

Original Article



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Randomized, Double-Blind, Placebo-Controlled Trial of Vitamin D Supplementation in the Build-up Phase of House Dust Mite-Specific Immunotherapy

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ABSTRACT

Purpose: Vitamin D (VitD) is an immunomodulatory molecule capable of alleviating allergic symptoms. However, the effectiveness of allergen-specific immunotherapy (AIT) is not commonly evidenced in the early build-up phase. The aim of the study was to determine the potential of VitD supplementation in this treatment phase.

Methods: Thirty-four house dust mite (HDM)-allergic adult patients treated with subcutaneous AIT were randomized to receive VitD2 60,000 IU/week or placebo for 10 weeks and followed up for 10 weeks. The primary endpoints were the symptom-medication score (SMS) and the treatment response rate. The secondary endpoints were eosinophil count and levels of plasma IL-10, Der p 2-specific IgG4, and dysfunctional regulatory T (CRTH2⁺ Treg) cells.

Results: Of 34 patients, 15 in each group completed the study. Patients with VitD deficiency receiving a VitD supplement showed significantly lower mean change SMS than the placebo group in weeks 10 (mean difference -54.54%, $P = 0.007$) and 20 (mean difference -42.69%, $P = 0.04$). The percentage of treatment responders reached 78% and 50% in the VitD and placebo groups, respectively, and the effect remained in week 20 (89% and 60%). No significant difference was observed for the tested immunological read-outs, with the exception of the frequency of CRTH2⁺ Treg cells, which was remarkably reduced in the VitD-treated patients. Moreover, improvement in SMS was correlated to the number of CRTH2⁺ Treg cells. Our *in vitro* experiment indicated that VitD downregulated activation markers, whereas it improved the function of CRTH2⁺ Treg cells.

Conclusions: VitD supplementation in the build-up phase of AIT could relieve symptoms and decrease Treg cell dysfunction, especially in patients with VitD deficiency.

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Disclosure

There are no financial or other issues that might lead to conflict of interest.

Keywords: Vitamin D; allergic rhinitis; immunologic desensitization; antigens, *Dermatophagoides*; treatment outcome; regulatory T-lymphocytes

INTRODUCTION

The prevalence of allergic diseases is increasing worldwide, particularly in low-and middle-income countries.¹ House dust mites (HDMs) are the most common sources of indoor allergens that cause perennial allergic symptoms.² Management of allergic diseases aims to avoid allergen exposure, relieve allergic symptoms and boost the immune system in order to develop allergen tolerance. Currently, allergen-specific immunotherapy (AIT) is a unique therapeutic method that could effectively relieve allergic symptoms.³ AIT induces long-term clinical tolerance, leading to a significant reduction in symptoms, medication, and ultimately improvement in the quality of life.^{4,5}

The potential mechanisms of immune tolerance induction include the development and maintenance of allergen-specific regulatory T (Treg) and B (Breg) cells together with regulatory dendritic cells (DCs) and innate lymphoid cells (ILCs).⁶⁻⁸ Interleukin (IL)-10 is an anti-inflammatory cytokine mainly produced by various regulatory cells.⁹ IL-10 inhibits the activation of eosinophils, basophils, T helper (Th)2, Th17, immunoglobulin (Ig) E-producing B cells, DC2, and ILC2 through the induction of Tr1, Br1 and IgG4-producing B cells.⁹ However, it must be pointed out that any population of Treg cells is not functional. In particular, Treg cells expressing CRTH2, immunoglobulin-like transcript 3 (ILT3), and soluble interleukin 1 receptor-like 1 (ST2) are described as dysfunctional Treg cells and their numbers are correlated with allergic symptoms.¹⁰ CRTH2⁺ Treg cells were not only defective in suppressive function but also expressed Th2 characters.^{11,12} Successful AIT has recently been reported to depend on the upregulation of activated allergen-specific Treg cells and the downregulation of dysfunctional allergen-specific Treg cells.¹³

Vitamin D (VitD) deficiency is a common medical condition worldwide, with more than one billion people suffering from VitD deficiency or insufficiency. The prevalence of VitD deficiency among adult populations varies from 5% to 60%, the Asian population being more affected than the Caucasian one.¹⁴ VitD is a pleiotropic immunomodulator as the VitD receptor is expressed in almost all cells of the immune system. Abnormal levels of VitD may influence the course of immune-mediated disorders, including allergic rhinitis, atopic dermatitis, food allergy, and asthma.^{15,16} Furthermore, VitD supplementation elicits different anti-inflammatory effects, including increasing the number and function of Treg cells.^{17,18} Moreover, VitD supplementation could improve rhinitis symptoms, eczema skin lesions, lung function, and asthma control.¹⁹

