

# Preventive and therapeutic effects of vitamin D in a mouse model of allergic asthma

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## Abstract

**Background:** Vitamin D produces an anti-allergic effect that prevents inflammation due to asthma.

**Objective:** We investigated whether vitamin D has an anti-inflammatory effect on the sensitization and challenge stages of asthma development in a murine model.

**Methods:** Mice were divided into the following five groups according to ovalbumin (OVA) and vitamin D (VD) administration: control group, OVA group, preventive VD group (VD injection before OVA sensitization), inhibitory VD group (VD injection after OVA challenge), and dual VD groups (VD injection before OVA sensitization and after OVA challenge). Each group was evaluated for airway hyperresponsiveness (AHR), cell counts, cytokines, total IgE, and OVA-specific IgE using bronchoalveolar lavage fluid (BALF). Cytokines in the lysate and eosinophils in the lung tissue were also evaluated.

**Results:** AHR occurred less in the groups to which VD was administered than in the OVA group. The eosinophils, neutrophils, IL-5 in BALF, IL-4, TGF- $\beta$ , and eosinophils in lysate decreased with the administration of VD in the preventive, inhibitory, and dual VD groups compared with the OVA group. Although the lymphocytes, macrophages, IL-4 in BALF, and IL-5 in lysate decreased with administration of VD in the inhibitory and dual VD groups, they were not affected by preventive VD administration. These anti-allergic effects of VD were most noticeable with VD administration for dual (preventive and inhibitory) purposes.

**Conclusions:** VD may produce preventive and inhibitory effects on the development and exacerbation of asthma in a murine model. These effects are most noticeable when VD is used for dual purposes.

**Key words:** vitamin D, asthma, mice, inflammatory markers, immune modulation

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## Introduction

Whereas the relationship between vitamin D deficiency and bone metabolic disorders is well-established, the effect of vitamin D deficiency and insufficiency on allergic disease has been studied only recently. A low level of vitamin D in asthma patients was associated with decreased lung function and increased airway hyperresponsiveness (AHR).<sup>1</sup> Some cohort studies reviewed the relationship between vitamin D replacement and allergic diseases such as asthma. The replacement of vitamin D in pregnant women resulted in a lower incidence of wheezing in their children at 3 years of age.<sup>2</sup> However, another study asserted that vitamin D replacement at 1 year of age was

related to an increased rate of atopy and allergic rhinitis in adult life.<sup>3</sup> To verify the different epidemiologic observations of the relationship between vitamin D and allergic diseases, studies have used animal allergy models. Some of these studies demonstrated the effect of vitamin D on the parameters of allergic diseases and others did not. Some works reported that vitamin D supplementation reduced AHR and inflammatory cells, such as eosinophils, in bronchoalveolar lavage fluid (BALF).<sup>4,5</sup> Interestingly, other studies reported that vitamin D deficiency did not affect the level of eosinophils, cytokines such as IL-4 and IL-5, or ovalbumin (OVA)-specific IgE and that

vitamin D replacement did not modify inflammatory changes in lung tissue.<sup>6,7</sup>

Some studies investigated the relationship between vitamin D supplementation and inflammatory markers of allergic disease. However, few studies focused on the effect of vitamin D on the sensitization and challenge stages of asthma development individually. In this study, we aim to evaluate whether vitamin D has a preventive or a therapeutic effect on asthma development by administering vitamin D individually at the sensitization and challenge stages in an OVA-induced allergic mouse model. We assume that vitamin D can have a preventive effect on allergic sensitization if the administration of vitamin D before OVA-induced sensitization reduces allergic inflammatory markers. If vitamin D administration after OVA challenge reduces the inflammatory markers, we can suppose that vitamin D has an inhibitory effect on allergic provocation and thus has some kind of therapeutic effect.<sup>8</sup>

## Methods

### *Mice and grouping*

Female BALB/c mice (4–6 weeks of age) were used to examine the effects of vitamin D on OVA-induced asthma. Mice were divided into five groups, with 20 mice in each of the control, OVA, and preventive VD groups and 15 in each of the inhibitory VD and dual VD groups. The control group was sensitized and challenged with phosphate-buffered saline (PBS). The OVA group was sensitized and challenged with OVA. The preventive VD group (Prev-VD group) was sensitized and challenged with OVA, and vitamin D was administered before OVA sensitization to show the preventive effect of vitamin D on allergic inflammation. The inhibitory vitamin D group (Inhib-VD group) was sensitized and challenged with OVA, and vitamin D was given after the OVA challenge to show the inhibitory effect of vitamin D on allergic activation. The dual-stage vitamin D group (Dual-VD group) was sensitized and challenged with OVA, and vitamin D was administered both before OVA sensitization and after the OVA challenge. Animal care and experimental procedures were conducted in accordance with the approval and guidelines of the Institutional Animal Care and Use Committee of the Medical School of Inha University (Incheon, Korea; approval ID, INHA 130318-198).

