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Vitamin D reduces the differentiation and expansion of Th17 cells in young asthmatic children

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ABSTRACT

Vitamin D [25(OH)D3] deficiency has been associated with asthma as in many inflammatory and autoimmune pathologies; however, there is still a lack of data about the effects of administration of vitamin D in immune regulation in young asthmatic patients. In this study, we investigated its inhibitory effect on the immune response in young asthmatic patients and the possible mechanisms involved.

Peripheral blood CD4⁺ T cells from 10 asthmatic patients and 10 healthy controls were cultured under Th17 polarizing conditions in the presence or absence of [25(OH)D3]. IL-17 cytokine production was determined by ELISA and flow cytometry. Messenger RNA (mRNA) expression of several factors related to Th17 cell function was determined by real-time PCR. The effect of [25(OH)D3]-treated dendritic cells (DCs) on CD4⁺ T cell response was determined by ELISA and flow cytometry.

Stimulation of naive CD4⁺ T cells under Th17 polarizing conditions showed a higher Th17 cell differentiation in asthmatic patients than healthy controls. The addition of [25(OH)D3] significantly inhibited Th17 cell differentiation both in patients [$P < 0.001$] and in normal controls [$P = 0.001$] in a dose-dependent way. [25(OH)D3] was able to inhibit the gene expression of RORC, IL-17, IL-23R, and CCR6. [25(OH)D3]-treated DCs significantly inhibited IL-17 production [$P = 0.002$] and decreased the percentage of CD4⁺IL-17⁺ [$P = 0.007$] in young asthmatics.

The findings suggest that the inhibitory effect of [25(OH)D3] on the Th17 response was mediated via both T cells and DCs. DCs pathway is involved in the direct inhibition of 25(OH)D3 on Th17 cell differentiation in young asthmatics.

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Introduction

Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation (GINA 2014, <http://www.ginasthma.org/>). Asthma phenotypes can be classified as mild, moderate, or severe according to the magnitude of the inflammation (Hamid and Tulic, 2009). Asthma was considered mainly as a T helper 2 (Th2) mediated disease, characterized by production of interleukin (IL)-4, IL-5, and IL-13 together with

eosinophilic infiltration of the bronchial mucosa. However, a CD4⁺ Th 17 mediated response has also been observed in patients that exhibit chronic inflammation (Pène et al. 2008; Al-Ramli et al. 2009; McKinley et al. 2008). The pro-inflammatory role of Th17 cells and Th17-associated cytokines, IL-17A and IL-17F, is widely recognized (Cosmi et al. 2011). The cytokine milieu has a decisive effect on the balance between developing immunosuppressive regulatory T cells or proinflammatory Th17 cells. Dysregulation of the cytokine balance can therefore contribute to autoimmunity and chronic inflammation (Hamzaoui et al. 2011; Silverpil and Linden 2012). In children with asthma, Th17 cells were increased in peripheral blood and induced sputum (Maalmi et al. 2014; Hamzaoui et al. 2011). Increased levels of IL-17 A/F were observed in asthmatic airways, and first candidate gene studies suggested single nucleotide polymorphisms (SNPs) in IL17A and IL17F to be associated with asthma (Hamzaoui et al. 2011; Chen et al. 2010). Th17 and regulatory T (Treg) cells are reciprocally deregulated in

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Table 1
Characteristics of patients and controls.

Characteristics	Mild asthma	Healthy controls
Number of subjects	10	10
Gender (female/male)	3/7	2/8
Age (range), years	8 (5–13)	6.5 (4–12)
Positive skin test results (%)	65.48%	Negative
FVC (%)	85.40 (57–117)	
FEV1 (%)	94.52 (53–127)	
FEV1/FVC ratio	0.9 (0.80–1)	

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity.

asthmatics. The finding of overexpression of RORC (Th17) and low expression of FOXP3 (Treg) in asthma suggests that these proteins have a significant role in this process (Hamzaoui et al. 2011).

A number of studies have demonstrated that Vitamin D (Vit D) could modulate the activity of dendritic cells (DCs). It was found that Vit D inhibited the differentiation and maturation of DCs reducing the expression of major histocompatibility complex (MHC) class II molecules and preventing the secretion of proinflammatory cytokines (Arnsen et al. 2007; Fujio et al. 2010).

In this study, we found that 25(OH)D3 inhibited the differentiation of Th17 from naive CD4⁺ T cells both in young asthmatic patients and in healthy controls. We also found that 25(OH)D3 inhibited the expression of Th17 cell effector molecules such as RORC, IL-17, IL-23R, and CCR6. Finally, we showed that DCs were involved in the 25(OH)D3-mediated suppression of the Th17 cell response.