Whereas AIT commonly lasts at least 2-3 years, the duration of AIT required to reach the treatment effect differs among individuals. Our previous studies reported that conventional AIT responders were usually found within 20–30 weeks of therapy.^{7,13,20} During this initial period, short-term adjunctive therapy could be combined to improve the reduction in allergic symptoms. VitD represents a safe, inexpensive adjuvant molecule and can modulate the immune system. However, few studies have been conducted to evaluate the effects of VitD in the build-up phase of AIT, particularly in adult patients.²¹ In the present study, we investigated the effects of VitD supplementation in HDM-allergic patients who were enrolled in the build-up phase of the AIT protocol in week 10 and week 20. Our results showed that VitD

supplementation alleviated composite scores in the early build-up phase of AIT, especially in patients with VitD deficiency through the down-regulation of dysfunctional Treg cells.

MATERIALS AND METHODS

Study design

A randomized, double-blind, placebo-controlled clinical study on the effect of VitD2 supplementation in AIT was conducted in the Allergy Outpatient Clinic, Department of Medicine, Phramongkutklao Hospital, Bangkok, Thailand, between December 2018 and July 2019. Randomization was done in blocks of 4 subjects to receive VitD2 treatment or placebo. The treatments were assigned for 10 weeks on a 1:1 basis: in each block, 2 patients received 3 capsules of VitD2 (calciferol) 60,000 IU/week, and the 2 other patients received placebo. The placebo capsules were indistinguishable in appearance from the supplement capsules prepared by Pharma Nueva (Bangkok, Thailand). Afterward, a 10-week follow-up period was included to evaluate the efficacy of AIT following the discontinuation of VitD supplementation. The treatment schedule included a screening period to determine eligibility, as described in **Fig. 1A**.

A sample size of 13 patients in each group achieving 95% power to detect significant mean differentiation was calculated by GPower 3.1 (Heinrich-Heine-Universität, Düsseldorf,

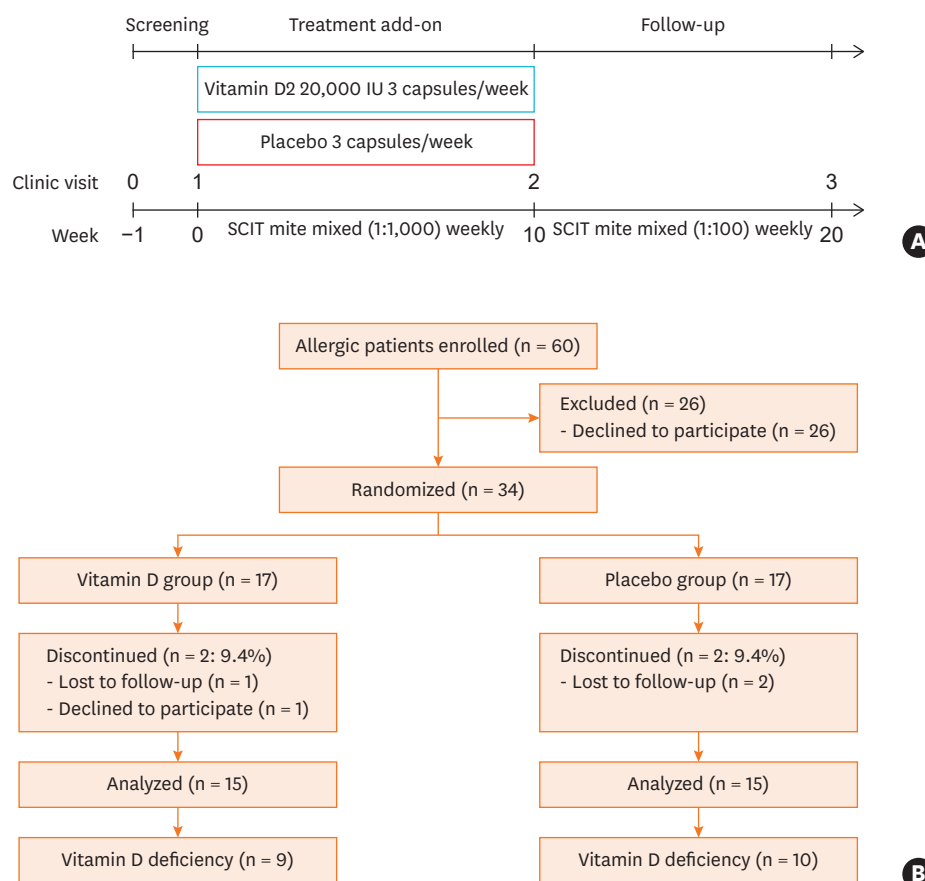


Fig. 1. (A) Study design flow chart. (B) The flow of subjects through the study showing the number of patients screened, excluded, randomized, withdrawn, and completed. SCIT, subcutaneous allergen-specific immunotherapy.

