

Original article

1,25-Dihydroxyvitamin D₃ pretreatment enhances the efficacy of allergen immunotherapy in a mouse allergic asthma model

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Keywords: asthma; airway inflammation; immunotherapy; vitamin D₃

Background Allergen-specific immunotherapy can induce immune tolerance to specific allergens by regulating immune status of individuals. However, its clinical application is limited due to individual differences in efficacy among patients and un-confirmed safety. 1,25 Dihydroxyvitamin D₃ (1,25(OH)₂D₃) has been shown to be involved in a variety of physiological processes, including immune response regulation. In the present study we explored the role of 1,25(OH)₂D₃ pretreatment for immunotherapy.

Methods Seventy-five BALB/c mice were randomly divided into five groups (15 mice per group). The mouse allergic asthma model was established by intra-peritoneal injection of ovalbumin (OVA, 10 µg) and aluminium hydroxide (2 mg) as an adjuvant. Intra-peritoneal injection of 50 ng of 1,25(OH)₂D₃ served as a pretreatment, subcutaneous injection of OVA (100 µg) as an immunotherapy, and 1% OVA inhalation as a challenge. Histopathological analysis was performed on four mice per group. The number of cells and their classification in bronchoalveolar lavage (BAL) fluid were assayed. Levels of serum OVA-specific immunoglobulin E (sIgE) and IFN-γ, IL-4, IL-5 and IL-10 in BAL fluid were measured by ELISA.

Results After 1,25(OH)₂D₃ pretreatment, immunotherapy could significantly inhibit the infiltration of inflammatory cells into lung tissues and BAL fluid of mice with allergic asthma when compared with un-treated animals (eosinophils: $(7.46 \pm 1.34) \times 10^4/\text{ml}$ vs. $(13.41 \pm 1.67) \times 10^4/\text{ml}$, $P < 0.05$). In addition, levels of IL-4 ((36.91 ± 7.87) pg/ml vs. (43.70 ± 6.42) pg/ml, $P > 0.05$) and IL-5 ((41.97 ± 7.93) pg/ml vs. (60.14 ± 8.35) pg/ml, $P < 0.05$) in BAL fluid and serum sIgE ((0.42 ± 0.05) vs. (0.75 ± 0.06) OD units, $P < 0.05$) were profoundly reduced. However, the IL-10 level in BAL fluid was significantly increased ((67.74 ± 6.57) pg/ml vs. (44.62 ± 8.81) pg/ml, $P < 0.05$).

Conclusions These results indicated that 1,25(OH)₂D₃ pretreatment enhanced the inhibitory effects of immunotherapy on allergic airway inflammation. In the treatment of allergic diseases, 1,25(OH)₂D₃ pretreatment may be beneficial for improving the efficacy of immunotherapy.

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Currently, allergen-specific immunotherapy can induce immune tolerance to specific allergens and is the only etiological treatment for allergy. It can alter the natural course of disease by regulating the immune status.¹ Subcutaneous immunotherapy (SCIT) has been an alternative therapy of allergies against insect venoms, house dust mites, pollens and animal dander for almost a century.² However, the need for repeated injections over several years makes this treatment inconvenient, particularly for children. In addition, SCIT may sometimes result in infrequent but severe side effects, including anaphylactic shock.³ Although administration of specific allergens is effective for rhinitis and insect venom allergy, the role of this intervention in allergic asthma remains controversial. Clinical trials demonstrate that allergen immunotherapy not only improves clinical symptoms, but inhibits allergic airway inflammation and airway hyperresponsiveness.^{4,5} Thus, improvement of SCIT should be explored for allergic asthma. The specific immunologic mechanisms by which immunotherapy achieves its effect have not been well elucidated,⁶ although the mechanisms underlying SCIT are related to antigen presenting cells, T cells, B cells and antigen-antibody reaction.⁷⁻¹⁰

The activated form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), is a steroid hormone and plays an important role in the metabolisms of calcium, phosphorus and bone. In addition, 1,25(OH)₂D₃ also regulates immune responses via special receptors on antigen presenting cells and activated T cells.¹¹⁻¹³ The inhibitory effects of 1,25(OH)₂D₃ on T lymphocyte activation was observed in experiments with autoimmune diseases and allogeneic transplantation. Furthermore, 1,25(OH)₂D₃ attenuates the activity of some diseases mediated by T cells including experimental allergic encephalomyelitis,¹⁴ collagen-induced arthritis,¹⁵ autoimmune thyroiditis,¹⁶ hereditary diabetes,¹⁷ Heymann nephritis,¹⁸ and rejection of heart transplantation.¹⁹

