

Vitamin D Increases Percentages of Interleukin-10 Secreting Regulatory T Cells in Children with Cow's Milk Allergy

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Cow's milk allergy (CMA) is known to be either IgE- or non-IgE mediated. Regulatory T (Treg) cell defect is involved in the pathogenesis of both types. Vitamin D has been suggested to improve the generation of allergen-specific Treg cell populations with the potential to provide safe and long-term alleviation of disease symptoms. This study aimed to assess Vitamin D status in children with physician-diagnosed CMA and to investigate the effect of *in vitro* cultivation with Vitamin D on the percentage of antigen-driven CD4⁺CD25^{high}Foxp3⁺IL10⁺ Treg cells following *in vitro* stimulation of cells with cow's milk allergen in culture. This cross-sectional study included 20 children with CMA and 20 healthy age and sex-matched children as a control group. All patients were subjected to clinical evaluation, cow's milk skin prick test (SPT), cow's milk elimination and oral re-challenge test in patients with negative cow's milk SPT and in those with gastrointestinal presentation, measurement of serum Vitamin D level and assessment of the percentage of antigen-driven CD4⁺CD25^{high}Foxp3⁺IL10⁺ Treg cells in response to stimulation with cow's milk allergen extract with and without Vitamin D in culture. Vitamin D deficiency was detected in 80% of children with CMA. Percentage of Foxp3⁺ and IL10⁺ co-expression on Treg cells was significantly increased after stimulation with cow's milk allergen extract in the presence of Vitamin D. A significant positive correlation was observed between serum Vitamin D level and percentage of antigen-driven CD4⁺CD25^{high}Foxp3⁺IL10⁺ Treg cells as well as level of Foxp3⁺ and IL10⁺ co-expression on Treg cells at baseline (control cultures without stimulation) and after PBMCs stimulation with cow's milk allergen extract in the presence of Vitamin D. Re-stimulation with cow's milk allergen extract was performed *in vitro* in order to evaluate milk-induced immune stimulation and regulation. In conclusion, patients with CMA whether IgE- or non-IgE mediated had Vitamin D deficiency with a decreased number of CD4⁺CD25^{high}Foxp3⁺IL10⁺ Treg cells which increased after *in vitro* addition of Vitamin D with increased Foxp3 and IL10 co-expression.

Cow's milk allergy (CMA) is the commonest food allergy during early childhood [1]. CMA designates objectively reproducible symptoms or signs initiated by exposure to cow's milk at a dose tolerated by normal persons and which involves specific immunologic mechanisms [2]. Milk allergy can be either antibody-mediated or cell-mediated, or occasionally both may be involved. If IgE is involved in the reaction, the term "atopic food allergy" is appropriate. If immunologic mechanisms other than IgE are predominantly involved,

the term "non-IgE-mediated food allergy" should be used [3].

The diagnosis of CMA has to be based on strict, well defined food elimination and challenge procedures establishing a causal relation between the ingestion of a particular food (or food protein) and a subsequent obvious clinical reaction [4, 5]. CMA results in dietary restriction that may impair children's growth [6]. Until now, the suitable therapy of CMA is strict avoidance of cow's milk and cow's milk products. Therefore, further studies are needed to develop effective and safe immunotherapies

that do not elicit severe adverse reactions [7].

Both naturally occurring CD4⁺CD25^{high} Treg cells and antigen-driven interleukin-10 (IL-10)-producing CD4⁺ Treg cells have been involved in the regulation of allergic manifestations [8,9,10] and also in successful allergen-desensitization therapy [11]. It has also been demonstrated that Treg cells have the ability to control and modify the development of CMA [12, 13]. Treg cells suppress allergic pathology via several mechanisms including: inhibition of mast cells, basophils, and eosinophils; generation of tolerogenic DCs, suppression of allergen specific TH2 cells, and reduction of IgE/IgG4 ratio [14].

It is worth noting that adaptive or induced Tregs comprise both Foxp3⁺ and Foxp3⁻ populations and synthesize anti-inflammatory mediators such as IL-10, transforming growth factor (TGF)- β , IL-35, and adenosine. The various Treg populations may additionally control immune responses through mechanisms including inhibitory cell surface ligands such as cytotoxic T-lymphocyte antigen (CTLA)-4 and programmed cell death 1 ligand 1 (PD-L1), cytotoxic mediators such as granzyme A and B. The range of inhibitory mechanisms used by Tregs has been the subject of several excellent reviews [15].

A range of experimental approaches have been used to highlight the capacity of vitamin D to modulate Foxp3⁺ and IL-10⁺ Treg numbers and/or function. These range from *in vitro* and *in vivo* murine models, to patient-based studies demonstrating associations between vitamin D status and Treg numbers and/or function, and studies assessing Treg status before and after delivery of vitamin D to patients through supplementation with the parent compound or pharmacologic delivery of the

active moiety. Together, these data have generated considerable interest in the therapeutic application of vitamin D to enhance Treg function from early life through to adulthood in a wide range of immune-mediated disorders [16].

