

Dose-response relationship of specific allergen exposure-induced immunological tolerance: a mouse model

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Background: It is believed that adequate allergen preimmunization exposure could induce immunologic tolerance. The purpose of this study was to investigate the dose-dependent mechanisms related to antigen-specific tolerance induction in a mouse model.

Methods: Mice were assigned to 5 groups: the control (Cont) group received phosphate-buffered saline (PBS) preimmunization exposure and PBS sham immunization; the other 4 groups were exposed preimmunization to PBS (PBS group) or ovalbumin (OVA) (first mucosal doses: 1.25%, 2.5%, or 5% wt/vol aerosol from days -3 to -1) prior to OVA immunization. The OVA-immunized mice received intraperitoneal doses of 20 μ g OVA (on days 1, 7, and 14), and then a second set of mucosal doses with 0.5% wt/vol OVA aerosol (on days 18 to 20). After assessment of airway hyperresponsiveness (AHR), the mice were euthanized and their blood, bronchoalveolar lavage fluids (BALFs), and lung tissues were collected for further analyses.

Results: OVA-immunized mice exposed to OVA preimmunization had reduced AHR and immunoglobulin E production when compared to the PBS group. OVA

preimmunization exposure inhibited eosinophilic inflammation in lung tissues. The proportions of BALF eosinophil counts from the groups exposed to OVA preimmunization were significantly decreased when compared with those exposed to PBS preimmunization. The balance of T helper 2 (Th2) and T regulatory (Treg) cytokines in BALFs were additionally observed in this mouse model.

Conclusion: Our results suggest that preimmunization exposure to an appropriate dose of a specific antigen could suppress allergic airway inflammation by induction of immunological tolerance. © 2015 ARS-AAOA, LLC.

Key Words:

aeroallergens; allergens; allergy immunotherapy; asthma; inhalation allergen challenge

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Asthma and allergic rhinitis present an important global health problem, affecting 20% to 30% of the population. According to the International Study of Asthma and Allergies in Childhood (ISAAC) epidemiological research

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Correction added on 3rd August 2015, after first online publication. At the end of the 7th page and continuing onto the 8th, the sentence reading "A similar situation exists with sublingual immunotherapy, which enjoys widespread popularity in European countries today, but has not been approved by the U.S. Food and Drug Administration because protocols for determining effective doses have not been established." has been changed to "A similar situation exists with sublingual immunotherapy, which enjoys widespread popularity in European countries today, but has limited use in the U.S. The Food and Drug Administration has only recently approved sublingual tablets for the U.S., but only for ragweed and grass allergens."

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program, the prevalence of asthma, allergic rhinoconjunctivitis, and eczema increased in most regions of the world.¹ Although the rising prevalence is of concern, effective preventive measures for allergy are lacking. One feasible method for allergy prevention in current use is environmental control to avoid allergen exposure. However, it is uncertain whether avoidance of exposure can either reduce the risk of developing allergy for high-risk children or achieve a reduction in clinical symptoms for patients with established allergic diseases.²

It is believed that preimmunization exposure to low-dose potential allergens in early life can prevent development of allergic diseases in high-risk subjects. Tsitoura et al.³ and Hansen et al.⁴ reported that mice intranasally exposed preimmunization to a protein antigen maintained immunological tolerance upon antigen re-stimulation. Hansen et al.⁴ compared the effect of 2 early-life interventions, maternal allergen immunization and postnatal intranasal exposure of an allergen to offspring, on the development of allergy in the offspring. They found that early mucosal allergen exposure suppressed the airway inflammation. Holt et al.⁵ reported inhibition of specific immunoglobulin E (IgE) responses to antigens in mice, which had been exposed preimmunization to the inhaled antigens. Two studies demonstrated that preimmunization exposure to allergens by subcutaneous injection prevents immediate hypersensitivity and airway hyperresponsiveness (AHR).^{6,7} Other studies have revealed that epicutaneous application of a low dose of contact allergens impedes the development of contact dermatitis.^{8,9} Previous animal studies had used different preimmunization exposure routes and antigen doses to induce immunologic tolerance. However, the mechanisms of the protective effect were not completely understood. In addition, the lack of information related to the dose-response relationship impedes its clinical use. It is thus apparent that the establishment and maintenance of immunological tolerance to an allergen are important primary strategies for allergy prevention.¹⁰ Introduction of an allergen in early life to prevent development of allergy is an attractive approach but remains controversial.^{11,12} According to previous studies, we hypothesized that inhalational allergen preimmunization exposure could induce immunologic tolerance, and that the preimmunization dose of the antigen affects the protective effect. The purpose of this study was to investigate the mechanisms of inhalational allergen preimmunization exposure-induced immunologic tolerance, and to assess the dose-response relationship of this effect.

Materials and methods

Animals and experimental design

The Institutional Animal Care and Use Committee of the study hospital approved the protocols for this animal experiment. Six-week-old male Balb/c mice were randomly assigned to 5 groups according to the preimmunization exposure mucosal dose and immunization protocols. The groups were as follows: (1) Cont group (preimmunization

exposure and immunization to phosphate-buffered saline [PBS]); (2) PBS group (preimmunization exposure to PBS and immunization to ovalbumin [OVA]); (3) OVA-1.25 group (preimmunization exposure to 1.25% wt/vol OVA and immunization with OVA); (4) OVA-2.5 group (preimmunization exposure to 2.5% wt/vol OVA and immunization to OVA); and (5) OVA-5 group (preimmunization exposure to 5% wt/vol OVA and immunization to OVA). Mice received aerosol inhalational of the preimmunization exposure reagents for 30 minutes twice daily for 3 days (first mucosal dose). Mice then received either OVA (Sigma, St. Louis, MO) immunization protocol to induce allergic airway inflammation or PBS immunization for comparison. Briefly, OVA-immunized mice received an intraperitoneal dose with OVA (20 μ g/mouse) and alum adjuvant (1 mg) dissolved in saline (final volume 200 μ L) on days 1, 7, and 14, followed by 30-minute second mucosal doses of 0.5% wt/vol solution of OVA in saline aerosol from days 18 to 20.^{13,14} The Cont group mice received a 200- μ L PBS injection intraperitoneally and a PBS second mucosal dose.