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Inhalation of aeroallergens in patients with allergic asthma may lead to a Th2-biased immune response and subsequent production of cytokines can induce representative features of allergic airway disease; such as eosinophilic airway inflammation, airway hyper-responsiveness and airway remodeling.²⁰ Independent studies revealed both allergen-specific immunotherapy and 1,25(OH)₂D₃ showed promise in regulating immune function. We hypothesized that 1,25(OH)₂D₃ pretreatment could enhance the efficacy of SCIT in a mouse allergic asthma model.

METHODS

Animals

Female BALB/c mice, 6–8 weeks old and weighing 19–22 g, were purchased from the Institute of Animal in the Third Military Medical University (Chongqing, China). During the experiments, mice were kept in specific pathogen-free animal facilities with controlled temperature (25±2°C) and humidity (55%). All animals were acclimated for at least one week before experiments. Experimental protocols were approved by the Institutional Animal Ethics Committee.

Sensitization, challenge and experimental procedures

BALB/c mice were divided into five groups (15 mice per group). Group A (negative control), mice were sensitized and challenged with phosphate buffered saline (PBS), which was then applied for pretreatment and immunotherapy. Group B (asthma control), mice were sensitized with ovalbumin (OVA) and aluminum hydroxide (Al(OH)₃), challenged with OVA, and PBS was applied for pretreatment and immunotherapy. Group C (1,25(OH)₂D₃), mice were sensitized with OVA and Al(OH)₃, challenged with OVA, pretreated with 1,25(OH)₂D₃, but PBS was used for immunotherapy. Group D (SCIT), mice were sensitized with OVA and Al(OH)₃, challenged with OVA, pretreated with PBS, and OVA was used for immunotherapy. Group E (1,25(OH)₂D₃+SCIT), mice were sensitized with OVA and Al(OH)₃, challenged with OVA, pretreated with 1,25(OH)₂D₃, and OVA was used for immunotherapy.

Sensitization and challenge were performed according to a previously described technique (Figure 1).²¹ In brief, all mice (except for those in Group A) were sensitized with 10 µg of OVA (grade V, Sigma, USA) adsorbed to 2 mg of Al(OH)₃ adjuvant (Imject Alum, Pierce, USA) in 0.1 ml of PBS by intraperitoneal injection on days 0 and 7. Mice of the negative control group were sensitized with 0.1 ml PBS. Two weeks after sensitization, mice in Group D and Group E were treated with 100 µg OVA in 0.2 ml of pyrogen-free PBS by subcutaneous injection on days 21, 23, and 25, serving as immunotherapy, while mice in Groups A, B and C were treated with 0.2 ml of PBS. Twenty four hours before each immunotherapy, mice of Groups C and E were pretreated with 50 ng of 1,25(OH)₂D₃^{22,23} in 96% (v/v) ethanol by intraperitoneal injection. Mice, except for those in Group A, were antigen

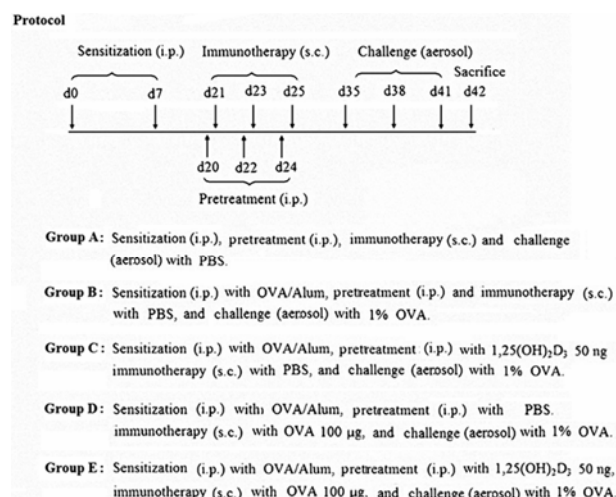


Figure 1. Experiment protocol. Mice were sensitized by intraperitoneal (i.p.) injections on days 0 and 7, pretreated by i.p. injections on days 20, 22 and 24 and immunotherapy was performed by s.c. injections on days 21, 23 and 25, followed by aerosol challenge on days 35, 38 and 41. All mice were sacrificed on day 42.

