Characterization of allergen-specific T cell subsets in allergy
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$1\alpha,25(OH)_2$VitD3 adjuvant increases IL-10+ Th cells after subcutaneous grass-pollen allergoid immunotherapy.
ABSTRACT

Background: Treg inducing adjuvants are a promising tool for the improvement of SIT. 1,25VitD3 was efficacious in the induction IL-10 and FOXP3 expressing Th cells *in vitro*, and potentiated SIT in a mouse model of immunotherapy. In a clinical trial of VitD3 adjuvant to SIT, changes in allergen-specific Immunoglobulins were present despite lack of clinical effectiveness. The levels of Immunoglobulins are thought to dependent on cytokines produced by Th cells. Therefore cytokine-production in allergen-specific Th cells was studied.

Method: PBMCs from patients treated with Placebo-Placebo, SIT-Placebo or SIT-VitD3 injections, were isolated before, 9 weeks, and 1 year after treatment. Grass-pollen responsive Th cells were detected by CFSE-dilution, and different Th cell subsets were analysed by the expression of IL-4, IFNγ, IL-10, FOXP3, IL-9, and IL-17 in allergen-responsive Th cells, using flow cytometry.

Results: After SIT, a temporary induction of Th cell proliferation was observed in the SIT-Placebo group, and not in the Placebo-Placebo and SIT-VITD3 Groups. Moreover, addition of VitD3 increased the percentage of grass-pollen specific IL-10 producing T cells. In both treatment groups a reduction in the percentage of IFNγ producing cells was observed, and the transient decrease of FOXP3 expressing Th-cells observed in patients treated with SIT, was abrogated in those receiving VitD3 adjuvant.

Conclusions: 1,25VitD3 as adjuvant for SIT potentiated the induction of allergen-specific IL-10 and FOXP3 expressing Th cells compared to SIT alone. The absence of clinical effective SIT precludes definite conclusions on the adjuvant activity of 1,25VitD3. As the induction of IL-10 and FOXP3 expressing Th cells is thought to be crucial for the induction of clinically successful SIT, 1,25VitD3 remains a promising adjuvant for the improvement of SIT.
INTRODUCTION

Allergen-specific Immunotherapy (SIT) is the only disease-modifying treatment for allergic disorders, inducing long-term relieve of symptoms[1]. The underlying mechanisms inducing this clinical improvement are not yet fully understood. It is well known that immunotherapy induces allergen specific IgG4 antibodies [2, 3], increases the ratio of allergen-induced Th1/Th2 cytokines, and induces allergen-specific regulatory T-cells (Tregs) [4-6]. These Tregs are characterized by the production of immunoregulatory cytokines IL-10 or TGFβ, or by expression of the transcription factor Forkhead box P3 (FOXP3). Moreover they are considered to be important, if not crucial for clinically successful SIT [4, 5]. Although the critical role of IL-10 in the suppression of allergic disease is difficult to determine in patients, it has been shown that blocking of IL-10 signaling in a mouse model of SIT reversed its improvement of allergic inflammation [7], and addition of anti-IL-10 to PBMC cultures prevented the SIT mediated downregulation of allergen-induced proliferation [8].

The clinical efficacy of SIT has been demonstrated in several trials, yet the clinical outcome is quite variable between patients. The unpredictable efficacy together with the long-term duration of the treatment (3-5 years) is a burden for clinical practice. Therefore, there is a strong unmet medical need to improve the clinical efficacy of SIT. Adjuvants are used in many vaccines to direct and enhance the favorable immune response. Likewise, Treg inducing adjuvants could be favorable in SIT. 1α,25 dihydroxyvitamin D3 (1,25VitD3), the active form of VitD3, has shown to inhibit dendritic cell (DC) maturation leading to the induction of IL-10 and FOXP3 expressing T cells [9]. Furthermore, 1,25VitD3 supplementation increased IL-10 gene expression in peripheral Th cells [10], and protein levels in serum [11]. Finally 1,25VitD3 was effective as an adjuvant in a mouse model of immunotherapy, where it potentiated the reduction of airway hyperresponsiveness, allergic inflammation, and specific IgE levels compared to SIT alone allowing lower doses of allergen to be used for injections [7].