Germany). The required final target sample size plus 20% of the loss to follow-up was estimated to contain 30 patients. The trial was approved by the Institutional Review Board of the Royal Thai Army's Ethics Committee (R151h/61) and registered in the Thai Clinical trials Registry (www.thaiclinicaltrials.in.th, TCTR20190813001). The study was conducted according to the Declaration of Helsinki and Good Clinical Practice guidelines. All patients provided written informed consent before participating in the present study.

Patients

We enrolled adults aged 18 to 70 years suffering from allergic rhinitis (AR) and treated with HDM AIT. Patients with AR were diagnosed according to Allergic Rhinitis and Its Impact on Asthma guidelines.²² HDM sensitization was diagnosed by skin prick tests (wheal diameter ≥ 6 mm than the negative control) with *Dermatophagoides pteronyssinus* extract (Alk-Abello, TX, USA). The HDM AIT build-up phase consisted in a weekly dose escalation protocol for 30 weeks and was based on subcutaneous administrations of standardized mite allergen extracts (mixture of *D. pteronyssinus* and *D. farinae* allergens, both at 5,000 Allergy Units/mL, Alk-Abello), as described elsewhere.²⁰ During the first 10 weeks, patients received a step-up protocol made of the mite mix vaccine at a 1:1,000 vol/vol dilution every week. Exclusion criteria were at least 1 year since the last VitD supplement intake, at least 1 month since the last systemic corticosteroid or immunosuppressive medication, pregnancy, and abnormal renal and liver function.

Measurement

The combined symptom-medication score (SMS) comprises the total nasal symptom score (TNSS) and the total medication score and is mostly used to analyze the primary endpoint in clinical trials.^{23,24} TNSS comprised the following symptoms of AR: sneezing, runny nose, and itchy nose; it was calculated using a 4-point scale (0 = none, 1 = mild, 2 = moderate, and 3 = severe). The total medication score was calculated by 1 = using beta-2 agonist, antihistamine, montelukast, pseudoephedrine; 2 = using inhaled or intranasal corticosteroid, and 3 = using systemic steroid.²⁰ SMS were recorded at baseline, in weeks 10 and 20.

Laboratory

Serum levels of 25(OH)D and absolute eosinophil count were assessed at 3 time points: baseline, week 10, and week 20. VitD deficiency and insufficiency were defined as serum 25(OH)D levels lower than 20 ng/mL and 20–30 ng/mL, respectively. Der p 2-specific IgG4 and Der p 2-specific IgE were measured by an enzyme-linked immunosorbent assay (ELISA) as described elsewhere.²⁵ Plasma IL-10 levels were measured with ELISA commercial kits (R&D, Minneapolis, MN, USA) according to the manufacturer's instructions.

Peripheral blood samples (10 mL) were collected into lithium heparin tubes. Fresh whole blood was stained with fluorescein isothiocyanate (FITC)-anti-CD3 (clone OKT3; Biolegend, San Diego, CA, USA), phycoerythrin (PE)-anti-CD25 (clone BC96, Biolegend), PE/Dazzle594-anti-CD4 (clone OKT4, Biolegend), Alexa Fluor 647 (AF647)-anti-CRTH2 (clone BM16; BD Biosciences, Franklin Lakes, NJ, USA) and PE-Cy7-CD127 (clone A019D5, Biolegend). The blood samples were mixed with 2 mL of BD FACS lysing solution (BD Biosciences) and washed with phosphate buffer saline. Flow cytometry was performed using Guava easyCyte (Merck, Darmstadt, Germany). T-cell subsets, including dysfunctional Treg cells (CD3⁺CD4⁺CD25⁺CD127⁺CRTH2⁺), CRTH2⁺ Treg cells (CD3⁺CD4⁺CD25⁺CD127⁺CRTH2⁺), and Th2 cells (CD3⁺CD4⁺CD25⁺CRTH2⁺), were analyzed by Kaluza software (Beckman Coulter, Indianapolis, IN, USA). For detailed *in vitro* experiment protocols, see the method section in **Supplementary Data S1**.

Study endpoints

All efficacy endpoints were analyzed in week 10. The primary study endpoint was defined as the change in SMS from baseline and the percentage of treatment responders. A treatment responder was defined by a reduction in SMS > 30% from baseline. Secondary endpoints included absolute eosinophil count, plasma Der p 2-specific IgG4 and IL-10 levels, and T cell subsets percentage. Adverse events were monitored throughout the treatment period to assess safety and tolerability.