challenged in a plexiglas chamber (10 cm × 30 cm × 15 cm) connected to the ultrasonic nebulizer (NE-U12, Omron, Tokyo, Japan). The chamber was infused with 10 mg/ml OVA aerosols in pyrogen-free PBS for 30 minutes on days 35, 38 and 41. For analysis, all mice were sacrificed 24 hours after the last challenge on day 42.

Examination of bronchoalveolar lavage (BAL) fluids

Mice were anaesthetized and the trachea was cannulated while gently massaging the thorax. Lungs were lavaged four times with 0.5 ml of 37°C PBS. The BAL fluid was kept on ice for later use. Cells in BAL fluid were washed with PBS (400 ×g, 5 minutes, 4°C), and the supernatant of the BAL fluid was used to measure cytokine levels. Cells were re-suspended in 150 µl of PBS. The number of cells in a 50 µl aliquot was determined with a haemocytometer and total numbers of BAL fluid cells were determined. Differential cell counting was performed on cytopspin preparations stained with Diff-Quick (International Reagents Corp., Kobe, Japan). Cells were identified and classified into mononuclear cells, lymphocytes, neutrophils, and eosinophils based on standard morphology. At least 200 cells were counted and the absolute number of each cell population was calculated. The remaining BAL fluid was centrifuged, and supernatant stored at -70°C for cytokine analysis.

Measurement of cytokines

The levels of cytokines in BAL fluid were determined by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (eBioscience, USA). ELISA kits were employed for the measurement of IL-4, IL-5, IL-10, and interferon-γ (IFN-γ) levels.

Determination of serum OVA-specific IgE level

Blood samples were obtained from the mice via a cardiac

puncture and centrifuged for 10 minutes at 2000 r/min. Serum was collected and samples were kept at -20°C until IgE levels were measured. OVA-specific serum IgE levels were measured by ELISA. Briefly, a 96-well microplate was coated with 10 mg of OVA in 50 μl coating buffer and kept overnight at 4°C . The wells were washed three times with PBS and blocked with 50 μl blocking buffer (50 mmol/L Tris, 2 mmol/L EDTA, 136.9 mmol/L NaCl, 0.05% Tween-20 and 0.5% bovine serum albumin (BSA)) and kept at 37°C for two hours. After washing three times with PBST (0.05% Tween 20 in PBS), 100 μl of serum (diluted 1:5 in blocking buffer) was added to the wells and kept overnight at 4°C . The wells were washed three times with PBST and 50 μl of biotinylated anti-mouse IgE (1:1000, Southern Biotech, USA) was added and kept at 37°C for two hours, followed by incubation with 0.33 $\mu\text{g}/\text{ml}$ peroxidase-conjugated streptavidin for one hour. The substrate phenylenediamine dichloride (0.4 mg/ml) in PBS containing 0.012% hydrogen peroxide was added. After 15 minutes, the reaction was stopped by adding 2.4 mol/L H_2SO_4 . Subsequently, optical density (OD) was determined at 490 nm using a Benchmark microplate reader (Bio-Rad, USA).

Histological analysis of lung tissues

Twenty four hours after the last challenge, mice were sacrificed and lungs were fixed by an intra-tracheal infusion of 10% formalin in PBS. Fixed lungs were removed and fixed in formalin for 24 hours followed by being embedded in paraffin. Tissues were sectioned into 5 μm sections and stained with hematoxylin and eosin (H&E) for histology.

