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Interplay of vitamin D with T regulatory cells (FOXP3+Treg) and thymic stromal lymphopoietin (TSLP) in children with atopic diseases

Abstract

Objective: Several observational studies have shown association between lower serum vitamin D levels and severe asthma outcomes. The present study is designed to determine the association between vitamin D levels and immunological parameters (T reg cells and TSLP levels) in children with atopic diseases.

Methods: Vitamin D, TSLP, FEV1% and % FOXP3+Treg cells were analyzed in enrolled subjects. 25-Hydroxy vitamin D levels were estimated with an electro chemiluminesence immunoassay. TSLP was analyzed by sandwich ELISA kit and the %FOXP3+Treg cells were determined by flow cytometric analysis.

Results: Eighty children with asthma and 20 healthy children (control group) were enrolled in a study of serum vitamin D levels, FOXP3+Treg cells, TSLP and pulmonary function (spirometry). Vitamin D levels were significantly lower in the patient group (22.6±13.9ng/ml) compared to control group (39.0±25.93ng/ml p <0.0001). % FOXP3+Treg cell expression was higher in the control group as compared with the patient group (63.54±32.6 versus 92.8±8.1) (p=0.0003). FEV1% was significantly lower in the patient group compared to control group (70.4±12.5 versus 97.4±14.1; p=0.0001). The spearman correlation coefficient between vitamin D levels and FOXP3+Treg cells was positively correlated (r= 0.48, p =0.08).

Conclusion: Lower Vitamin D status is associated with lower FOXP3+Treg cells and higher TSLP levels in children with atopic diseases.

Keywords: allergy, asthma, atopic disease, vitamin D, FOXP3+T reg cells, FEV1%, TSLP, allergic rhinitis, atopic dermatitis

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Abreviations: CD4+T, cells cluster differentiation; ELISA, enzyme linked immuno sorbent assay; FEV, forced expiratory volume; FOXP3, forkhead box P3; IL, Interleukin; Treg cells, T regulatory cells; TSLP, thymic stromal lymphopoietin; TLR, toll like receptor; TGF-β, transforming growth factor-β.

Introduction

In recent years, vitamin D has been postulated as a risk factor for asthma and evidence suggests a connection between vitamin D deficiency with allergy and asthma. There is also an association between the dysregulated immune response marked by an increase in FOXP3+ and IL-10 T-regulatory (Treg) cells with the inflammatory processes of asthma.[1] Severe asthma was associated with lower vitamin D levels in one of the observational study.[2] An *in vitro* study noted an increase in the synthesis of IL-10 from Treg and dendritic cells was seen in the presence of exogenous vitamin D.[3] In cultured steroid resistance T cells, vitamin D restored the immunosuppressive ability of dexamethasone.[4] Low vitamin D levels, i.e. serum 25-OH-D3 less than 30ng/ml as insufficient and less than 20ng/ml as deficient, have been positively correlated with atopic diseases.[5] there is negative correlation between asthma exacerbation and vitamin D levels.[6] The inverse association between serum vitamin D levels and need for corticosteroid use in patients with asthma has also been observed.[7]

Decreased expression of FOXP3 (Forkhead boxP3) is associated with increased exacerbation of asthma and steroid sensitivity, and decreased formation, production and differentiation of FOXP3+Treg cells.[8–10] Upregulated expression of FOXP3+Treg cells by vitamin D supplementation reverses steroid resistance.[11–12] In what? Furthermore, increased expression of FOXP3+Treg cells in allergen specific immunotherapy correlated with higher serum vitamin D levels.[13] Vitamin D potentiates the efficacy of allergen immunotherapy by increasing the anti-inflammatory cytokines (IL-10 and TGF-beta) response in an animal model of OVA challenged and sensitized BALB/C mice.[14] The association of TSLP with vitamin D and % FOXP3+Treg cells is yet to be fully explored. It has been observed that when 16 human bronchial epithelial cell- lines were exposed to 50 and 500nM of vitamin D, inactive 25-OH- D3 is converted to active 1,25 D3 and there is increase in TSLP mRNA and protein expression levels.[15–16] In our previous report, we showed that higher concentrations of TSLP and IL-33 correlate negatively with Treg cells in children with asthma.[17] Until now, no study has demonstrated an association of vitamin D levels with TSLP and T regulatory cells in children with atopic disease.