### *Schedule of immunization and nasal challenge with OVA and administration of vitamin D*

OVA was given to induce sensitization and elicit an allergic reaction. Vitamin D was administered to each group according to a schedule. The control group was injected with 200  $\mu$ l of phosphate buffered saline (PBS) subcutaneously on days 0 and 14. About 100  $\mu$ l of distilled PBS (D-PBS) was inhaled intranasally on days 21, 22, and 23. The OVA group was injected subcutaneously on days 0 and 14 with 25  $\mu$ g of OVA combined with 1 mg of aluminum hydroxide and 200  $\mu$ l of D-PBS. Intranasal inhalation of 20  $\mu$ g of OVA mixed with 50  $\mu$ l of D-PBS was conducted on days 21, 22, and 23. The Prev-VD group was injected subcutaneously with 100 ng of 1,25(OH)<sub>2</sub>D<sub>3</sub> (Calcijex®, Abbott) prior to a 25  $\mu$ g OVA injection on days 0 and 14. Intranasal OVA inhalation was performed on days 21, 22, and 23. The Inhib-VD group was injected with 25  $\mu$ g of OVA subcutaneously on days 0 and 14. After OVA inhalation

on days 21, 22, and 23, about 100 ng of 1,25(OH)<sub>2</sub>D<sub>3</sub> was injected subcutaneously. The Dual-VD group was injected with 100 ng of 1,25(OH)<sub>2</sub>D<sub>3</sub> subcutaneously prior to OVA injection on days 0 and 14 and after OVA inhalation on days 21, 22, and 23 (**Figure 1**).

### *Measuring AHR with a noninvasive enhanced pause system (Penh)*

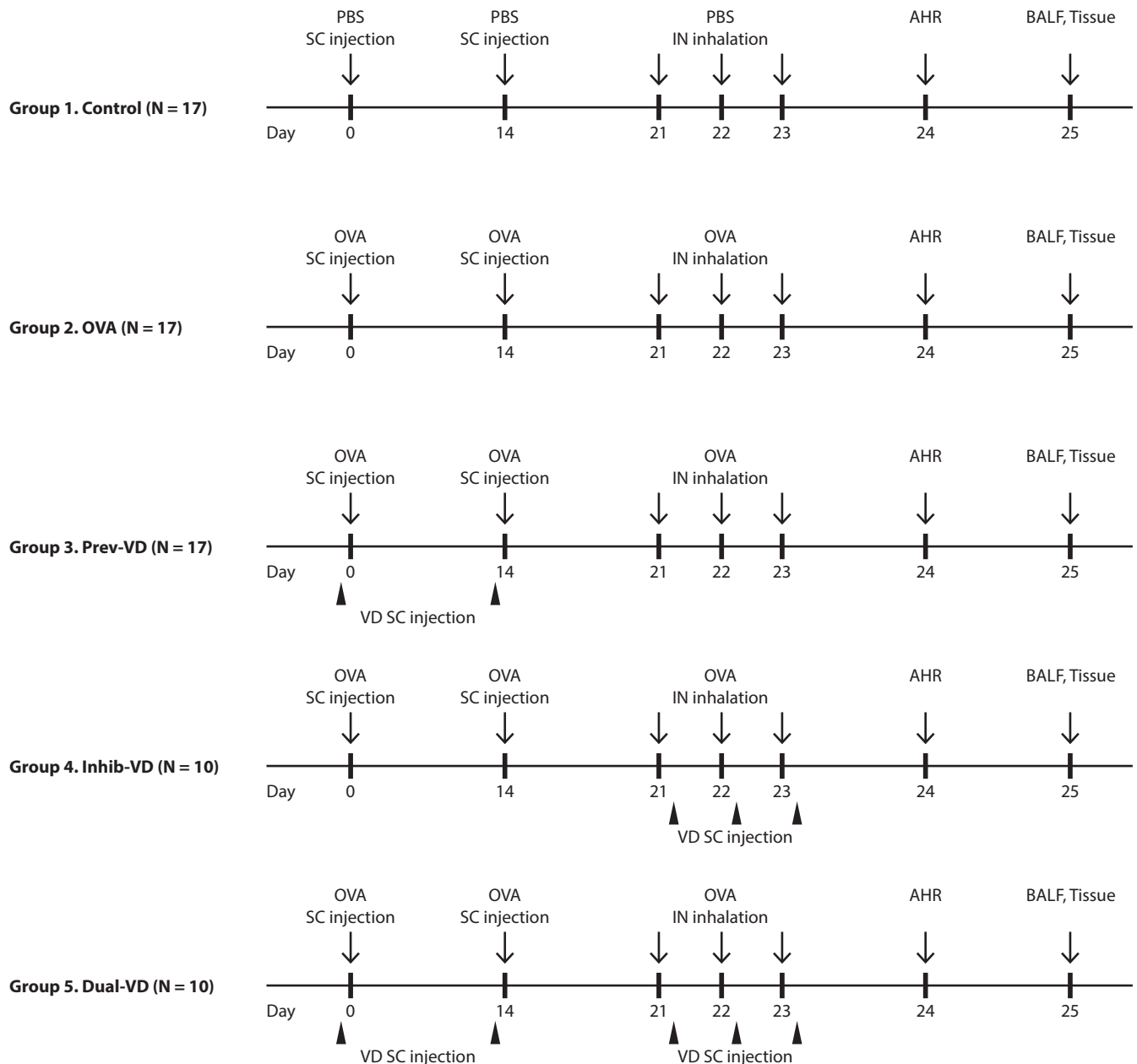
We examined the AHR of the mice on day 24 by employing barometric whole-body plethysmography (OCP 2000, Allmedicus, Korea). The mice in each group inhaled 3 ml of three different concentrations of methacholine mixture (5, 20, and 50 mg/ml) for 3 min using an Ultra-Neb nebulizer (3650p, DeVilbiss, Pennsylvania, PA). AHR was evaluated for each concentration of methacholine as an enhanced pause (Penh) for 3 min. The Penh is a value that is a function of the proportion of maximal expiratory to maximal inspiratory pressure signals and of the duration of expiration. The Penh was calculated as  $[Te / Tr - 1] \times PEF / PIF$ , where Te = expiratory duration (s); Tr = relaxation duration(s), defined as the duration of pressure decay to 30% of the total expiratory pressure signal; PEF = peak expiratory flow rate (ml/s); and PIF = peak inspiratory flow rate (ml/s).<sup>9</sup>