Statistical analysis

Statistical analyses were performed using SPSS 16.0 statistical software package (SPSS, Inc., Chicago, USA). Data are expressed as means $\pm$ standard error (SE). One-way analysis of variance (ANOVA), followed by a least-significant difference (LSD) test was performed for comparisons between two groups or Wilcoxon rank sum test used for data not normally distributed. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Inflammatory cells in BAL fluid were reduced by $1,25(\text{OH})_2\text{D}_3$ pretreatment and SCIT

To determine the effect of $1,25(\text{OH})_2\text{D}_3$ pretreatment and SCIT on airway inflammation, we examined the number of inflammatory cells in airway lumen 24 hours after the last OVA aerosol challenge. OVA sensitization and challenge induced marked increases in the number of eosinophils in BAL fluid. When compared with the $1,25(\text{OH})_2\text{D}_3$ alone group (Group C) and the SCIT alone group (Group D), the numbers of eosinophils in the $1,25(\text{OH})_2\text{D}_3$ pretreatment in combination with SCIT group (Group E) were significantly reduced; Group E vs. Group D: $(7.46 \pm 1.34) \times 10^4/\text{ml}$ vs. $(13.41 \pm 1.67) \times 10^4/\text{ml}$, P

< 0.05 . In addition, eosinophilia in the lung was also markedly improved by $1,25(\text{OH})_2\text{D}_3$ pretreatment and SCIT (Figure 2).

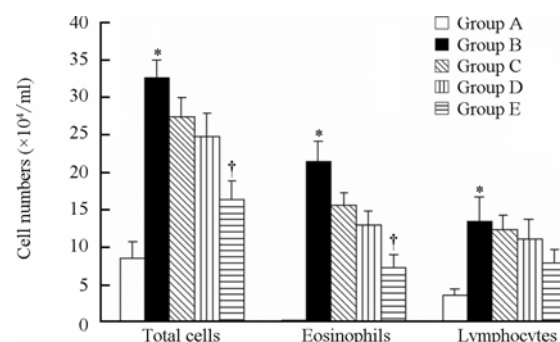


Figure 2. Total cells, eosinophils and lymphocytes in BAL fluid. Total cells, eosinophils and lymphocytes were significantly increased in BAL fluid of mice in Group B (asthma control), when compared with Group A (negative control). The numbers of all cell types were profoundly decreased in mice in Group E ($1,25(\text{OH})_2\text{D}_3$ +SCIT) when compared with Group C ($1,25(\text{OH})_2\text{D}_3$ alone) and Group D (SCIT alone). * $P < 0.05$, Group B vs. Group A; † $P < 0.05$, Group E vs. Group D.

Pathological changes were improved by $1,25(\text{OH})_2\text{D}_3$ pretreatment and SCIT

The changes in numbers of inflammatory cells shown by histology of the lungs were similar to those seen in BAL fluid. The results of histological examination of lung tissues paralleled the cell numbers in the BAL fluid. A significant inflammatory response was observed in OVA-sensitized and challenged mice. Of note, inflammatory cells could be observed between epithelial cells and in lamina propria, and goblet-cell hyperplasia was also noted in the airways of OVA-challenged mice (Group B), which was not found in the airway of Group A. Additionally, airway epithelial cells were disrupted and base membrane significantly thickened, suggesting remodeling. However, $1,25(\text{OH})_2\text{D}_3$ pretreatment in combination with SCIT (Group E) notably reduced airway inflammation (Figure 3).

Cytokine levels in BAL fluid were reduced by $1,25(\text{OH})_2\text{D}_3$ and SCIT

Th2 cytokines such as IL-4 and IL-5 are important in the initiation and propagation of allergic inflammation responses. Several studies have also demonstrated that IFN- γ plays an important role in the pathogenesis of asthma. The BAL fluid was obtained 24 hours after the last airway challenge. The levels of IL-4, IL-5, IL-10, and IFN- γ in the BAL fluid were determined by ELISA. The levels of all cytokines in the BAL fluid of Group B were significantly increased after airway OVA challenge when compared with those of the negative control (Group A). When compared with SCIT alone group (Group D), $1,25(\text{OH})_2\text{D}_3$ pretreatment in combination with SCIT (Group E) reduced the IL-4 levels ((36.91 ± 7.87) pg/ml vs. (43.70 ± 6.42) pg/ml, $P > 0.05$), and IL-5 levels ((41.97 ± 7.93) pg/ml vs. (60.14 ± 8.35) pg/ml, $P < 0.05$). However, SCIT pretreatment resulted in increases in the