Materials & methods

Subjects: Eighty children suffering from asthma (1-12 yrs) from

the Allergy and Asthma Clinic of Advanced Pediatrics Centre, Postgraduate Institute of Medical Education and Research, PGIMER, Chandigarh were enrolled in the study after obtaining informed consent from parents/guardians. Twenty healthy children, not having any symptoms, were enrolled as the control group. The study was approved by Institute Ethics committee of PGIMER. The diagnosis of asthma was made by clinical history, physical examination and other parameters according to GINA guidelines.[18]

Vitamin D & TSLP estimation

The quantitative estimation of serum 25-Hydroxy vitamin D (3 only or 2 + 3?) levels was done in both patient and control group with an electro chemiluminesence immunoassay, Cobas, Roche Diagnostics. TSLP was analyzed by sandwich ELISA kit (Ray Bio Human TSLP ELISA kit) according to the manufacturer's instructions.

Flow cytometric analysis for FOXP3+ Treg cells

FOXP3+Treg cells were analyzed after extracting PBMCs from buffy coat from the fresh blood samples. The PBMCs were stained with anti CD4+, anti CD25+ and FOXP3+ antihuman antibody and subjected to flow cytometric analysis. The determination was based on forward and side scatter properties captured by FACS Canto (Becton Dickinson, Mountain View, CA, USA) and analyzed using FACS Diva Software. Spirometry was done in children >5 years of age who were able to perform spirometry using a Spirolab II (Medical International Research SN A23-050.0814) standard protocol.[19] The presence of symptoms of asthma, such as cough, wheezing, respiratory difficulty were also noted.

Statistical analysis

The mean values and standard deviation were calculated using GraphPad Prism. A Student T test was performed between patient and control group. The Spearman correlation coefficients were used to evaluate the relationship between variables. Data were analyzed with GraphPad Prism v6.2; Graphpad Software Inc, La Jolla, CA, USA.

Results

Of the 80 patients enrolled in the study; there were children with asthma (n=42), asthma with allergic rhinitis (n=13), asthma with allergic rhinitis and atopic dermatitis (n=10), asthma with atopic dermatitis (n=10) and atopic dermatitis alone (n=5) (Figure 1). Children in the control group were matched for average? Mean age and gender distribution with the control group. Approximately 52% of patients had a family history of allergy and asthma. Other demographic and clinical details of enrolled children are given in (Table 1). Out of the total enrolled patients, 62 patients were on inhaled glucocorticoid therapy (budesonide 400µg/day), 6 were taking Fluticasone of 200µg/day, 2 patients used Fluticasone 200µg with Salmeterol 50µg twice a day and the remaining 10 patients were not taking any medications at the time of presentation. None of the children were taking anti-leukotrienes at the time of inclusion. The mean Vitamin D level was significantly lower in the patient group compared to the control group (22.6±13.9ng/ml versus 39.0±25.9ng/ml; p <0.001). More than seventy percent of children with asthma had serum vitamin D levels that were below 30ng/ml (deficient or insufficient) whereas only 1 (5%) of the control children did.

TheFEV1% was significantly lower in the patient group compared to control group (70.4±12.5 versus 97.4±14.1; p=0.0001). The percentages of Treg cells expressing FOXP3+ was significantly lower in the patient group in comparison to control group (63.5±32.6 versus 92.8±8.1; p < 0.0003) (Table 1). The Asthma Control Test score (ACT) determined in the patient group was 20 which is above the cutoff value of 19 (20; 95% CI 19-21).

