

Novel Recombinant Interleukin-13 Peptide-based Vaccine Reduces Airway Allergic Inflammatory Responses in Mice

Yanbing Ma^{1,2}, Kent T. HayGlass^{1,3}, Allan B. Becker^{1,2,3}, Yijun Fan⁴, Xi Yang^{3,4}, Sujata Basu^{2,5}, Ganesh Srinivasan^{1,2}, F. Estelle R. Simons^{1,3}, Andrew J. Halayko^{1,2,5}, and Zhikang Peng^{1,2,3}

¹Department of Pediatrics and Child Health, University of Manitoba, Winnipeg, Manitoba, Canada; ²Biology of Breathing Theme, Manitoba Institute of Child Health, Winnipeg, Manitoba, Canada; ³Department of Immunology, ⁴Department of Medical Microbiology, and ⁵Department of Physiology, University of Manitoba, Winnipeg, Manitoba, Canada

Rationale: Interleukin (IL)-13 plays a pivotal role in the pathogenesis of allergic asthma. Passive administration of its monoclonal antibody or soluble receptor to block overproduced IL-13 has been proven to be effective in controlling airway allergic responses in animal models, but these approaches have disadvantages of short half-lives, high costs, and possible adverse effects.

Objectives: We sought to develop a novel therapeutic strategy through constructing an IL-13 peptide-based vaccine for blocking IL-13 on a persistent effect basis and to evaluate its *in vivo* effects using a murine model.

Methods: To break self-tolerance, truncated hepatitis B core antigen was used as a carrier. Vaccine was prepared by inserting a peptide derived from the receptor binding site of mouse IL-13 into the immunodominant epitope region of the carrier using gene recombination methods. Mice received vaccine subcutaneously three times, and then subjected to intraperitoneal sensitization and intranasal challenge with ovalbumin. Control animals received carrier or saline in place of vaccine.

Measurements and Main Results: The vaccine presented as virus-like particles and induced sustained and high titered IL-13-specific IgG without the use of conventional adjuvant. Vaccination significantly suppressed ovalbumin-induced inflammatory cell number, and IL-13 and IL-5 levels in bronchoalveolar lavage fluids. Serum total and ovalbumin-specific IgE were also significantly inhibited. Moreover, allergen-induced goblet cell hyperplasia, lung tissue inflammatory cell infiltration, and pulmonary hyperresponsiveness to inhaled methacholine were significantly suppressed in vaccinated mice.

Conclusions: Our data indicate that IL-13 peptide-based vaccines could be an effective therapeutic approach in the treatment of asthma.

Keywords: interleukin-13; vaccine; airway allergic responses; Th2 cytokine; asthma

Interleukin (IL)-13 is a critical mediator in the development and maintenance of allergic asthma (1). Polymorphisms of IL-13 gene are associated with increased asthma susceptibility (2). Direct administration of recombinant IL-13 to the airways (3, 4) or its overexpression in the lungs of transgenic mice (5) leads to airway inflammation and hyperresponsiveness, goblet cell hyperplasia, and subepithelial fibrosis. IL-13 blockade with soluble receptor

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Interleukin (IL)-13 is a key mediator in asthma. To date, the neutralizing of excessive endogenous IL-13, using a vaccine, has not been reported.

What This Study Adds to the Field

Administration of a recombinant virus-like IL-13 peptide-based vaccine effectively suppresses airway allergic responses in mice, suggesting a novel approach for long-term treatment of asthma.

$\alpha 2$ (6) or neutralizing antibodies (7) reduces or even abolishes such responses in animal models of asthma. Given its central role in asthma, IL-13 presents an attractive target for new treatment options.

A variety of monoclonal antibodies (mAb) and soluble receptors have been developed to neutralize the overproduced endogenous cytokines associated with severe disease (8). However, in humans these approaches are hampered by the requirement for frequent high-dose injections, as positive effects are lost rapidly after treatment discontinuation. For instance, 200 to 400 mg of mAb against human immunoglobulin (Ig)E is required every 2 to 4 weeks (9). Soluble receptors of tumor necrosis factor (TNF)- α (10) or IL-4 (11) must also be administered weekly or bi-weekly. Such passively administered therapies are inconvenient and costly. In addition, infusion reactions occurred in 17% of patients treated with mAb against TNF- α (Infliximab) (12). Antibodies against therapeutic mAbs, which reduce the effectiveness of the treatment, can also be induced, as reported in 28 to 68% of patients receiving Infliximab therapy (12).

To overcome these disadvantages, a new strategy that may offer long-term efficacy with fewer adverse effects is being investigated, in which auto-antibodies against self-cytokines are induced using vaccines. Based on providing effective T cell help, two broad experimental strategies have been used to design such vaccines. One aims to modify the intact self-protein by inserting a foreign peptide containing Th epitopes (13). The second aims to link the intact self-protein or a considerable part thereof to an heterologous carrier protein (14). Polyclonal antibodies induced by such vaccines can have excellent neutralizing capacity, but because they are raised against multiple self-antigen determinants, their use may be hindered by undesirable cross-reactions with other self-proteins that contain similar epitopes. This is a particular concern when this strategy is used in humans. Furthermore, for induction of high titers of auto-antibody with such vaccines, the use of adjuvant has

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Correspondence and requests for reprints should be addressed to Dr. Zhikang Peng, Department of Pediatrics and Child Health, University of Manitoba, 532-715 McDermot Avenue, Winnipeg, MB, R3E 3P4 Canada. E-mail: zpeng@ms.umanitoba.ca

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always been required (13). These issues largely limit their application, efficacy and safety.