Materials and methods

Patients

Ten children with well-defined asthma were recruited from the Department of Paediatric Respiratory Diseases, Abderrahman Mami Hospital (Ariana, Tunisia), using the criteria set by the Global Initiative for Asthma guidelines (Van Weel et al. 2008). Detailed definitions of the inclusion and exclusion criteria for the enrolment of asthmatic subjects were reported previously (Hamzaoui et al. 2010a,b, 2011, 2013). The protocols for the study were reviewed and approved by the ethics committee of our Hospital, and informed consent was obtained from all participating subjects. Children with mild asthma were treated with regular inhaled glucocorticoids (ICS), but variable daily doses were required to control the symptoms (at the time of evaluation daily ICS dose ranged 200–800 µg/day). Table 1 describes the characteristics of the asthmatic patients in the study. Ten healthy control children (female/male: 2/8) were recruited (aged 4–12 years, means 6.5) with no respiratory or allergic manifestations.

Serum vitamin D measurement

Serum concentrations of 25(OH)D3 were assayed with a radioimmunoassay kit (Dia-Sorin, Stillwater, MN) as we previously reported (Maalmi et al. 2012). Vitamin D level values were used as a continuous variable and were categorized in descriptive analyses as desirable (or sufficient) when scores were at least 30–40 ng/mL (75–100 nmol/L), insufficient between 20 and 30 ng/mL (50 and 75 nmol/L) and deficient when <20 ng/mL, as previously recommended (Chinellato et al. 2011).

Cell culture

Peripheral blood mononuclear cells (PBMC) were obtained from 10 mL of peripheral venous blood by Ficoll-Hypaque density

centrifugation, then isolated and suspended in 25 mL medium RPMI 1640 (Sigma–Aldrich, USA) containing 2 mM glutamin (Gybcu, USA), 10 mM HEPES (Gybcu, USA), Penicilin-streptomycin 10,000 U/mL (Gybcu, USA), and 10% Foetal Bovine Serum (Gybcu, USA), and incubated for 48 h. Naive CD4⁺ T cells were separated from PBMCs by magnetic microbead negative selection according to the manufacturer's instructions (the purity of CD4⁺CD45RA⁺ T cells was 93%; Miltenyi Biotec, Palo Alto, CA). To study the effect of Vit D on Th17 polarization, naive CD4⁺ T cells were stimulated at 1×10^6 /mL with anti-CD3 and anti-CD28 (2 µg/mL) (eBioscience, San Diego, CA) in Th17 polarizing conditions (IL-6: 50 ng/mL; IL-23: 10 ng/mL; IL-1β: 10 ng/mL; anti IL-4: 10 µg/mL; and anti-IFN-γ: 10 µg/mL) in the presence or absence of 25(OH)D3 (Sigma–Aldrich, St. Louis, MO) at different concentrations. 25(OH)D3 was dissolved in ethanol at an initial 1 nM concentration and serially diluted to the working concentrations with RPMI 1640 medium. Naive CD4⁺ cells were incubated with different concentrations of [25(OH)D3] doses: (control/0 nM), (1 nM), (10 nM) and (50 nM). All antibodies (Abs) and recombinant cytokines used in the Th17 cell polarization experiments were purchased from R&D Systems (Minneapolis, MN). On day 7, the supernatants were obtained and IL-17 concentration measured by ELISA (R&D Systems). The cells were harvested for intracellular detection of cytokines by flow cytometry and gene expression assays.

CD14⁺ monocytes and CD4⁺ T cells were separated from PBMCs using human microbeads (both purity >90%; Miltenyi Biotec). Dendritic cells (Cs) were generated from monocytes plated at 1×10^6 cells/mL in 96-well plates, in RPMI 1640 complete medium supplemented with 10% foetal bovine serum (FBS), L-glutamine (2 mM), and penicillin G/streptomycin (100 U/mL; Invitrogen, Carlsbad, CA) in the presence of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (100 ng/mL; R&D Systems) and IL-4 (50 ng/mL; R&D Systems). Vit D was added at the beginning of the culture. On day 3, half of the culture medium including recombinant cytokines and 25(OH)D3 was refreshed. On day 6, the cells were stimulated with lipopolysaccharide (LPS) for 24 h, and matured DCs were washed three times before co-culturing with CD4⁺ T cells. Co-cultures were performed with a DC:T cell ratio of 1:5 in 96-well plates for 4 days. The culture supernatants were assayed for IL-17 levels by ELISA. The cells were harvested for intracellular detection of IL-17 using flow cytometry. The Supernatants from naive CD4⁺ T cells and co-cultures of dendritic/T (DC/T) cells were collected and stored at –80 °C for IL-17 cytokine measurements. IL-17 secretion was measured by enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's instructions using reagent kits of human IL-17 (purchased from R&D Systems, Minneapolis, MN, USA).

