The Role of Vitamin D in Regulating Immune Responses

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Interest in vitamin D seems to be surging due to the increased number of studies suggesting that it could prevent a variety of chronic diseases. Many na-tional surveys have demonstrated a growing proportion of the population presenting with serum concentrations below the lower limit of 10 ng/ml, whereas less than 5% reached 30 ng/ml, a level generally recommended for avoiding vitamin D insufficiency [1-3].

Vitamin D receptor has been found on many immune cells, such as macrophages, dendritic cells, T and B cells, mainly after activation. The engagement of VDR on DCs was shown to shape DC phenotype and function, enhancing their tolerogenicity in adaptive immune responses. Tolerogenic DCs induced by a short treatment with VDR agonists promote CD4+CD25+FoxP3+ T regulatory cells, which are able to mediate transplantation tolerance and arrest the development of autoimmune diseases [4]. In addition, it has been shown that vitamin D inhibits pro-inflammatory processes by suppressing the over-activity of CD4+ Th1, Th2 and Th17 cells and the production of their related cytokines such as interleukin-2, interferon-gamma and tumor necrosis factor-alpha [5,6].

The receptor for the biologically active metabolite of vitamin D appears to be a key player in these associations, as a mediator not only of the biological effects of vitamin D, but of the regulation of vitamin D metabolism itself, as well [7].

The possible involvement of vitamin D deficiency in the development of auto-immune diseases has recently gained interest. Epidemiological studies present evidence linking vitamin D deficiency with autoimmune diseases, such as rheumatoid arthritis, systemic sclerosis and systemic lupus erythematosus [8-10]. Prospective studies on the involvement of vitamin D in SLE are limited, but most of the existing cross-sectional studies show an inverse relationship between levels of vitamin D and disease activity [11,12]. When vitamin D was added *in vitro*, many immunological abnormalities characteristic of SLE were reversed, suggesting that vitamin deficiency skews the immunological response towards the loss of tolerance [13]. In another study, although vitamin D deficiency was common among SLE patients and was found to be associated with both sun avoidance and extreme fatigue, this had no relation to SLE severity. Here, the authors were able to show that along with its beneficial effect in SLE, hydroxychloroquine therapy prevented vitamin D deficiency [14].

When immunomodulatory mechanisms of vitamin D are discussed, many studies point to their ability to enhance the anti-inflammatory loop, namely, their ability to modulate T regulatory cell function. In this issue of *IMAJ*, Prietl et al. [15] question whether vitamin D supplementation increases Treg cell frequency (% Tregs) of circulating CD4+ T cells in apparently healthy individuals. Following a supplementation of 140,000 U at baseline, volunteers were assessed 4 weeks (visit 1) and 8 weeks after baseline (visit 2). The authors demonstrated that in 46 study participants who completed the trial, 25(OH) D levels increased from 23.9 ± 12.9 ng/ml at baseline to 45.9 ± 14.0 ng/ml at visit 1 and 58.0 ± 15.1 ng/ml at visit 2. Compared to baseline levels of %Tregs (4.8 ± 1.4), vitamin D supplementation induced a significant %Tregs increase at study visit 1 and visit 2 (5.8 ± 1.7, $P < 0.001$; and 5.6 ± 1.6, $P < 0.001$) respectively.

RECENT STUDIES LINKING VITAMIN D AND TREG CELLS

Allergen-specific immunotherapy was shown to suppress allergen-induced airway manifestations in a mouse mo-del of allergic asthma. Moreover, allergen immunotherapy induced IL-10-dependent longlasting tolerance of ovalbumin-induced asthma manifestations, pointing to a role for Treg cells. Since immature tolerogenic DCs play a critical role in Treg cell generation and peripheral tolerance, it was intriguing to explore whether allergen immunotherapy could be improved by adding vitamin D, inhibiting the DC maturation. In this regard Taher and colleagues [16] were able to demonstrate that 1.25(OH)2 D3 potentiates the efficacy of immunotherapy and that the regulatory cytokines IL-10 and transforming growth factor-beta play a crucial role in the effector phase of this mouse model.