Statistical analyses

Statistical analyses and figures were performed with GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Baseline patient characteristics data were analyzed using either the χ^2 test or the unpaired *t* test. Data are presented as mean $\pm$ standard deviation. A repeated ANOVA with a *post hoc* test was used to study differences between time points and between groups. Spearman's test was performed for correlation analysis. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Demographic characteristics

We screened 60 HDM AR patients who met the inclusion criteria and initiated HDM AIT. Of 60 patients, 26 withdrew because they declined to participate in the study (**Fig. 1B**). Thirty-four patients were randomized to receive VitD supplements or placebo. Two patients dropped out in each group because of the inconvenience induced by AIT and did not want to continue the study drug. Finally, 15 patients in both the VitD and placebo groups completed the 20-week study. The clinical and demographic characteristics of the randomized patients were comparable for VitD versus placebo groups (**Table**).

Table. Baseline patient characteristics

Variables	Vitamin D (n = 15)	Placebo (n = 15)	<i>P</i> value
Sex (female)	10 (66.7)	10 (66.7)	> 0.99
Age (years)	41.7 $\pm$ 14.6	38.7 $\pm$ 12.4	0.54
Allergic disease			
Allergic rhinitis	15 (100)	15 (100)	> 0.99
Allergic asthma	5 (33.3)	6 (40)	> 0.99
Atopic dermatitis	1 (6.6)	0 (0)	> 0.99
Allergic conjunctivitis	0 (0)	2 (13.3)	0.48
Allergen sensitization			
<i>Dermatophagoides pteronyssinus</i>	15 (100)	15 (100)	> 0.99
Pollen	1 (6.6)	3 (20)	0.59
Fungus	8 (53.3)	4 (26.7)	0.26
Cockroach	5 (33.3)	4 (26.7)	> 0.99
Polysensitization	10 (66.6)	9 (60)	> 0.99
Absolute eosinophil (cells/ μ L)	260.7 $\pm$ 217.7	211.5 $\pm$ 162.4	0.48
TNSS	8.0 $\pm$ 2.7	6.7 $\pm$ 3.7	0.28
Total medication score	1.7 $\pm$ 0.6	2 $\pm$ 0	0.16
SMS	9.7 $\pm$ 2.9	8.7 $\pm$ 3.7	0.77
Vitamin D (ng/mL)	20.3 $\pm$ 6.0	18.4 $\pm$ 5.6	0.78
Vitamin D insufficiency	5 (33.3)	5 (33.3)	> 0.99
Vitamin D deficiency	9 (60)	10 (66.6)	> 0.99

Values are presented as number (%) or mean $\pm$ standard deviation.
TNSS, total nasal symptom score; SMS, symptom-medication score.

Serum 25(OH)D levels

The mean serum levels of 25(OH)D of patients at baseline were 19.28 ± 5.73 ng/mL. Of 30 patients, 10 and 19 displayed VitD insufficiency and deficiency, respectively. In the VitD group, mean levels of 25(OH)D increased significantly in week 10 (20.36 ± 6.04 to 41.91 ± 8.01 ng/mL, $P < 0.0001$) and decreased in week 20 of stopping VitD supplementation (24.86 ± 3.84 ng/mL, $P = 0.002$), whereas the low vitamin concentration remained stable (approximately 18 ng/mL) in patients receiving placebo (**Fig. 2A**). VitD sufficiency was achieved in 92.8% of patients with suboptimal VitD levels receiving a VitD2 supplement for 10 weeks. Compliance was similar in both groups, with 100% of the patients taking $\geq 95\%$ of their study drug. The serum calcium levels were within the normal range among patients during the study (data not shown). No serious adverse events related to VitD supplementation were reported.

Primary endpoints

At baseline, mean SMS was comparable between the VitD and placebo groups. A significant improvement in allergic symptoms in week 10 was only found in patients receiving a VitD2 supplement as assessed by decreased SMS from baseline (9.73 ± 2.9 to 4.86 ± 2.58 , $P < 0.0001$) (**Fig. 2B**). At the end of the study, SMS was reduced remarkably from baseline in both groups. The number of AIT-treated responders was similar (9/15 patients, 60%) in both groups in week 10 (**Fig. 2C**). Following VitD2 supplement discontinuation, the frequency of responders was higher in the VitD group (93.3%) than in the placebo group (73.3%), but did not reach statistical significance (**Fig. 2C**).