### *Bronchoalveolar lavage fluid analysis*

Twenty-four hours after AHR measurement, both lungs were lavaged twice with 2 ml of PBS. Cytospin samples were prepared by centrifuging the lavaged fluid at 2000 rpm for 7 min. The precipitate was mixed with 1 ml of PBS, and then 10  $\mu$ l of the mixture was once again mixed with 10  $\mu$ l of trypan blue. The total number of cells in the final mixture was counted with a hemocytometer, and the mixture was further diluted to a concentration of  $1 \times 10^6$  cells/ml. Then, we cytocentrifuged (Cytospin 2, Shandon) and stained the precipitate with Diff-Quick stain. Cell differentials were counted in the 400 HPF. Cell types were identified according to standard hemocytologic procedures as neutrophils, eosinophils, lymphocytes, or macrophages. The supernatant was analyzed by an enzyme-linked assay to detect IL-4, 5, 10, and IFN- $\gamma$ , the cytokines that are known to be inflammatory markers in asthma.<sup>10</sup> The total IgE and OVA-specific IgE in BALF were also measured by this method.

### *Examination of lung tissue*

Lung tissue lysate was prepared by homogenization in an ice-cold modified radio-immunoprecipitation assay lysis buffer with a cocktail of protease inhibitors. Cell debris was removed by centrifugation.<sup>11</sup> A reverse transcription polymerase chain reaction (RT-PCR) was performed to check for cytokines such as IL-4, 5, and 10. TGF- $\beta$  in the lung lysate was analyzed to evaluate the inflammatory state of the lung tissue of OVA-induced allergic mice. IL-4, 5, and 10 and TGF- $\beta$  were measured by RT-PCR using commercial products made by QIAGEN (Hilden, Germany). The commercial kits used were Mm\_IL4\_1\_SG Quanti Tect Primer Assay (QT00160678) for IL-4, Mm\_IL5\_1\_SG Quanti Tect Primer Assay (QT00099715) for IL-5, Mm\_IL10\_1\_SG Quanti Tect Primer Assay (QT00106169) for IL-10, Mm\_Tgfb1\_1\_SG Quanti Tect Primer Assay (QT00145250) for TGF- $\beta$ , and Mm\_Gapdh\_3\_SG Quanti Tect Primer Assay (QT01658692) for the control.



**Figure 1. Outline of the protocol in a murine model of asthma and the vitamin D intervention studies.**

A total of 25 µg of OVA was injected on days 0 and 14 for sensitization, and 20 µg of OVA was inhaled on days 21, 22, and 23 for the asthma challenge in OVA, preventive vitamin D, inhibitory vitamin D, and dual vitamin D group. About 100 ng of 1,25(OH)<sub>2</sub>D<sub>3</sub> was injected on days 0 and 14 in the preventive vitamin D and dual vitamin D groups and on days 21, 22, and 23 in the inhibitory vitamin D and dual vitamin D groups.

OVA, ovalbumin; PBS, phosphate-buffered saline; VD, vitamin D; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; SC, subcutaneous; IN, intranasal; Prev-VD, preventive vitamin D; Inhib-VD, inhibitory vitamin D; Dual-VD, preventive and inhibitory vitamin D.