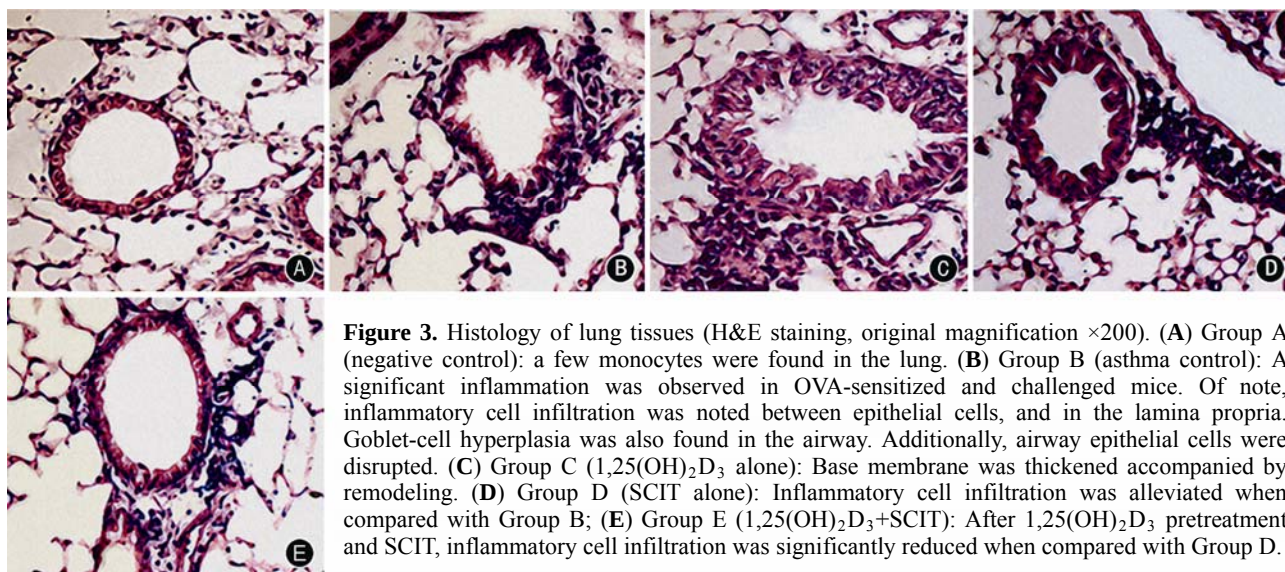


Figure 3. Histology of lung tissues (H&E staining, original magnification $\times 200$). (A) Group A (negative control): a few monocytes were found in the lung. (B) Group B (asthma control): A significant inflammation was observed in OVA-sensitized and challenged mice. Of note, inflammatory cell infiltration was noted between epithelial cells, and in the lamina propria. Goblet-cell hyperplasia was also found in the airway. Additionally, airway epithelial cells were disrupted. (C) Group C ($1,25(\text{OH})_2\text{D}_3$ alone): Base membrane was thickened accompanied by remodeling. (D) Group D (SCIT alone): Inflammatory cell infiltration was alleviated when compared with Group B; (E) Group E ($1,25(\text{OH})_2\text{D}_3$ +SCIT): After $1,25(\text{OH})_2\text{D}_3$ pretreatment and SCIT, inflammatory cell infiltration was significantly reduced when compared with Group D.

IL-10 levels of Group E to Group D ((67.74 ± 6.57) pg/ml vs. (44.62 ± 8.81) pg/ml, $P < 0.05$) (Figure 4). In addition, $1,25(\text{OH})_2\text{D}_3$ pretreatment in combination with SCIT reduced IFN- γ level in the mouse allergic asthma model, but the difference was not significant when compared with the SCIT alone group (Group D).