Real-time SYBR-green PCR on asthmatic and healthy controls samples

Total RNA was extracted from stimulated naive CD4⁺ T cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The first-strand cDNA was synthesized for each RNA sample using the Superscript III Reverse Transcriptase system (Invitrogen). Real-time quantitative PCR was performed on AB 7500 Fast System (Applied Biosystems, Foster City, CA). The following primers were used for real-time PCR: **IL-23R**: forward 5'-AACAGCAATGTTGTGAAAATGC3', reverse 5'-AGGCTTGTTCTCTGGGATGA-3'; **IL-17**: forward 5'-ACCAATCCC AAAAGGTCCTC-3', reverse 5'-TGGATGGGGACAGAGTTCAT-3'; **RORC2**: forward 5'-CAGTC ATG AGAACACAAATGAAGTG-3', reverse 5'-CAGGTGATAACCCGTAGTGGAT-3'; **CCR6** forward 5'-GGCTGCAAATTT GGGTAAA-3', reverse 5'-CACAGGAGAAG CCTGAGGAC-3'; **β-actin** forward 5'-GGATGCAGAAGGAG ATCACTG-3', reverse 5'-CGATCCACA CGGAGTACTTG-3'. The

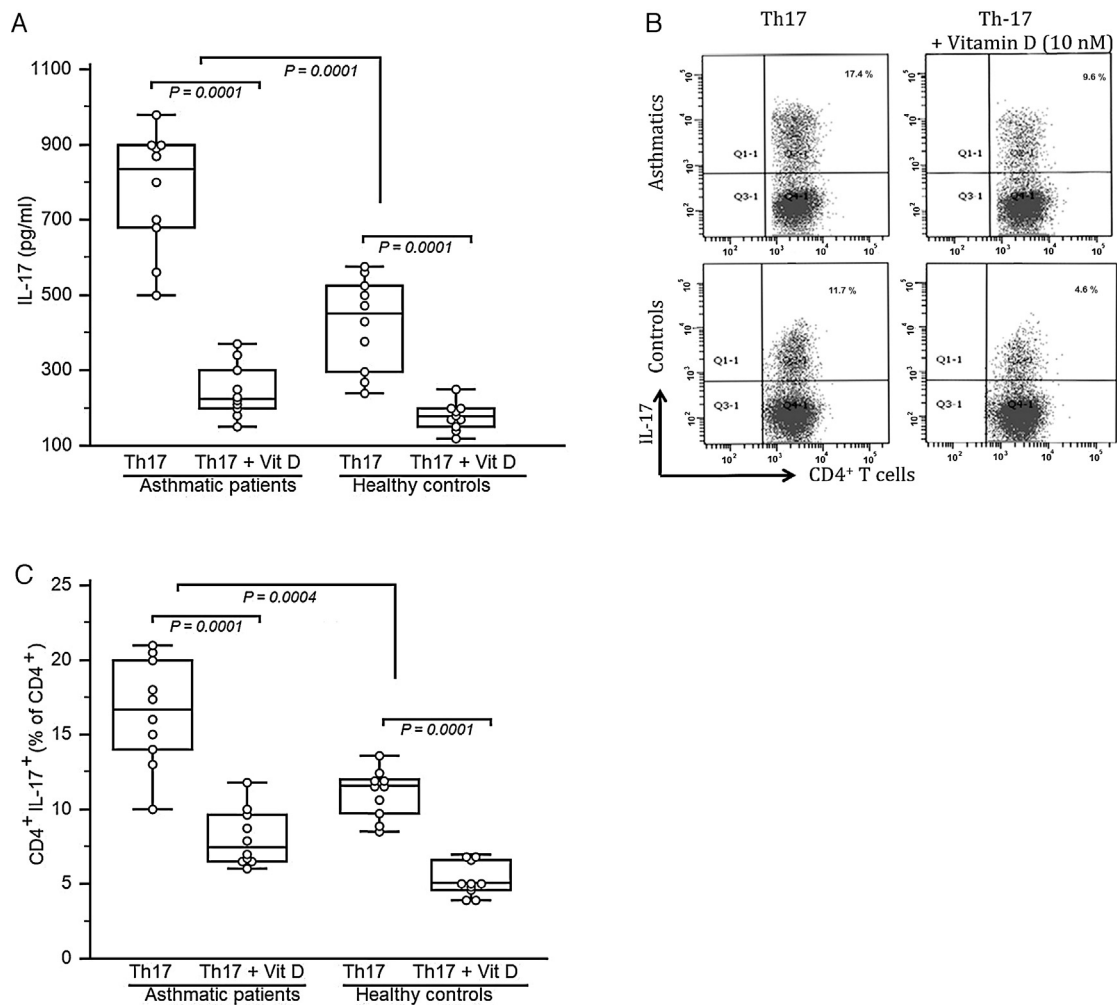


Fig. 1. Vitamin D inhibits Th17 cell differentiation in BD patients and normal controls. Naive CD4⁺ T cells from asthmatics ($n = 10$) and healthy controls ($n = 10$) were cultured under Th17 polarizing with Vitamin D at different concentrations (1 nM; 10 nM and 50 nM) for 6 days. (A) ELISA of IL-17 production in the supernatants. (B) Flow cytometric analysis of IL-17 on the stimulated naive CD4⁺ T cells. Representative dot plots in asthmatics and healthy controls before and after addition of Vitamin D. (C) Quantitative analysis of the frequency of IL-17-producing CD4⁺ T cells after PMA/ionomycin stimulation. The medians are indicated by a line inside each box. The 25th and 75th percentiles are indicated by the box limits, the lower and upper error bars represent the 10th and 90th percentiles, respectively.

expression of each gene was normalized to the expression of β -actin using the $2^{-\Delta\Delta CT}$ method as described previously (Livak and Schmittgen, 2001).