To investigate the infiltration of eosinophils into the lungs of OVA-induced allergic mice, the histopathology of the lung tissue was examined by staining the major basic protein (MBP), a well-known eosinophil marker.<sup>12</sup> The lung tissue was extracted from the experimental mice on day 25, fixed with neutralized buffered formalin, and embedded in paraffin. The tissue was soaked in a fluid containing an anti-MBP antibody for 24 h. After soaking, the tissue was exposed to a secondary antibody (anti-rabbit IgG) for 30 min. The tissue that had been treated

with anti-MBP antibody was stained with 3, 3'-diaminobenzidine and hematoxylin. The MBP-stained cells from at least 10 different sites in the peribronchial area were counted.<sup>13</sup>

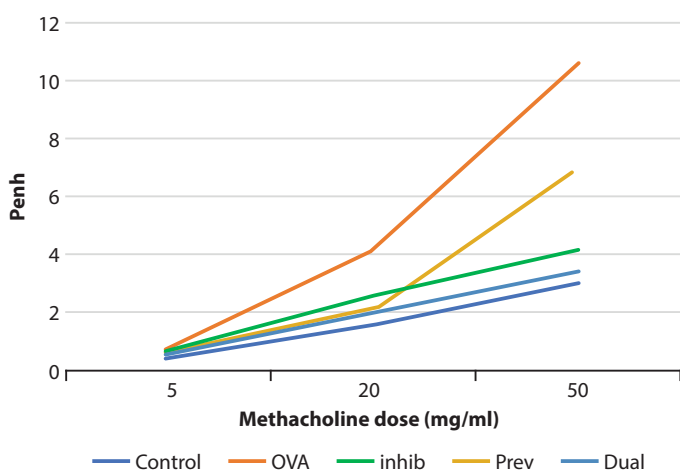
#### Statistical analysis

Data are expressed as the mean ± standard deviation. The results were analyzed by the Wilcoxon–Mann–Whitney test using PASW statistics 18. Results were considered statistically significant at  $p < 0.05$ .

## Results

### Level of Penh after methacholine inhalation

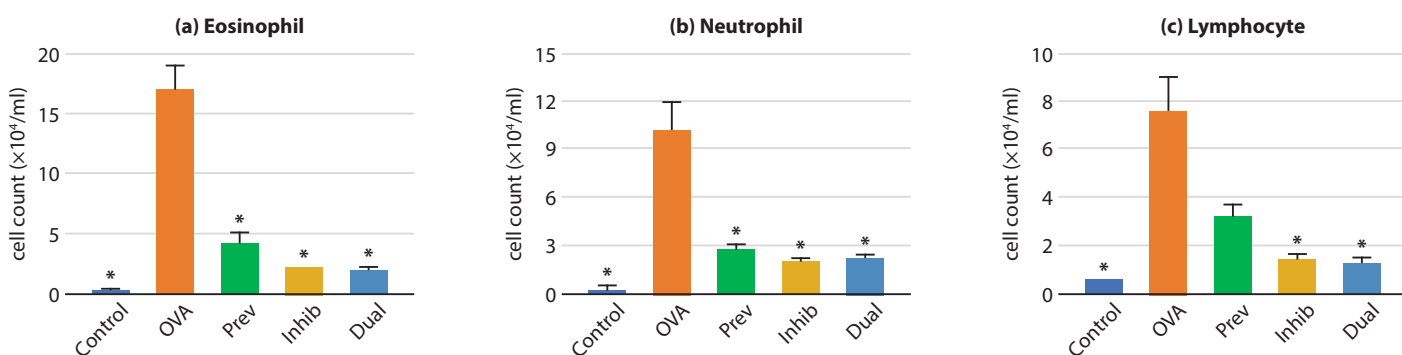
At a concentration of 5 mg/ml methacholine, the Penh value of the control group was lower than those of the other groups ( $p < 0.05$ ). The Penh value of the OVA group at a concentration of 20 mg/ml methacholine was higher than those of the other groups ( $p < 0.05$ ). No significant difference was found in the Penh value at these concentrations of methacholine among the other four groups, namely, the control, Prev-VD, Inhib-VD, and Dual-VD groups. By contrast, at a concentration of 50 mg/dl methacholine, the Penh values of the control, Inhib-VD, and Dual-VD groups were lower than that of the OVA group ( $p < 0.05$ ) but not that of the Prev-VD group ( $p = 0.88$ ) (Figure 2).



**Figure 2. Level of the Penh value after the bronchial provocation test with methacholine in the five groups.**

The Penh value was evaluated after the mice from each group inhaled 3 ml of different concentrations of the methacholine mixture (5, 20, and 50 mg/ml) for 3 min.

OVA, ovalbumin; Prev-VD, preventive vitamin D; Inhib-VD, inhibitory vitamin D; Dual-VD, preventive and inhibitory vitamin D. \*  $P < 0.05$  vs. the OVA group



**Figure 3. Effect of vitamin D on the cell counts, cytokines, total IgE, and OVA-specific IgE levels in the bronchoalveolar lavage fluid.**

The cell counts (a)–(d), cytokines (f) – (i), total IgE (j), and OVA-specific IgE (k) in the bronchoalveolar lavage fluid were measured on day 25.