In addition to its recognized multiple physiological functions, vitamin D is now accepted as an immunomodulator. Active Vitamin D (1,25(OH)₂VD₃) can affect human immune responses by regulating Foxp3 expression in CD4⁺ T cells through direct Vitamin D binding to the Foxp3 gene, which is necessary for the inhibitory function of Vitamin D-induced Treg (Vitamin D-iTreg) cells [17]. However, there are contradictory results about the association of allergic diseases with Vitamin D level. Some studies reported a positive relationship between hypovitaminosis D and increased prevalence of allergy [18, 19, 20], while other studies did not [21, 22]. The involvement of Vitamin D in food allergy has been of huge concern in the last five years [23, 24]. Vitamin D is thought to preserve epithelial barrier integrity by maintaining cell junction complexes. It has been hypothesized that Vitamin D deficiency increased the incidence of gastrointestinal infections leading to defective barrier functions and increased risk of food allergy [25].

To our knowledge, the effect of Vitamin D supplementation on antigen specific IL-10-producing Treg cells has not been demonstrated before in children with CMA. This study aimed to investigate the role of Vitamin D in children with CMA and to assess the effect of *in vitro* cultivation with Vitamin D on antigen-driven CD4⁺CD25^{high}Foxp3⁺IL10⁺ Treg cells in peripheral blood mononuclear cells (PBMCs) isolated from those patients, upon

stimulation with cow's milk allergen in culture.

Materials and Methods

Subjects and sample collection

This was a cross sectional study which was conducted at the Pediatric Allergy and Immunology Unit, Pediatric Gastroenterology Unit at Children's Hospital and Department of Clinical Pathology and Immunology Unit, Ain Shams University. It included 20 infants and children who have physician-diagnosed CMA and 20 age and sex-matched healthy children as a control group.

Inclusion criteria: Patients with symptoms and/or signs that are related to cow's milk intake (e.g. atopic dermatitis, asthma, gastrointestinal symptoms like vomiting or diarrhea) with positive skin prick test (SPT) and /or open oral challenge test with cow's milk.

Exclusion criteria: Patients with symptoms suggestive of cow's milk allergy but with negative SPT to cow's milk and negative oral challenge test. Patients with chronic diseases to avoid any bias in the assessment of serum Vitamin D and the Treg cells.

An informed consent was taken from parents of all study participants after explaining the aim and procedures of the study and ensuring the confidentiality of the data. The study was carried out after the approval of Ain Shams University Ethics Committee.

Patients were classified according to type of allergy into 3 groups:

1- IgE mediated CMA group included 11 patients (55%) with a history of allergic symptoms within few minutes to 2 hours after consuming foods that contains cow's milk proteins. Also, they had a positive SPT to cow's milk. Six patients (30%) had bronchial asthma with a history of exacerbation shortly after milk ingestion. Four patients (20%) had acute urticaria and one patient (5%) experienced vomiting and diarrhea shortly after milk consumption.

2- Non-IgE mediated CMA group included 6 patients (30%) with a history of unexplained gastrointestinal manifestations which were prominent with consuming food containing cow's milk protein. They were diagnosed by a positive oral cow's milk challenge test (after cow's milk elimination for 6

weeks) with a negative SPT to cow's milk. Four patients (20%) had abdominal distension and constipation, one patient (5%) had bloody diarrhea and one patient (5%) had watery diarrhea and failure to thrive.

3- Mixed (IgE and non-IgE mediated) CMA group included 3 patients (15%). They had atopic dermatitis with a positive SPT to cow's milk.

Two venous blood samples (5 mL each) were withdrawn by venipuncture from all patients and controls. The first sample was collected into a gel vacutainer tube (Becton Dickinson, Oxford, UK) to be clotted and centrifuged. The yielding serum was used for the detection of 25 OH cholecalciferol (25 (OH) D) levels. The second sample was collected on K3EDTA vacutainer tube (Becton Dickinson, Oxford, UK) and blood was used for separation of PBMCs for measurement of the percentages of CD4⁺CD25^{high}Foxp3⁺IL10⁺ Treg cells by flow cytometry (FCM) in the peripheral blood and after culture with allergen in the presence or absence of Vitamin D.

Methods

Individuals included in the study were subjected to the following:

- Cow's milk skin prick test (SPT)

SPT with the commercial cow's milk allergen extract (Allergopharma, Germany). First generation antihistamines were avoided for 72 hours before SPT, while second generation antihistamines were avoided at least 5 days before the procedure. The volar aspect of the patient's forearm was cleaned with alcohol, positive (histamine) and negative (saline) control solutions were applied, the test site on the volar aspect of the forearm was marked and labeled at least 3 cm apart from the positive control. The marked site was dropped by the allergen and gently pricked by sterile skin test lancet. The patient was asked to wait for twenty minutes before interpretation of the results. A response of at least 3-mm diameter (with equivalent erythema) more than diluent control done at the same time was required [26].