Flow cytometric analysis

For analysis of intracellular cytokine levels in naive CD4⁺ T cells and CD4⁺ T cells co-cultured with 25(OH)D₃-treated DCs or control DCs by flow cytometry, we used the following fluorochrome-conjugated Abs: anti-human CD3-(PerCP)-Cy5.5, anti-human CD8-APC, anti-human IL-17A-PE, and mouse anti-human IgG isotype control Ab. All fluorescent Abs were obtained from BD Biosciences (Sunnyvale, CA). The cells were stimulated by addition of 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and ionomycin (1 μ g/mL; Sigma) for 1 h at 37 °C. Then, brefeldin A (10 μ g/mL; Sigma) was added for another 4 h. The cells were fixed and permeabilized using the eBioscience Cytofix/Cytoperm kit according to the manufacturer's instructions and then incubated with the above fluorescent Abs. The cells were washed and analyzed on a FACScan flow cytometer (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

Statistical analysis

Student's *t*-test and ANOVA were applied using SPSS 10.0 software (SPSS Inc., Chicago, IL). Data are shown as mean $\pm$ SD. *P* values less than 0.05 were considered statistically significant.

Results

Serum vitamin D levels

Ten patients with asthma and ten healthy controls are examined in this work. Data of asthmatic patients and control subjects are depicted in Table 1. The severity of asthma in this group of patients was presented according to GINA guidelines. The severity of asthma was depicted as mild in our patients group. No difference was observed in the body weight between asthmatic children [30.7 kg; range: 24–37] and the healthy control group [31.5 kg; range: 22–40]. Deficient values of vitamin D were observed in all 10 patients [16.19 ± 2.20 ng/mL; range: 12–19 ng/mL]. Healthy controls ($n = 10$) were sufficient in vitamin D [37.38 ± 2.15 ng/mL; range: 30–37 ng/mL; $P = 0.0001$].