VitD deficiency

Next, we analyzed a subgroup of 19 patients with VitD deficiency. Of 19 patients, 9 in the VitD group and 10 in the placebo group were surveyed. Here, we found that significantly decreased SMS from baseline (9.79 ± 1.64) to week 10 (4.33 ± 2.87 , $P = 0.0002$) and week 20 (4.8 ± 3.73 , $P < 0.0001$) only in the patients receiving a VitD2 supplement (**Fig. 3A**). Also, the mean percentage change in SMS dropped markedly in the VitD group in comparison with the placebo group (mean different -54.54% , $P = 0.007$) in week 10 (**Fig. 3B**). Moreover, the average SMS change from baseline to week 20 was significantly lower in the VitD group than in the placebo group (mean different -42.69% , $P = 0.04$) (**Fig. 3B**). The success rate of AIT was higher in patients receiving a VitD2 supplement than in those with placebo in week 10 (78% vs. 50%) and the effect was still ongoing in week 20 (89% vs. 60%) (**Fig. 3C**).

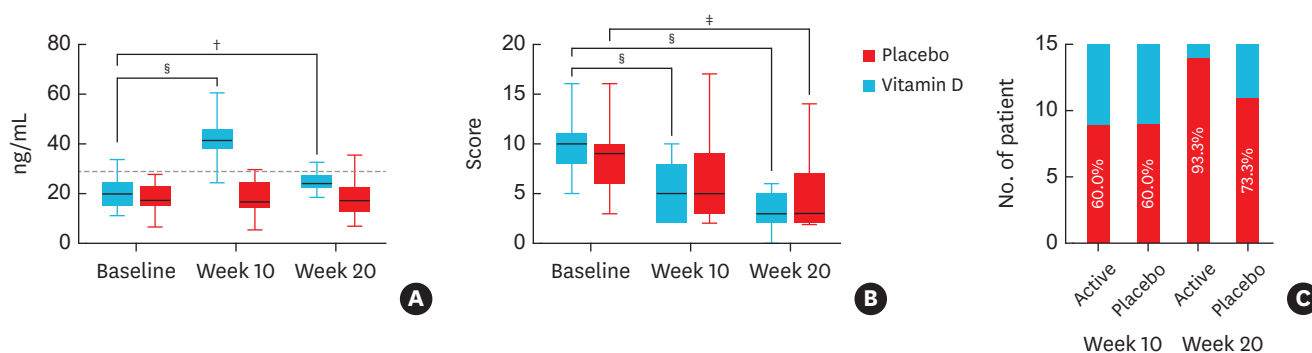


Fig. 2. (A) Serum 25(OH)D levels at baseline, week 10, and week 20 in the vitamin D (n = 15) and placebo (n = 15) groups. (B) Symptom-medication score. (C) Number of patient responders and non-responders. Percent of treatment responders presented in the stacked bars.

$^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$, $^{\S}P < 0.0001$.

Laboratory results

No significant differences in eosinophil counts, plasma IL-10 levels and Der p 2-specific IgG4 between the groups were observed and whatever the time points were (**Fig. 4A-C**). The characterization of dysfunctional Treg cells and other T cell subtypes (**Supplementary Fig. S1**) evidenced that VitD2 supplementation significantly reduced the frequency of CRTH2⁺ Treg cells in week 10 and 20 compared with baseline (**Fig. 4D**). Mean changes dropped from $0.37\% \pm 0.21\%$ at baseline to $0.18\% \pm 0.09\%$ ($P = 0.0003$) in week 10 and $0.09\% \pm 0.03\%$ ($P < 0.0001$) in week 20. Meanwhile, the frequency of CRTH2⁺ Treg cells slightly decreased during

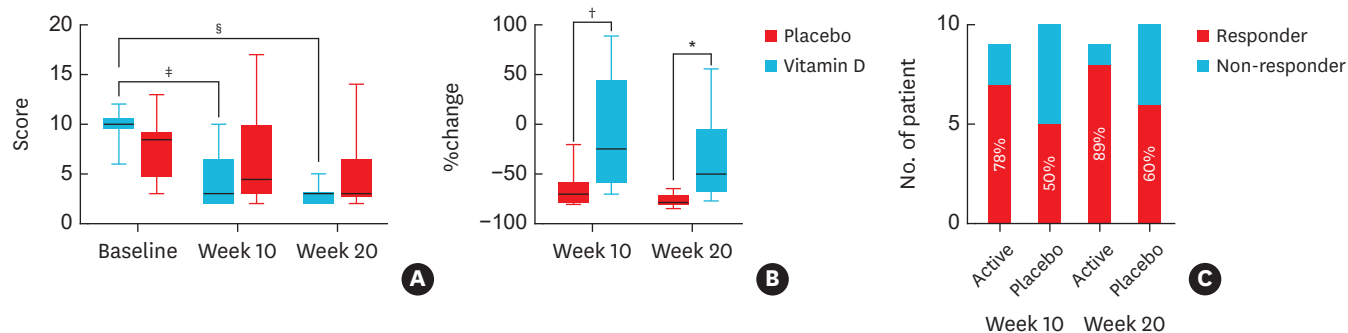


Fig. 3. (A, B) Symptom-medication score and %change from baseline in patients with vitamin D deficiency. Vitamin D ($n = 9$) and placebo ($n = 10$) groups. (C) The number of patient responders and non-responders in patients with vitamin D deficiency. Percent of treatment responders presented in the stacked bars.