The total cell count and percentages of the cells are shown in (e). Blue color stands for eosinophil, red for neutrophil, green for lymphocytes and yellow for macrophage.

OVA, ovalbumin; Prev-VD, preventive vitamin D; Inhib-VD, inhibitory vitamin D; Dual-VD, preventive and inhibitory vitamin D. Data are shown as mean  $\pm$  SEM. \*  $P < 0.05$  vs. the OVA group, #  $P < 0.05$  vs. the control group

### Analysis of bronchoalveolar lavage fluid

Differential cell counts were performed in BALF. The eosinophil counts in all three administered groups that had been given vitamin D (Prev-VD, Inhib-VD, and Dual-VD) and in the control group were lower than that of the OVA group ( $p < 0.01$ ). No significant difference in the eosinophil count was found among the three groups that had received vitamin D. Neutrophils showed a similar pattern to that of eosinophils. The lymphocyte counts of the Inhib-VD and the Dual-VD groups were lower than that of the OVA group ( $p < 0.01$ ). No statistical difference was observed between the Prev-VD group and the OVA group in lymphocyte counts ( $p = 0.078$ ). The macrophage counts of the control, Inhib-VD group, and Dual-VD group were higher than that of the OVA group ( $p < 0.05$ ). The total cell counts of the OVA group were higher than those of the other groups ( $p < 0.05$ ) (Figure 3, a-e).

The IL-4 levels in BALF of the Inhib-VD and Dual-VD groups were lower than that of the OVA group ( $p < 0.05$ ), but no significant difference was found between the Prev-VD and OVA groups at the IL-4 level ( $p = 0.264$ ). The IL-5 levels of the three groups that had received vitamin D were lower than that of the OVA group ( $p < 0.01$ ). The IL-5 level of the Dual-VD group was low and not significantly different from that of the control group ( $p = 0.248$ ). The IFN- $\gamma$  levels of the control group and the Dual-VD group were higher than that of the OVA group ( $p < 0.05$ ), but no difference was found among the Prev-VD, Inhib-VD, and OVA groups at the IFN- $\gamma$  level. The IL-10 level was higher in the Prev-VD and Inhib-VD groups than in the other groups ( $p < 0.01$ ) (Figure 3, f-i).

The total IgE levels of the three groups that had received vitamin D and of the OVA group were higher than that of the control group ( $p < 0.01$ ). However, no statistically significant difference in the total IgE level was found between these three groups and the OVA group. The OVA-specific IgE showed a similar pattern to that of the total IgE (Figure 3, j, k).