- Elimination of cow's milk and re-challenge

Cow's milk was eliminated for 6 weeks from the diet of the infants with persistent gastrointestinal symptoms suggestive of cow's milk (gastroesophageal reflux disease not appropriately responding to antireflux measures, watery diarrhea with failure to thrive, distension, colics and

constipation with early introduction of milk and milk products). Milk was introduced in gradually increasing amounts as follow: Day 1: 5, 10, 20, and 30 mL at 30 minutes intervals. Day 2: 30, 60 and 120 mL at 2 hours interval. Day 3: normal volumes of milk, i.e., 450 mL per day. Expected reaction if CMA is positive: within one day and up to one week [27].

- Measurement of serum of 25(OH) D levels

As 25 (OH) D is the main circulating form of Vitamin D, it is widely used to assess Vitamin D status. Vitamin D deficiency was defined as a 25 (OH) D serum level <20 ng/mL, while Vitamin D insufficiency was defined as 25(OH) D level 20–30 ng/mL and finally, Vitamin D sufficiency was defined as >30 ng/mL [28]. Serum 25(OH) D levels were estimated using ELISA kit supplied by CALBIOTECH Inc, USA (Catalog #: VD220B).

- Isolation of PBMCs

The whole blood was diluted 1: 1 with phosphate buffered saline (PBS) (Lonza, Walkersville, USA). PBMCs were isolated from buffy coats by Ficoll-Hypaque (Lonza) density-gradient centrifugation. The separated cells were washed twice with RPMI 1640 medium (Lonza). Then, cell pellets were suspended in 1 ml PBS buffer then counted and tested for viability. Viability was exceeding 90% in all cases. Then, PBMCs were suspended in complete medium which consisted of RPMI 1640 supplemented with 2mM L-glutamine, 15% fetal calf serum (Biowest, Nuaille, France), 100µ/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml amphotericin –B (Biowest).

- Cell culture

Culture optimization: vitamin D concentration was chosen due to former experiment [29]. Regarding cow's milk allergen extract, PBMCs of three patients were cultured in the presence of 0 (control), 10, 20, 40, and 80 µg/mL of cow's milk allergen extract in the presence or absence of vitamin D. Then, Treg cells were analyzed using flow cytometry. 10 µg/mL of cow's milk allergen extract was selected as optimum for this study.

PBMCs were cultured at a density of 5×10^5 /well in 200 µL of culture media in 96 wells U-bottom culture plates (Costar Corp., Cambridge, MA, USA) and stimulated with 10 µg/mL of cow's milk allergen extract (Allergopharma, Germany) in the presence or absence of 10,000 pmol/L vitamin D3 (Memphis, Pharmaceutical Chemical Industries, Egypt). The cow's milk allergen extract used in culture is the

same used for SPT(the total volume of the vial is 5 mL (500 µg/mL), so 20 µL was added for each 1ml of complete medium to reach a final concentration of 10µg/ml). Re-stimulation with cow's milk allergen extract was performed *in vitro* in order to evaluate milk-induced immune stimulation and regulation. Prior to use, 1µl of vitamin D vial (200,000IU) (Memphis, Egypt) was diluted in 1 ml of RPMI, then 200µl of the preparation were added to 800µl of RPMI. (N.B 40IU=1µl, 1ng/ml=2.5nmol/ml). 1.6 µl of vitamin D preparation were added to 200ul of complete medium to reach a final concentration of 10,000 pmol/L of vitamin D. Simultaneously, parallel control cultures were run without stimulation to obtain a baseline.

Subsequently, cells were incubated for 48 h in a humidified atmosphere at 37°C and 5% CO₂. After the incubation time, cells were washed and centrifuged at 1500g (3200rpm) for 3 minutes then re-suspended in 500 µL PBS for FCM analysis.

- Flow cytometric analysis of separated PBMCs stained by monoclonal antibodies against CD4, CD25, FOXP3 and IL-10

All monoclonal antibodies (MoAbs) were supplied by eBioscience, USA and stored in refrigerator at 4°C. 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 intracellular staining of IL-10 and Foxp3: PE conjugated anti- IL-10 and anti-human foxp3 staining set: Fluorescein Isothiocyanate (FITC) conjugated anti- foxp3, Fixation/ permeabilization concentrate, Fixation/ permeabilization diluents and permeabilization buffer. Respective isotype control antibodies were used supplied by eBioscience, USA (Two plastic test tubes were used; one for test and the other for isotypic control according to manufacturer instruction). One hundred µL of freshly isolated PBMCs suspension was delivered to a sample wassermann plastic tube with cell count adjusted to 1×10^6 / 100 µL. 10 µL of ECD anti-CD4 and 5 µL of PE-Cy5 anti-CD25 were added to the suspended cells and incubated for 30 min in the refrigerator at 4°C. Washing was done once by adding 2 mL of cold PBS to the tube then centrifuged at 2500 rpm for 5 minutes and the supernatants were discarded. The cell pellet was suspended in 1 ml of freshly prepared fixation/permeabilization working solution and incubated at 4°C for 30 min. This was followed by washing once with PBS then washing once again with 1 ml of 1X permeabilization buffer. Then the pellet