To determine whether 1,25VitD3 is useful as adjuvant for SIT in allergic patients, a double blind placebo-controlled clinical trial was performed, in which grass-pollen allergic patients were treated with subcutaneous grass-pollen allergoid SIT (SCIT) supplemented with 1,25VitD3 or carrier control (Chapter 6). Unfortunately allergoid SCIT did not result in improvement of symptom and medication scores, either in the presence of absence of 1,25VitD3. The lack of clinical efficacy after grass-pollen allergoid SCIT precludes a definite conclusion on the adjuvant activity of 1,25VitD3. Despite the absence of clinical effectiveness, a significant increase in the levels of slgG4 was observed in both SIT treatment groups but not in the placebo group. As IL-10 has shown to be involved in the induction of slgG4 [12], we aimed to characterize the GP-specific Th-
cell responses in patients during SIT treatment with or without 1,25VitD3 adjuvant. We find that addition of 1,25VitD3 adjuvant increased the numbers of grass-pollen-specific IL-10 producing T cells. Moreover in both SIT treatment groups decreased numbers of IFNγ producing Th-cells were observed, and a transient decrease of FOXP3+ Th-cells was observed in patients treated with only SIT, not in those receiving 1,25VitD3 adjuvant.

**MATERIAL & METHODS**

**Patients**

Patients suffering from allergic rhino-conjunctivitis with or without mild asthma and grass-pollen sensitization were randomized in 3 groups. The treatment is schematically depicted in figure 1: The Placebo-Placebo group (n=9) was treated with 2 placebo injections, The SIT-Placebo group (n=9) with Grass-pollen-allergoid (Purethal Gras, HAL Allergy, Leiden, The Netherlands), and Placebo injections, and the SIT-VitD3 group (n=9) with Grass-pollen-allergoid (Purethal Gras), and 1,25VitD3 (Calcijex, Abbott, Illinois) injections. Grass-pollen allergy was defined by a positive history, in addition to a positive skin prick test and nasal challenge with a standardized allergen extract (Phleum pratense, ALK-Albelló BV, Almere). Positive skin prick tests for perennial allergens, like house dust mite and pets, were allowed as long as the clinical symptoms of the patient were restricted to the grass pollen season, and these patients were not exposed to these pets. Sensitization to tree pollen was allowed as long as clinical manifestations were restricted to the grass-pollen season. All patients gave written informed consent. From each group samples from 9 patients were taken for T-cell analysis. Age and sex of the different groups from which T cell and antibody determinations were done are described in table 1.

**Figure 1:** Dosage scheme of induction and maintenance phase of GP immunotherapy applied within the three treatment groups. Arrows above the time line display the subcutaneous injections of GP-allergoid. Arrows below the time line display the blood drawing before the subcutaneous injection.
Table 1: Patient Characteristics

<table>
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<th>Placebo-placebo</th>
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</tr>
<tr>
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<td>31.4 (20-50)</td>
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**PBMCs**

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by ficoll gradient (Lymphoprep, Axis-Shield, Norway), washed twice, and frozen and thawed as described previously [13]. In short, isolated PBMCs were resuspended in a 50% FCS, 40% HBSS (Lonza/Bio-Whittaker) and 10% DMSO (Sigma-Aldrich, St Louis, MO, USA) freezing medium, and placed within a Cryo preservation module at -80°C. After 24h, frozen vials were transferred into liquid nitrogen. Frozen PBMCs were thawed quickly, and gradually diluted with 10ml of PRMI-1640, supplemented with 50μM β-Mercaptoethanol, and washed a second time with 20ml of medium, before culturing.

**PBMC cultures with GP-extract**

We previously showed that we were able to detect GP-specific T cells with the highest accuracy using 8-day cultures of CFSE labeled PBMCs in the presence of GP-extract [14]. Therefore allergen-induced T cell proliferation was detected by flow cytometry, 10 x 10⁶ PBMCs were stained in 0.5ml 10μM CFSE solution for 15 minutes at 37°C. To stop the reaction, cells were washed 3 times with RPMI 1640 (Bio-Whittaker) supplemented with 10% Fetal Calf Serum (FCS). CFSE labeled PBMCs were cultured in a 24 well plate (Costar, Cambridge, Mass, USA) in 500μl of Ultra Culture Medium (BioWhittaker) in the presence of 30μg/ml of GP-extract. Wells without GP-extract served as a control. At day 7 cells were harvested and restimulated in flat bottom 96 well plates with plate bound anti-CD3 (overnight, 30μl of 5μg/ml, OKT-3), and soluble anti-CD28 (1μg/ml, clone? BD biosciences) for 16h in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich).