Measurement of airway responsiveness by unrestrained whole-body plethysmography

Airway responsiveness was measured by methacholine (Mch)-induced airflow obstruction in conscious unrestrained mice placed in a whole-body plethysmograph (Buxco, Troy, NY). Pulmonary resistance was evaluated and expressed as an enhanced pause (Pen h). Mice were exposed to PBS first and then challenged with a series of aerosolized Mch at doses from 6.25 to 50 mg/mL with an ultrasonic nebulizer (Pari Proneb UltraII compressor nebulizer system; PARI Respiratory Equipment, Inc., Midlothian, VA). Readings were collected for 3 minutes after a 3-minute rest in the chamber.

Collection and analyses of bronchoalveolar lavage fluids

Mice were deeply anesthetized and placed in a supine position. The trachea was surgically exposed and cannulated with a catheters pointing toward the lung. Bronchoalveolar lavage fluids (BALFs) were obtained from mice by instilling two 1-mL aliquots of PBS via the catheter, followed by 2 aspirations of BALF into the syringe. The BALFs were centrifuged at 6000 rpm (3420 g) at 4°C for 5 minutes and the supernatants were stored at -80°C until analysis. The cell pellets of BALFs were resuspended in 200 μ L saline: 100 μ L of the cell suspension was mixed with trypan blue solution to determine total cell counts using a hemocytometer (Burjer-Turk, Marienfeld, Germany). The other 100 μ L of BALF was used to prepare Cytospin® slides (Shandon Inc., Pittsburgh, PA): samples were centrifuged at 600 rpm (30 g) for 6 minutes. The deposited cells were then fixed to the microscope slides and stained with Diff-Quik (Baxter, McGaw Park, IL) then assessed under a microscope at $\times 200$ magnification. In each case, 10 randomly chosen high-power fields were selected from the Cytospin® slides. Then 200 cells were counted and the percentage of each

type of cell was calculated. White blood cells were classified as eosinophils, neutrophils, macrophages, and lymphocytes based on cellular staining and morphology characteristics. Two laboratory technicians identified cellular compositions without reference to each other's results.

Measurements of total and OVA-specific IgE and IgG2a

Sera and supernatant of BALFs were used for measurement of total IgE and IgG2a levels with commercial mouse IgE and IgG2a isotype-specific enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (BD Biosciences, San Diego, CA). Serum OVA-specific IgE or IgG2a levels were also detected by ELISA as described.¹⁴ Briefly, 96-well ELISA plates were coated with 100 $\mu\text{g/mL}$ OVA in 0.1 mol/L carbonate buffer, at pH 8.3, blocked with PBS-T (0.05% Tween-20), and then incubated with mouse sera diluted 1:50 or 1:100, followed by addition of biotin-conjugated rat anti-mouse IgE or IgG2a (BD Biosciences). Avidin-horseradish peroxidase (HRP) solution was then added to each well and optical density was read at 405 nm. The values of standards were calculated as 100 ng/mL for IgE and 200 ng/mL for IgG2a.

Cytokine and chemokines measurements in BALFs: IL-4, IL-5, IL-13, IL-10, and transforming growth factor β

The levels of IL-4, IL-5, IL-13, IL-10, and transforming growth factor β (TGF- β) in BALFs were determined using ELISA kits (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions and as described.¹⁴ Samples (100 μL supernatant of BALFs) were assayed in duplicate and the mean absorbance was calculated with comparison to the standard curve by interpolation.

Histopathological and morphological examinations of lung samples

Mice lungs were fixed overnight in 10% neutral buffered formalin (Surgipath, Richmond, IL). Following fixation, the tissues were embedded in paraffin, cut into 5- μm sections, mounted on poly-L-lysine-coated glass slides, and stained with hematoxylin and eosin (H&E). Eosinophils, which are histologically the predominant cell types, are believed to be important effector cells, capable of mediating tissue damage and contributing to the development of AHR. Therefore, the presence and localization of eosinophils in samples from each group were detected by immunohistochemistry (IHC) stain using antibody against a peptide mapping near the C-terminal of eosinophil major basic protein (eMBP) (Santa Cruz Biotechnology, Inc., Dallas, TX). Lymphocyte/neutrophil infiltration was identified in samples from the same mouse as well by antibody against T cell/neutrophil marker (Santa Cruz Biotechnology).

In brief, the paraffin sections were deparaffinized, washed, immersed in 3% bovine serum albumin for 1 hour, and then incubated with primary antibodies against eMBP

or T cell/neutrophil marker at dilutions of 1:400 for 18 hours at 4°C. After washing twice in PBS/Tween-20 solution, these slides were incubated with HRP-conjugated secondary antibody for 1 hour at room temperature and detected with chromogenic substrates 3,3' diaminobenzidine (DAB). Slides were then examined using a bright-field microscope, and corresponding digital images were captured for later analyses.