was suspended in 100 μ L permeabilization buffer. 5 μ L of PE anti-IL10 and 20 μ L of FITC anti-Foxp3 were added to the test tube. The samples were incubated at 4°C for 30 min. Washing was done for 2 times then cells were suspended in 500 μ L PBS with adjustment of cell count to 100,000/ml to be ready for acquiring data by the FCM. Tregs were identified by combined expression of CD4⁺, CD25^{high} and Foxp3, while the T effectors cells were identified by the combined expression of CD4⁺, CD25^{low}. Data acquisition and analysis were performed on EP-ICS XL flow cytometry using UAC40004 version with a standard four-color filter configuration. Isotype - matched controls were used to set up the gating. Figures (1, 2) represent dot plots of flow cytometry and the gating strategy in both stimulated PBMCs and unstimulated (base line) control PBMCs in culture respectively. Lymphocytes were identified according to their size and complexity on the forward and side scatter plot Fig. (1-A). CD4⁺T cells “gate B” were identified based on ECD anti-CD4⁺ expression Fig. (1-B). Different co-expression of CD4 and CD25 on lymphocytes was identified by using ECD anti CD4 and PE Cy5 anti CD25. PE-Cy5 isotype control was used to set gates separating CD4⁺/CD25⁺ cells from CD4⁺/CD25⁻ cells, Fig. (1-C); area (C1) for CD4⁺/CD25⁺ cells, area (C2) for CD4⁺/CD25⁺ cells, area (C3) for CD4⁺/CD25⁻ cells and area (C4) for CD4⁺/CD25⁻

cells. Two electronic gates were done to divide CD25⁺ cells into 2 parts; “E” gate(bright one) for CD25^{hi} and “F” gate (dim area)for CD25^{lo}, whereas CD25^{lo} were identified relative to the intensity of CD25 on CD4⁺ cells. Co-expression of Foxp3 and IL10 by using FITC anti Foxp3 and PE anti IL10 were identified on CD25^{hi} cells, respective isotype control antibodies were used, Fig. (1-D); area (D1) IL10⁺/Foxp3⁺, area (D2) IL10⁺/Foxp3⁺, area (D3) IL10⁻/Foxp3⁺, and area (D4) IL10⁻/Foxp3⁺. Percent of CD4⁺CD25^{high} Foxp3⁺ IL-10⁺(Treg cells)cells within gated lymphocytes was calculated by: Percent of co-expression on D2 X percent of cells on E gate / 100

Statistical Analysis

Statistical analysis was performed using Statistical Package for Social Sciences software computer program version “PASW 23.0, IBM Corp., USA, 2015”.Data were expressed as median percentiles for quantitative non-parametric measures and both number and percentage for categorized data. Wilcoxon signed rank test was used for comparison between two independent groups for non-parametric data. Ranked Spearman correlation test was used to study the possible association between each of the two variables among each group for non-parametric data. All p-values are two-sided. A *P* value <0.05 indicated statistical significance.

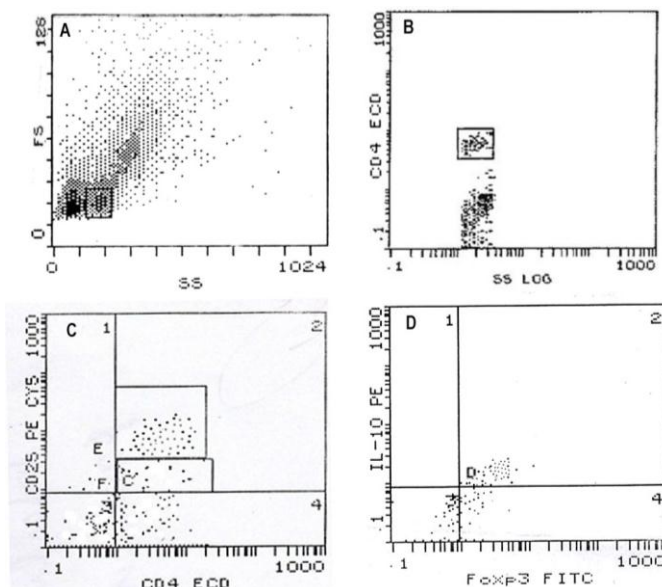


Figure 1. Representative dot plots of flow cytometry and the gating strategy used (A to D) in patients with CMA after PBMCs stimulation with cow's milk allergen extract in the presence of vitamin D3: (A) Show forward and side scatter to gate lymphocytes. (B) CD4⁺ cells were acquired after gating the lymphocyte population by forward- and side-scattered properties. (C) Show co- expression of CD4 and CD25 on lymphocytes; “E” for CD25^{hi} and “F” for CD25^{lo} cells. (D)Show co-expression of Foxp3 and IL10 on CD4⁺/CD25^{hi} lymphocytes.

