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Role of Vitamin D in the Induction of Regulatory T Cells Producing Interleukin 10 in Children with Cow Milk Allergy

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ABSTRACT

Background: Various populations of regulatory T cells play a central role in the development of peripheral tolerance to allergens. Culturing of $CD4^+$ T cells isolated from peripheral blood of allergic patients with vitamin D induces the generation of stable IL-10 producing $CD4^+CD25^+$ Treg cells suppressing the proliferation of T helper cells obtained from the same patients. The immune regulatory role of vitamin D in allergic patients has been controversial and obviously needs a more clarifying research work. **Aim of the work:** to determine the percentage of induced T regulatory cells producing interleukin 10 after stimulation of T regulatory cells with cow milk allergen in the presence of vitamin D in culture. This aims to further in-vitro study the immune regulatory role of vitamin D in cow milk allergic patients. **Results:** there is association between decreased level of vitamin D and milk-allergy, as serum level of 25(OH) D3 was insufficient in 16 (80 %) patients (10- 29.9 ng/ml) while 4 (20%) patients were sufficient (30-100 ng/ml). Addition of vitamin D, in culture, induces the production of $CD4^+$ $CD25^{hi}$ $Foxp3^+$ $IL10^+$.

Treg cells within peripheral blood mononuclear cells (PMNCs) isolated from allergic children who had insufficient vitamin D, but not in allergic children who had normal level of vitamin D.

Conclusion: this work provides further evidence for an important role of 1,25(OH)2D3 as an immune-modulatory molecule and suggests that supplementation of vitamin-D-deficient individuals, who are reported to have reduced numbers of circulating and $Foxp3^+$ $IL10^+$ Treg cells, may represent an attractive therapy for enhancing endogenous populations of Treg cells in allergy.

Keywords: Regulatory T cells, calcitriol, food allergy.

INTRODUCTION

Various populations of regulatory T cells play a central role in the development of peripheral tolerance to allergens and also in successful clinical improvement in allergen-specific immunotherapy which represents the single curative treatment in allergic diseases[1]. T regulatory cells are classified to natural T regulatory cells and induced T regulatory cells. The natural T regulatory cells (nTreg) are self antigen specific $CD4^+$ T cells that express $CD25^+$ in high levels and forkhead box protein (FOX) that are selected in the thymus, and become regulatory T cells in the periphery. The induced-T reg cells (Treg-1) are converted from naïve T cells after encountering specific antigen in the periphery and are characterized by elevated production of interlukin-10 [2]. Regulatory T cells inhibit the activation of T-helper cells (Th), mast cells, basophils and eosinophils thus minimizing the production of interlukin-4 (IL-4) and IL-5 which are essential cytokines during the allergic reactions. In addition, Treg also act on B lymphocytes to suppress the production of allergen-specific immunoglobulin-E [3]. Cow milk allergy is believed to be either IgE-mediated in which activation of milk-specific Th cells leads to the production of milk-specific IgE, or non IgE-mediated that may include T cell/mast cell interaction with secretion of inflammatory cytokines including IL-4 and IL-5. Decreased Treg activity has been identified as a factor in both allergy mechanisms [4]. **Shreffler and colleagues (2009)** [5] showed that children, who were allergic to cow milk and become tolerated, had high percentage of milk-specific $CD4^+$ $CD25^+$ Treg cells in their peripheral blood with high in-vitro proliferation activity when stimulated with cow milk protein. **Xystrakis *et al*.** [6] previously reported that culturing of $CD4^+$ T cells isolated from peripheral blood of atopic patients with vitamin D induces the generation of stable IL-10 producing $CD4^+CD25^+$ Treg cells suppressing the proliferation of T helper cells obtained from the same patients. They confirmed the immune regulatory role of vitamin D in atopic patients. However the role of vitamin D in

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allergy has been controversial and obviously needs a more clarifying research work[7].

Subjects and methods:

Twenty children with cow's milk allergy from (one month to five years) from the Pediatric Allergy and Immunology Unit, Children's Hospital, Ain Shams University.

A-Diagnosis of cow milk allergy:

1. Positive skin prick test (SPT) for cow milk (≥3 mm above negative control) using commercial cow's milk allergens extract purchased from allergy pharma.
2. Oral challenge with cow's milk in patients with negative SPT

B-Sampling:

Four ml of venous blood were drawn from each patient: 2 ml were placed into
sterile tube containing K-ethylene diamine tetra-acetic acid (K2-EDTA) at a concentration of 1.2 mg/ml for performing PBMCs separation, analysis and culture, and 2 ml was collected into plain vacutainers to be clotted and centrifuged, the yielding serum was kept at -20 until the time of vitamin D analysis.

C- **Flow Cytometric Analysis of Separated PBMCs Stained by Monoclonal Antibodies Against CD4, CD25, FOXP3 and IL-10**

The separated PBMCs from all patients were subjected to immune-phenotypic analysis by flow cytometry (FCM) to detect the percent of $CD4^+$ $CD25^{hi}$ $Foxp3^+$ $IL10^+$ (Treg) cells and $CD4^+$ $CD25^{lo}$ $Foxp3^+$ $IL10^+$ (Teff) cells within gated lymphocytes. Percent of $Foxp^+$ and $IL10^+$ co-expression on both Treg and Teff cells was also determined to know the difference between co-expression of both markers on Treg and Teff cells (figure:1).

All MoAbs were supplied by eBioscience, USA and stored in refrigerator at 4^oC. For surface staining of CD4 and CD25: Phycoerythrin Texas Red-X (also known as ECD) conjugated anti-CD4 and Phycoerythrin-cyanine 5 (PE-Cy5) conjugated anti-CD25. For intercellular staining of IL-10 and Foxp3: PE conjugated anti- IL-10 and anti-human FOXP 3 staining set: Fluorescein Isothiocyanate (FITC) conjugated anti- FOXP3, Fixation/*permeabilization* concentrate, Fixation/ *permeabilization* diluents and *permeabilization* buffer.