Immunoblotting analyses of eosinophil major basic protein and T cell/neutrophil marker

Lung tissues were homogenized with lysis buffer and were centrifuged at 10,000g at 4°C for 20 minutes to obtain the cellular proteins in the supernatant. The protein concentrations were determined by BCA Protein Assay (Pierce Chemical Co., Rockford, IL). Equal amounts of proteins from each sample were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membranes, and blocked in blocking buffer for 1 hour at room temperature. The membranes were incubated with primary antibodies at 1:1000 dilution (eMBP, T cell/neutrophil marker, and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]; Santa Cruz Biotechnology). Overnight at 4°C, washed 3 times, incubated with HRP-conjugated secondary antibodies (1:5000 dilution) for 1 hour at room temperature, washed 3 times, and then detected with electrochemiluminescence (ECL) (Pierce Chemical Co., Rockford, IL). The density of each protein band was scanned using Science Lab Image-Gauge version 4.0 Software (Fujifilm, Tokyo, Japan) and compared by densitometry.

Statistical analysis

All experiments were repeated with multiple mice in each experiment. Data are expressed as mean $\pm$ SEM of each group. The Kruskal-Wallis test was used to compare differences in each inflammatory parameter among the experimental groups and the PBS group. The Mann-Whitney *U* test was used to compare differences between 2 groups if Kruskal-Wallis test demonstrated significant differences. All statistical analyses were carried out using SPSS 17.0 software (SPSS, Chicago, IL) and a value of $p < 0.05$ was considered significant.

Results

Assessment of the AHR

The OVA-immunized mice had increased AHR compared to the PBS-immunized mice when they were exposed to serial concentrations of Mch challenge. Interestingly, OVA-immunized mice exposed to OVA preimmunization resulted in reduced AHR (Fig. 1, $n = 7$ for Cont group; $n = 9$ for experimental groups). The AHR was significantly reduced in mice exposed to OVA preimmunization when compared with that of the PBS group at Mch dose of 50 mg/mL (PBS vs OVA-1.25, OVA-2.5, OVA-5; $p = 0.019$, 0.03, and 0.024, respectively).

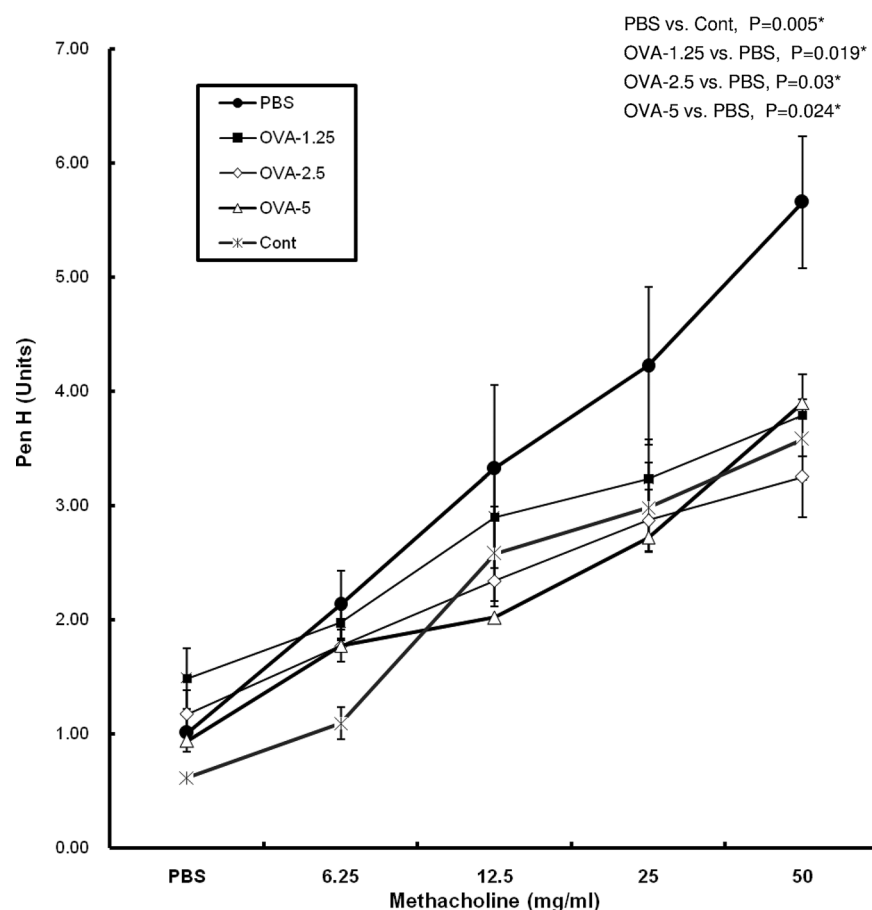


FIGURE 1. Measurement of airway responsiveness by unrestrained whole-body plethysmography. Six-week-old male Balb/c mice were assigned to 5 groups: the control (Cont) group received PBS preimmunization exposure and immunization; the other 4 groups were exposed to PBS or OVA preimmunization (1.25%, 2.5%, or 5% wt/vol aerosol from days -3 to -1) prior to OVA immunization. All mice received a pulmonary function test on day 20. The corresponding Pen h was recorded for 3 minutes following each challenge of methacholine and values are calculated and presented as mean $\pm$ SEM ($n = 7$ for Cont group; $n = 9$ for experimental groups). The Mann-Whitney U test was used to compare differences between the 2 groups when the result of Kruskal-Wallis test was significant. $^*p < 0.05$. Cont = control; OVA = ovalbumin; PBS = phosphate-buffered saline; Pen h = pulmonary resistance evaluated and expressed as an enhanced pause; SEM = standard error of the mean.

Total IgE, OVA-IgE, total IgG2a, and OVA-IgG2a in sera, BAL, and nasal lavage fluids

All 3 OVA-immunized groups exposed to OVA preimmunization had decreased total IgE from sera, BALFs, and sera OVA-specific IgE compared to the PBS group ($n = 7$ for each group). There were significantly decreased serum total and OVA-specific IgE levels from mice in the OVA-1.25 and OVA-2.5 groups when compared with those of the PBS group ($p = 0.008$ and 0.004 for total IgE, and $p = 0.007$ and 0.005 for OVA-specific IgE, respectively) (Fig. 2A, C). The total IgE significantly decreased in BALFs from mice in the OVA-2.5 group compared to that of the PBS group ($p = 0.003$) (Fig. 2E).