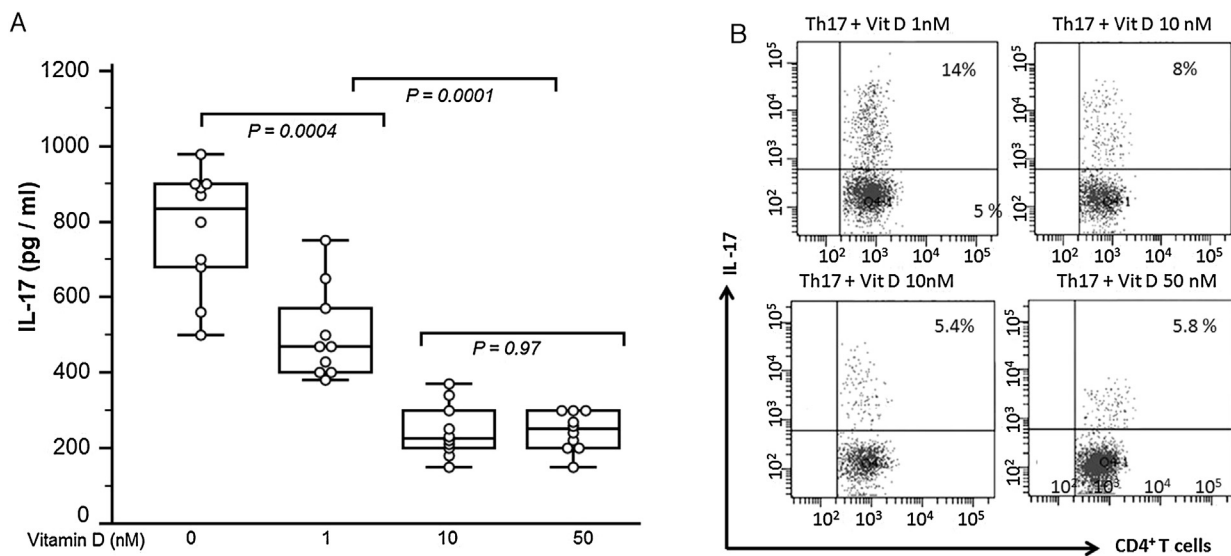


Fig. 2. Vitamin D inhibits Th17 cell differentiation in a dose-dependent manner. Naïve CD4⁺ T cells from asthmatic patients ($n = 10$) were cultured under Th17 polarizing conditions with different concentrations of Vitamin D (0, 1, 10, 50 nM) for 6 days. (A) ELISA of the IL-17 production in the supernatants. Flow cytometric analysis of IL-17 on the stimulated naive CD4⁺ T cells. Box plot indicating IL-17 level quantified by ELISA in the culture supernatants. The medians are indicated by a line inside each box. The 25th and 75th percentiles are indicated by the box limits, the lower and upper error bars represent the 10th and 90th percentiles, respectively. (B) Dot plots of a representative patient.

Vitamin D inhibits IL-17 and Th17 cell differentiation

IL-17 production in the supernatants of stimulated naive CD4⁺ T cells was significantly higher in asthmatic patients than in healthy controls [777.9 ± 160.8 pg/mL v.s. 424.7 ± 122.6 pg/mL; $P < 0.0001$]. Addition of 25(OH)D3 (10 nM) significantly inhibited the production of IL-17 in both asthmatics and healthy controls [245.0 ± 70.7 pg/mL; 177.5 ± 36.4 pg/mL; $P = 0.0152$] (Fig. 1A). Consistent with ELISA data, intracellular cytokine analysis revealed that a significantly increased percentage of IL-17-producing CD4⁺ T cells was observed in asthmatic patients as compared with healthy controls (Fig. 1B and C). Our results also showed that the inhibitory effect of 25(OH)D3 on IL-17 extracellular and intracellular IL-17 production was higher at a concentration of 10 nM than 1 nM (Fig. 2A and B). No further reduction in IL-17 production was observed when 25(OH)D3 concentration was increased to 50 nM (Fig. 2A and B).

Vitamin D regulates associated molecules of Th17 cells

Vitamin D significantly inhibited the gene expression of the molecules related to differentiation of Th17 cells *RORC* (Fig. 3A) and *IL-17* (Fig. 3B). Similarly, it was also able to inhibit the gene expression of molecules related to the function of Th17 cells such as *IL-23R* (Fig. 3C) and *CCR6* (Fig. 3D).

Effect of 25(OH)D3 treatment on dendritic cells maturation

The expression of IL-17 was significantly decreased in the supernatants of naive CD4⁺ T cells co-cultured with 25(OH)D3-treated DCs [291.0 ± 83.5 pg/mL] as compared with those co-cultured with untreated DCs [452.0 ± 116.0 pg/mL; $P = 0.0022$] (Fig. 4A). Flow cytometry (Fig. 4B and C) showed that the percentage of IL-17-staining CD4⁺ T cells was also decreased in the naive CD4⁺ T cells co-cultured with 25(OH)D3-treated DCs [$4.35 \pm 1.12\%$] as compared with those cultured with control DCs [$5.91 \pm 1.17\%$; $P = 0.0072$] (Fig. 4B).

Discussion

This study showed that Vit D decreases the expression of Th17 mediated cytokines through DC modulation in young asthmatic patients. Several studies have reported associations of low vitamin D status with poor pulmonary function and increased incidence of respiratory infections, asthma, and chronic obstructive pulmonary disease (Brehm et al., 2010). Low levels of vitamin D have been associated with asthma severity and loss of control, together with recurrent exacerbations (Berraies et al. 2014). Children with moderate asthma expressed lower vitamin D levels than mild asthmatics did (Maalmi et al. 2012). Consequently, clinical trials are ongoing to assess the effect of Vitamin D supplementation on asthma control.