* $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$, § $P < 0.0001$.

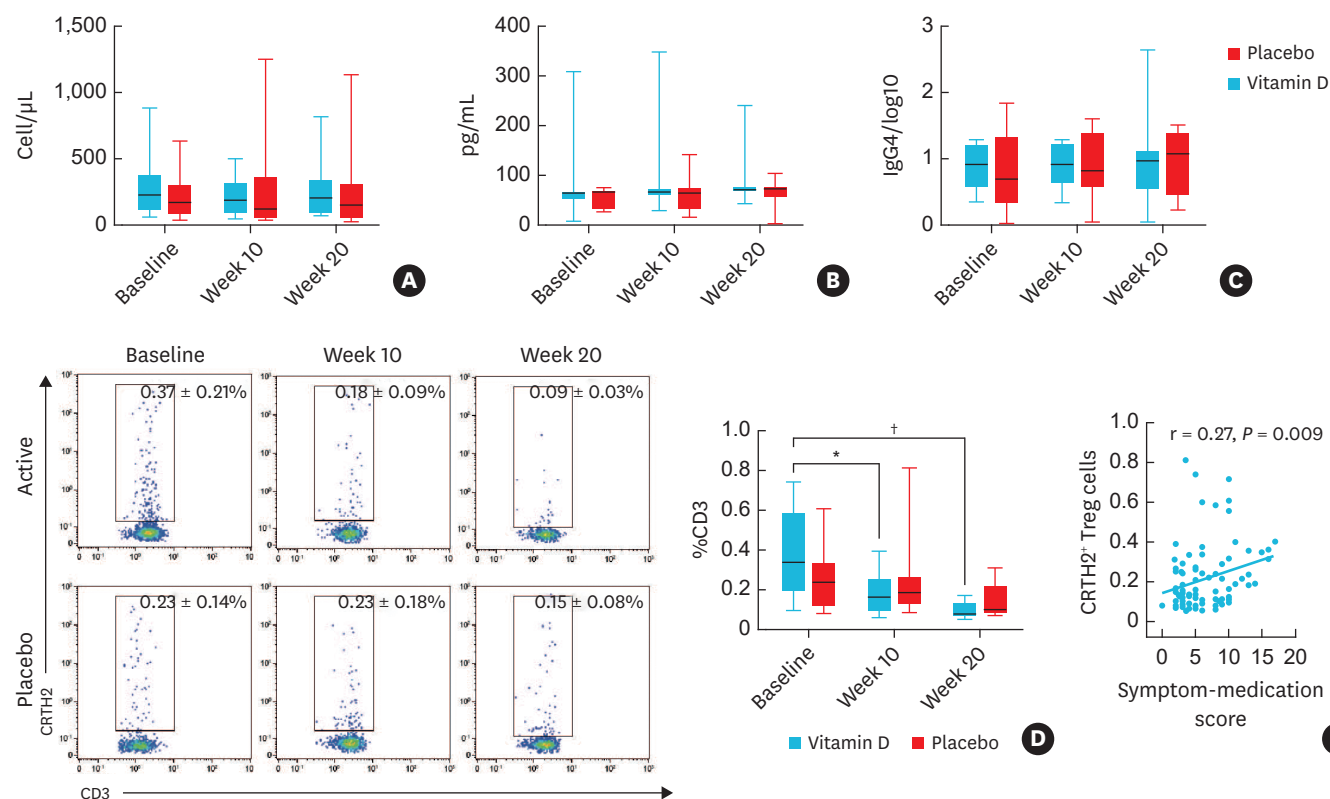


Fig. 4. Absolute eosinophil count (A), plasma IL-10 levels (B), and Der p 2-specific IgG4 (C) in the vitamin D ($n = 15$) and placebo ($n = 15$) groups. (D) Representative dot plot (left), frequency (right) of CRTH2⁺ Treg cells during AIT. Data are presented as mean $\pm$ SD. (E) Correlation of the frequency of CRTH2⁺ Treg cells with the symptom-medication score.

* $P < 0.001$, † $P < 0.0001$.