In contrast, the total and OVA-specific IgG2a from sera and BALFs increased in OVA-1.25 and OVA-2.5 groups when compared with those of the PBS group ($p = 0.01$ and 0.037 for sera total IgG2a, $p = 0.008$ and 0.004 for sera OVA-specific IgG2a, $p = 0.003$ for BALF total IgG2a of OVA-1.25, respectively) (Fig. 2B, D, F).

Cellular and cytokines changes in BALFs

The total and differential cellular counts from BALFs are displayed in Figure 3A ($n = 4$ for each group). The representative Cytospin® slides from each group are shown in Figure 3B. There were significantly decreased eosinophil counts in BALFs from the OVA-2.5 and OVA-5 groups when compared with those from the PBS group (both $p = 0.021$). In addition, macrophages in BALFs significantly increased in the OVA-2.5 and OVA-5 groups. All OVA-immunized mice exposed to OVA preimmunization demonstrated the tendency of decreased Th-2 cytokine (IL-4, IL-5, and IL-13) expression and increased Treg cytokine (IL-10 and TGF- β) expression in their BALFs (Fig. 4A, B; $n = 4$ for each group). IL-4 and IL-5 levels were significantly decreased in BALFs from mice in the OVA-1.25 and OVA-2.5 groups when compared with those of mice in the PBS group (both $p = 0.021$ for IL-4; $p = 0.021$ and 0.043 for IL-5, respectively). All 3 groups exposed to OVA preimmunization had significantly increased IL-10

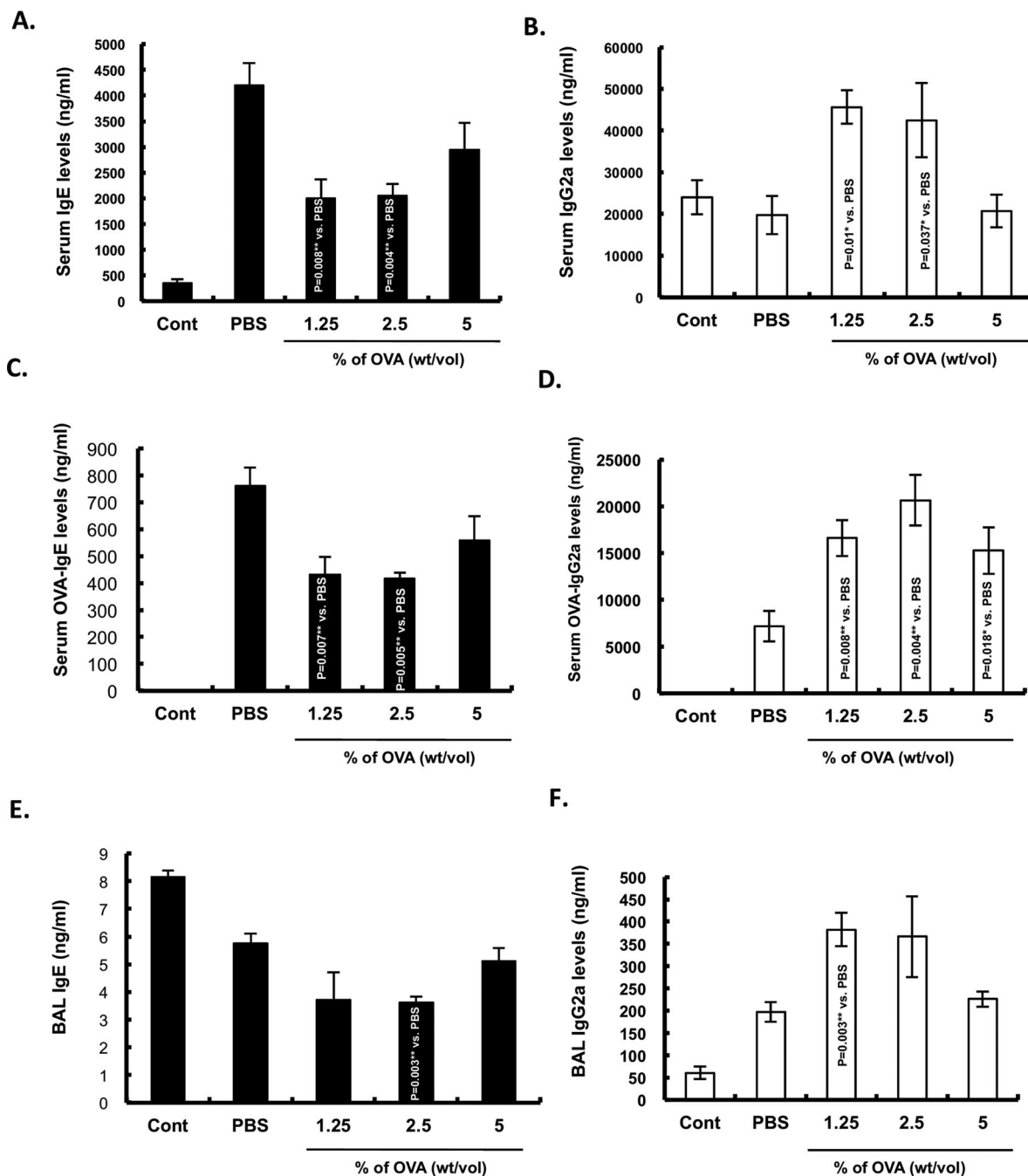


FIGURE 2. IgE and IgG2a levels in sera and BAL fluids. The total IgE and IgG2a (mean $\pm$ SEM) detected in sera (A-D) and bronchoalveolar lavage fluids (E, F) for each group of mice by ELISA ($n = 7$ for each group). OVA-specific IgE and IgG2a levels (C, D) measured in sera from the same mice from (A, B). The Mann-Whitney U test was used to compare differences between 2 groups when the result of Kruskal-Wallis test was significant. * $p < 0.05$, ** $p < 0.01$. BAL = bronchoalveolar lavage; ELISA = enzyme-linked immunosorbent assay; Ig = immunoglobulin; OVA = ovalbumin; PBS = phosphate-buffered saline; SEM = standard error of the mean.