25(OH)D3 inhibited dendritic cells maturation and Th17 cells activation in autoimmune/inflammatory diseases (Wahono et al. 2014; Galowitz and Chang, 2014; Hamzaoui et al. 2010a,b). Our data strongly suggest that 25(OH)D3 treated CD4⁺ T cells has ability to modulate DC maturation in asthma patients inhibiting IL-17 over-expression. We also found that 25(OH)D3 inhibited the expression of various molecules associated with differentiation and function of Th17 cells such as *RORC*, *CCR6*, and *IL-23R*.

Th17 cells constitute a third subset of effector helper T cells, which functions are distinct from those of Th1 and Th2 cells. Th17 cells and IL-17 play a critical role in the pathogenic mechanisms of inflammation in animal model and human diseases (Li et al. 2013; Manni et al., 2014).

We studied the effect of 25(OH)D3 on the differentiation of Th17 cells. The results showed that 25(OH)D3 could inhibit the frequency of IL-17-producing CD4⁺ T cells and IL-17 secretion by naive CD4⁺ T cells under Th17 polarizing conditions. Our findings validate the suppressive effect of 25(OH)D3 on IL-17 secretion, as reported by Tang et al. in an animal model (Tang et al., 2009). Moreover, naive CD4⁺ T cells from asthmatic patients were more sensitive to 25(OH)D3 as compared to healthy controls. Our study showed that percentage of Th17 was decreased by 25(OH)D3 treatment at 1 nM and 10 nM.

Chang et al. (2010) reported that a dose dependent reduction in IL-17A production was observed when naive CD4⁺ T cells were