Human IL-10-secreting Tregs (IL-10-Tregs), which express low levels of CD4+CD25+ Treg-associated transcription factor FoxP3, can be induced following activation, through either polyclonal stimuli or a specific antigen presentation, in the presence of the glucocorticoid dexamethazone and the active form of vitamin D (1α,25-dihydroxyvitamin D3;1α,25VitD3). In a very recent study the stimulation of 1α,25VitD3-induced IL-10-secreting Tregs with toll-like receptor-9 agonists, CpG oligonucleotides, resulted in

VDR = vitamin D receptor
DCs = dendritic cells
SLE = systemic lupus erythematosus

Tregs = regulatory T cells
IL = interleukin

acute coronary syndromes had significantly reduced numbers of Tregs when compared to patients with stable angina pectoris who had a similar extent of coronary atherosclerosis [23]. Hence, immunomodulatory effects of vitamin D might be relevant for several chronic diseases and this may hypothetically underlie the increased mortality in persons with a poor vitamin D status [24,25].

The main limitation of our work is the lack of a placebo group in this uncontrolled pilot trial. It should also be noted that we measured circulating Tregs and it is still unclear whether an increase of these peripheral Tregs is also associated with an increase of Tregs effects at the site of local inflammation. Apart from this, it still remains to be elucidated whether changes also occur in the immunosuppressive activity of Tregs after vitamin D supplementation. This could be measured in cell culture experiments by demonstrating reduced activity of Teffs (autoreactive effector T cells) in the presence of Tregs, and it remains an interesting research question for future studies [14].

In conclusion, our study results suggest that vitamin D supplementation increases %Tregs in apparently healthy subjects. This proposed immunomodulatory effect of vitamin D might be a key mechanism by which vitamin D exerts protective effects against autoimmunity. Our data might therefore serve as a rationale for further placebo-controlled trials to substantiate the beneficial effects of vitamin D supplementation on autoimmunological processes related to dysfunctions of Tregs.

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References

1. Holick MF. Vitamin D deficiency. *N Engl J Med* 2007; 357: 266-81.
2. Shoenfeld N, Amital H, Shoenfeld Y. The effect of melanism and vitamin D synthesis on the incidence of autoimmune disease. *Nat Clin Pract Rheumatol* 2009; 5: 99-105.
3. Arnson Y, Amital H, Shoenfeld Y. Vitamin D and autoimmunity: new aetiological and therapeutic considerations. *Ann Rheum Dis* 2007; 66: 1137-42.
4. Bouillon R, Carmeliet G, Verlinden L, et al. Vitamin D and human health: lessons from vitamin D receptor null mice. *Endocr Rev* 2008; 29: 726-76.
5. Adorini L, Penna G. Dendritic cell tolerogenicity: a key mechanism in immunomodulation by vitamin D receptor agonists. *Hum Immunol* 2009; 70: 345-52.
6. Kamen DL, Cooper GS, Bouali H, Shaftman SR, Hollis BW, Gilkeson GS. Vitamin D deficiency in systemic lupus erythematosus. *Autoimmun Rev* 2006; 5: 114-17.
7. Cutolo M, Otsa K, Uprus M, Paolino S, Seriolo B. Vitamin D in rheumatoid arthritis. *Autoimmun Rev* 2007; 7: 59-64.
8. Doria A, Arienti S, Rampudda M, Canova M, Tonon M, Sarzi-Puttini P. Preventive strategies in systemic lupus erythematosus. *Autoimmun Rev* 2008; 7: 192-7.
9. Smolders J, Peelen E, Thewissen M, et al. The relevance of vitamin D receptor gene polymorphisms for vitamin D research in multiple sclerosis. *Autoimmun Rev* 2009; 8: 621-6.
10. Alele JD, Kamen DL. The importance of inflammation and vitamin D status in SLE-associated osteoporosis. *Autoimmun Rev* 2010; 9(3): 137-9.
11. La Cava A. Tregs are regulated by cytokines: implications for autoimmunity. *Autoimmun Rev* 2008; 8: 83-7.
12. Askenasy N, Kaminitz A, Yarkoni S. Mechanisms of T regulatory cell function. *Autoimmun Rev* 2008; 7: 370-5.
13. Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nat Rev Immunol* 2007; 7: 585-98.
14. Tang Q, Bluestone JA. The Foxp3$^+$ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* 2008; 9: 239-44.
15. Penna G, Roncari A, Amuchastegui S, et al. Expression of the inhibitory receptor ILT3 on dendritic cells is dispensable for induction of CD4$^+$Foxp3$^+$ regulatory T cells by 1,25-dihydroxyvitamin D_3. *Blood* 2005; 106: 3490-7.
16. Gorman S, Kuritzky LA, Judge MA, et al. Topically applied 1,25-dihydroxyvitamin D_3 enhances the suppressive activity of CD4$^+$CD25$^+$ cells in the draining lymph nodes. *J Immunol* 2007; 179: 6273-83.
17. Daniel C, Sartory NA, Zahn N, Radeke HH, Stein JM. Immune modulatory treatment of trinitrobenzene sulfonic acid colitis with calcitriol is associated with a change of a T helper (Th) 1/Th17 to a Th2 and regulatory T cell profile. *J Pharmacol Exp Ther* 2008; 324: 23-33.
18. Urry Z, Xystrakis E, Richards DF, et al. Ligation of TLR9 induced on human IL-10-secreting Tregs by 1α,25-dihydroxyvitamin D_3 abrogates regulatory function. *J Clin Invest* 2009; 119: 387-98.
19. Ghoreishi M, Bach P, Obst J, Komba M, Fleet JC, Dutz JP. Expansion of antigen-specific regulatory T cells with the topical vitamin D analog calcipotriol. *J Immunol* 2009; 182: 6071-8.
20. Gerli R, Nocentini G, Alunno A, et al. Identification of regulatory T cells in systemic lupus erythematosus. *Autoimmun Rev* 2009; 8: 426-30.
21. Alonso N, Soldevila B, Sanmarti A, Pujol-Borrell R, Martinez-Caceres E. Regulatory T cells in diabetes and gastritis. *Autoimmun Rev* 2009; 8: 659-62.
22. Ardalan MR, Maljaei H, Shoja MM, et al. Calcitriol in the donor expands the population of CD4$^+$CD25$^+$ T cells in renal transplant recipients. *Transplant Proc* 2007; 39: 951-3.
23. George J. Mechanisms of disease: the evolving role of regulatory T cells in atherosclerosis. *Nat Clin Pract Cardiovasc Med* 2008; 5: 531-40.
24. Pilz S, Dobnig H, Nijpels G, et al. Vitamin D supplementation and total mortality: a meta-analysis of randomized controlled trials. *Clin Endocrinol (Oxf)* 2009; 71: 666-72
25. Autier P, Gandini S. Vitamin D supplementation and total mortality. *Arch Intern Med* 2007; 167: 1730-7.