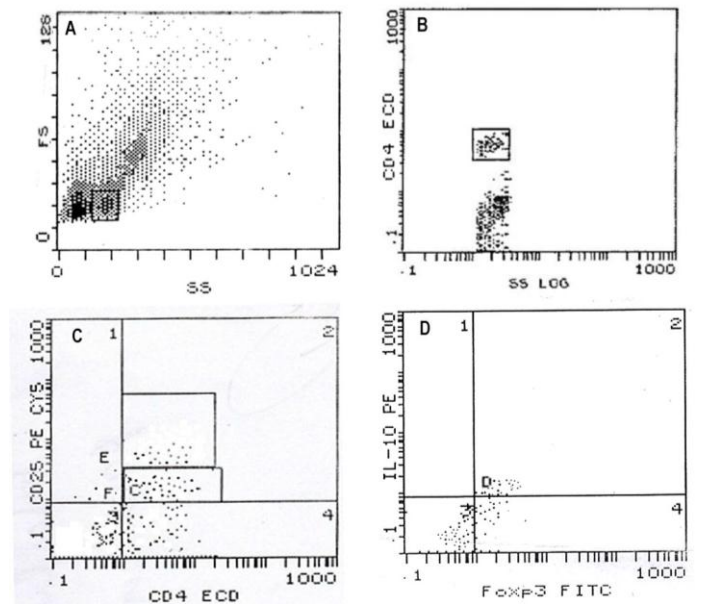


Figure 2. Representative dot plots of flow cytometry and the gating strategy used of control unstimulated cells (baseline).

Results

Laboratory results of the study groups showed that the percentage of CD4⁺CD25^{high}Foxp3⁺IL10⁺ Treg cells and percentage of Foxp3⁺ and IL10⁺ co-expression on Treg cells were significantly

lower in children with CMA as compared to controls ($P<0.05$). Also 25 (OH) VD levels were significantly lower in patients than controls ($P<0.001$), (table 1).

Table 1. Demographic characteristics and laboratory results of the study groups.

Variable	Cases (N=20)	Controls (N=20)	P value
Age:Median(IQR)	2 years(6 months-5years)	2 years(1-4years)	NS
Gender: n (%)	11(55%) males 9(45%) females	7(35%) males 13(65%)females	NS
Serum 25(OH)D level (ng/ml): Median(IQR)	16.5 (8-37)	32.5(17-40)	<0.001
% of CD4 ⁺ CD25 ^{high} Foxp3 IL-10 ⁺ (Treg) cells: Median(IQR)	2.04 (2.0-3.4)	3.1(3.01-4.8)	<0.001
% of CD4 ⁺ CD25 ^{low} (Teff):Median(IQR)	3.68 (3.06-4.9)	2.3(2.02-2.1)	<0.001
Treg/Teff ratio: Median(IQR)	0.58 (0.42-0.75)	0.91(0.71-1.1)	<0.001
% of Foxp3 ⁺ and IL10 ⁺ on Treg: Median (IQR).	39 (21-73)	48.5(28-73)	0.03

*Data compared together using Wilcoxon Signed Rank Test, IQR; interquartile range, 25(OH)D =25-hydroxy vitamin D, Teff; T effectors cells, $P>0.05$ is not significant (NS).

Clinical, demographic characteristics and laboratory results of patients according to the different types of CMA are summarized in (table 2).

Patients were divided according to the serum 25 (OH) D level into the following groups: 1-Sufficient serum 25 (OH) D level group included 4 patients (20%), their 25 (OH) D level ranged from 31 to 37 ng/ml, median 32 ng/ml. 2-Deficient serum 25 (OH) D level group included 16 patients (80%), their 25 (OH) D level ranged from 8 to 19 ng/ml, median 15 ng/ml.

There was no significant difference in serum 25 (OH) D levels between patients with IgE and non IgE mediated allergy ($P=0.43$). NB: As patients with atopic dermatitis (mixed IgE and non-IgE) were only 3 cases, they were excluded from statistical tests. A significant positive correlation was observed between serum vitamin D levels and percentage of $CD4^+CD25^{high}Foxp3^+IL10^+$ Treg cells ($r=0.51$, $P=0.02$) as well as percentage of $Foxp3^+$ and $IL10^+$ co-expression on Treg cells ($r=0.54$, $P=0.01$) in CMA patients.

Table 2. Clinical and demographic characteristics and laboratory results of patients according to different types of CMA non- IgE and mixed.

Variable	IgE mediated CMA N(%)=11(55%)	Non-IgE CMA N(%)=6(30%)	Mixed (IgE-non-IgE) mediated CMA N(%)=3(15%)
Age Median(IQR)	2 years(8.5 months-5years)	7 months(6 months-4years)	3 years(2-5years)
Gender	7 males 4 females	2 males 4 females	2 males 1 female
Diagnosis: n(%)	-Bronchial asthma: 6 patients (30%). -Urticaria: 4 patients (20%). -Vomiting and diarrhea: one patient (5%).	-Abdominal distension and constipation: 4 patients (20%). -Bloody diarrhea: one patient (5%). - Watery diarrhea and failure to thrive: one patient (5%).	Atopic dermatitis: 3 patients (15%)
serum 25(OH)D (ng/ml): Median(IQR)	19 (10-37)	24(10.5-33)	17.5 (16.5 -17.5)
% of $CD4^+CD25^{high}Foxp3^+IL10^+$ (Treg) cells: Median(IQR)	2.06(2.0-3.43)	2.04(2.01-2.18)	2.02(2.01-2.04)
% of $CD4^+CD25^{low}$ (T eff)	3.7(3.06-3.56)	3.6(3.13-4.9)	3.7(3.4-3.8)
Treg/Teff ratio: Median(IQR)	0.62(0.47-0.75)	0.55(0.42-0.69)	0.54(0.53-0.59)
% of $Foxp3^+$ and $IL10^+$ on Treg cells: Median(IQR)	40(21-73)	38(21-65)	42(41-44)