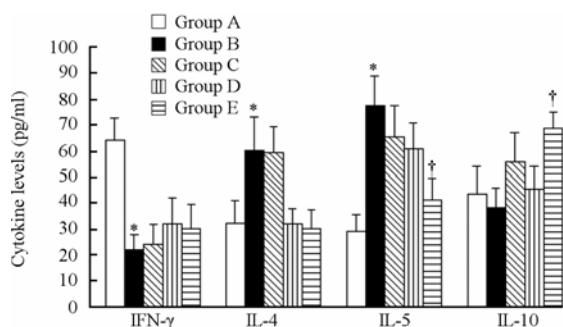


Figure 4. IL-4, IL-5, IFN- γ and IL-10 cytokine levels in BAL fluid. Levels of IL-4 and IL-5 were significantly increased in BAL fluid of Group B (asthma control) when compared with Group A (negative control). However, the IL-5 level was dramatically decreased in the BAL fluid of Group E ($1,25(\text{OH})_2\text{D}_3$ + SCIT) as compared with Group D (SCIT alone) and Group C ($1,25(\text{OH})_2\text{D}_3$ alone). The IL-10 level in Group E was profoundly increased compared with Group D. * $P < 0.05$, Group B vs. Group A; † $P < 0.05$, Group E vs. Group D.

$1,25(\text{OH})_2\text{D}_3$ and SCIT reduced serum OVA-specific IgE level

The cross-linking of allergen-specific IgE on the surface of mast cells upon allergen challenge is relevant to the initiation of the early asthma response. The serum levels of OVA-specific immunoglobulins were measured 24 hours after the last airway challenge. Serum level of OVA-specific IgE in Group B was markedly increased when compared with the negative control group (Group A). $1,25(\text{OH})_2\text{D}_3$ pretreatment in combination with SCIT (Group E) resulted in a reduction in OVA-specific IgE levels when compared with the $1,25(\text{OH})_2\text{D}_3$ alone group (Group C) and the SCIT alone group (Group D); Group E

vs. Group D was (0.42 ± 0.05) OD units vs. (0.75 ± 0.06) OD units, $P < 0.05$ (Figure 5). These results suggest that $1,25(\text{OH})_2\text{D}_3$ pretreatment in combination with SCIT suppressed the Th2-type immune response in the animal asthma model.

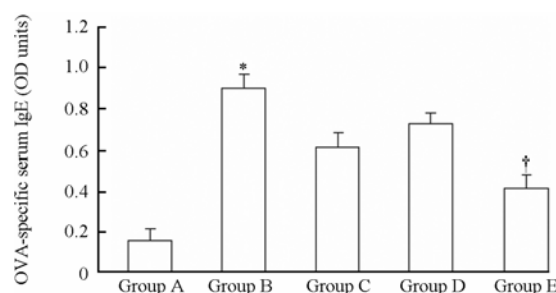


Figure 5. Serum sIgE level. sIgE level was significantly increased in the serum of Group B (asthma), as compared with Group A (negative control). The sIgE level was decreased markedly in Group E ($1,25(\text{OH})_2\text{D}_3$ + SCIT), when compared with mice of Group D (SCIT alone). * $P < 0.05$, Group B vs. Group A; † $P < 0.05$, Group E vs. Group D.

DISCUSSION

In the present study, we showed that mice receiving 50 ng of $1,25(\text{OH})_2\text{D}_3$ pretreatment in combination with 100 μg of OVA as a SCIT had markedly inhibited OVA-induced airway inflammation in an asthma model. It implies that $1,25(\text{OH})_2\text{D}_3$ pretreatment enhanced the effect of SCIT by augmenting the suppression of the allergen-specific IgE level and eosinophilic airway inflammation, reducing IL-4 and IL-5 levels and increasing the IL-10 level in BAL fluid.

There are a variety of protocols for the clinical application of SCIT. Generally, the concentration of allergen gradually increases from a low concentration to a maximum tolerated dose. The course of SCIT is usually up to 3 to 5 years,²⁴ which makes it difficult to conduct an animal study. Rush immunotherapy can achieve similar

efficacy as traditional SCIT, and the course is reduced to six months or less.²⁵ Based on experience from clinical rush-immunotherapy, a mouse asthma model in which 1 mg of OVA was injected three times weekly showed that SCIT was also effective in reducing allergic airway inflammation and levels of IL-4.²⁶ In another study, SCIT was performed by subcutaneous injection with 10 µg, 100 µg and 1000 µg three times weekly. A low dose of OVA-IT (10 µg) failed to counteract the asthma-like symptoms after OVA inhalation challenge. In contrast, suboptimal OVA-IT (1000 µg) partially suppressed the airway eosinophilia in the BAL fluid and OVA-specific IgE levels in serum but not the AHR, an optimal response was achieved at the dose of 1000 µg of OVA followed by 100 µg of OVA.²³ To determine the synergistic effect of 1,25(OH)₂D₃ pretreatment and SCIT in our study, 100 µg of OVA were administered by subcutaneous injection injected three times in one week. The dose of 1,25(OH)₂D₃ (50 ng) was determined as previously described.^{22,23,27}