D- In-vitro culture:

Isolated PBMCs from all patients were cultured with milk allergen alone at a dose of 10 ug/ml RPMI (as a stimulator for T cells) in one set and in presence of both vitamin D at a dose of 20 IU/ml RPMI and 10 ug/ml RPMI of milk allergen in another set. After 2 days of culture, MNCs that were collected from both culture sets and were subjected to FCM analysis.

E- Statistical Methods: The data were coded, entered and analyzed using Statistical Package for Social Sciences **(SPSS)** software computer program version ***"PASW 23.0, IBM Corp., USA, 2015)".*** Description data were expressed as median & percentiles for quantitative non-parametric measures. Wilcoxon signed rank test for Comparison between two dependant groups for non-parametric data. The probability of error > 0.05was considered non significant (NS), at 0.05 was considered significant (S), while at 0.01 and 0.001 are highly significant (HS).

RESULTS

This study was conducted on 20 well documented allergic infants and children who were diagnosed as having cow milk allergy. They were recruited from outpatient Pediatric Allergy and Immunology Clinic at Children's Hospital, Ain Shams University. They were 9 females and 11 males, their ages ranged from 6 months to 5 years [median (25 percentile; 75percentile): 2 years (9 months; 3.3 years)].

They were divided according to their serum Vitamin D level into :

(a) Sufficient vitamin D level: they were 4 (20%) patients their VD level ranged from 31 to 37 ng/ml [median (25 percentile; 75 percentile): 32 ng/ml (31; 36 ng/ml)]. They were all females and their ages ranged from 9 months to 2.5 years [median (25 percentile; 75 percentile): 2 years (1; 2.3 years)]. One (5%) patient had Non-IgE allergy, represented by abdominal distension and constipation and the other 3 patients had IgE allergy; two (10%) patients represented by bronchial asthma (BA) and one (5%) patient represented by urticaria.

(b) Insufficient vitamin D level: they were16 patients (80%). their VD level ranged from 10 to 28 ng/ml [median (25 percentile; 75 percentile): 17 ng/ml (11; 22 ng/ml)]. They were 11 males and 5 females and their age ranged from 6 months to 5 years [median (25 percentile; 75 percentile): 2 years (9 months; 3.8 years) (table 1)]. Eight (40%) patients had IgE allergy; 4 (20%) of them represented by BA and the other 4 (20%) patients represented by urticraia. Five (25%) patients had non-IgE allergy; 3 (15%) patients represented by abdominal distension and constipation, one (5%) patient represented by bloody diarrhea and one (5%) patient complained from failure to thrive. Three (15%) patients had mixed type of allergy represented by atopic dermatitis.

In our studied patients, serum level of 25 (OH) VD ranged from 8 to 37 ng/ml. Reference range is shown in (table 2).

In all patients, percent of Teff cells was higher than percent of Treg cells with decreased Treg/Teff ratio (median=0.05). $Foxp3^+$ and $IL10^+$ co-expression on Treg/ $Foxp3^+$ and $IL10^+$ co-expression on Teff cells ratio (median=1.05) (table 3).

Statistical comparison between sufficient and insufficient VD patients groups as regard percent of Treg cells showed no significance difference (p=0.3) (table 4).

Statistical comparison of percent of $Foxp3^+$ and $IL10^+$ co-expression on Treg cells between sufficient and insufficient VD patients groups showed no significant difference (p=0.74) (table 5).

Statistical comparison of percent of Teff cells between sufficient and insufficient VD patients groups showed no significant difference (p=0.6) (table 6).

Statistical comparison of percent of $Foxp3^+$ and $IL10^+$ co-expression on Teff cells between sufficient and insufficient VD patients groups showed no significant difference (p=0.63) (table 7).

Statistical comparison of Treg/Teff ratio between sufficient and insufficient patients groups showed no significant difference (p=0.82) (table 8).

Statistical comparison of $Foxp3^+$ and $IL10^+$ co-expression on Treg/ $Foxp3^+$ and $IL10^+$ co-expression on Teff ratio between sufficient and insufficient VD patients groups showed no significant difference (p=0.7) (table 9).

Statistical comparison of percent of Treg cells between base line (BL) and after culture with allergen (A) only and after culture with A and VD showed that a highly significant difference in the percent of Treg cells between BL and after culture with A and VD (p=0.00). Also, it showed a highly significant difference between Treg percent after culture with A only and culture with A and VD (p=0.00) (table 10).

Statistical comparison between patients groups according to serum VD level as regard percent of Treg cells, we found, in VD insufficient group, a highly significant increase in the percent of Treg cells after culture with A and VD in comparison to their percent at BL (p=0.001) and their percent after culture with A only (p=0.001) (tatble10).

Statistical comparison of percent of Teff cells between BL and after culture with A only and after culture with A and VD showed that: within all patients groups, there was no significant difference (p>0.05) (table 11).

Statistical comparison between different sets of culture as regard Treg/Teff ratio among all cases showed that, Treg/Teff ratio was significantly increased after culture with A and VD in comparison to culture with A only and in comparison to BL. In patients with sufficient VD level where Treg/Teff ratio showed no significant difference after culture with A and VD in comparison to culture with A only (table 12).