*Data compared together using Wilcoxon Signed Rank Test, IQR; interquartile range, 25(OH)D =25-hydroxy vitamin D. Teff; T effectors cells

Compared to baseline values, the percentage of $CD4^+CD25^{high}Foxp3^+IL10^+$ Treg cells and that of $Foxp3^+$ and $IL10^+$ co-expression on Treg cells were significantly increased after PBMCs stimulation with cow's milk allergen extract in the presence of vitamin D in cell culture of patients with IgE, non-IgE mediated CMA, Vitamin D-deficient patients and healthy controls (table 3 and table 4). The percentage of $CD4^+CD25^{low}T$

effector cells did not differ significantly among the different sets of culture (table 5). A significant positive correlation was observed between serum 25 (OH) D level and percentage of $CD4^+CD25^{high}Foxp3^+IL10^+$ Treg cells ($r=0.45$, $P=0.04$) as well as that of $Foxp3^+$ and $IL10^+$ co-expression on Treg cells ($r=0.53$, $P=0.02$), after PBMCs stimulation with cow's milk allergen extract in the presence of vitamin D.

Table 3. Comparison between different sets of culture as regards % of $CD4^+CD25^{high}Foxp3^+IL10^+$ Treg cells

	Base line/ after culture with allergen only		Base line/ After culture with allergen and VD		In culture with allergen only/ After culture with allergen and VD	
	Median	(IQR)	Median	(IQR)	Median	(IQR)
All patients	2.04 / 2.06	2.0-3.4/ 2.0-3.6	2.04 / 2.6	2.0-3.4/ 2.11-5.3	2.06 / 2.6	2.0-3.6/ 2.11-5.3
<i>P</i> value	NS		0.001		0.002	
Healthy controls	3.1/ 3.4	3.01-4.8/ 3.04-4.7	3.1/ 3.75	3.01-4.8/ 3.09-4.9	3.4/ 3.75	3.04-4.7/ 3.09-4.9
<i>P</i> value	NS		0.002		0.04	
Patients with IgE CMA	2.06/ 2.05	2.0-3.4/ 2.0-3.6	2.06/ 2.5	2.0-3.4/ 2.11-5.3	2.05/ 2.5	2.0-3.6/ 3.11-5.3
<i>P</i> value	NS		0.009		0.005	
Patient with non IgE CMA	2.04/ 2.1	2.01-2.18/ 2.0-2.4	2.04/ 3.2	2.01-2.18/ 2.3-3.9	2.1/ 3.2	2.0-2.4/ 2.3-3.9
<i>P</i> value	NS		0.02		0.02	
VD-sufficient patients	2.07/ 2.07	2.01-3.4/ 2.0-3.6	2.07/ 3.1	2.01-3.4/ 2.25-5.3	2.07/ 3.1	2.0-3.6/ 2.25-5.3
<i>P</i> value	NS		0.06		0.06	
VD-deficient patients	2.04/ 2.06	2.0-2.35/ 2.0-2.4	2.04/ 2.6	2.0-2.35/ 2.11-3.9	2.06/ 2.6	2.0-2.4/ 2.11-3.9
<i>P</i> value	NS		0.001		0.001	

*Data compared together using Wilcoxon Signed Rank Test, VD=vitamin D, $P>0.05$ is not significant (NS).

Table 4. Comparison between different sets of culture as regards percentage of Foxp3⁺ and IL10⁺ co-expression on Treg cells

	Base line/After culture with allergen only		Base line/ After culture with allergen A and VD		After culture with allergen only/ After culture with allergen and VD	
	Median	(IQR)	Median	(IQR)	Median	(IQR)
All patients	39.5 /	21-73/	39 /	21-73/	42/	21-100/
	42	21-100	80.5	53-100	80.5	53-100
<i>P</i> value	NS		<0.001		<0.001	
Healthy controls	48.5/	28-73/	48.5/	28-73/	50.5/	30-73/
	50.5	30-73	58.5	39-77	58.5	39-77
<i>P</i> value	NS		0.01		0.03	
Patients with IgE CMA	40/	21-73/	40/	21-73/	40/	21-100/
	40	21-100	83	53-100	83	53-100
<i>P</i> value	NS		<0.001		<0.001	
Patient with non IgE CMA	38/	21-65/	38/	21-65/	41.5/	21-68/
	41.5	21-68	76	66-100	76	66-100
<i>P</i> value	NS		<0.001		<0.001	
VD-sufficient patients	40/	21-73/	40/	21-73/	42/	21-100/
	42	21-100	69	56-100	69	56-100
<i>P</i> value	NS		0.08		0.3	
VD-deficient patients	38/	21-65/	38/	21-65/	46.5/	21-68/
	46.5	21-68	82.5	53-100	82.5	53-100
<i>P</i> value	NS		<0.001		<0.001	

*Data compared together using Wilcoxon Signed Rank Test, IQR; interquartile range, VD=vitamin D *P*>0.05 is not significant (NS).