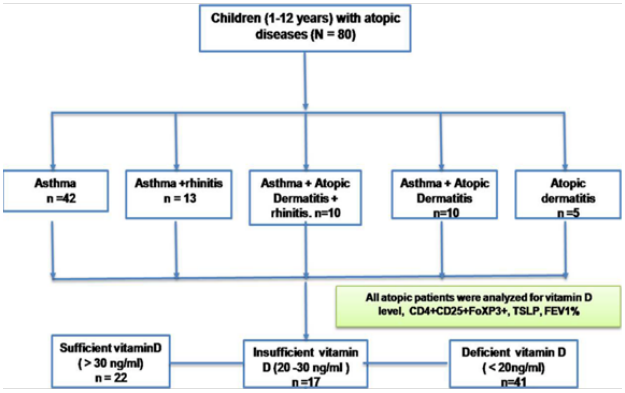

Figure 1 Patient enrollment and study design.

There was a higher level of TSLP in the patient group when compared with control group (583.4±567.9pg/ml versus 206±94.0pg/ml; p=0.05) Table 1 Serum vitamin D and the percentage of FOXP3+Treg cells were positively correlated among the patient group. (r=0.48, p=0.08) Figure 2A, while there was a negative correlation between vitamin D and TSLP (r=-0.16, p=0.18) Figure 2B. There were 38 asthma patients having concomitant allergic rhinitis, atopic dermatitis and 42 children with only asthma. No significant differences were observed in vitamin D, FOXP3+Treg cells, FEV1% and TSLP between those diagnosed with asthma only and those having more complex disease presentations (asthma with atopic dermatitis or rhinitis) (p>0.05)(Table 2).

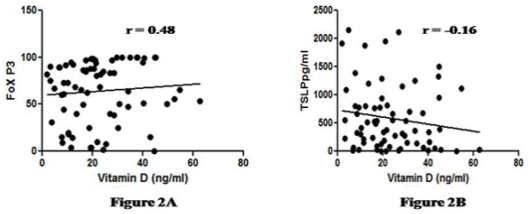

Figure 2A Figure 2B

Figure 2 (A) Spearman's correlation of vitamin D with FOXP3+T reg in children with atopic diseases, (B) Spearman's correlation of vitamin D with TSLP in children with atopic diseases.

Table 1 Demographic and clinical profile of patient and control group

	Patients (n=80)	Control (n=20)	p value
Age (Y)	8.1±2.1	8.9±2.4	0.2
	95%CI (7.5–8.8)	95% CI (7.7-10.0)	
Gender (M:F)	3.5:1	2.3:1	0.99
Height (cm)	126.2±18	127.7±21	0.77
	95%CI (120-131.2)	95%CI (117.8-137.5)	
Weight (kg)	25.4±8.3	30.1±12.6	0.07
	95%CI(11.0-29)	95%CI (24.2–36)	

Table Continued

	Patients (n=80)	Control (n=20)	p value
BMI (kg/m^2)	15±2..3	16.88±3.059	0.07
	95% CI(14.6-16))	95% (15.4-18.35)	
Non Vegetarian	25%	68%	0.0003
*Duration of sunshine (hrs)	1.469±0.86	2.52±0.96	0.0001
	(95%CI 1.27–1.66)	(95% CI 2.06-2.99)	
Breast fed (months)	17.06±11.38	10.42±3.80	0.0181
	(95% CI 14.53-19.59)	(95%CI 8.58-2.26)	
Family history -52.5%	0.52±0.5	---	---
	(95% CI 0.41–0.63)		
Asthma Control Test (ACT) score	20.05±2.58	---	---
	(95%CI 19.31-20.48)		
Dry cough at night (54/75)	75%	---	---
On inhaled Glucocorticoids (62/75)	82.60%	---	---
Number of children requiring hospitalizations for nebulization (68/75)	91%	---	---
Vitamin D ng/ml	22.6±13.9	39.0±19.57	< 0.0001
	(95%CI 19.5–25.8)	(95%CI 33.8–44.0)	
% FOXP3+	63.54±32.6	92.8±8.1	0.0003
	(95% CI 56–71.4)	(95%CI 88.9–96.8)	
TSLP pg/ml	583.4±567.9	206± 94	0.054
	(95%CI 446-719)	(95%CI 139-273)	
FEV1 %	70.4±12.5	97.4±14.1	< 0.0001
	(95%CI 66.4±77.2)	(95%CI 90.1-104.7)	