**Flow cytometry**

PBMCs were washed with cold (4°C) dPBS, and fixed using 2% formaldehyde (Merck KGaA, Darmstadt, Germany) in PBS during 20 minutes at RT in dark. Subsequently cells were permeabilized using 0.1% saponin (Sigma-Aldrich) and 0.5% BSA (Sigma-Aldrich) in PBS, and stained using following antibodies: CD4-PerCP (SK3), IL-4-PE (8D4-8) (BD Pharmingen), IFNg-PE-Cy7 (4S.B3) (eBioscience), FOXP3-AlexaFluor647 (206D), IL-10-
PeCy7 (JES3-9D7), IL17-AlexaFluor647 (BL168), IL-9-PE (MH9A4) (all Biolegend, San Diego, USA). Identically stained, non-restimulated cells served as control. Measurements were performed using BD LSR-II flow Cytometer (BD Pharmingen), and analysed using Flowjo 9.2 software (Tree Star, Inc, Ashland, Ore).

**Statistics**

Differences between the Th-cell responses from the two groups are detected using non-parametric Mann-Whitney Tests, and results are expressed as median and range. When groups were normally distributed, students T test was used to detect differences, and results are expressed as mean ± SEM. A p-value < 0.05 was considered to be statistically significant (GraphPad Prism 4 for Mac; Inc, La Jolla, USA).

**RESULTS**

**After 9 weeks of SIT treatment the percentage of proliferating Th cells increased transiently**

The proliferative responses of the Th cells to the GP allergens were studied using CFSE labeled PBMCs cultured in the presence of 60µg/ml GP-extract. Proliferating Th cells, detected by reduced CFSE-intensity, were not observed in PBMCs cultured in medium alone (Fig 2A). Within the Placebo group, no differences were detected in the percentages of proliferating Th cells between baseline (12.3 ± 2.3 %), 9 weeks (12.2 ± 1.8%), and 1 year of treatment (11.2 ± 1.9 %) (Fig. 2B, left). Within the SIT-placebo group, a significant increase in the percentage of proliferating Th cells was observed from 10.6 ± 2% at baseline to 15 ± 1% after 9 weeks of treatment (p=0.02), which decreased again to baseline levels after one year of treatment (11.9 ± 1.5%) (Fig. 2B, middle). Within the SIT-VitD3 group, no differences were detected in the percentages of proliferating Th cells between baseline (9.4 ± 1.4 %), 9 week (11 ± 1.7%), and 1 year of treatment (8.6 ± 1.7 %) (Fig. 2B, right).

Overall, a transient increase in the percentage of allergen-responsive Th cells was observed after 9 weeks of SIT-Placebo treatment, which was not observed within the Placebo-Placebo, and the SIT-VitD3 groups.
**Chapter 7**

The cytokine production of GP-allergen responsive Th cells was studied within the CFSE-low Th cells as previously described [14]. Th cells were stained for IL-4, IL-9, IL-10, IL-17 and IFNγ. First, we analyzed IL-4 and IFNγ as hallmark cytokines for Th2 and Th1 cells, respectively.

Fig 3A shows a dot plot of the CD4⁺CFSE<sub>low</sub> T cells expressing IL-4 from a representative Placebo-Placebo treated patient. The percentage of GP-responsive IL-4 expressing Th cells within the Placebo-Placebo group did not change compared to baseline levels (2.4 ± 0.5%) after 9 weeks (2.2 ± 0.3%) or 1 year (2 ± 0.2%) of treatment. Also within the SIT-
Placebo and the SIT-VitD3 groups, no differences were detected for the fraction of IL-4-producing cells within the allergen-responsive Th cell population between baseline (resp. 2.4 ± 0.6%, and 2.3 ± 0.4%), 9 week (resp. 2.5 ± 0.3% and 2.1 ± 0.2%), and 1-year (resp. 2.5 ± 0.3% and 2.3 ± 0.5%) of treatment (Fig 3B).