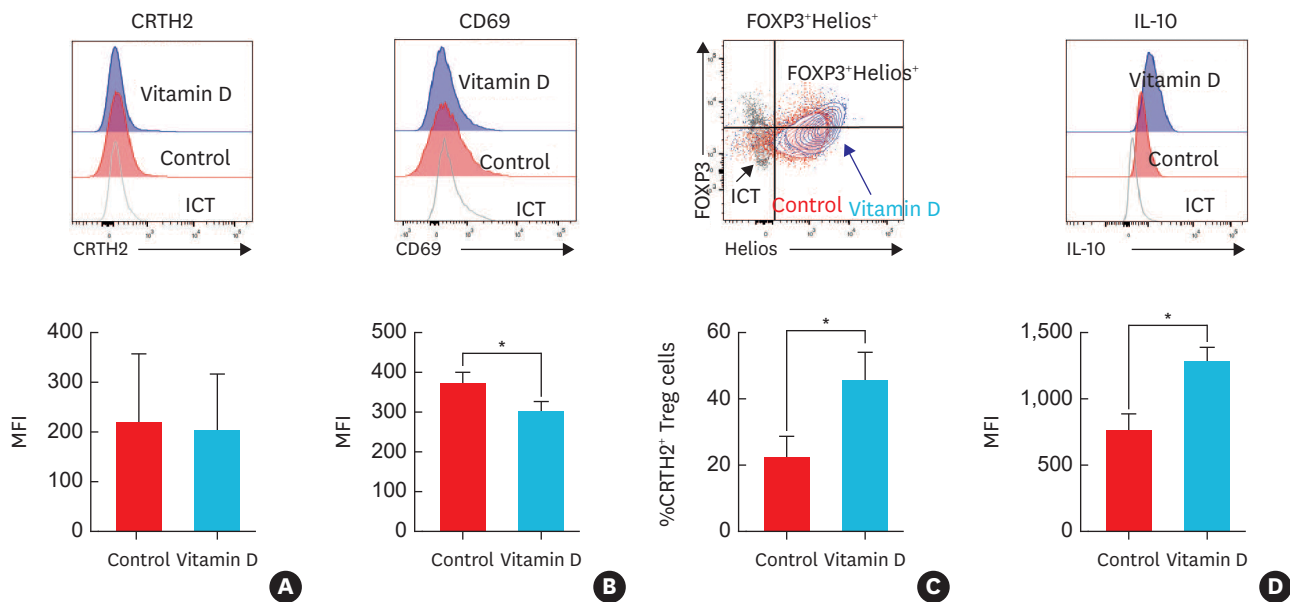


Fig. 5. (A) Effect of vitamin D on the CRTH2 expression on Treg cells ($n = 10$). Vitamin D reduced CD69 expression on CRTH2⁺ Treg cells (B), whereas it increased the percentage of FOXP3⁺Helios⁺ (C) and IL-10 expression (D) in CRTH2⁺ Treg cells. ICT, isotype control.

* $P < 0.05$.

AIT, but there was no statistically significant difference between the groups. The frequency of CRTH2⁺ Treg showed a slight increase, whereas Th2 cells showed a slight decrease but no significant change during AIT (**Supplementary Fig. S2A and B**). Moreover, the frequency of CRTH2⁺ Treg cells correlated with SMS ($r = 0.27$, $P = 0.009$) (**Fig. 4E**), but did not correlate with VitD levels (data not shown).

In vitro experiment

The effect of VitD on cultured CRTH2⁺ Treg cells was further investigated (**Supplementary Fig. S3**). The VitD did not influence CRTH2 expression on Treg cells (**Fig. 5A**). Meanwhile, VitD influenced CRTH2⁺ Treg cells by decreasing cell activation (CD69 expression), increasing Treg cell function (FOXP3⁺Helios⁺) and IL-10-production (**Fig. 5B-D**).

DISCUSSION

AIT is the treatment option that could modify the pathogenesis of allergic diseases, but the initial treatment requires time to improve patients' clinical and laboratory results. VitD supplementation could improve allergic disease symptoms such as rhinitis, eczema skin lesions, lung function, and asthma control.¹⁹ Our results demonstrated that VitD supplementation could improve allergic symptoms and reduce dysregulated Treg cells in the build-up phase of AIT. A 10-week of VitD supplementation increased 25(OH)D serum levels which were related to a reduction in allergic symptoms. Once VitD2 supplementation was discontinued, serum 25(OH)D levels decreased, but reached a value higher than baseline. The composite score decreased significantly from baseline in both groups in week 20, and this effect was attributed to AIT. However, the efficacy of VitD in AIT was obviously seen in patients with VitD deficiency. There was a significantly decreased mean change in SMS compared to patients without VitD. Also, the number of treatment responders was higher in

patients with VitD-treated than in controls. The efficacy of AIT was effective when the serum VitD was sufficient.^{26,27} Recent studies showed a significant reduction in skin test reactivity to grass pollen following grass pollen-AIT with VitD supplementation compared to placebo.²¹ However, SMS was comparable between both groups.²¹ Both AIT and VitD can modify the immune system.²⁸ In a murine model of OVA sensitization, VitD3 could promote a long-term effect of AIT characterized by reduced allergic airway inflammation and responsiveness to OVA challenge.²⁹ Also, co-administration of VitD3 and AIT induced higher levels of blocking IgG antibodies, reduced eosinophilia, and increased IL-10 levels in lung tissue in the animal model of grass pollen allergy.³⁰ Moreover, VitD3 adjuvanted with the allergoid vaccine could reduce airway eosinophils and Th2 cytokines, concomitantly increase the number of Treg cells, IL-10 levels in the lung and Der p 2-specific IgG2a in the serum of Der p 2-sensitized BALB/c mice.³¹