($p = 0.021$, 0.021 , and 0.043 , respectively) and TGF- β (both $p = 0.021$ for OVA-2.5 and OVA-5) from their BALFs when compared with those of mice in the PBS group (Fig. 4).

Histopathological changes of lung

Five mouse lungs for each group were used for histopathological studies. Sections with H&E staining of lung and

from allergic mice (OVA immunization) demonstrated inflammatory changes when compared to those of nonallergic mice (Cont group). Various inflammatory cells including macrophage, lymphocyte, neutrophil, and eosinophils infiltrated in nasal mucosa and alveolar spaces in lung specimens. Loss of epithelial lining, reticular basement membrane thickening, and increased vascularity in the subepithelial of bronchioles were also observed. Comparison among the 4 groups of allergic mice revealed that the

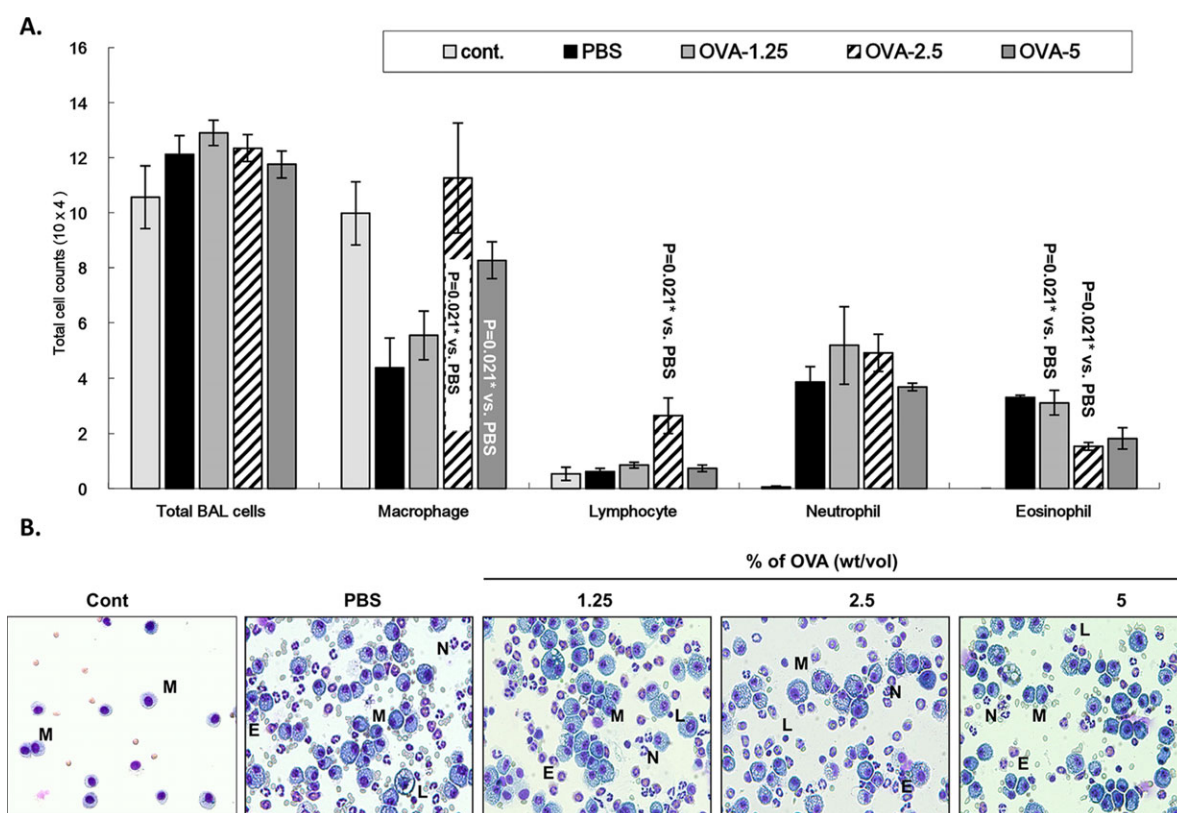


FIGURE 3. Total cell counts and differentiations of BALFs. (A) The total and differential cell counts (mean $\pm$ SEM) from BALFs. Total cells were counted using trypan blue stain under light microscope ($n = 4$ for each group). (B) Illustration of the representative Cytospin[®] slide from eluted BAL cells (magnification $\times 400$). Cell differentials were done using Cytospin[®] slides to the BAL stained with Diff-Quik (Baxter, McGaw Park, IL). BALF = bronchoalveolar lavage fluid; E = eosinophil; L = lymphocyte; M = macrophage; N = neutrophil.

inflammatory changes were reduced in mice exposed to OVA preimmunization (Fig. 5A). Sections with IHC staining for eMBP demonstrated decreased eosinophil infiltration in the lung tissues of mice exposed to OVA preimmunization. The sections with T cell/neutrophil marker IHC staining did not demonstrate a difference among the 4 OVA-immunized groups (Fig. 5B).