Table 2 Comparison of vitamin D, %CD4+CD25+FOXP3+Treg, FEV1%, TSLP, IL-33 between children with asthma and asthma with other allergies

Note: values are expressed as mean and 95% CI

	Asthma (n=39)	Asthma with other allergies (AD and rhinitis)(n=41)	p value
Vitamin D (ng/ml)	22.88	30.9	0.39
	(18.1-27.6)	(12.6-49.6)	
%CD4+CD25+	59.76	68.01	0.24
FOXP3+Treg	(48.33-71)	(57.5-78.4)	
FEV1%	71.76	69.1	0.47
	(65.8-77.6)	(64.2-73.9)	
TSLPpg/ml	634.5	541.6	0.46
	(430–839)	(350.9–732.4)	

Discussion

The present study demonstrated prevalence toward lower vitamin D levels in children with atopic diseases. The % FOXP3+Treg cells and TSLP in the atopic disease group is associated with vitamin D deficiency. A correlation between Vitamin D deficiency and increase in respiratory infections, asthma, and chronic obstructive pulmonary disease has been observed,[20] as has a reduction in risk of upper respiratory tract infection.[21] The increased markers of asthma severity are associated with lower levels of vitamin D.[22] The present study observed significant difference of vitamin D levels between the patient group and control group. Our study demonstrated 71.5% of the patients with deficient or insufficient vitamin D levels.[23] Vitamin D has an immunomodulatory role, a direct effect on T cells and also on antigen presenting cells especially dendritic cells.[23–25] Our study demonstrates the significant difference in vitamin D level between patient and control group, which is in agreement with a previous study done in southern Mediterranean countries demonstrating deficient levels of vitamin D in 43.59% asthmatic patients.[26] FOXP3+ expression is regulated by binding of vitamin D on FOXP3 in T cells? Which potentiates immunosuppressive? Function of T reg cells.[27] This was also suggested in our study showing positive correlation between vitamin D and FOXP3+Treg cells. Previous study reported that the number of FOXP3+Treg cells is significantly lower in steroid resistant asthma, when compared to steroid sensitive asthmatic patients. In our study, there was lower %FOXP3+Treg cells expression in the patient groups which are mostly on inhaled steroids when compared with control group. There was also positive correlation of %FOXP3+Treg cells expression with the vitamin D levels in our study. In asthmatic patients with higher vitamin D levels on steroid immunotherapy, there is significant reduction of TGF alpha, asthma symptom score and a steroid sparing effect.[13] There are several studies which implicate the association of vitamin D deficiency with poor pulmonary function targeting therapeutic role of vitamin D.[21] The role of vitamin D in reversing the inhibitory action of T reg cells in glucocorticoid resistant asthma has also been observed.[27,28] Another recent study has reported lower levels of vitamin D and T reg cells in children with asthma, but did not report a association between them.[29] All these studies support the therapeutic potential of vitamin D as a steroid-enhancing agent. In our study, we had explored the diagnostic relevance of vitamin D in atopic diseases, but have yet to demonstrate its optimum levels in our population. Such an analysis awaits large scale clinical trials data. TSLP has been implicated in the regulation of suppressive activity of % FOXP3+Treg cells. In our study negative correlation of TSLP with vitamin D status, and at the same time a higher level in atopic diseases implies that vitamin D supplementation may have therapeutic potential in the treatment of atopic diseases. If vitamin D is adopted as an alternative to steroid therapy, it could change modern therapy for atopic disease and could spare patients from adverse effects of steroids. Hours of sunlight were also significantly, different between the patient and control groups, further supporting the possibility that Vitamin D activated *in situ* could play a protective role.

Conclusion

Data from children with atopic disease showed that Lower serum Vitamin D levels ng/ml is associated with lower FOXP3+Treg cells and higher TSLP levels in children with atopic diseases. There is need to evaluate the vitamin D status in pediatric population and consider vitamin D supplementation in cases of deficiency or risk of deficiency.

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Conflict of interest

The author declares that there is no conflict of interest.

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