In an effort to overcome the inherent disadvantages of the above therapeutic approaches, we designed an IL-13 peptide-based vaccine and tested its *in vivo* effect in the down-regulation of airway allergic inflammatory responses in a murine model of asthma. Some of the results of these studies have been previously reported in the form of an abstract (15).

METHODS

Selection of Antigenic Peptides

Antigenic peptide prediction was performed based on the occurrence of amino acid residues in experimentally known segmental epitopes (<http://bio.dfci.harvard.edu/Tools/antigenic.html>) and the hydrophilicity/hydrophobicity analyses of amino acids (<http://arbl.cvmbs.colostate.edu/molkit/hydropathy/index.html>). Using the information from the high-resolution solution structure of IL-13 (16), priority was given to the peptide sequences located in the receptor binding sites and/or at the terminal regions.

Preparation and Identification of Recombinant Vaccine

Two plasmids were prepared: (1) plasmid HBcAg, which expressed "carrier" composed of truncated hepatitis B virus core antigen (HBcAg) (amino acids 1–149); and (2) plasmid HBcAg-La, which expressed "vaccine" consisting of a fusion protein with the selected IL-13 peptide inserted between amino acids 78 and 79 of the immunodominant epitope of HBcAg. Recombinant protein was expressed using *Escherichia coli* DH5 α cells, purified using ultrasonic lysis, 40% ammonium sulfate precipitation, ultracentrifugation with 10 to 40% sucrose density gradient, and chromatography with Sepharose CL-4B (Sigma-Aldrich, St. Louis, MO), and identified by SDS-PAGE and immunoblotting using a polyclonal goat anti-mouse IL-13 antibody (R&D Systems Inc., Minneapolis, MN).

Animals

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

Immunization, Sensitization, and Challenge

In the IL-13-specific IgG time course study (Figure 1A), mice were immunized subcutaneously with 100 μ g vaccine with or without adjuvant. In the asthma study (Figure 1B), four groups of mice were included: (1) vaccine (n = 12): immunized subcutaneously with 100 μ g vaccine (HBcAg-La), then subjected to intraperitoneal sensitization and intranasal challenge with ovalbumin (OVA) (grade IV; Sigma-Aldrich); (2) carrier (n = 12): immunized with 100 μ g carrier (HBcAg)

and sensitized/challenged with OVA; (3) phosphate-buffered saline (PBS) (n = 4): injected with PBS (0.02 mol/L sodium phosphate buffer, 0.15 mol/L NaCl, pH 7.2) and sensitized/challenged with OVA; and (4) normal (n = 4): injected with PBS and no exposure to OVA.

Analysis of Breathing Response to Inhaled Methacholine

Changes in work of breathing in response to inhaled methacholine were measured in unrestrained mice using whole body plethysmography (model PLY 3211; Buxco Electronics, Troy, NY) (17–19). The percent increase in enhanced paused (Penh) above a saline challenge baseline value was compared between groups.

Bronchoalveolar Lavage Fluids and Differential Cell Counts

Bronchoalveolar lavage fluid (BALF) was collected with three repeated washes of excised lungs using 1 ml PBS. After cytospin the slides were stained with HEMA 3 (Fisher Diagnostics, Pittsburgh, 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 Enzyme-Linked Immunosorbent Assay

Cytokine levels in BALF supernatants, serum OVA-specific IgE, IgG1, IgG2a, total IgE, and IL-13-specific IgG were assayed by enzyme-linked immunosorbent assay (ELISA) using techniques established in our laboratory (20, 21) or following manufacturer's instructions (PharMingen, San Diego, CA).

Histologic Assessment

Lungs were fixed in formalin, then embedded in paraffin and sectioned. Specimens were stained with hematoxylin and eosin (H&E) or periodic acid Schiff (PAS) and examined using an Olympus IX51 light microscope equipped with CCD camera under control of Image Pro Plus software (Media Cybernetics, Inc., Bethesda, MD). As previously described (22), peribronchiolar and perivascular inflammation in H&E-stained slides was assessed using an indexed scale: 0 = normal; 1 = infrequent inflammatory cells; 2 = a ring of inflammatory cells 1 cell layer deep; 3 = a ring of inflammatory cells 2 to 4 cells deep; 4 = a ring of inflammatory cells of more than 4 cells deep. Goblet cell abundance was measured as percentage of PAS-positive cells in the total airway epithelia of medium-sized airways. Scoring was performed at a magnification of $\times 200$ by examining at least 40 consecutive fields. The sections were assigned a random code to blind the examiner to the identity of each specimen.

Statistical Analysis

Differences between experimental groups were assessed by one-way ANOVA followed by Newman-Keuls multiple comparison test (GraphPad Prism 3.03; GraphPad Software, Inc., San Diego, CA). Values are reported as the mean $\pm$ SEM.

RESULTS

Vaccine Without Adjuvant Induces Sustained and High Titered IL-13-Specific IgG

A 15-amino acid peptide (hereafter named "La") that includes ¹²LTLKELIEELSNITQ²⁶ from helix B at the receptor binding site of mouse IL-13 was selected to generate a vaccine. NCBI BLAST searching has confirmed that there is no similar sequence to the IL-13 peptide in other known molecules. The plasmid HBcAg-La expressed a recombinant protein in *E. coli* DH5 α cells that was recognized by polyclonal rabbit anti-mouse IL-13 antibody in Western-blotting analyses. The fractionation patterns of recombinant HBcAg-La and HBcAg proteins were similar in sucrose gradient centrifugation, as each was enriched in fractions 6 to 8, as verified by SDS-PAGE. This indicates that the chimeric HBcAg-La protein assembles into particles that are similar to native HBcAg. The presence of virus-like particles of the HBcAg-La protein was further confirmed by scanning electron microscopy.