Changes of eosinophil major basic protein and T cell/neutrophil marker protein expressions in lung tissues

The results of T cell/neutrophil marker and eMBP protein expression are shown in Figure 6 ($n = 4$ in each group). Semiquantitative analyses of the Western blotting results were performed: There were significant decreases in eMBP protein expressions from lung tissues in the OVA-2.5 and OVA-5 groups when compared to that of the PBS group (both $p = 0.021$; Fig. 6B). However, there were no significant differences in T cell/neutrophil marker protein expression between the experimental groups and the PBS group in lung tissues (Fig. 6C).

Discussion

Healthy subjects maintain immunological tolerance to innocuous environmental allergens. In contrast, atopic

individuals have aberrant immune responsiveness to environmental allergens, resulting in asthma, allergic rhinoconjunctivitis, or eczema. Allergic inflammatory reactions involve mast cells, eosinophils, lymphocytes, immunoglobulin, and associated cytokines or chemokines.¹⁵ The importance of T helper 2 (Th2) cytokines including IL-4, IL-5, and IL-13 are stressed in most studies of murine allergic model or human allergy.^{16–18} The actions of IL-4 include B-cell activation, IgE switching, and induction of Th2 cell differentiation.¹⁹ IL-5 critically regulates expression of genes involved in proliferation, cell survival, and maturation and effector functions of B cells and eosinophils.²⁰ IL-13 triggers macrophage and eosinophil activation to accelerate inflammation, IgE production by B cells, smooth muscle cell activation contributing to airway hyperresponsiveness, mucus, and growth factor production in airway epithelial cells, and activation of airway fibroblasts to promote airway remodeling.²¹ The ideal treatment or preventive approach for an allergy is to correct the Th2-skewed immune responses induced by allergens and to achieve a status of immunologic tolerance to the offending allergens. Currently, pharmacotherapy is the main treatment modality for allergies. Regimens for treatment of allergic diseases include antihistamines, corticosteroids, bronchodilators, and antileukotrienes, and although they have been shown to be safe and effective, they are primarily for

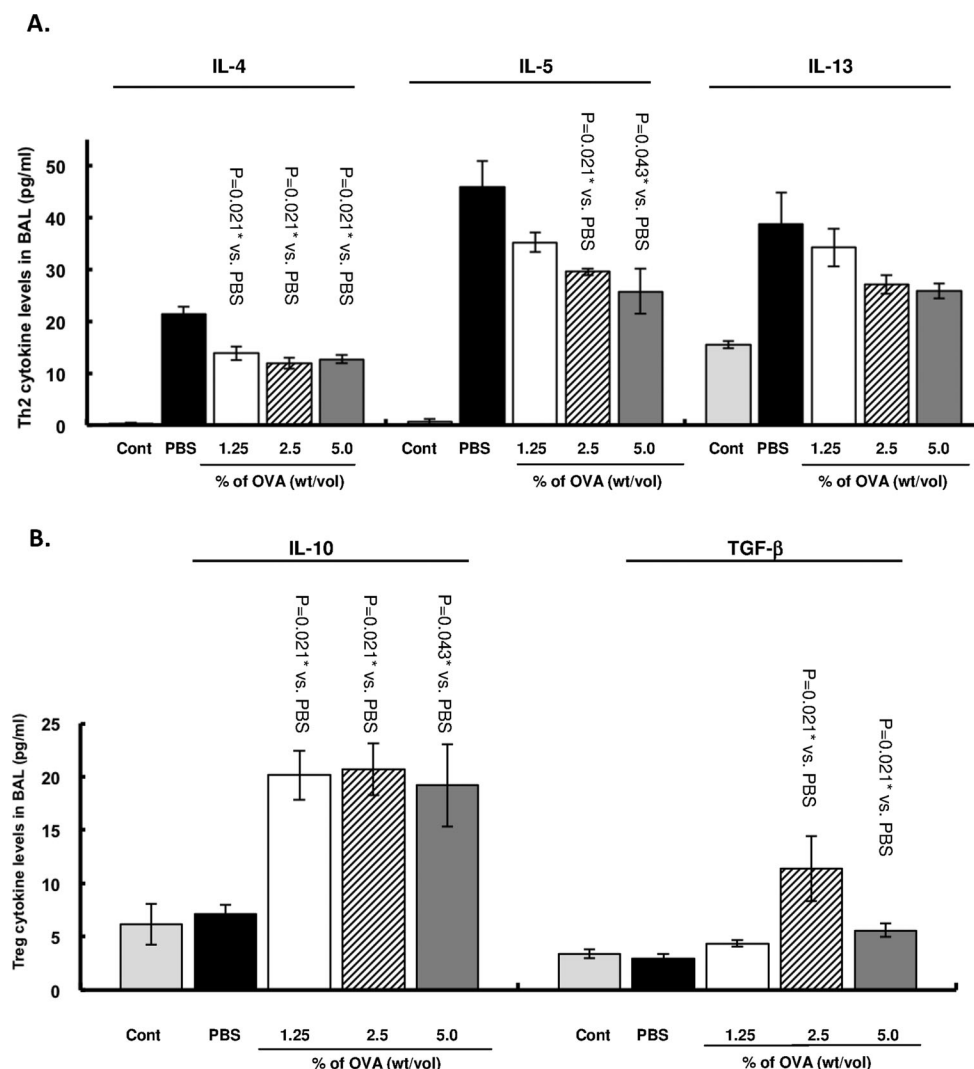


FIGURE 4. Expression levels of IL-4, IL-5, IL-13, IL-10, and TGF- β in bronchoalveolar lavage fluids. ELISA measured the cytokine levels in BALFs for IL-4, IL-5, and IL-13 (A), and IL-10 and TGF- β (B) ($n = 4$ for each group). The Mann-Whitney U test was used to compare differences between 2 groups when the result of Kruskal-Wallis test was significant. * $p < 0.05$. BALF = bronchoalveolar lavage fluid; ELISA = enzyme-linked immunosorbent assay; IL = interleukin; TGF = transforming growth factor.