Allergic asthma has been recognized as a disease characterized by infiltration of inflammatory cells into the lungs, mucus hyper-secretion, airway obstruction, and thickening of bronchial walls and smooth muscle. Asthma severity varies from mild asthma to potentially fatal asthma. Currently, treatments of asthma usually aim to inhibit inflammation with glucocorticoids and reverse bronchoconstriction with bronchodilators, these are only palliative therapies.²⁸ Allergen-specific immunotherapy is a highly attractive strategy. At present, studies have shown that the effects of immunotherapy are related to induction of blocking IgG antibodies,²⁹ induction of T cell anergy,⁷ a shift from Th2 to Th1 cytokine profiles,^{8,9} or induction of *in vivo* regulatory T cells (Treg).¹⁰ However, the need for repeated injections over several years makes this treatment inconvenient. SCIT may also be associated with infrequent but severe side effects including anaphylactic shock.³ Our study investigated the adjuvant effect of 1,25(OH)₂D₃ during SCIT with the intention of reducing the duration of treatment.

1,25(OH)₂D₃ not only regulates metabolism of calcium and phosphorus, but acts on cell proliferation, differentiation, apoptosis and immune function. 1,25(OH)₂D₃ can bind to vitamin D receptors on the cell membrane and in the cytoplasm of target cells (such as dendritic cells, monocytes macrophages, B cells, T cells, etc) affecting cell function and secretion of Th1/Th2 cytokines. Previous experiments showed 1,25(OH)₂D₃ in combination with SCIT played an important role in regulating immune function. Dendritic cells are a target cell of 1,25(OH)₂D₃ mediators of immune function. Besides promoting a shift to Th1 or Th2 cells, dendritic cells also provide directions for the generation of adaptive Treg cells, such as Th3 and Tr1 cells.^{30,31} As regulators of immune suppression, the effect of 1,25(OH)₂D₃ on dendritic cells is central in understanding its adjuvant effect during SCIT.

Inhalation of aeroallergens in sensitized individuals leads

to a Th2-biased immune response and subsequent production of cytokines resulting in major manifestations of allergic airway disease.^{20,24} In the present study, 1,25(OH)₂D₃ pretreatment resulted in a significant decrease in BAL levels of Th2 cytokines IL-4 and IL-5 while the Th1 cytokine IFN-γ was not markedly changed. This implies that treatment partially rectified Th1/Th2 imbalance. In asthma, IL-10 plays a key role in negative feedback regulation. The increased IL-10 level in BAL fluid after 1,25(OH)₂D₃ pretreatment might be related to the decreased IgE level and the production of Treg cells.^{10,32} The serum sIgE level was reduced significantly after 1,25(OH)₂D₃ pretreatment, which suggested it might be beneficial for treatment of early stage asthma. Because it is an allergen penetrating into an allergic organism, the antigen presentation results in a Th2 differentiation of specific T cells. Th2 cells trigger the IgE synthesis, while by producing IL-5 they attract and activate eosinophil polymorphonuclear cells. Then IgEs bind their high affinity receptor (FcεRI) on effector cells such as mast cells and in case of continuous exposure or re-exposure induce mast cell degranulation, histamine and leukotriene release and the early phase of the allergic reaction.

In conclusion, 1,25(OH)₂D₃ pretreatment significantly enhanced the efficacy of SCIT, and markedly reduced airway inflammation in a mouse allergic asthma model. These findings indicate that 1,25(OH)₂D₃ may serve as an adjuvant therapy for allergic airway inflammation by inhibiting the Th2 response. To date, 1,25(OH)₂D₃ has been safely applied in clinical practice for many years. We postulated that 1,25(OH)₂D₃ pretreatment could provide a favorable clinical adjuvant strategy for the treatment of asthma with SCIT. Our study may provide a basis for further investigation of SCIT in the treatment of asthma. However, it is important that we continue to investigate the exact mechanism underlying the effects of adjuvant SCIT therapy for treatment of the aberrant allergic immune response in asthmatics.

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