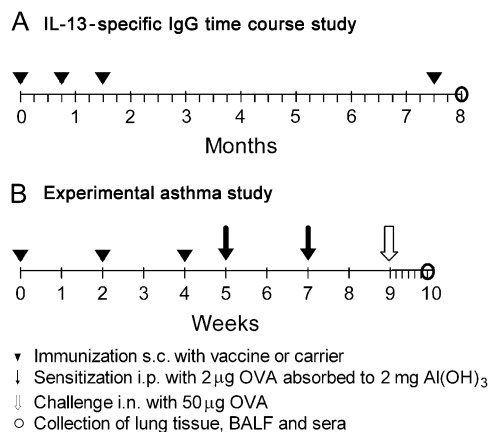


Figure 1. Immunization and sensitization protocols.

We first tested the ability of the vaccine (HBcAg-La) in inducing an IL-13-specific IgG response and the time course of antibody responses (Figure 1A) after immunization with the vaccine alone or in the presence of adjuvant (Novasome or alum or complete/incomplete Freund's adjuvant, CFA/IFA). Mice immunized with vaccine alone produced strong and long-lasting IL-13-specific antibody responses that were comparable to those induced by vaccine emulsified with any of the adjuvants tested. In all groups, IL-13-specific autoantibodies reached high levels (titer up to 256,000) and sustained for 1 month before any detectable decrease was measured (Figure 2). Although the IgG levels decreased gradually, the titers above 10,000 were sustained for 6 months (Figure 2). When a booster was given at Month 7, the antibodies in all groups increased rapidly to high levels, indicating the generation of effective immune memory for autoantibody production. Collectively, these data demonstrate that the recombinant IL-13 peptide-based vaccine is immunogenic, even in the absence of conventional adjuvant.

Vaccine Reduces Accumulation of Inflammatory Cells in BALF

Based on the effective immune response elicited by vaccine in the absence of adjuvant, for all subsequent studies immunization was performed using vaccine alone. Six days after nasal challenge, mice were killed, and BALF, lung tissues, and sera were obtained for analyses. Total inflammatory cell number in BALF of IL-13-vaccinated mice was reduced by 62.5% compared with the carrier group ($P < 0.001$), and by 48.6% compared with the PBS group ($P < 0.05$) (Figure 3). Moreover, compared to the carrier or PBS groups, immunization with IL-13 vaccine markedly inhibited eosinophil recruitment to BALF by 74.0% ($P < 0.001$) and 80.9% ($P < 0.01$), respectively (Figure 3). Figure 3 also shows that vaccination reduced the number of BALF lymphocytes by 72.7% and 64.3%, and neutrophils by 82.4% and 83.3% compared to the carrier or PBS groups, respectively. Collectively, our data reveal that IL-13-based vaccination suppressed the accumulation of total inflammatory cells, eosinophils, neutrophils, and lymphocytes that is typically induced by OVA sensitization and intranasal challenge.

IL-13 and IL-5 Levels in BALF Are Inhibited by Vaccination

To examine the effects of the IL-13 vaccine on cytokine levels in BALF, T helper (Th)2 (IL-4, IL-5, IL-13), Th1 (interferon [IFN]- γ), and pro-Th1 (IL-12) cytokines were measured using ELISA. When compared to the carrier or PBS groups, the mean

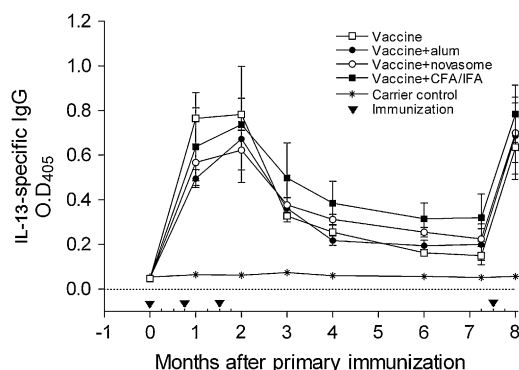


Figure 2. Immunization with interleukin (IL)-13 vaccine alone induces sustained IL-13-specific IgG. Mice were immunized subcutaneously with IL-13 vaccine alone or with one of the following adjuvants: (1) Novasome, (2) alum, or (3) CFA/IFA. IL-13-specific IgG levels were measured by enzyme-linked immunosorbent assay (ELISA) at the serum dilution of 1:10,000. The values were read at OD₄₀₅ and expressed as mean $\pm$ SEM ($n = 4$).

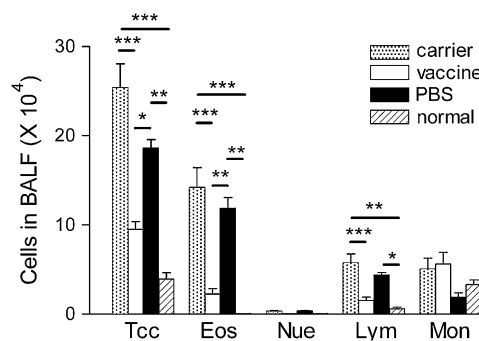


Figure 3. Immunization with IL-13 vaccine suppresses inflammatory cell accumulation in bronchoalveolar lavage fluid (BALF). Eos = eosinophil; Lym. = lymphocytes; Mon. = monocytes; Neu = neutrophils; Tcc = total cell count. Significant differences were shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

level of IL-13 in vaccine group was decreased by 40.6% and 61.9% respectively, and IL-5 level was decreased by 51.6% and 72.7%, respectively (P values < 0.05) (Figure 4). However, vaccination did not significantly affect the mean levels of IL-4, IL-12, or IFN- γ when compared with the carrier group. Immunization with the carrier protein did not produce significant effects on cytokine levels, compared with the PBS group.