Statistical comparison between different sets of culture as regard the percent of $Foxp3^+$ and $IL10^+$ co-expression on Treg cells showed that the percent of $Foxp3^+$ and $IL10^+$ co-expression on Treg cells was significantly increased after culture with A and VD in comparison to their percent of co-expression at BL (p=0.00) or their percent of co-expression after culture with A only (p=0.00). Besides, percent of $Foxp3^+$ and $IL10^+$ co-expression on Treg cells was significantly increased after culture with A only in comparison to their percent of co-expression at BL (p=0.009) (table 13).

Among patients with VD insufficiency, the percent of $Foxp3^+$ $IL10^+$ co-expression was highly significantly increased after culture with A and VD when compared to their percent of co-expression at BL (p=0.001) and their percent of co-expression after culture with A only (0.001) (table 13).

Statistical comparison of percent of $Foxp3^+$and $IL10^+$ co-expression on Teff cells between BL and after culture with A only and after culture with A and VD showed that, a significant increase in their percent of co-expression after culture with A and VD in comparison to their percent of co-expression at base line (p=0.005) or their percent of co-expression after culture with A only (p=0.001) (table 14)

Among patients with VD insufficiency, a significant rise observed only in percent of $Foxp3^+$ and $IL10^+$ co-expression on Teff cells after culture with A and VD in comparison to their percent of co-expression after culture with A only (p=0.01) (table 14).

Statistical comparison between different sets of culture as regard $Foxp3^+$ and $IL10^+$ co-expression on Treg/$Foxp3^+$ and $IL10^+$ co-expression on Teff ratio among all cases showed that, this ratio was significantly increased after culture with A and VD in comparison to culture with A only and in comparison to BL. In patients with sufficient VD level where this ratio showed no significant difference after culture with A and

VD in comparison to culture with A only (table 15).

DISCUSSION

Allergic reaction is characterized by activation of Th2 cells, IgE production and eosinophilia .[8] $CD4^+CD25^{high}$ Foxp3 Treg cells are known to play a key role in balancing immune responses to maintain peripheral tolerance against harmless antigens or allergens [9]. Peripheral blood Treg cells were found to suppress Th2 cytokine production from both atopic and non atopic individuals [10]. Many studies have focused on the role played by T-cell counteractive processes, and have revealed a decreased frequency of T reg cells in the peripheral blood of patients with allergy, compared with that in healthy control subjects [11].

Mucosal or oral tolerance is characterized by the deletion or suppression of T reactive specific-antigen cells and by production of Treg cells that suppress the inflammatory response against benign antigens. A dysfunction on Treg activity seems to be a necessary background for the spread of both reactions (IgE- and not-IgE mediated CMPA), as the induction of mucosal tolerance in children is linked with an increase of Treg lymphocytes [12]

Vitamin D shares in the process of the development of $CD4^+$ $CD25^+Foxp3^+$Treg cells [13] and enhances their suppressive function [14] Vitamin D has the ability to establish homeostasis between regulatory and effector T cell functions to modulate inflammatory process [15]

This study tried to evaluate and investigate the role of VD in children with CMPA and to assess the effect of VD on induction of $CD4^+CD25^{hi}Foxp3^+IL10^+$ Treg cells in peripheral blood MNCs isolated from those patients, upon stimulation with milk allergen in culture.

The present study included 20 infants and children with a clinically active cow milk allergy. Most cases (55%) had IgE-mediated allergy symptoms e.g bronchial asthma and urticaria, diagnosed by positive skin prick test (SPT) to commercially extracted milk allergen. while (30%) of cases had non-IgE mediated allergy symptoms e.g abdominal distension and constipation, diagnosed by negative SPT and positive diet elimination test. The other (15%) of cases had atopic dermatitis in which both (IgE and non-IgE) mechanisms of allergy are involved.

In studied patients, serum level of 25(OH)D (measured by ELISA) was insufficient (10-29.9 ng/ml) in 16 (80%) patients; and sufficient (30-100 ng/ml) in 4(20%) patients. VD status was not differing between patients represented by different allergic symptoms. Obviously, all patients with atopic dermatitis had insufficient VD level.

The potential mechanisms for the hypothesized link between VDD and food allergy in children were studied by **Vassallo and Camargo *et al.*** [16] who hypothesized that the hormone deficiency at a particular time, such as the postnatal period, can increase the susceptibility to colonization by abnormal intestinal microbial flora, that contributes to increased intestinal permeability, which in turn leads to excessive and inappropriate exposure of the immune system to dietary allergens.

Probably, VDD could contribute to a non effective tight junction at the skin level which may result in abnormal cutaneous sensitization to food allergens leading to eczema such in cases of atopic dermatitis [17] Moreover, vitamin D also directly suppresses skin inflammation by increasing IL-10 production by cutaneous mast cells [18]

The present study was in agreement with a cross sectional study done by **Allen *et al.*** [19] who found that infants with VD insufficiency were more likely to have food allergies than those with adequate VD level. Another cross sectional study done by **Baek *et al.*** [20] found that the degree and prevalence of food allergen sensitization increased in infants with VDD.

On the other hand further studies showed no association between VD level and development of food allergy. In a pilot study, **Mullins *et al.*** [21] found that the relationship between neonatal 25(OH)D levels and childhood peanut allergy was nonlinear with slightly higher levels (30-39.9 ng/ml) associated with lower risk than those in the reference group. The risk of peanut allergy at the lower levels of neonatal vitamin D, levels present in very few children of this cohort, was not significantly different from the reference group.

One retrospective, case control study did not find any association between asthma severity and serum 25(OH) VD concentrations. In this study, 263 subjects with asthma were compared to 284 normal subjects (ages: 2 to 19 years). No significant difference in VD concentrations was found between the asthmatic group and the control group, and the severity of asthma symptoms was not correlated with the vitamin D

concentrations [22]. Furthermore, in a birth cohort study, [23] reported no overall association between cord blood vitamin D levels and food-specific IgE.