Fig 4C shows a dot plot of the CD4+ CFSElow T cells expressing IFNγ from a representative Placebo-Placebo treated patient. The percentage of GP-responsive IFNγ expressing Th cells within the Placebo-Placebo group did not change compared to baseline levels (11.2 ± 1.9%) after 9 weeks (8.8 ± 2.5%) or 1 year (7.7 ± 1.1%) of treatment. Within the SIT-Placebo group, the percentage of IFNγ expressing Th cells decreased significantly from 10 ± 1.5% at baseline to 7.5 ± 1.2% (p=0.02), and 7.7 ± 1.4% (p=0.04) after respectively 9 weeks and one year of treatment. Also within the SIT-VitD3 group, the percentage of IFNγ expressing cells decreased from 14.2 ± 1.5% at baseline to 10 ± 1.2% (p=0.003) and 10.9 ± 2 (p=0.02) after respectively 9 weeks and 1 year of treatment (Fig 3D).

Overall, no changes in the percentage of grass-pollen responsive IL-4 producing cells were observed, while the percentage of IFNγ producing cells, significantly decreased after 9 weeks and 1 year of treatment in both the SIT-Placebo, and SIT-VITD3 group.

Figure 3: Th1/Th2 cytokine expression in PBMCs cultured in the presence of GP-extract during 8 days. Cultured PBMCs were restimulated during the last 16h of culturing using plate-bound anti-CD3, and soluble anti-CD28. Representative dot plots from the CD4+ CFSE low PBMCs expressing IL-4, and IFNγ are shown in A and C, respectively. Graphs B and D represent respectively the IL-4 and IFNγ expression from each single patient measured at different time points during treatment from the Placebo-Placebo (left), SIT-Placebo (middle), and SIT-VitD3 (right). Each point represents a single patient measurement, within patient measurements are connected. *: p < 0.05, **: p < 0.01.
IL-9 and IL-17 expression by allergen-responsive Th cells

The percentages of IL-9 and IL-17 expressing T cells were studied as described earlier [14, Chapter 2]. Fig. 4A shows a dot plot of the CD4$^+$CFSE$^{low}$ T cells expressing IL-9 from a representative Placebo-Placebo treated patient. The percentage of GP-responsive IL-9 expressing Th cells within the Placebo-Placebo group did not change compared to baseline levels (0.12 ± 0.03%) after 9 weeks (0.11 ± 0.03%) and 1 year (0.13 ± 0.04) of treatment. Also within the SIT-Placebo, and SIT-VitD3 groups, no differences were detected between baseline (resp 0.1 ± 0.02% and 0.12 ± 0.04%) 9 weeks (resp. 0.08 ± 0.02% and 0.11 ± 0.03%), and 1-year (resp 0.09 ± 0.02% and 0.12 ± 0.02%) of treatment (Fig 4B).

Fig. 4C shows a dot plot of the CD4$^+$CFSE$^{low}$ T cells expressing IL-17 from a representative Placebo-Placebo treated patient. The percentage of GP-responsive IL-17 expressing Th cells within the Placebo-Placebo group did not change compared to baseline levels (1.7 ± 0.5%) after 9 weeks (1.3 ± 0.4%) or 1 year (1.5 ± 0.5%) of treatment. Also within the SIT-Placebo and the SIT-VitD3 groups, no differences were detected between baseline (resp. 1.6 ± 0.2% and 2.2 ± 0.5%) 9 weeks (resp. 1.1 ± 0.2% and 1.9 ± 0.4%), and 1-year (resp. 1.2 ± 0.3% and 1.8 ± 0.4%) of treatment (Fig 4D).

Overall, no differences were detected in the fraction of IL-9 and IL-17 producing allergen-responsive Th cells after treatment with both SIT-Placebo, and SIT-VitD3 treatment groups.