Total and OVA-specific IgE Are Reduced by Vaccine

In this study, total IgE and OVA-specific IgE, IgG1, and IgG2a levels in endpoint sera were quantified via ELISA. Although both total and OVA-specific IgE antibodies in the vaccine-treated group remained higher than those in normal mice, they were markedly reduced compared with those seen in the carrier or PBS groups (Figure 5A). Both the mean levels of serum OVA-specific IgG1, also driven by Th2 cytokines, and those of OVA-specific IgG2a, driven by Th1 cytokines, decreased significantly. In a replicate experiment, sera were collected from the vaccine and carrier groups at Weeks 0, 3, 5, 7, 9 and again at the endpoint of the experiment. Results are shown as Figure 5B to Figure 5F. Titers of serum mouse IL-13-specific IgG were significantly increased after the second immunization with

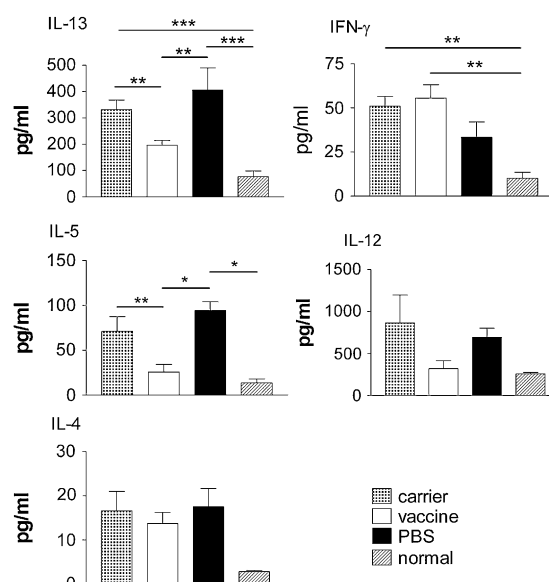


Figure 4. Effects of IL-13 vaccine on cytokine accumulation in BALF. Significant differences were shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

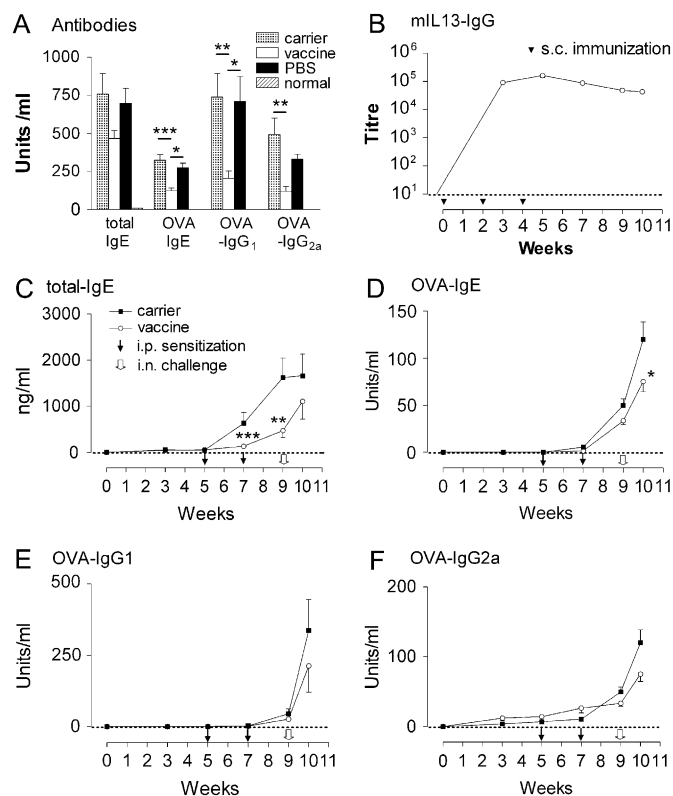


Figure 5. Total and ovalbumin (OVA)-specific IgE in serum are significantly reduced by IL-13 vaccine. (A) Antibodies levels in the sera collected at the endpoint of the experiment were measured using ELISA. (B–F) In replicate experiments, serum samples were collected at the times indicated, and dynamic antibody responses are shown. To quantitatively measure OVA-specific antibodies, a reference serum defined as 1,000 units/ml for OVA-specific antibodies was used. Data are reported as mean $\pm$ SEM. Significant differences were shown as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

the vaccine, and remained high for the duration of the study. Total IgE and OVA-specific antibody levels were significantly boosted after the second intraperitoneal administration and intranasal challenge with OVA, while total IgE and OVA-IgE responses in vaccine group were suppressed significantly compared with those in carrier and PBS control groups.

Vaccine Suppresses Airway Goblet Cell Hyperplasia and Inflammation

We used PAS staining and histochemical methods to assess airway goblet cell hyperplasia, as this is a common manifestation of allergic airways inflammation (Figure 6). Mice that received IL-13 vaccine showed markedly fewer goblet cells after allergen sensitization and challenge compared with mice that received carrier (Figure 6A). Consistent with BALF cytology, H&E staining of lung sections confirmed that the accumulation of inflammatory cells in the airways was also suppressed in mice that received IL-13 vaccine (Figure 6A). Notably, in control mice receiving only carrier or PBS, there was evidence of significant perivascular and peribronchial accumulation of inflammatory cells, which appeared to be mainly comprised of eosinophils, as shown by the *magnified inserts* in Figure 6A. We performed semiquantitative analyses of goblet cells and tissue inflammation in four mice in each group. The results further confirmed that IL-13 vaccine immunization significantly suppressed goblet cell hyperplasia and lung tissue inflammation (Figure 6B).