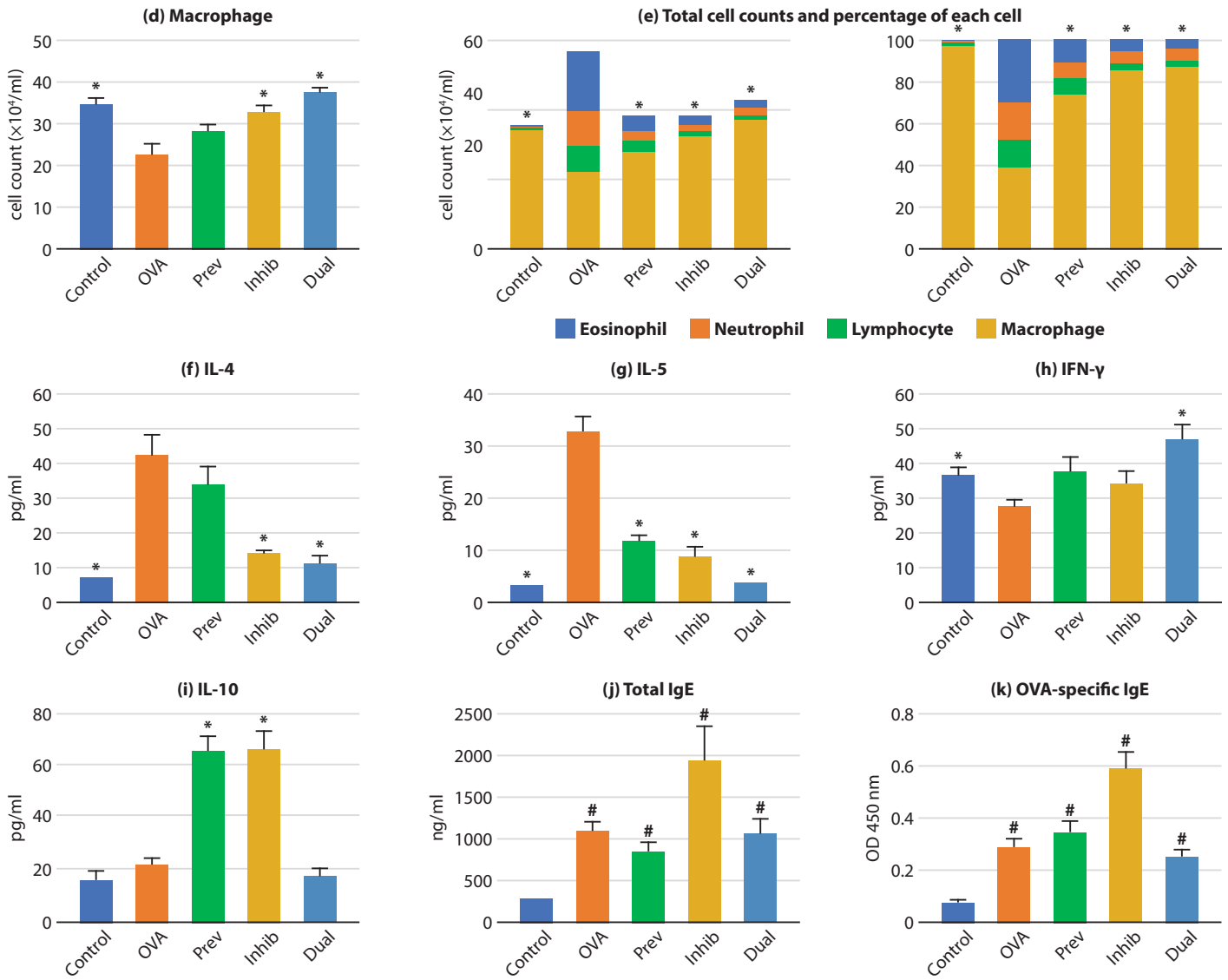


Figure 3. (Continued)

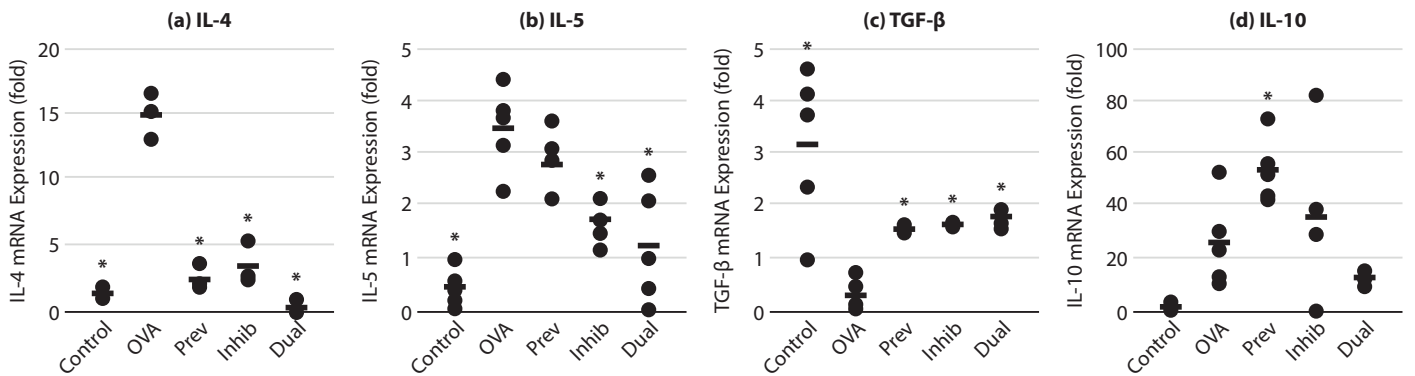
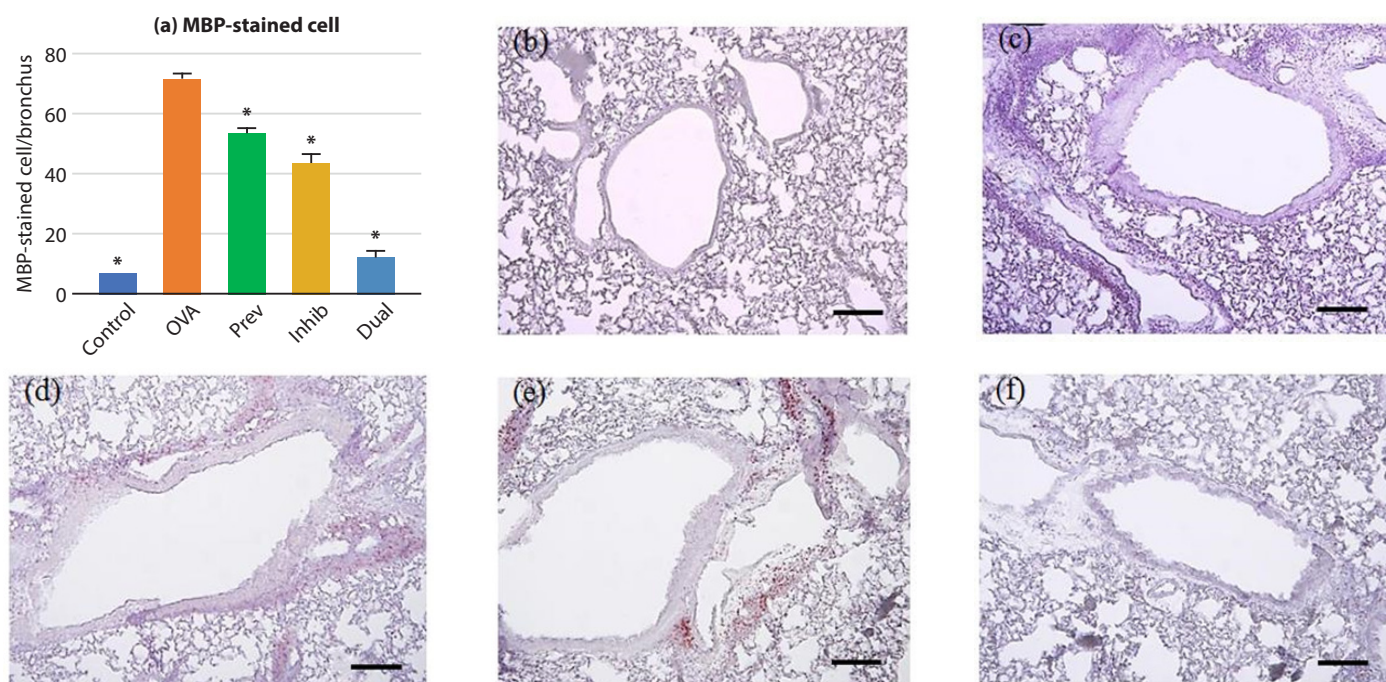