![Figure 4](image_url)  
**Figure 4:** Additional T cell subsets were studied in PBMCs cultured in the presence of GP-extract during 8 days. Cultured PBMCs were restimulated during the last 16h of culturing using plate-bound anti-CD3, and soluble anti-CD28. Representative dot plots from the CD4$^+$ CFSE low PBMCs expressing IL-9 and IL-17 are shown in A and C respectively. Graphs B and D represent respectively the IL-9 and IL-17 expression from each single patient measured at different time points during treatment from the Placebo-Placebo (left), SIT-Placebo (middle), and SIT-VitD3 (right). Each point represents a single patient measurement, within patient measurements are connected.
**FOXP3 and IL-10 expression by allergen responsive T cells**

Finally, the percentages of FOXP3 and IL-10 expressing Th cells were studied within the GP-responsive Th cells as previously described by staining for FOXP3, and IL-10.

Fig 5A shows a dot plot of the CD4+CFSE<sup>low</sup> T cells expressing FOXP3 from a Placebo-Placebo treated patient. The percentage of GP-responsive FOXP3 expressing Th cells within the Placebo-Placebo group did not change compared to baseline levels (3 ± 0.9%) after 9 weeks (2.3 ± 0.6%) or 1 year (2.8 ± 0.8%) of treatment. Within the SIT-Placebo group, the percentage of FOXP3 expressing Th cells decreased significantly from 1.7 ± 0.4% at baseline to 1.1 ± 0.15% (p=0.04) after 9 weeks of treatment, and increased again to before treatment levels after 1 year of treatment (1.9 ± 0.5%). Within the SIT-VitD3 group no differences were found between baseline (2.4 ± 0.4%), 9 week (2 ± 0.2%), and 1-year (2 ± 0.3%) of treatment (Fig 5B). In addition, no differences in FOXP3 expressing Th cells were detected in the non-dividing population (data not shown).

Fig 5C shows a dot plot of the CD4+CFSE<sup>low</sup> T cells expressing IL-10 from a representative Placebo-Placebo treated patient. The percentage of GP-responsive IL-10 expressing Th cells within the Placebo-Placebo group did not change compared to baseline (0.15 ± 0.07%), after 9 weeks (0.1 ± 0.04%), and 1-year (0.25 ± 0.17%) of treatment. Also within the SIT-Placebo group, no differences were detected between baseline levels (0.09 ± 0.02%),

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**Figure 5:** Treg cytokine expression in PBMCs cultured in the presence of GP-extract during 8 days. Cultured PBMCs were restimulated during the last 16h of culturing using plate-bound anti-CD3, and soluble anti-CD28. Representative dot plots from the CD4+ CFSE low PBMCs expressing FOXP3 and IL-10 are shown in A and C respectively. Graphs B and D represent respectively the FOXP3 and IL-10 expression from each single patient measured at different time points during treatment from the Placebo-Placebo (left), SIT-Placebo (middle), and SIT-VitD3 (right). Each point represents a single patient measurement, within patient measurements are connected. *: p < 0.05.
after 9 weeks (0.1 ± 0.02%) or 1 year (0.13 ± 0.04) after treatment. Remarkably, within the SIT-VitD3 group, the percentage of IL-10 expressing Th cells increased significantly from 0.1 ± 0.02% at baseline to 0.16 ± 0.03% (p=0.03), and 0.18 ± 0.05% (p=0.1) respectively after 9 weeks and 1 year of treatment (Fig 5D).

Overall, a temporary decrease in the percentage FOXP3 expressing cells was observed after 9 weeks of treatment within the SIT-Placebo group. Within the SIT-VitD3 group, a significant increase in the percentage of IL-10-producing cells was observed after 9 weeks, which remained after 1 year of treatment although not statistically significant.

**DISCUSSION**

Adjuvants are a promising tool to improve the clinical efficacy of allergen immunotherapy. In this study we used 1,25VitD3 to enhance the induction of a regulatory Th cell response, known to occur after SIT. As observed for the clinical parameters (Chapter 6), no differences were observed in the fraction of IL-4 expressing allergen-responsive Th cells after SIT treatment, irrespective of the addition of 1,25VitD3 as adjuvant. However, we found that by in the presence of 1,25VitD3, increased numbers of grass-pollen-specific IL-10 producing Th cells were induced compared to SIT alone. Moreover, 1,25VitD3 seemed to abrogate the transient decrease in FOXP3 expressing cells observed after SIT alone.

