *J Immunol* 2004;173:4020–4029.
34. Blease K, Jakubzick C, Westwick J, Lukacs N, Kunkel SL, Hogaboam CM. Therapeutic effect of IL-13 immunoneutralization during chronic experimental fungal asthma. *J Immunol* 2001;166:5219–5224.
35. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, Elias JA. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 1999;103:779–788.
36. Wagers SS, Haverkamp HC, Bates JH, Norton RJ, Thompson-Figueroa JA, Sullivan MJ, Irvin CG. Intrinsic and antigen-induced airway hyperresponsiveness are the result of diverse physiological mechanisms. *J Appl Physiol* 2007;102:221–230.
37. Wagers SS, Norton RJ, Rinaldi LM, Bates JH, Sobel BE, Irvin CG. Extravascular fibrin, plasminogen activator, plasminogen activator inhibitors, and airway hyperresponsiveness. *J Clin Invest* 2004;114:104–111.
38. Lundblad LK, Thompson-Figueroa J, Allen GB, Rinaldi L, Norton RJ, Irvin CG, Bates JH. Airway hyperresponsiveness in allergically inflamed mice: the role of airway closure. *Am J Respir Crit Care Med* 2007;175:768–774.
39. Corren J, Lemanske RF, Hanania NA, Phillip E, Korenblat PE, Parsey MV, Arron JR, Harris JM, Scheerens H, Wu LC, *et al.* Lebrikizumab treatment in adults with asthma. *N Engl J Med* 2011;365:1088–1098.
40. Payne DN, Rogers AV, Adelroth E, Bandi V, Guntupalli KK, Bush A, Jeffery PK. Early thickening of the reticular basement membrane in children with difficult asthma. *Am J Respir Crit Care Med* 2003;167:78–82.

Sustained Suppression of IL-13 by a Vaccine Attenuates Airway Inflammation and Remodeling in Mice

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Online supplement

METHODS

Histological Assessment

Left lungs were carefully separated and fixed in 10% PBS-buffered formalin, then embedded in paraffin and serially sectioned (5 μ m) sagittally at the anterior-posterior midline. Specimens were stained with Hematoxylin and Eosin (H&E) to examine inflammation, periodic acid Schiff (PAS) to detect mucus production or Masson's trichrome solution to detect collagen deposition. Slides were then examined using an Olympus IX51 light microscope equipped with CCD camera under the control of Image Pro Plus software (Media Cybernetics, Inc.). Semi-quantitative analyses for peribronchiolar inflammation and airway goblet cell hyperplasia were performed double-blinded (10). At least three separate sections and four fields in each section were evaluated in 200 $\times$ microscopic magnifications per mouse. Only medium-sized airways (0.5 mm to 1.5 mm diameter) were assessed. Each airway was assigned a value for inflammatory cell infiltration as follows: 0 = normal; 1 = few cells; 2 = a ring of inflammatory cells one cell layer deep; 3 = a ring of inflammatory cells 2–4 cells deep; 4 = a ring of inflammatory cells of >4 cells deep. PAS-stained sections were used to analyze mucosa production semi-quantitatively (10). The scores for the abundance of goblet cells in each airway were determined by the ratio of PAS-positive cells in total epithelial cells as follows: 0: <5% goblet cells; 1: 5–25%; 2: 25–50%; 3: 50–75%; 4: >75%. Each lung was assigned a score calculated by total scores of counted airways/airway numbers.