Figure 4. Effect of vitamin D on the cytokine levels in the lung lysate.

Cytokines in the lung lysate were measured on day 25. IL-4 (a), IL-5 (b), TGF- $\beta$  (c), and IL-10 (d).

OVA, ovalbumin; Prev-VD, preventive vitamin D; Inhib-VD, inhibitory vitamin D; Dual-VD, preventive and inhibitory vitamin D. Data are shown as mean  $\pm$  SEM. \*  $P < 0.05$  vs. the OVA group.



**Figure 5. MBP-stained cell counts in the lung tissue.**

MBP-stained cell counts in the lung lysate were measured on day 25 (a). Histologic findings were obtained with hematoxylin and 3,3'-diaminobenzidine staining on day 25. Representative magnification,  $\times 400$ . Scale bars = 50  $\mu\text{m}$ . Control group (b), OVA group (c), preventive VD group (d), inhibitory VD group (e), and dual VD group (f).

MBP, major basic protein; OVA, ovalbumin; Prev-VD, preventive vitamin D; Inhib-VD, inhibitory vitamin D; Dual-VD, preventive and inhibitory vitamin D. Data are shown as mean  $\pm$  SEM. \*  $P < 0.05$  vs. the OVA group.

#### Cytokine analysis of lung lysate

The IL-4 levels of all the other groups were lower than that of the OVA group ( $p < 0.05$ ). The IL-5 levels of the control, Inhib-VD, and Dual-VD groups were also lower than that of the OVA group ( $p < 0.05$ ), but the IL-5 level of the Prev-VD group was not statistically significantly different from the OVA group ( $p = 0.29$ ). The TGF- $\beta$  levels were higher in the control and in the three groups that had received vitamin D than in the OVA group ( $p < 0.05$ ). The IL-10 levels were higher in the Prev-VD and Inhib-VD groups than in the OVA group ( $p < 0.05$ ). No difference was observed between the Dual-VD group and the OVA group in the IL-10 level (Figure 4).

#### MBP staining of lung tissue

The control and the three groups that had received vitamin D yielded fewer MBP-stained cells than did the OVA group ( $p < 0.05$ ). Among the three groups that had received vitamin D, the mean count of the MBP-stained cells of the Dual-VD group was lower than those of the Prev-VD and Inhib-VD groups (Figure 5).

#### Discussion

An increase in AHR is one of the characteristic features of asthma. Vitamin D deficiency is associated with AHR, and vitamin D supplementation decreases AHR.<sup>4,14,15</sup> Our study demonstrated that vitamin D in the preventive, inhibitory, and dual vitamin D groups reduced the Penh.