Table 5. Comparison between different sets of culture as regards percentage of CD4⁺CD25^{low}Teff cells

	Base line/ After culture with allergen only		Base line/After culture with allergen and VD		After culture with allergen only/ After culture with allergen and VD	
	Median	(IQR)	Median	(IQR)	Median	(IQR)
All patients	3.7 / 3.5	3.06-4.9/ 3.03-5.8	3.7 / 3.6	3.06-4.9/ 3.17-6.6	3.5/ 3.6	3.03-5.8/ 3.17-6.6
<i>P</i> value	NS		NS		NS	
Healthy controls	2.3/ 2.7	2.02-3.1/ 2.04-3.1	2.3/ 2.6	2.02-3.1/ 2.07-3.3	2.7/ 2.6	2.04-3.1/ 2.07-3.3
<i>P</i> value	NS		NS		NS	
Patients with IgE CMA	3.7/ 3.4	3.06-4.5/ 3.03-5.8	3.7/ 3.6	3.06-4.5/ 3.17-6.6	3.4/ 3.6	3.03-5.8/ 3.17-6.6
<i>P</i> value	NS		NS		NS	
Patient with non IgE CMA	3.6/ 3.4	3.13-4.9/ 3.3-4.4	3.6/ 3.6	3.13-4.9/ 3.2-3.9	3.4/ 3.6	3.3-4.4/ 3.2-3.93
<i>P</i> value	NS		NS		NS	
VD-sufficient patients	3.7/ 3.7	3.33-4.5/ 3.35-4.1	3.7/ 3.7	3.3-4.5/ 3.4-5.1	3.7/ 3.7	3.3-4.1/ 3.4-5.1
<i>P</i> value	NS		NS		NS	
VD-deficient patients	3.6/ 3.4	3.06-4.9/ 3.03-5.8	3.6/ 3.6	3.06-4.9/ 3.17-6.6	3.4/ 3.6	3.03-5.8/ 3.17-6.6
<i>P</i> value	NS		NS		NS	

*Data compared together using Wilcoxon Signed Rank Test, IQR; interquartile range, VD=vitamin D
P>0.05 is not significant (NS).

Discussion

Regulatory T cells (Treg cells) are requisite for the maintenance of tolerance to self- and environmental proteins [30]. A dysfunction of Treg cellular activity seems to be essential for the increase of both IgE- and non-IgE mediated CMA, as the generation of mucosal tolerance in children is associated with an increase of Treg cells[31]. Milk-tolerant children have a higher frequency of milk allergen-driven

Treg cells than those who have allergic symptoms to all forms of milk protein [32].

In the present study, we observed a significant reduction of the percentage of CD4⁺CD25^{high}Foxp3⁺IL10⁺ Treg cells as well as the percentage of Foxp3⁺ and IL10⁺ co-expression on Treg cells in the CMA group when compared to the control group. In a previous study conducted by Perezabad *et al.* (2017) [33] on infants with newly diagnosed CMA, they demonstrated a

significant reduction in the percentage of Treg cells in infants with CMA when compared to controls. Association between lower percentage of Treg cells and development of allergic diseases was documented by Karlsson *et al.* (2004) [12] who reported that children who had clinically active CMA had lower number of Treg cells than those who were cured from their CMA. Furthermore, Smith *et al.* (2008) [34] looked at Treg number in cord blood from infants who later developed egg allergy by 12 months of age, he reported that Infants who developed allergies had lower cord blood Treg number than infants who were tolerant to egg, suggesting that a defect in Tregs may predispose to food allergy. This is consistent with those reported in two studies done on Egyptian asthmatic children, the first study conducted by Ismail *et al.* (2015) [18] in which they determined a significant decrease in CD4⁺CD25^{hi} Treg cells in asthmatic group compared to a control group. The second study was done by Bakr *et al.* (2013) [35] revealed a significant decrease in the percentage of Foxp3⁺Treg cells, decreased Treg/ T effector ratio and decreased Foxp3 expression on Treg cells in peripheral blood in asthmatic patients when compared to a healthy control group.

An additional aspect of our study was to evaluate the role of Vitamin D in children with CMA and to assess the effect of Vitamin D on induction of CD4⁺CD25^{high}Foxp3⁺IL10⁺ Treg cells in PBMCs isolated from those patients upon stimulation with cow's milk allergen in culture, since Treg cells differentiation and activity can be influenced by vitamin D. It is worth noting that several authors have suggested that adequate concentrations of 25(OH) D in childhood are usually protective of allergic diseases [36-38],

although not all studies agree to that conclusion [39, 40].