symptom relief.² Immunotherapy is the only feasible modality for modulation of allergic inflammatory reactions. Immunotherapy builds up allergen tolerance through repeated exposure of patients to progressively increasing doses of a specific allergen extract.^{22–24} Although the precise mechanisms associated with immunologic tolerance are complex and remain to be elucidated, there is evidence showing that T regulatory (Treg) cells exert a protective effect.²⁵ Treg cells produce cytokines including IL-10, IL-12, and TGF- β , as well as suppress recruitment of Th2 cells and eosinophils.^{26–31} In our results, inhalational antigen preimmunization exposure resulted in downregulation of IL-4, IL-5, and IL-13, reduced eosinophil infiltration, and upregulation of IL-10 and TGF- β cytokine expression in lung.

Given the potential of genetic techniques to help identify children at risk for development of asthma, a recent review of advances in pediatric asthma suggests that the

future therapeutic modalities should move toward preventive management.³² Several factors including infection in early life and environmental microbial exposure are postulated to protect children from development of allergic airway diseases.^{33,34} Early-life mucosal allergen preimmunization exposure might also play a role in the prevention of allergy, according to the results of animal studies.^{3–5} Microbial exposure is difficult to implement as a protective management because the assessment of optimal exposure to microorganisms is challenging. Inadequate exposure might result in infectious diseases. However, it is feasible to preimmunization-expose high-risk children to potential allergens in their early life based on the clinical efficacy of immunotherapy. Although it is a highly attractive modality, early-life antigen preimmunization exposure as a preventive management remains controversial because the mechanisms and dose-response relationships have not yet been clearly elucidated. A similar situation exists with

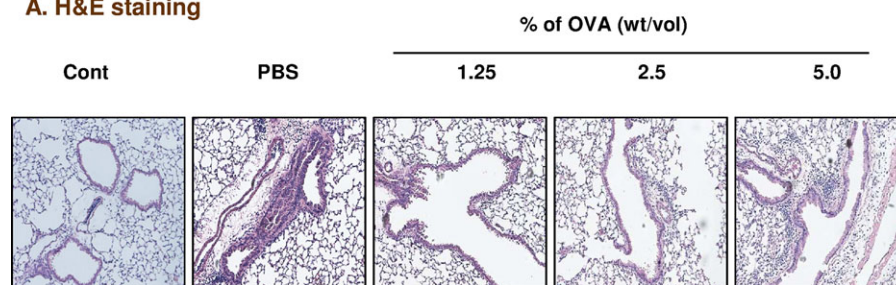
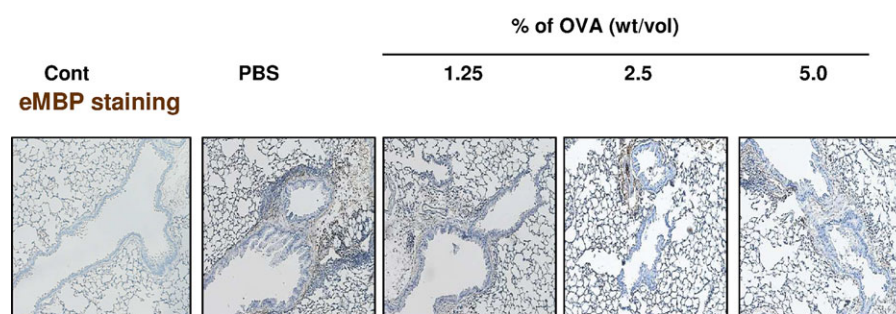
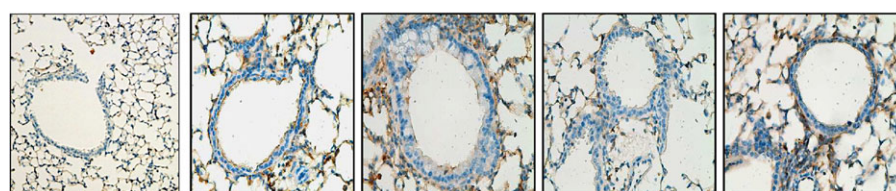
A. H&E staining**B. Immunocytochemistry staining****T cell/Neutrophil marker staining**

FIGURE 5. Histological and morphological observations in lung tissues. (A) Representative sections from lung tissues of Cont, PBS, OVA-1.25, OVA-2.5, and OVA-5 groups, respectively. The representative paraffin sections were stained with H&E and the digital images were captured using a light microscope (magnification $\times 100$). The inflammatory changes were significantly reduced in OVA-2.5 group when compared with those of the PBS group. (B, C) eMBP (magnification $\times 100$) and T cell/neutrophil marker immunocytochemistry staining ($\times 200$) of representative sections from lung tissues of 5 groups. eMBP = eosinophil major basic protein; H&E = hematoxylin and eosin; OVA = ovalbumin; PBS = phosphate-buffered saline.

sublingual immunotherapy, which enjoys widespread popularity in European countries today, but has limited use in the U.S. The Food and Drug Administration has only recently approved sublingual tablets for the U.S., but only for ragweed and grass allergens.