This work showed that the number of inflammatory cells in BALF decreased as a result of vitamin D administration. This

effect was seen after both preventive and inhibitory vitamin D administration. MBP is an eosinophil-specific protein, and eosinophils may have an important role in allergic and inflammatory reactions in asthma.<sup>15</sup> Vitamin D administration decreased the number of MBP-stained cells in the lung tissue, implying that vitamin D decreased eosinophil infiltration into the lungs. The effect of vitamin D on eosinophil infiltration may be mediated by the effect of vitamin D on IL-4.<sup>16</sup>

In our study, this effect of vitamin D in reducing eosinophil infiltration was most noticeable when vitamin D was used for both preventive and inhibitory purposes. Matheu et al. asserted that both therapeutic vitamin D administration and combined preventive and therapeutic vitamin D administration reduced eosinophil infiltration into the lung parenchyma but that preventive vitamin D alone did not.<sup>5</sup> Agrawal et al. found that vitamin D supplementation reduced eosinophil infiltration but did not affect neutrophil and lymphocyte infiltration.<sup>4</sup> Chen et al. reported that both the timing and the amount of vitamin D administration were key factors in modulating the eosinophil count. A low dose (2  $\mu\text{g}/\text{kg}$  for 27 days) of vitamin D supplementation reduced the eosinophil count and other inflammatory markers, whereas a high dose (20  $\mu\text{g}/\text{kg}$  for 27 days) of vitamin D supplementation aggravated inflammation.<sup>17</sup>

Asthma patients generate high serum levels of cytokines that are secreted by Th2 cells, and these cytokines are known to affect eosinophils and mast cells in the pathogenesis of asthma.<sup>18,19</sup> In this study, we evaluated the effect of vitamin D on asthma development by evaluating the effect of vitamin D on Th1 and Th2 cell cytokines and on other inflammatory markers

of asthma. The effect of vitamin D on the cytokine level associated with asthma remains controversial. Filley et al. reported that vitamin D reduced IL-4 in the BALF, and Pichler et al. found that vitamin D decreased IL-5.<sup>15,20,21</sup> By contrast, Agrawal et al. reported that vitamin D reduced the IL-5 levels but did not affect the IL-4 levels.<sup>4</sup> Interestingly, Mahon et al. showed that vitamin D increased the IL-4 levels.<sup>22,23</sup> In our study, we observed that IL-4 in lysate and IL-5 in BALF decreased when we administered preventive and inhibitory vitamin D. IL-4 in BALF and IL-5 in lysate decreased with the administration of inhibitory vitamin D but did not decrease with the administration of preventive vitamin D. The decrease in these cytokines was most noticeable when vitamin D was administered for both preventive and inhibitory purposes.

In this study, TGF- $\beta$  in lung lysate and IL-10 in BALF increased with the administration of preventive and inhibitory vitamin D in comparison with the OVA group. Cantorna et al. reported similar results.<sup>23</sup> Regulatory T cells (Tregs) produce TGF- $\beta$  and IL-10. Vitamin D is known to increase IL-10, thus producing Tregs. IL-10 has a suppressive effect on Th2 cells.<sup>24</sup> We observed an increase in TGF- $\beta$  and IL-10 and a decrease in IL-4 and 5 after vitamin D administration. Therefore, we can assume that vitamin D induces Tregs and that suppression of Th2 cell by Tregs results in a decrease in IL-4 and 5.<sup>23,25</sup> Additional studies are needed for direct confirmation of the effect of vitamin D on the Treg cell count using cytometry. Unlike other cytokines on which a cumulative effect of vitamin D was observed, IL-10 levels decreased more when vitamin D was administered for both preventive and inhibitory purposes than for either a preventive purpose or a therapeutic purpose only. Further studies to explain these results are also needed.

IFN- $\gamma$  in the BALF was increased by dual vitamin D administration in comparison with the OVA group. We can assume that vitamin D stimulates IFN- $\gamma$ -secreting Th1 cells. However, Matheu et al. reported that vitamin D suppressed Th1 cells, resulting in a decrease in IFN- $\gamma$ .<sup>5</sup>

The total IgE level of the four OVA-induced groups was higher than that of the control group, and no differences in IgE levels were noted between the three groups administered vitamin D and the OVA group. The OVA-specific IgE level showed similar results. Vitamin D seemed to have no effect on the total IgE and OVA-specific IgE levels. However, total IgE and allergen-specific IgE levels require a long period (months) to adjust after immune therapy.<sup>26</sup> Further studies using chronic allergic mouse models to reveal the effect of vitamin D on total IgE and OVA-specific IgE in OVA-induced allergy in mice are needed.

In summary, the eosinophils, neutrophils, IL-5 in BALF, and IL-4 in lysate were decreased, and TGF- $\beta$  in lysate was increased by employing preventive and inhibitory vitamin D therapy. The lymphocytes, macrophages, IL-4 in BALF, and IL-5 in lysate were decreased only by inhibitory and dual vitamin D administration. These anti-allergic and anti-inflammatory effects of vitamin D on asthma were most pronounced when vitamin D was used for inhibitory purposes.

## Conclusion

We hypothesized that vitamin D would have a preventive effect on the development of asthma and some kind of therapeutic effect on allergic inflammation in an OVA-induced allergic mouse model. This immunomodulatory effect was most noticeable when vitamin D was administered for dual (preventive and inhibitory) purposes. Further investigations should be conducted to reveal the mechanism of these effects of vitamin D.

## Acknowledgement

This study is supported by Inha Research Grant.

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