In humans, Baris *et al.*³² demonstrated that combining AIT with VitD promoted higher FOXP3 expression in Treg cells than AIT alone. Also, the frequency of CD38⁺ naïve B cells, grass-specific B cells, and Breg cells increased after AIT with VitD but not in AIT alone.²¹ Moreover, AIT with VitD3 supplementation could inhibit the expression of miR-19a and increase IL-10 levels in the B cells of AR patients.³³ As presented above, VitD has been encouraged for allergic immune tolerance along with AIT. There are many reports that VitD can help prevent or treat allergic diseases. However, recent studies have shown that VitD has no effect on suppressing the immune response to allergies or preventing allergic diseases.³³ VitD supplementation may not help alleviate allergic symptoms in all patients.

Our results showed that the percentage of CRTH2⁺ Treg cells was shown to significantly increase in allergic asthmatic patients in comparison to healthy controls.^{12,34,35} The frequency of CRTH2⁺ Treg cells correlated with the severity of allergic symptoms and lung function, the same as in our previous study.^{11,12} Our study showed that VitD supplementation during the build-up phase of AIT markedly reduced dysfunctional Treg cells at a higher level than AIT alone in weeks 10 and 20. The positive effect of VitD was mediated by enhanced FOXP3 expression and IL-10 secretion in CRTH2⁺ Treg cells. However, CRTH2⁺ Treg cells were a tiny population, and the increased function of dysfunctional Treg cells might be less important. It was difficult to judge whether the improvement of clinical symptoms with an increase in VitD was due to CRTH2⁺ Treg cells. Other immunomodulatory mechanisms from increasing VitD that were not investigated in this study might also have an effect on clinical symptoms.

The limitation of the study was the low number of participants available for the efficacy analysis. However, the number of patients who completed the study (15 patients in each group) was the same as the sample size calculation. In the subgroup analysis of VitD deficiency, the number of participants was less than ten. The future clinical trial will include more participants and be restricted to AR patients with VitD deficiency in order to investigate the clinical and therapeutic effects of VitD supplementation. Moreover, the group receiving a VitD supplement without AIT should also be included. Another important limitation is the short duration of the study period (20 weeks), which might be attributed to the insignificant change in immunologic response.

In summary, this study shows the efficacy and safety of VitD2 supplementation in the build-up phase of AIT. There was an obvious association between increased VitD levels and the improvement of AR symptoms/the down-regulation of dysfunctional Treg cells. Moreover, the rate of AIT treatment responders was higher with VitD2 supplementation in patients with

VitD deficiency. The analysis of serum 25(OH) would be recommended before the initiation of AIT. We recommend VitD supplementation for patients with VitD deficiency to optimize their early AIT treatment outcomes.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIALS

Supplementary Data S1

Supplementary methods.

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Supplementary Fig. S1

Gating strategy of CRTH2⁺, CRTH2⁻ Treg cells, and Th2 cells in fresh whole blood. Identified cells were first gated on lymphocytes. CD3⁺CD4⁺ cells were gated after gating lymphocytes. CRTH2⁺ Treg cells were identified by the expression of CD4⁺CD25⁺CD127⁻ and then gated on CRTH2. Th2 cells were gated on CRTH2⁺CD4⁺CD25⁻ T cells.

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Supplementary Fig. S2

(A, B) Frequency of Th2 and CRTH2⁻ Tregs at baseline, in weeks 10 and 20, in the vitamin D (n = 15) and placebo (n = 15) groups. Data were presented as mean ± SD.

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Supplementary Fig. S3

Flow cytometry gating strategy of CD69⁺ and FOXP3⁺Helios⁺ CRTH2⁺Treg cells. Identified cells were first gated on lymphocytes and single cells. Alive CD3⁺CD4⁺ cells were gated after gating out dead cells (viability⁺). The CD4⁺ T cells population was gated into CD25⁺CD127⁻ (Treg cells) and CRTH2⁺ Treg cells. Then CRTH2⁺ Treg cells were gated on CD69⁺, FOXP3⁺Helios⁺, and IL-10⁺ cells.

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