Inhalational exposure to protein antigen is the primary route of immunization in allergic airway diseases. In 1981, Holt et al.³⁵ first reported inhibition of specific IgE responses by preimmunization exposure to an inhaled antigen weekly on 6 to 8 occasions. Seymour et al.³⁶ confirmed that mice exposed preimmunization to 10 days of OVA aerosol induced IgE nonresponsiveness to subsequent OVA challenges, and showed that the underlying mechanisms were through the loss of both Th1 and Th2 responses. Very low-dose OVA (from 1% to 0.01% wt/vol) aerosol for preimmunization was used in the 2 aforementioned studies,^{35,36} and the dose-response relationship was not explored in detail in their experiments. Antigen dose is an important factor in the polarized development of helper T cell subsets (ie, Th1 or Th2). Shin et al.³⁷ fed mice

25 mg or 6 mg OVA before immunization. They found that both groups had reduced inflammatory changes, and the low-dose group had less airway remodeling. Ishida et al.³⁸ reported that both 100 mg and 2.5 mg OVA feeding induced oral tolerance in their experimental allergic conjunctivitis mouse model. The authors interpreted that the prophylactic effect of high-dose antigen feeding is likely to be mediated by the clonal anergy/deletion of OVA-reactive T cells, and the effect of low-dose OVA feeding is mediated by enhancing regulatory T cell effects. The 2 forms of tolerance are not mutually exclusive and may overlap.³⁸ We designed a rush prophylactic treatment protocol with 3 inhalation doses (1.25%, 2.5%, and 5% wt/vol). When we extended the preimmunization exposure OVA doses, we found that higher OVA (10% wt/vol) preimmunization exposure could exacerbate the airway hyperresponsiveness and enhance IL-13 production (Supporting Fig. 1). An optimal preimmunization exposure antigen dose is important in successful induction of immunological tolerance. Our results showed that medium doses (1.25%, 2.5%, and 5%

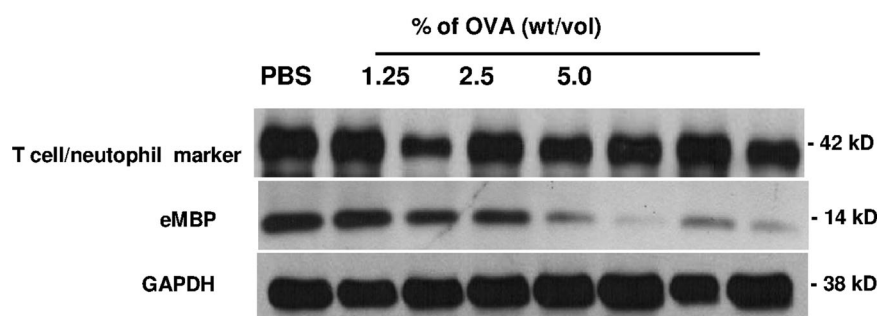
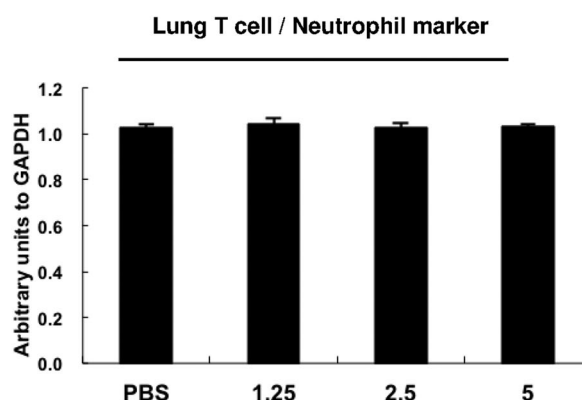
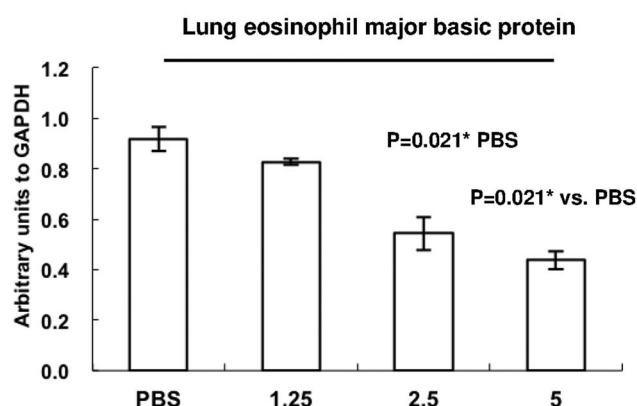
A.**B.****C.**

FIGURE 6. The eMBP and T cell/neutrophil marker protein expression levels in lung tissues. (A) Results of immunoblotting analyses of eMBP and T cell/neutrophil marker. (B, C) Data of relative eMBP and T cell/neutrophil marker proteins from densitometry to internal control, GAPDH, and are presented as mean $\pm$ SEM ($n = 4$ in each group). The Mann-Whitney U test was used to compare differences between 2 groups when the result of Kruskal-Wallis test was significant. $*p < 0.05$. eMBP = eosinophil major basic protein; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; SEM = standard error of the mean.

wt/vol) were effective to balance cytokine changes, reduce cellular infiltration and IgE, and suppress AHR. A lower dose (0.625% wt/vol) generated a subtle effect, whereas a higher dose (10% wt/vol) exacerbated AHR. Upon application of prophylactic preimmunization exposure to humans, careful selection of an adequate dose is very important to provide efficacy and avoid adverse reactions. In our study

design, mice received immediate immunization right after preimmunization exposure to a specific antigen. It would be more clinically valuable if the preimmunization exposure were able to induce tolerance far in advance of immunization. Seymour et al.³⁶ had reported that mice exposed to 10 days of very-low-dose OVA aerosol preimmunization (0.001% to 0.1 wt/vol) can keep IgE nonresponsiveness to

subsequent OVA challenges until day 120. Further investigation is necessary to evaluate how long the protective effect can last using our methods.

Conclusion

Our results suggest that preimmunization exposure to an appropriate dose of a specific antigen could effectively suppress the allergic airway inflammation by induction of im-

munological tolerance. Antigen preimmunization exposure may have value as a primary prevention strategy for allergic airway diseases. 

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