Vaccine Suppresses Allergic Hyperresponsiveness to Inhaled Methacholine

We assessed the ability of IL-13 vaccination to prevent hyperresponsiveness to inhaled methacholine, 2 days after intranasal challenge with OVA. For these studies we used whole body plethysmography of unrestrained animals to measure Penh. This approach measures changes in expiration and expiration time, and breathing rate; therefore, it provides evidence for a change in work of breathing, which can be related to a number of specific parameters of respiratory mechanics, including tissue resistance, tissue compliance, and airway resistance. Though Penh is not a reliable marker for airway resistance in all strains of mice, a number of studies have reported that it can correlate with airway responsiveness in the BALB/c strain that we used in our studies (18, 19). Consistent with our experiments showing that IL-13 vaccination prevented allergic airway inflammation, mice immunized with IL-13 vaccine ($n = 6$) exhibited markedly reduced Penh responses to maximum concentrations of inhaled methacholine (25 mg/ml) compared with carrier ($n = 6$) and PBS ($n = 4$)-exposed animals ($P < 0.05$) (Figure 7).

DISCUSSION

Since the introduction of vaccines against microbes, many infectious diseases have become well controlled. However, diseases reflecting maladaptive immunoregulatory responses such as allergic and autoimmune diseases have increased significantly. Innovative application of vaccine concepts represents a novel approach to treatment of these severe, often lifelong, chronic human diseases. Prior studies have demonstrated that targeting of a single molecule, if the molecule is sufficiently critical to the pathogenesis or maintenance of the disease, can be effective. Anti-IgE therapy is useful in treatment of moderate to severe asthma (23, 24). Anti-TNF- α therapy with its monoclonal antibodies or soluble receptors is used in the treatment of rheumatoid arthritis (25), inflammatory bowel disease (12, 26), and may have some applicability in severe asthma (27, 28). Although blocking only one molecule may be ineffective (11, 29) or insufficient to attain significant benefit in all clinical manifestations in all patients (24, 27, 28), the clinical experience provides proof of concept about the potential utility of targeting key molecule(s) for therapy (30).

IL-13 is a key mediator of asthma (1). We have developed a novel IL-13 peptide-based vaccine to induce specific antibodies directed at a key sequence in IL-13 that is not found to be similar to any known molecules through NCBI BLAST research, thereby avoiding the increased likelihood of cross-reactions with vaccines carrying large-to-full-length peptide sequences. Our decision to use HBcAg to develop the IL-13 peptide-based vaccine was based on some key features related to the particles that are produced. First, the safety of using HBcAg as a carrier has been confirmed in a phase I clinical trial for a malaria vaccine (31). Second, it is effective in activating naive B cells as primary antigen-presenting cells, which are 10^5 -fold more efficient than what is typically associated with dendritic cell and macrophage interactions (32). Third, a single virus-like HBcAg particle consists of 180 or 240 HBcAg molecules, each of which is inserted with one IL-13 peptide. Therefore, a total of 180 or 240 IL-13 peptides are displayed on the surface of a single vaccine particle in highly ordered, optimally spaced repeats. This structural feature likely explains the high immunogenicity found with our vaccine. The highly repetitive ordered array of inserted self-polypeptides on the surface of virus-like particles may also abrogate the ability of the immune system to distinguish between foreign and self (33),