Another study found that cord blood $25(OH)D_3$ highest levels were positively associated with the risk of children presenting food allergy in the first 2 years of life. Therefore, the study's message was the demonstration that high vitamin D levels at birth may contribute to a higher risk of food allergy and therefore that no supplementation is required to protect against allergy[24].

In the meantime, it should consider that all the previous shown evidences and demonstrations could help us to evaluate the opportunity for VD supplementation in children, keeping in mind that there is more evidence for the positive effects of supplementation. It is also very important in this regard to remember **Liu *et al***. [23] who reported that VDD may increase the risk of food allergy and sensitization among individuals with certain genotypes, providing evidence of gene–vitamin D interaction on food allergy.

$CD4^+CD25^+$ were identified as Treg markers in humans. The percentage of $CD4^+CD25^+$ can range from 2% to 13%. However, each study used a different reference WBC population to calculate the percentage of $CD4^+CD25^+$ such as PBMC, lymphocytes, total WBC,$CD3^+$, and $CD4^+$ cells. This is one reason why the percentage of Treg varied between the studies. Since $CD25^+$ cells include activated T cells, researchers tried to exclude this population from Treg by selecting cells with a high expression of CD25.Again, because of the use of different cell gating criteria for the calculations as mentioned above, there is a range of $CD4^+CD25^{hi}$ from 0.25% to 6% in the Caucasian population [25]

For the purpose of this study, PB was collected from our patients and PMNCs were separated and stained with Abs against CD4 CD25 Foxp3 and IL10 to detect the percent of $CD4^+$ $CD25^{hi}$ $Foxp3^+$ $IL10^+$ (Treg) cells and $CD4^+$ $CD25^{lo}$ $Foxp3^+$ $IL10^+$ (Teff) cells within gated lymphocytes by FCM analysis and correlated their percentages with different patients groups.

In the present study, all patients had lower level of Treg cells than level of Teff cells with decreased Treg/Teff ratio. Relatively, co-expression of $Foxp3^+$ and $IL10^+$ wasn't differing, between Treg cells and Teff cells; this evidenced by that co-expresion of $Foxp3^+$ and $IL10^+$ on Treg/ co-expression of $Foxp3^+$ and $IL10^+$ on Teff ratio was (1.05). These findings supporting the hypothesis that allergic disease may develop as a result of dysfunction and/or decreased number of PB $Foxp^+$ Treg cells

These finding may be explained by other findings from previous studies which determine that, stable and high expression of Foxp3 on Treg cells depends on DNA de-methylation at Treg-specific de-methylated region (TSDR) within Foxp3 locus [26]. Furthermore, decreased Foxp3 expression converts Treg cells into pathogenic effector cells which may contribute to the development of TH cell-associated responses [27].

Results obtained from this study were in agreement with **Karlsson *et al.*** [28]who reported that children who cured from cow's milk allergy had higher numbers of Tregs with more potent function than children who had remained allergic. In a very interesting study, **Smith *et al.*** [29] looked at Treg function in cord blood from infants who later developed egg allergy by 12 months of age. Infants who developed allergies had lower Treg function than infants who were tolerant to foods, suggesting that a defect in Tregs may predispose to food allergy. Similarly, infants with higher numbers of allergen-specific Tregs had milder disease and a more favorable outcome than those who had lower frequencies of Tregs [30]

The same findings observed in two studies done on Egyptian asthmatic children, the first study done by **Aly *et al.*** [31] who determined a significant decrease in $CD4^+CD25^{hi}$ Treg cells in asthmatic group compared to a control group, although Foxp3 expression in $CD4^+$ $CD25^{hi}$ cells was higher in patients compared to controls. They explained the higher Foxp3 expression by the evidence that, Foxp3 marker renders more accurate and reliable identification of Treg cells. The second study done by **Bakr *et al.*** [32] who revealed a highly significant decrease in $CD4^+$ $CD25^{hi}$ Foxp3+ Treg, Treg/ Teff ratio, Foxp3 determination in asthmatic group compared to a healthy control group.

Taken together, the evidence is accumulating that differences in Treg activity may be a factor in the development and resolution of food allergies. Importantly, Treg modulates the allergic response by exerting a direct effect on B cells, suppressing the production of allergen-specific IgE and inducing IgG4. Treg also suppress allergic inflammation through direct action on mast cells, basophils and eosinophils [33]

The findings from this study were against the findings revealed from a study done by **Zhang *et***

al. [34] who carried out their study on 15 asthmatic children; they compared the percentage of T reg cells in the peripheral blood, and in bronchio-alveolar lavage fluid (BALF) of asthmatic children with 10 healthy controls .They found a significant decrease in T reg cells in BALF of asthmatic children than the controls while the T reg cells in peripheral blood were equally in both groups. They mentioned that measuring T reg cells at site of inflammation is more accurate than in peripheral blood as normal percentage of T reg in peripheral blood do not exclude its impairment capacity to maintain immune tolerance at diseased site.

In the present study, percent of $CD4^+$ $CD25^{hi}$ $Foxp3^+$ $IL10^+$ Treg cells didn't show any difference between allergic patients with sufficient VD and allergic patients with insufficient VD. Also, increased Teff/Treg ratio with no difference in $Foxp^+$ and $IL10^+$ co-expression on both cells between sufficient and insufficient VD patients groups hypothesized that, VD wasn't the only factor affecting function/number of Treg cells in these patients.