In the present study, we found a significant lower serum level of 25(OH) vitamin D in the children with CMA than in the control group. Additionally, Vitamin D deficiency was observed in 83% of patients with non-IgE mediated CMA and in 72% of patients with IgE-mediated CMA. No significant difference was observed between patients with IgE-associated CMA and those with non-IgE associated CMA regarding serum Vitamin D levels. Consistent with our findings, Jones *et al.* (2012) [41] found an association between lower Vitamin D level at birth and increased risk of eczema in infants at 12 months of age. Also, they demonstrated a non significant difference between IgE- and non-IgE-associated eczema regarding Vitamin D level.

Allergen immunotherapy might work via the generation of IL-10-secreting Treg cells. Nevertheless, such therapy can be associated with a great risk of developing side-effects and needs to be administered for a period of several years for maximal efficacy. There remains, for that reason, an obvious need for novel treatments that change the underlying immune response to an allergen [42]. Accumulating data from experimental studies underlines the value of Vitamin D for Treg cell differentiation, but data from clinical studies in humans are sparse[43,44]. Vitamin D has been shown to induce IL-10-secreting Treg cell generation [45]. As a result, secreted IL-10 inhibits improper allergen specific Th2-driven immune responses and regulates allergic sensitization [46, 47].

In the present study, a positive correlation was observed between serum vitamin D levels and % T regs as well as % of Foxp3⁺ and IL10⁺ co-expression on Treg cells. This finding was in partial agreement with several

studies which demonstrated a strong correlation between the scarcity of Treg cells and reduced serum Vitamin D levels in different forms of allergy [33,48,49].

In the current study, the percentage of $CD4^+CD25^{high}Foxp3^+IL10^+$ T reg cells and percentage of $Foxp3^+$ and $IL10^+$ co-expression on Treg cells were significantly increased in culture after PBMCs stimulation with cow's milk allergen extract in the presence of vitamin D in patients with IgE and non-IgE mediated CMA as well as among Vitamin D-deficient patients and healthy controls. This finding was not observed among patients with Vitamin D sufficiency, this could be attributed to the small number of Vitamin D-sufficient CMA patients. The importance of vitamin D for Treg cell differentiation was reinforced by a positive correlation between serum vitamin D levels and percentage of $CD4^+CD25^{high}Foxp3^+IL10^+$ Treg cells as well as percentage of $Foxp3^+$ and $IL10^+$ co-expression on Treg cells after PBMCs stimulation with cow's milk allergen extract in the presence of vitamin D. In this context, several studies have shown that activation of human $CD3^+CD4^+$ T cells *in vitro* in the presence of vitamin D3, induced a sizable population of $CD4^+IL10^+$ Treg cells [46,50].

In contrast to our results, Vieira *et al.* (2004) [51] observed that homogeneous populations of mouse IL-10-secreting Treg cells do not express $Foxp3$ but have comparable regulatory function to naturally occurring $CD4^+CD25^+$ Treg cells. They designed a strategy of antigenic stimulation of naive $CD4^+$ T cells using immunosuppressive drugs that reproducibly gave rise to a homogeneous population of IL-10- secreting Treg cells. Source of $CD4^+$ T cells and possible contamination with effectors, different antigenic stimulus and number used may explain such discrepancies. On the

other hand, our results agreed with (Uhlig *et al.*, (2006) [52]; Maynard *et al.*, (2007) [53]; Rubtsov *et al.*, (2008) [54] who demonstrated that $Foxp3^+$ Tregs are able to secrete IL-10 and this appears to be mainly essential in regulating immune responses at the body's environmental interfaces. Additionally, Kornete *et al.*, (2012) [55] observed that the secretion of IL-10 by $Foxp3^+$ Treg cells is also important in regulating immune responses against self-antigens in some animal models. They found that in the non-obese diabetic (NOD) mouse model of type1 diabetes, disease progression is associated with gradual loss of pancreatic IL-10-secreting $Foxp3^+$ Tregs [55]. However interpretation of our results in terms of a requirement for extrathymic development of adaptive $Foxp3^+$ Treg cells is complex and will require further study.

In conclusion, we demonstrated that almost 80% of patients with CMA whether IgE-or non-IgE mediated had Vitamin D deficiency with decreased number of antigen-driven $CD4^+CD25^{high}Foxp3^+IL10^+$ Treg cells in comparison to age and sex-matched healthy children. The percentage of $CD4^+CD25^{high}Foxp3^+IL10^+$ Treg cells and that of $Foxp3^+$ and $IL10^+$ co-expression on Treg cells was significantly increased *in vitro* after PBMCs stimulation with cow's milk allergen extract in the presence of vitamin D, in cell cultures of patients with IgE, non-IgE mediated CMA, Vitamin D-deficient patients and healthy controls. Future research should focus on the effect of Vitamin D supplementation on Treg cells function and number in allergic patients with Vitamin D deficiency and evaluation of the clinical outcome of the allergic diseases. Future challenges include optimization of the conditions for the induction of allergen-driven IL-10- secreting regulatory T cells with minimal adverse reactions.

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