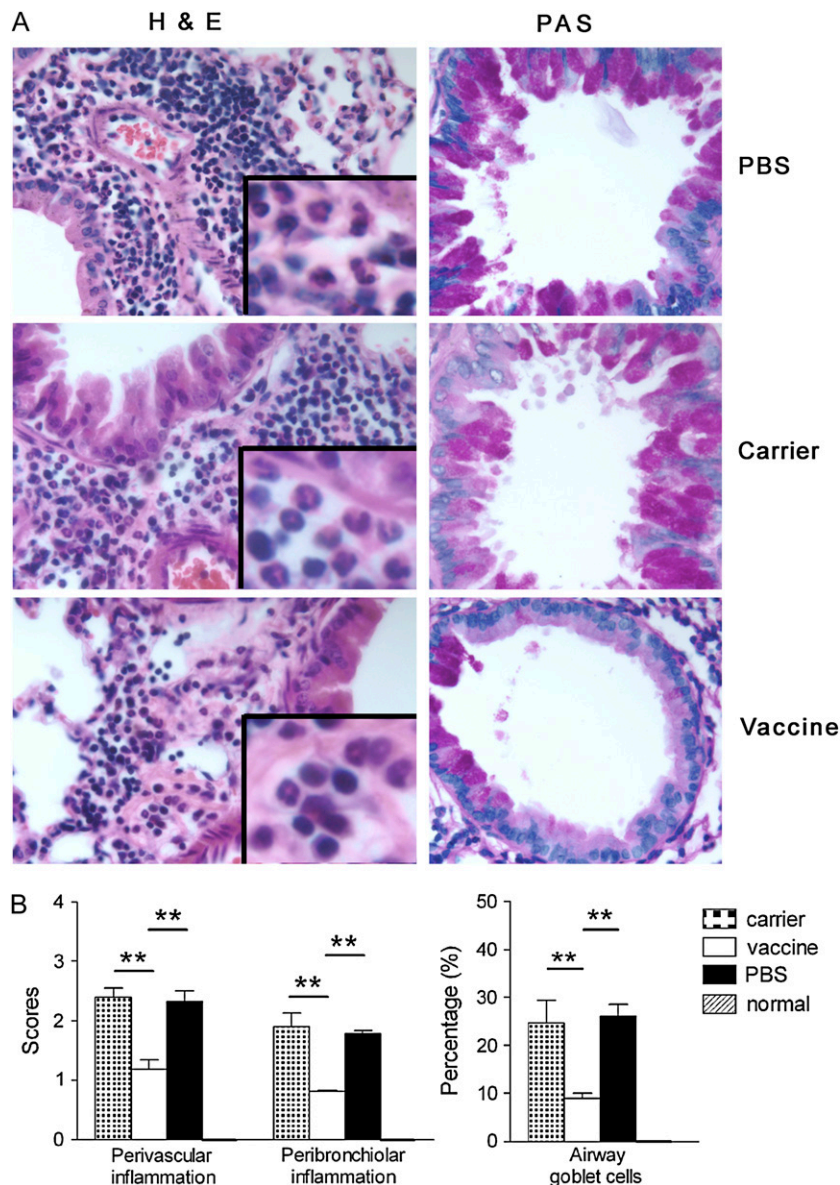


Figure 6. Airway tissue inflammation and goblet cell hyperplasia are significantly decreased by IL-13 vaccine. (A) Representative photomicrographs are shown. *Top panels:* mice vaccinated with PBS followed by OVA sensitization/challenge; *middle panels:* mice vaccinated with the carrier; *bottom panels:* mice immunized with IL-13 vaccine. *Left panels:* H&E staining; the *insert* is powered magnification showing infiltrated inflammatory cells. *Right panels:* PAS staining. (B) Semi-quantification analysis was performed to assess histologic changes ($n = 4/\text{group}$). Significant differences were shown as $**P < 0.05$.

thus providing a unique benefit that leads to breaking of B cell tolerance. Our vaccine was successful in breaking self-immune tolerance and inducing high titers of auto-antibodies for IL-13 (up to 256,000), comparable to those elicited by a foreign peptide in the same carrier system (data not shown). We also demonstrated that the IL-13-specific auto-antibodies were maintained for at least 6 months without use of conventional adjuvant.

The overall goal of our investigation was to assess the potential of IL-13 peptide-based virus-like particle vaccines to suppress features of airway allergic inflammatory response. For this purpose, we used a well-established acute model of allergic airways inflammation to induce asthma-like features that have been reported to include AHR, airway and lung tissue eosinophilia, mucus overproduction, and IgE elevation (34). Although some reports in mouse models have failed to confirm a direct association between AHR and airway eosinophilia (4, 6), there is evidence that eosinophils may play a unique and important effector role in the pathogenesis of asthma (35, 36). Our results show that IL-13 vaccine suppresses airway inflam-

matory cell recruitment, in particular eosinophils. This is consistent with current published reports that IL-13 regulates the expression of chemokines, adhesion molecules, and metalloproteinases associated with inflammatory cells (1), and that airway administration (3, 4) or overexpression (5) of IL-13 in the lungs is sufficient to induce cellular infiltration.

In addition to the critical effect of IL-4, IL-13 also showed a positive role in Th2 development and responses (37). Our experiments measured the effects of IL-13 vaccine on the BALF Th1/Th2 cytokine profile in mice after sensitization and challenge with allergen. Notably, accumulation of IL-13 and IL-5 was markedly suppressed. As IL-5 is crucial in regulating eosinophil development, differentiation, recruitment, activation, and survival (38), it is likely that its suppression is involved with the ability of our IL-13 vaccine to suppress airway eosinophilia. Anti-IgE therapy has confirmed a central role for IgE in the allergic inflammatory process in asthma (24). Studies indicate that IL-13 contributes to IgE synthesis (39), due to its role in the Th2 cell development (37) and the survival of B cells (40). Therefore, down-regulation of IL-13 can also modulate

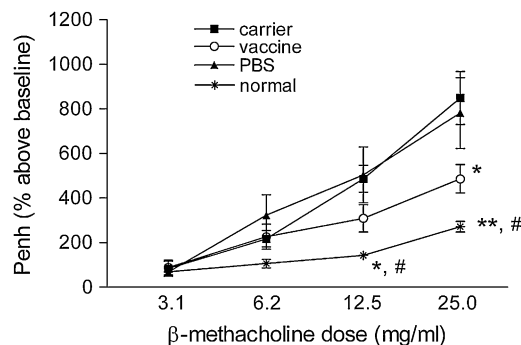


Figure 7. IL-13 vaccine prevents hyperresponsiveness to inhaled methacholine. Breathing responses to increasing concentrations of β -methacholine (0–25 mg/ml) nebulized for 3 minutes. Changes in the ease of breathing were assessed by whole body plethysmography. Temporal changes in body box pressures were used to calculate enhanced pause (Penh). Percent change in Penh for each mouse was used as an index of the magnitude of the change in breathing response and is plotted as increase above baseline values obtained from an initial saline challenge. * $P < 0.05$, compared with the carrier group; ** $P < 0.01$; # $P < 0.001$, compared with the PBS group.

Th2 responses and levels of IgE and other antibodies. These effects likely provide an explanation for our findings that vaccination with IL-13 vaccine suppressed allergen-induced total and OVA-specific IgE, and also had an effect in suppressing OVA-specific IgG1 and IgG2a (Figure 5).

IL-13 appears to promote goblet cell hyperplasia and mucus production, as its airway administration (3, 4) or overexpression in lung (5) rapidly induces mucus hyperplasia and production. *In vitro* and *in vivo* experiments further demonstrate that IL-13 induces the gene expression of Ca^{2+} -activating chloride channel, stimulates the differentiation of epithelial cells, and promotes mucin gene expression and release (1). Notably, IL-13 gene knockout (41) or blockade (6, 42) in mice prevents mucous hypersecretion, and can reverse established mucus metaplasia. Our results using IL-13 vaccine are consistent with these observations, as inhibition of IL-13 effects by vaccination attenuated the accumulation of mucus-secreting airway epithelial goblet cells that is typically induced in mice after allergen challenge.