In disagreement of these results, **Chambers *et al.*** [35] found a strong positive correlation between serum 25(OH) D levels and $CD3^+$ $CD4^+$ $Foxp3^+$ T cell number in otherwise asthmatic adult patients. VD assessed in this study by high performance liquid chromatography system. Also, **Maalmi *et al.*** [36] observed a significant positive correlation between $CD4^+$ $CD25^{hi}$ $Foxp3^+$ Treg cells and VD values in asthmatic patients (using Pearson's correlation coefficient). VD concentrations were assayed with a radioimmunoassay and included asthmatic children in this study were aged (6-16) years old.

In contrary, **Wiesse *et al.*** [24] found a negative correlation between cord blood regulatory T-cell numbers and levels of cord blood $25(OH)D_3$, which could be considered a potential contributing mechanism for allergy development. Different assay methods of VD, different age groups and different identification markers for Treg cells may make these different results.

In the earlier, ingestion of oral calcitriol by steroid-refractory asthmatics enhances the responsiveness to glucocoticoids for induction of IL 10 synthesis from $CD4^+$ cells ***6.*** Previous studies also reported that 1α25VitD3 promoted FOXP3 expression in $CD4^+$ T cells in the presence of anti-CD3/CD28 Abs and IL-2 [37]

The present study, aimed to investigate the capacity of 25(OH) D3 to promote induction of Treg cells producing IL10 *in vitro* upon culturing of isolated MNCs obtained from our patients in presence of cow milk allergen (as a stimulant for T cells), for 48 hours.

It was previously evidenced that VDR expression peaked at 48 hours post stimulation [38]

It was found that percent of $CD4^+CD25^{hi}$ $Foxp3^+$ $IL10^+$ Treg cells was increased after culture in presence of A and VD than BL. Whereas percent of $CD4^+CD25^{lo}$ $Foxp^+IL10^+$ Teff cells wasn't differ after culture with A and VD. Terg/Teff (ratio) was significantly increased after culture in presence of VD than BL

In addition, percent of $Foxp3^+$ and $IL10^+$ co-expression on Treg was significantly increased after culture with VD, and percent of co-expression of $Foxp3^+$ and $IL10^+$ on Teff was also significantly increased after culture with VD but not to the same percent as occurred on Treg cells; co-expression of $Foxp3^+$ and $IL10^+$ on Terg/co-expression on Teff (ratio) was significantly increased after culture in presence of VD

These findings may be explained by that, upon stimulation, transient and low expression of Foxp3 may occur on CD4 $CD25^{lo}$ or CD4 $CD25^{hi}$ cells. This expression may be due to partial methylation of Foxp3 gene which may be associated with a non-suppressive capacity [39]

In addition, T cells express CYP27B1 enzyme that allow the conversion of 25(OH) D3 to its active form calcitriol**.** Calcitriol increased the transcription of human Foxp3 gene by direct binding to the VDREs that were identified in Foxp3 gene and functioned as calcitriol-dependent enhancers [40]

Based on these findings, it may be hypothesized that VD has little or no effect on suppressing Teff cells but it promotes the proliferation of Treg cells and maintain sustainable and high expression of Foxp3 on Treg cells, may be, by inducing de-methylation of (TSDR) within Foxp3 gene.

Findings from this study were in agreement with another observations documented by **Urry *et al.*** [41] and subsequent study done by **Chambers *et al.*** [15] who found that, activation of human $CD3^+$ $CD4^+$ T cells *in vitro* in presence of calcitriol, induced a sizable population of $CD4^+$ $IL10^+$ Treg cells. Again, frequency and absolute number of $Foxp3^+$ T cells was strongly enhanced by addition of 25(OH) D3 (inactive form) upon culturing $CD4^+$ $CD25^-$ T cells in presence of APCs [42]

This is in consistent with the work done by **chambers *et al.*** [43] who also observed that culturing of isolated human PB $CD4^+$ T cells while adding calcitriol at a level (100 nmol/L)

increased the frequency of $CD4^{+/}Foxp3^{-}$ /$IL10^{+}$ Treg cells, whereas higher calcitriol (1000 nmol/L) increased the frequency of $CD4^{+}/FoxP3^{+}/IL10^{-}$ T cells.

Obviously, all these studies documented the effect of VD on $CD4^{+}$ T cells isolated from healthy volunteers. One study by **Xystrakis *et al*.** [6] showed the same effect of calcitriol on inducing $CD4^{+}$ $IL0^{+}$ Treg cells isolated from asthmatic patients in combination with dexamethasone. Unfortunately, VD level in those patients wasn't done.

In the present study, it was observed that, better response of VD in induction of Treg occurred in patients with insufficient VD level than in patients with sufficient VD level. This finding may hypothesized that VDRs on T cells and VDREs on Foxp3 gene may have high accessible affinity to VD in status of its deficiency than in its presence in a sufficient state. This observation requires subsequent evaluation.

The concentration of 25(OH) D3 used in this study was 20 IU/mL RPMI. This is equal to (80 ng or 200 nmol). Health care professionals need to keep in mind that in general, 100 IU (2.5 mcg) of VD per day can raise the VD blood test only 1 ng/ml or just 2.5 nmol/L after 2 to 3 months [44]

Two small trials have addressed the impact of vitamin D supplementation on allergic diseases. A study of 11 children with winter-related atopic dermatitis was enrolled in a pilot study of vitamin D supplementation. The children were randomized to 1000 IU per day of ergocalciferol or placebo for 1 month. There was a trend for improvement of atopic dermatitis scores, but due to the small number and short duration of the trial, the results did not achieve statistical significance [45]

Another study examined the effect of either inhaled budesonide + 500 IU of cholecalciferol or inhaled budesonide + placebo in asthmatic children for about 6 months. The authors found a reduction in attacks of asthma exacerbations in children who took VD supplementation [46]

To ensure an adequate intake of vitamin D, the American Academy of Pediatrics has raised the daily recommended intake for children and adolescents, to a dose of 400 IU up to 12 months of age and 400–600 over 12 months, recommending that this supplementation should begin during the first days of life [47]