IL-10 was shown to be involved in the induction of IgG4, therefore the early induction of IL-10 producing Th-cells was thought to precede the increase of slgG4 [12]. Within our study increased serum levels of slgG4 were observed in both SIT-treated groups, although increased numbers of IL-10 producing Th-cells were only detected within the SIT-VitD3 group. Moreover no significant differences in IL10+ Th-cell numbers were found between the SIT-placebo and SIT-VitD3 group. Therefore, our data do not support a strict relation between specific IgG4 levels and allergen-responsive IL10+ Th cells since we did not observe increased IgG4 levels in the SIT-VitD3 group while increased numbers of IL10+ Th cells were observed. Notwithstanding, the fraction of IL-10+ cells within the allergen-responsive Th cell subset was very small, indicating that our method of detecting allergen-specific IL-10+ Th cells might not be sensitive enough.

Peripheral T cell tolerance is known to occur early after SIT, and is characterized by decreased allergen induced T cells proliferation [6], probably caused by the induction of IL-10 and FOXP3 expressing T cells [2, 5, 15, 16]. In our study grass-pollen induced T cell proliferation increased transiently within the SIT-Placebo group compared to the Placebo-Placebo and the SIT-VitD3 groups. In line herewith, the SIT-Placebo group showed no increase in the number of IL-10 producing T cells, and a temporary reduction in FOXP3 expressing allergen-responsive Th cells was observed. The absence of de novo
induced Tregs, in combination with a temporary reduction of FOXP3 expressing cells can explain the increased T cell proliferation in the SIT-Placebo group. In line herewith, the transient reduction in FOXP3 expressing T cells was absent in the SIT-VitD3 group, while also no induction of grass-pollen induced proliferation was observed. In line with our results several studies reported no reduction in allergen-induced T cell proliferation after SIT [5, 17, 18], while in other studies a reduction of T cell proliferation was observed [8, 19–21]. The absence of reduced T cell proliferation, and moreover the transient induction thereof seen in the SIT-Placebo group could be important for the absence of clinical success in the study.

Although several studies observed increased numbers of FOXP3 expressing Th cells after SIT [16, 22], a transient decrease of FOXP3 expressing cells was also observed after VIT [23], and was associated with enhanced activation and lymph node homing phenotype of FOXP3 expressing Th cells. Therefore the temporary decreased numbers of FOXP3 expressing T cells do not necessarily indicate a reduced number of Tregs. In contrast increased numbers of FOXP3 were reported one year after treatment, probably overlooking the early effects of SIT.

Within both SIT-treated groups, no decrease in the numbers of IL-4 producing allergen-specific Th cells were detected. However, a significant decrease in the numbers of IFNγ producing cells was observed over the course of SIT treatment in both SIT-Placebo and SIT-VitD3 groups. Within studies of clinically successful SIT, decreased Th2, and increases in Th1 cytokines were detected [17, 18, 21]. In our study no clinical effects were detected (Ref Hfst). Lack of clinical efficacy in our study is therefore paralleled with lack of decrease in allergen-specific Th2 cells after immunotherapy, indicating that successful suppression of Th2 cells could be of major importance for clinical effectiveness. Nevertheless, changes reported in literature in T-cell cytokine profiles after successful SIT were mostly detected locally [17, 18], indicating that detection of allergen-specific Th cells in PBMCs might not be the most sensitive approach to detect the alterations induced by SIT on the adaptive T cell-mediated immune response. The numbers of IL-9 and IL-17 producing cells did not change during treatment, and no differences were observed between the different treatment groups. In contrast to our results, decreased numbers of IL-9 producing cells after SIT have been shown in nasal biopsies during the pollen season [24], and we previously showed decreased numbers of IL-9 expressing cells after VIT (Hfst). Furthermore, the number of IL-17 producing cells did not change during treatment. Increased numbers of IL-17-producing T cells are associated with neutrophil inflammation in severe asthma [25–28]. In line with our results, no changes in IL-17 cells have been reported after sublingual pollen immunotherapy [29].
Overall, 1,25VitD3 as adjuvant for SIT seemed to potentiate the induction of allergen-specific IL-10 and FOXP3 expressing Th cells compared to SIT alone. As the induction of IL-10 and FOXP3 expressing Th cells is thought to be crucial for the induction of clinically successful SIT, 1,25VitD3 could be a promising adjuvant for the improvement of SIT.
REFERENCES