IL-13 appears to be both necessary and sufficient for the development of AHR in mice (3, 6, 41). Our results using an acute challenge protocol are consistent with this paradigm, as IL-13 vaccination, in contrast to the carrier protein, significantly reduced allergen-driven changes in breathing responses to inhaled methacholine. A limitation of our study is that we used a barometric method to assess breathing responses, so it is not possible to attribute the increased work of breathing caused by aerosolized methacholine solely to increased airway responsiveness. Nonetheless, our results clearly demonstrate that IL-13 vaccination suppresses the increased work of breathing induced by methacholine in allergen-challenged mice. The effects of IL-13 on AHR, which may be those inhibited by our vaccine, are likely due to enhancement of inflammatory cell infiltration, by direct induction of biomolecule release from airway epithelial cells (43), and on modulation of airway smooth muscle cell contraction or proliferation (44). These data suggest IL-13 may have a role in airways remodeling in prolonged allergic inflammation. Recently, Leigh and coworkers (45) found that inhibition of IL-13 with soluble receptors only partially reversed sustained airway hyperresponsiveness associated with airway remodeling in a mouse model. Therefore, it will be of interest in future experiments using our IL-13 vaccine to establish whether

sustained neutralization of IL-13 is able to modulate airway remodeling and persistent AHR in response to chronic allergen challenge.

A potential concern of active immunization with cytokine vaccines is that it could induce a permanent autoimmune condition, eliminating all target cytokine, including that required for normal physiological processes. Under normal conditions, cytokine release occurs within a so-called “immune synapse” that requires intimate association of the cells involved (46). Previous studies indicate that auto-antibodies induced by vaccine had little impact at the “immune synapse,” and appear only to neutralize excess cytokine that accumulates ectopically in the extracellular compartment (47). The safety of cytokine vaccines is also supported by animal experiments and human clinical trials (47). In our present experiments, vaccinated mice that developed abundant IL-13-specific auto-antibodies showed no evidence of physiologic or growth abnormalities for up to one year after vaccination.

In summary, we have, for the first time, designed an IL-13 peptide-based vaccine that effectively breaks immune tolerance and elicits sustained high titers of auto-antibodies to endogenous IL-13. Using a murine model of acute allergic airway inflammation, we demonstrate that immunization with the vaccine markedly suppresses allergic responses, including inflammation, mucus hypersecretion, and the development of hyperresponsiveness to inhaled methacholine. These observations support the notion that IL-13 likely plays a key role in the pathogenesis of asthma. The administration of cytokine peptide-based vaccines has the potential to be an effective therapeutic tool in treating allergic asthma and, by extension, other disorders in which elevated cytokines are involved in the pathogenesis.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

- Wills-Karp M. Interleukin-13 in asthma pathogenesis. *Immunol Rev* 2004;202:175–190.
- Vercelli D. Genetics of IL-13 and functional relevance of IL-13 variants. *Curr Opin Allergy Clin Immunol* 2002;2:389–393.
- 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.
- Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science* 1998;282:2258–2261.
- 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.
- Liossis SN, Tsokos GC. Monoclonal antibodies and fusion proteins in medicine. *J Allergy Clin Immunol* 2005;116:721–729; quiz 730.
- Holgate ST, Chuchalin AG, Hebert J, Lotvall J, Persson GB, Chung KF, Bousquet J, Kerstjens HA, Fox H, Thirlwell J, et al. Efficacy and safety of a recombinant anti-immunoglobulin E antibody (omalizumab) in severe allergic asthma. *Clin Exp Allergy* 2004;34:632–638.
- Tyring S, Gottlieb A, Papp K, Gordon K, Leonardi C, Wang A, Lalla D, Woolley M, Jahreis A, Zitnik R, et al. Etanercept and clinical outcomes,