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Table 1: Demographic data of patient groups according to serum VD levels

Patients groups	Age Median (25^{th}-75^{th} perc.)	Gender	Clinical diagnosis	VD level (ng/ml) Median (25^{th}-75^{th} perc.)
Patients with Sufficient VD 30 – 100 ng/ml (n=4)	2 years (1-2.3 y)	All Females (20%)	- One patient (5%) had non-IgE allergy represented by abdominal distension and constipation. - Three patients had IgE allergy; 2 of them (10%) represented by BA and one patient (5%) represented by urticaria.	32 ng/ml (31-36)
Patients with Insufficient VD 10 – 29.9 ng/ml (n=16)	2 years (9 m -3.8 y)	11 Males (55%) 5 Females (25%)	- Eight patients had IgE allergy; 4 (20%) of them represented by BA and 4 (20%) patients represented by urticraia. - Five patients had non-IgE allergy; 3 (15%) of them represented by abdominal distension and costipation, one (5%) patient represented by bloody diarrhea and one (5%) patient represented by failure to thrive. - Three patients had mixed type (15%) represented by atopic dermatitis.	17 ng/ml (11-22)

BA: bronchial asthma

Table 2: Reference range of serum 25 (OH) VD.

Deficient	< 10 ng/ml
Insufficient	10 – 29.9 ng/ml
Sufficient	30 – 100 ng/ml
Intoxication	> 100 ng/ml

Table 3: Percent of Treg and Teff cells and percent of Foxp3 and IL10 co-expression on both cells in all patients

Variable	Median (25^{th}-75^{th} perc.)
% Treg	0.04 (0.01-0.17)
% Teff	0.68 (0.33-1.01)
% $Foxp3^+$ and $IL10^+$ on Treg	19 (11-23)
% $Foxp3^+$ and $IL10^+$ on Teff	20.5 (13.7-29.5)
Treg/Teff ratio	0.05 (0.03-0.3)
$Foxp3^+$ and $IL10^+$ on Treg/ $Foxp3^+$ and $IL10^+$ on Teff ratio	1.05 (0.69-1.39)

Table 4: Statistical comparison between sufficient and insufficient VD patient groups as regard the percent of Treg cells

Patients groups	Treg cells (%) Median (25^{th}-75^{th} perc.)	*Z	P
Sufficient VD (n=4)	0.07 (0.02-1.09)	0.902	0.3 (NS)
Insufficient VD (n=16)	0.04 (0.01-0.17)		

Table 5: Statistical comparison between sufficient and insufficient VD patient groups as regard the percent of foxp3+ and $IL10^{+}$ co-expression on Treg cells

Patients groups	$Foxp3^{+}$ and $IL10^{+}$ co-expression on Treg cells (%) Median (25^{th}-75^{th} perc.)	*Z	P
Sufficient VD (n=4)	20 (5-44)	0.332	0.74 (NS)
Insufficient VD (n=16)	18 (11-23)		

Table 6: Statistical comparison between sufficient and insufficient VD patient groups as regard the percent of Teff cells

Patients groups	Teff cells (%) Median (25^{th}-75^{th} perc.)	*Z	P
Sufficient VD (n=4)	0.72 (0.33-1.4)	0.473	0.6 (NS)
Insufficient VD (n=16)	0.68 (0.2-0.8)		

Table7: Statistical comparison between sufficient and insufficient VD patients groups as regards $Foxp3^{+}$ and $IL10^{+}$ co-expression on Teff cells

Patients groups	Foxp3+ and IL10+ co-expression on Teff Cells (%) Median (25^{th}-75^{th} perc.)	*Z	P
Sufficient VD (n=4)	19 (13-34)	0.473	0.63 (NS)
Insufficient VD (n=16)	21 (13-29)		

Table 8: Statistical comparison between sufficient and insufficient VD patients groups as regard Treg/Teff ratio

Different allergic patients groups	Treg/Teff ratio Median (25^{th}-75^{th} perc.)	*Z	P
Sufficient VD (n=4)	0.08 (0.03-0.5)	0.227	0.82 (NS)
Insufficient VD (n=16)	0.08 (0.03-0.3)		

Table 9: Statistical comparison between sufficient and insufficient VD patients groups as regard Foxp3$^+$ and IL10$^+$ co-expression on Treg/ Foxp3$^+$ and IL10$^+$ co-expression on Teff ratio

Different allergic patients groups	Foxp3$^+$ and IL10$^+$ on Treg / Foxp3$^+$ and IL10$^+$ on Teff ratio Median (25th-75th perc.)	*Z	p
Sufficient VD (n=4)	1.05 (0.1-1.2)	0.284	0.7 (NS)
Insufficient VD (n=16)	1.04 (0.6-1.4)		

Table 10: Statistical comparison between different sets of culture as regard percent of Treg cells in studied patients

Treg cells in studied patients	Base line/ after culture with A only		Base line/ After culture with A and V D		In culture with A only/ After culture with A and V D	
	Median	(25th-75th perc.)	Median	(25th-75th perc.)	Median	(25th-75th perc.)
All cases	0.04/ 0.06	0.01-0.17/ 0.03-0.18	0.04/ 0.6	0.01-0.17/ 0.3-1.4	0.06/ 0.6	0.03-0.18/ 0.3-1.4
P value	*0.5 (NS)*		***0.001(HS)***		***0.001 (HS)***	
Sufficient VD	0.07/ 0.07	0.02-1/ 0.01-1.2	0.07/ 1.1	0.02-1/ 0.3-2.8	0.07/ 1.1	0.01-1.2/ 0.3-2.8
P value	*0.4 (NS)*		*0.06(NS)*		*0.06(NS)*	
Insufficient VD	0.04/ 0.06	0.01-0.1/ 0.03-0.1	0.04/ 0.6	0.01-0.1/ 0.3-1.2	0.06/ 0.6	0.03-0.1/ 0.3-1.2
P value	*0.6 (NS)*		***0.001 (HS)***		***0.001 (HS)***	