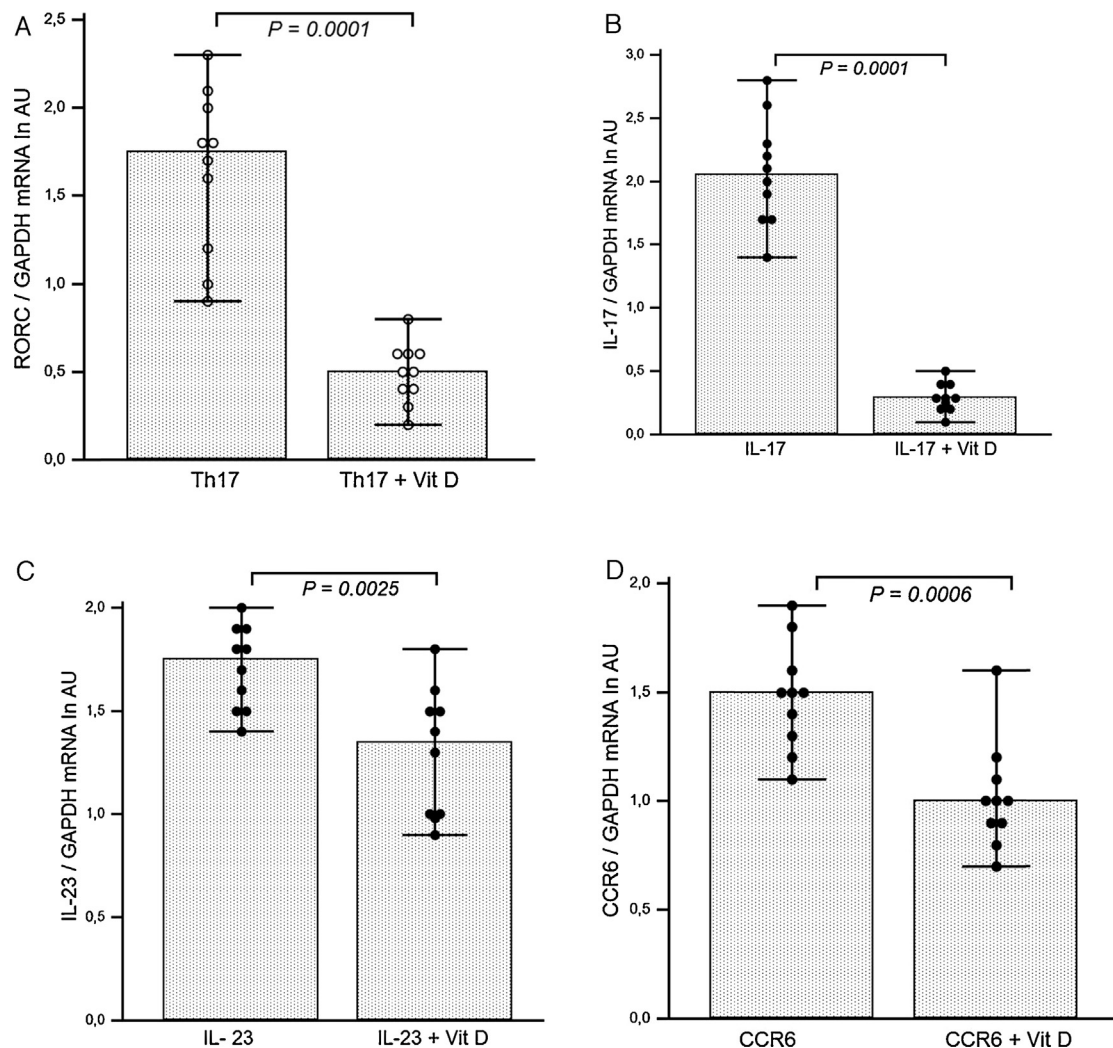


Fig. 3. Vitamin D inhibits the molecules associated with Th17 cell effector function. Naive CD4⁺ T cells from BD patients ($n=10$) were cultured under Th17 polarizing conditions with 25(OH)D3 (10 nM) or without 25(OH)D3 for 6 days. The cells were harvested for mRNA analysis for (A) RORC expression, (B) IL-17 expression, (C) IL-23R expression and (D) CCR6 expression by real-time PCR.

cultured with TGF β , IL-6 and increasing concentrations of Vitamin D. In our study naive CD4⁺ T cells from asthmatic patients were more sensitive to 25(OH)D3 at 10 nM compared to normal controls. The concentration of 25(OH)D3 used in our study is relevant to the physiological level of 25(OH)D3 in children.

The exact mechanism whereby 25(OH)D3 affects Th17 cell differentiation is not well known. The transcriptional factor IRF-8 is expressed in Th17 cells and is involved in Th17 cell differentiation (Ouyang et al. 2011; Tian et al. 2012). 25(OH)D3 could significantly induce the gene expression of IRF-8. The specific inhibition of IRF-8 gene expression using IRF-8 siRNA abrogated the effect of 25(OH)D3-mediated inhibition of Th17 differentiation (Tian et al. 2012). Previous studies reported that several mechanisms might be involved in the 25(OH)D3-mediated inhibition on Th17 cells. C/EBP homologous protein (CHOP), a molecule involved in endoplasmic reticulum stress and translational inhibition, has been shown to be involved in the inhibitory effect of 25(OH)D3 on Th17 cytokine production (Chang et al., 2010). Furthermore, nuclear factor for activated T cells (NFAT), histone deacetylase (HDAC), and Runt-related transcription factor 1 (Runx1) were all involved in the controlling effect of 1,25(OH)(2)D(3) on IL-17A production (Joshi et al. 2011).

As numerous molecules, such as RORC, CCR6, and IL-23R, are related to the differentiation and function of Th17 cells, it is interesting to investigate the influence of 25(OH)D3 on these factors.

The nuclear receptor RORC is a key transcription factor in the Th17 lineage commitment process (Manel et al. 2008; Fauber and Magnuson, 2014; Yu et al. 2013). Furthermore the addition of 25(OH)D3 to naive CD4⁺ T cells under Th17 polarizing conditions resulted in a significant decrease in the gene expression of RORC and IL-17. Human Th17 cells express constitutively IL-23R on their surface (Annunziato et al. 2007). We found that 25(OH)D3 markedly inhibited the gene expression of IL-23R, suggesting that 25(OH)D3 can inhibit the expansion and pathogenicity of the Th17 cells. CCR6 is expressed on human Th17 cells and contributes to their recruitment to sites of inflammation (Wang et al. 2009). We found that the addition of 25(OH)D3 to naive CD4⁺ T cells cultured under Th17 polarizing conditions inhibited the gene expression of CCR6, suggesting that 25(OH)D3 can decrease the number of pathogenic Th17 cells at the site of inflammation by influencing the migration of Th17 cells to the site. Therefore, the results showed that VitD3 not only inhibited the Th17 cell differentiation, but also inhibited the molecules that are relevant to Th17 cell function and migration in asthmatics.