- fatigue, and depression in psoriasis: double-blind placebo-controlled randomised phase III trial. *Lancet* 2006;367:29–35.
11. Borish LC, Nelson HS, Corren J, Bensch G, Busse WW, Whitmore JB, Agosti JM. Efficacy of soluble IL-4 receptor for the treatment of adults with asthma. *J Allergy Clin Immunol* 2001;107:963–970.
 12. Sandborn WJ. New concepts in anti-tumor necrosis factor therapy for inflammatory bowel disease. *Rev Gastroenterol Disord* 2005;5:10–18.
 13. Zuany-Amorim C, Manlius C, Dalum I, Jensen MR, Gautam A, Pay G, Mouritsen S, Walker C. Induction of TNF- α autoantibody production by AutoVac TNF106: a novel therapeutic approach for the treatment of allergic diseases. *Int Arch Allergy Immunol* 2004;133:154–163.
 14. Rad FH, Le Buanec H, Paturance S, Larcier P, Genne P, Ryffel B, Bensussan A, Bizzini B, Gallo RC, Zagury D, et al. VEGF kinoid vaccine, a therapeutic approach against tumor angiogenesis and metastases. *Proc Natl Acad Sci USA* 2007;104:2837–2842.
 15. Peng Z, Ma Y, Fan Y, Srinivasan G, Yang X, Becker A, Simons F, HayGlass K. Novel interleukin-13 peptide-based vaccine downregulates murine allergic airway responses. *J Allergy Clin Immunol* 2006;117:S250–S251.
 16. Moy FJ, Diblasio E, Wilhelm J, Powers R. Solution structure of human IL-13 and implication for receptor binding. *J Mol Biol* 2001;310:219–230.
 17. Idzko M, Hammad H, van Nimwegen M, Kool M, Vos N, Hoogsteden HC, Lambrecht BN. Inhaled iloprost suppresses the cardinal features of asthma via inhibition of airway dendritic cell function. *J Clin Invest* 2007;117:464–472.
 18. Borchers MT, Biechele T, Justice JP, Ansay T, Cormier S, Mancino V, Wilkie TM, Simon MI, Lee NA, Lee JJ. Methacholine-induced airway hyperresponsiveness is dependent on Galphq signaling. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L114–L120.
 19. Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, Gelfand EW. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* 1997;156:766–775.
 20. Wang H, Mao X, Simons FER, Peng Z. Induction of IgE responses using a recombinant mosquito salivary allergen rAed 2 without adjuvant in mice. *Int Arch Allergy Immunol* 1999;120:135–140.
 21. Chen YL, Simons FER, Peng Z. A mouse model of mosquito allergy for study of antigen-specific IgE and IgG subclass responses, lymphocyte proliferation, and IL-4 and IFN- γ production. *Int Arch Allergy Immunol* 1998;116:269–277.
 22. 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.
 23. Fahy JV. Anti-IgE: lessons learned from effects on airway inflammation and asthma exacerbation. *J Allergy Clin Immunol* 2006;117:1230–1232.
 24. Holgate S, Casale T, Wenzel S, Bousquet J, Deniz Y, Reisner C. The anti-inflammatory effects of omalizumab confirm the central role of IgE in allergic inflammation. *J Allergy Clin Immunol* 2005;115:459–465.
 25. Scott DL, Kingsley GH. Tumor necrosis factor inhibitors for rheumatoid arthritis. *N Engl J Med* 2006;355:704–712.
 26. Rutgeerts P, Sandborn WJ, Feagan BG, Reinisch W, Olson A, Johanss J, Travers S, Rachmilewitz D, Hanauer SB, Lichtenstein GR, et al. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med* 2005;353:2462–2476.
 27. Berry MA, Hargadon B, Shelley M, Parker D, Shaw DE, Green RH, Bradding P, Brightling CE, Wardlaw AJ, Pavord ID. Evidence of a role of tumor necrosis factor α in refractory asthma. *N Engl J Med* 2006;354:697–708.
 28. Howarth PH, Babu KS, Arshad HS, Lau L, Buckley M, McConnell W, Beckett P, Al Ali M, Chauhan A, Wilson SJ, et al. Tumour necrosis factor (TNF α) as a novel therapeutic target in symptomatic corticosteroid dependent asthma. *Thorax* 2005;60:1012–1018.
 29. Kips JC, O'Connor BJ, Langley SJ, Woodcock A, Kerstjens HA, Postma DS, Danzig M, Cuss F, Pauwels RA. Effect of SCH55700, a humanized anti-human interleukin-5 antibody, in severe persistent asthma: a pilot study. *Am J Respir Crit Care Med* 2003;167:1655–1659.
 30. O'Byrne PM. Cytokines or their antagonists for the treatment of asthma. *Chest* 2006;130:244–250.
 31. Nardin EH, Oliveira GA, Calvo-Calle JM, Wetzel K, Maier C, Birkett AJ, Sarpotdar P, Corado ML, Thornton GB, Schmidt A. Phase I testing of a malaria vaccine composed of hepatitis B virus core particles expressing Plasmodium falciparum circumsporozoite epitopes. *Infect Immun* 2004;72:6519–6527.
 32. Milich DR, Chen M, Schodel F, Peterson DL, Jones JE, Hughes JL. Role of B cells in antigen presentation of the hepatitis B core. *Proc Natl Acad Sci USA* 1997;94:14648–14653.
 33. Chackerian B, Lenz P, Lowy DR, Schiller JT. Determinants of autoantibody induction by conjugated papillomavirus virus-like particles. *J Immunol* 2002;169:6120–6126.
 34. Epstein MM. Do mouse models of allergic asthma mimic clinical disease? *Int Arch Allergy Immunol* 2004;133:84–100.
 35. Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, McKenna EE, Ghiran S, Gerard NP, Yu C, Orkin SH, et al. A critical role for eosinophils in allergic airways remodeling. *Science* 2004;305:1776–1779.
 36. Lee JJ, Dimina D, Macias MP, Ochkur SI, McGarry MP, O'Neill KR, Protheroe C, Pero R, Nguyen T, Cormier SA, et al. Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* 2004;305:1773–1776.
 37. McKenzie GJ, Emson CL, Bell SE, Anderson S, Fallon P, Zurawski G, Murray R, Grecis R, McKenzie AN. Impaired development of Th2 cells in IL-13-deficient mice. *Immunity* 1998;9:423–432.
 38. Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 1996;183:195–201.
 39. Emson CL, Bell SE, Jones A, Wisden W, McKenzie AN. Interleukin (IL)-4-independent induction of immunoglobulin (Ig)E, and perturbation of T cell development in transgenic mice expressing IL-13. *J Exp Med* 1998;188:399–404.
 40. Lai YH, Mosmann TR. Mouse IL-13 enhances antibody production in vivo and acts directly on B cells in vitro to increase survival and hence antibody production. *J Immunol* 1999;162:78–87.
 41. Walter DM, McIntire JJ, Berry G, McKenzie AN, Donaldson DD, DeKruyff RH, Umetsu DT. Critical role for IL-13 in the development of allergen-induced airway hyperreactivity. *J Immunol* 2001;167:4668–4675.
 42. Bleas K, Jakubzik 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.
 43. Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, Erle DJ. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 2002;8:885–889.
 44. Shore SA. Direct effects of Th2 cytokines on airway smooth muscle. *Curr Opin Pharmacol* 2004;4:235–240.
 45. 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.
 46. Kourilsky P, Truffa-Bachi P. Cytokine fields and the polarization of the immune response. *Trends Immunol* 2001;22:502–509.
 47. Zagury D, Le Buanec H, Bizzini B, Burny A, Lewis G, Gallo RC. Active versus passive anti-cytokine antibody therapy against cytokine-associated chronic diseases. *Cytokine Growth Factor Rev* 2003;14:123–137.