Table 11: Statistical comparison between different sets of culture as regard percent of Teff cells in studied patients

Teff cells in studied patients	Base line/ After culture with A only		Base line/ After culture with A and VD		After culture with A only/ After culture with A and VD	
	Median	25th-75th perc.	Median	25th-75th perc.	Median	25th-75th perc.
All cases	0.68/ 0.47	0.33-1.01/ 0.32-1	0.68/ 0.6	0.33-1.01/ 0.25-0.9	0.47/ 0.6	0.32-1/ 0.25-0.9
P value	*0.2 (NS)*		*0.9 (NS)*		*0.1 (NS)*	
Sufficient VD	0.7/ 0.7	0.3-1.4/ 0.3-1	0.7/ 0.7	0.3-1.4/ 0.4-1.8	0.7/ 0.7	0.3-1/ 0.4-1.8
P value	0.7(NS)		0.2(NS)		0.4(NS)	
Insufficient VD	0.6/ 0.4	0.2-0.8/ 0.3-0.9	0.6/ 0.6	0.2-0.8/ 0.2-0.8	0.4/ 0.6	0.3-0.9/ 0.2-0.8
P value	*0.5 (NS)*		*0.9 (NS)*		*0.3 (NS)*	

Table 12: Statistical comparison between different sets of culture as regard Treg/Teff ratio in studied patients

Treg/Teff ratio in studied patients	Base line/ After culture with A only		Base line/ After culture with A and VD		After culture with A only/ After culture with A and VD	
	Median	25^{th}-75^{th} perc.	Median	25^{th}-75^{th} perc.	Median	25^{th}-75^{th} perc.
All cases	0.05/ 0.14	0.03-0.3/ 0.02-0.35	0.05/ 1.25	0.03-0.3/ 0.5-2.5	0.14/ 1.25	0.02-0.35/ 0.5-2.5
P value	*0.3 (NS)*		***0.001 (HS)***		***0.001 (HS)***	
Sufficient VD	0.08/ 0.12	0.03-0.5/ 0.06-0.79	0.08/ 1.3	0.03-0.5/ 0.6-1.7	0.12/ 1.3	0.06-0.79/ 0.6-1.7
P value	*0.48(NS)*		***0.04(S)***		*0.1(NS)*	
Insufficient VD	0.08/ 0.17	0.03-0.3/ 0.08-0.4	0.08/ 1.2	0.03-0.3/ 0.5-2.5	0.17/ 1.2	0.08-0.4/ 0.5-2.5
P value	*0.2(NS)*		***0.001 (HS)***		***0.001 (HS)***	

Table 13: Statistical comparison between different sets of culture as regard percent of $Foxp3^+$ and $IL10^+$ co-expression on Treg cells in studied patients

Co-expression of $Foxp3^+$ and $IL10^+$ on Treg cells in studied patients	Base line/After culture with A only		Base line/ After culture with A and VD		In culture with A only/ After culture with A and VD	
	Median	25^{th}-75^{th} perc.	Median	25^{th}-75^{th} perc	Median	25^{th}-75^{th} perc
All cases	19/ 24	11-23/ 11-30	19/ 62	11-23/ 46-100	24/ 62	11-30/ 46-100
P value	***0.009 (HS)***		***0.001 (HS)***		***0.001 (HS)***	
Sufficient VD	20/ 22	5-44/ 5-81	20/ 49	5-44/ 38-88	22/ 49	5-81/ 38-88
P value	*0.1(NS)*		*0.06(NS)*		*0.1(NS)*	
Insufficient VD	18/ 25	11-23/ 11-30	18/ 63	11-23/ 51-100	25/ 63	11-30/ 51-100
P value	*0.2 (NS)*		***0.001 (HS)***		***0.001 (HS)***	

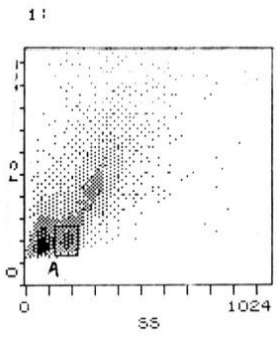
Table 14: Statistical comparison between different sets of culture as regard percent of $Foxp3^+$ and $IL10^+$ co-expression on Teff cells in studied patients

Co-expression of $Foxp3^+$ and $IL10^+$ on Teff cells in studied patients	Base line/ After culture with A only		Base line/ After culture with A and VD		In culture with A only/ After culture with A and VD	
	Median	25^{th}-75^{th} perc.	Median	25^{th}-75^{th} perc.	Median	25^{th}-75^{th} perc.
All cases	20.5/ 20	13.7-29.5/ 12-29	20.5/ 27	13.7-29.5/ 22-41	20/ 27	12-29/ 22-41
P value	*0.5 (NS)*		***0.005 (HS)***		***0.001 (HS)***	
Sufficient VD	19/ 26	13-34/ 14-41	19/ 21	13-34/ 18-42	26/ 21	14-41/ 18-42
P value	*0.06(NS)*		*0.1(NS)*		*1(NS)*	
Insufficient VD	21/ 19	13-29/ 10-27	21/ 29	13-29/ 22-41	19/ 29	10-27/ 22-41
P value	*0.5 (NS)*		*0.1 (NS)*		***0.01 (S)***	