IL-17 plays a critical role in inducing airway inflammation. In asthmatic patients, IL-17 expression is increased in sputum, lung cells, bronchoalveolar lavage fluid (BALF), and peripheral blood (Molet et al. 2001). The increased IL-17 in asthmatic airways stimulates human bronchial fibroblasts to produce cytokines inducing

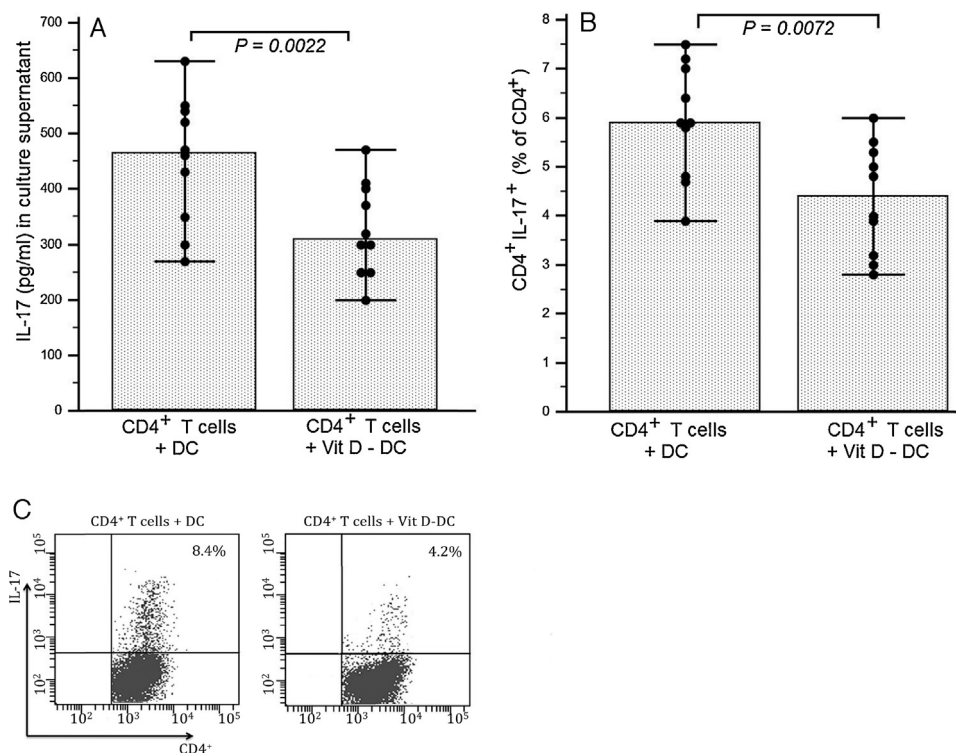


Fig. 4. Vitamin D-treated dendritic cells (DCs) have a decreased ability to induce Th17 cell response. Allogenic CD4⁺ T cells were isolated from asthmatics (10 patients) and cocultured with control DCs and VitD3-treated DCs for 7 days (25(OH)D3: 10 nM). (A) ELISA of IL-17 in the supernatants. (B) Representative dot plots. Quantitative analysis of the frequency of IL-17-producing CD4⁺ T cells after PMA/ionomycin stimulation. Data are expressed as mean $\pm$ SD.

airway remodelling (Molet et al. 2001; Berraies et al. 2014). The up-regulation of IL-17 mRNA levels in the airways of asthmatic mice (Nakae et al. 2002), supports our finding of increased IL-17 mRNA expression in the peripheral blood of young asthmatics. We recently reported that IL-17A and IL-17F are new pro-inflammatory cytokines implicated in neutrophilic inflammation and thus, involved in the pathogenesis of asthma. Haplotype analysis revealed a significant association between GA and AG haplotypes and asthma, suggesting that SNPs in IL-17A and IL-17F confer susceptibility to childhood asthma in Tunisia (Maalmi et al. 2014). In severe adult asthma, glucocorticoids did not inhibit IL-17A cytokine expression whereas treatment with vitamin D with or without dexamethasone significantly reduced both IL-17A and IL-22 levels (Nanzer et al. 2013).

Our data showed that 25(OH)D3 directly reduced the expression of Th17 cells. It is not yet known whether 25(OH)D3 could exert its effect on Th17 cells in humans via an interaction with DCs. DCs are critical to the differentiation of Th17 and Th1 cells and their function (Lanzavecchia and Sallusto, 2001; Sallusto and Lanzavecchia, 2002). Our results showed that 25(OH)D3-treated DCs could significantly inhibit Th17 cell response from CD4⁺ T cells. Our study therefore suggests that 25(OH)D3 acts as a negative modulator of Th17 cell response via an effect on DCs.

Vit D demonstrated anti-inflammatory and corticosteroid-enhancing effects in monocytes of patients with steroid resistant asthma (Searing et al. 2010). Our results suggest that addition of Vit D may be of interest to improve the control of inflammatory pathways in childhood asthma, even in mild cases.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contributions

AH and KH designed the experiments; AH, AB, JA and BH selected the patients; WK and KH did the experiments; KH, WK, and AB analyzed the data; KH, AH and AB wrote the manuscript. All authors read and approve the final manuscript.

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