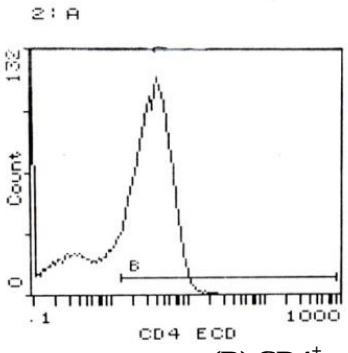
Table 15: Statistical comparison between different sets of culture as regard $Foxp3^+$ and $IL10^+$ on Treg/$Foxp3^+$ and $IL10^+$ on Teff ratio in studied patients

$Foxp3^+$ and $IL10^+$ on Treg/ $Foxp3^+$ and $IL10^+$ on Teff ratio in studied patients	Base line/ After culture with A only		Base line/ After culture with A and VD		After culture with A only/ After culture with A and VD	
	Median	25^{th}-75^{th} perc.	Median	25^{th}-75^{th} perc.	Median	25^{th}-75^{th} perc.
All cases	1.05/ 1.1	0.6-1.3/ 0.4-1.6	1.05/ 2.3	0.6-1.3/ 1.8-3.0	1.1/ 2.3	0.4-1.6/ 1.8-3.0
P value	*0.5(NS)*		***0.001 (HS)***		***0.001 (HS)***	
Sufficient VD	1.05/ 0.8	0.1-1.2/ 0.1-1.7	1.05/ 2.2	0.1-1.2/ 1.5-2.8	0.8/ 2.2	0.1-1.7/ 1.5-2.8
P value	*0.5(NS)*		***0.02(S)***		*0.08(NS)*	
Insufficient VD	1.04/ 1.1	0.6-1.4/ 0.4-1.6	1.04/ 2.2	0.6-1.4/ 1.8-2.7	1.1/ 2.2	0.4-1.6/ 1.8-2.7
P value	*0.4(NS)*		***0.001(HS)***		***0.001(HS)***	

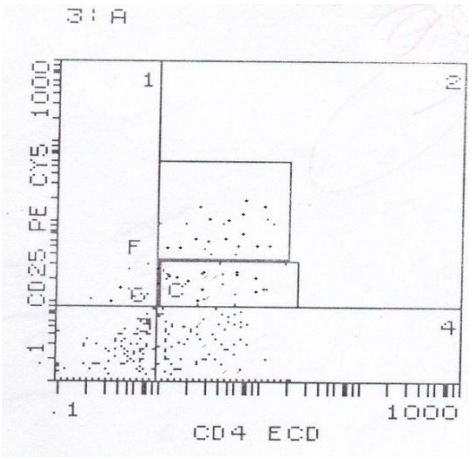
Figure (1): Representative dot plots of flow cytometry and the gating strategy used (A to H): (A) Show forward and side scatter to gate lymphocytes. **(B)** Show $CD4^+$ cells were acquired after gating the lymphocyte population by forward- and side-scattered properties. **(C)** Show coexpression of CD4 and CD25 on lymphocytes; "G" for $CD25^{lo}$ and "F" for $CD25^{hi}$ cells. **(D)** Show coexpression of Foxp3 and IL10 on $CD4^+/CD25^{lo}$. **(H)** Show coexpression of Foxp3 and IL10 on $CD4^+/CD25^{hi}$ lymphocytes.



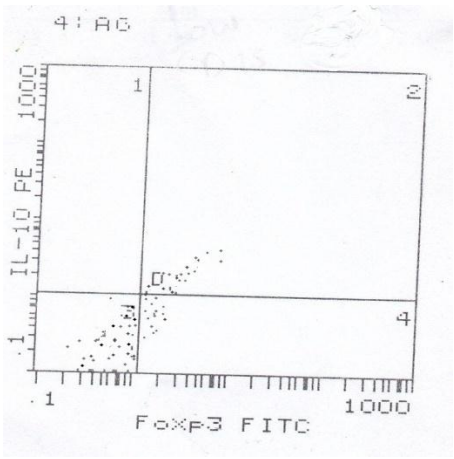
(A) Lymphocyte gating using forward scatter versus side scatter



(B) $CD4^+$ cells gating



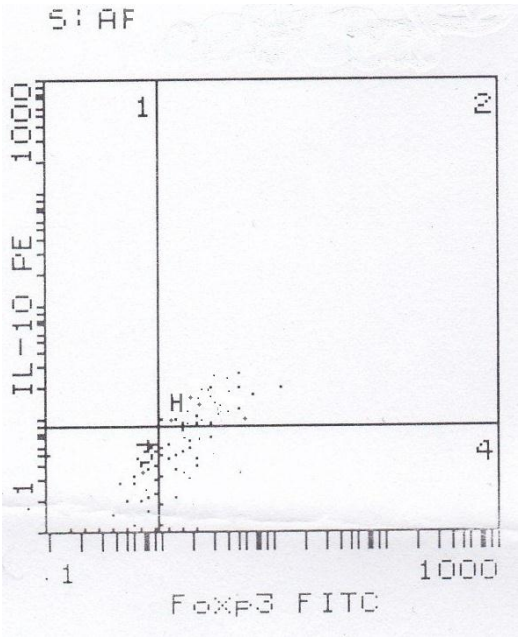
(C) Coexpression of CD4 and CD25 on lymphocytes;



(D) Different Coexpression of Foxp3 and IL10 on $CD4^+/CD25^{lo}$

"F" gate on C2 for $CD4^+/CD25^{hi}$ and
"G" gate on C2 for $CD4^+/CD25^{lo}$

Figures C & D don't correlate with comment below figures.



(H) Different Coexpression of Foxp3 and IL10 on $CD4^+/CD25^{hi}$
Figure not correct