Table 1. Baseline characteristics

No. of patients	50
Females (%)	64
Age (yrs)	31 ± 8
Height (cm)	171.7 ± 8.8
Weight (kg)	68.8 ± 13.7
Body mass index (kg/m²)	23.3 ± 4.3
C-reactive protein (mg/L)	2.2 ± 2.7
Serum calcium (mmol/L)	2.4 ± 0.99
25-hydroxyvitamin D (ng/ml)	24.1 ± 12.6

Continuous variables are presented as means ± standard deviation (SD)

insufficient vitamin D status. 25(OH)D levels increased from 23.9 ± 12.9 ng/ml (mean ± SD) at baseline to 45.9 ± 14.0 ng/ml at visit 1 ($P < 0.001$) and to 58.0 ± 15.1 ng/ml at visit 2 ($P < 0.001$). At visit 2, all study subjects had a sufficient vitamin D status (25[OH] D levels > 30 ng/ml).

%Tregs within 20,000 CD4+ cells were 4.8 ± 1.4 at baseline. Compared to baseline values %Tregs were significantly increased at visit 1 (5.9 ± 1.7, $P < 0.001$) and at visit 2 (5.6 ± 1.6, $P < 0.001$). %Tregs were significantly lower at visit 2 compared to visit 1 ($P = 0.011$). Pearson correlation coefficients of %Tregs and 25(OH) D levels were 0.223 ($P = 0.120$) at baseline, 0.092 ($P = 0.530$) at visit 1 and 0.170 ($P = 0.259$) at visit 2. When %Tregs and 25(OH) D levels of all study visits were used for correlation analysis the Pearson correlation coefficient was 0.315 ($P < 0.001$).

CRP (normal range 0 to 8 mg/l) was 2.3 ± 2.8 at baseline, 2.0 ± 1.9 at visit 1, and 2.4 ± 2.7 at visit 2. There was no statistically significant difference in CRP levels between any of the study visits. In addition, there was no significant difference between serum calcium levels at baseline (2.37 ± 0.09) and at visit 1 (2.36 ± 0.11, $P = 0.486$). Compared to baseline, however, we observed a significant decrease in serum calcium at visit 2 (2.30 ± 0.09, $P < 0.001$).

DISCUSSION

This 8 week pilot trial demonstrated that vitamin D supplementation of 140,000 IU at baseline and after 4 weeks was associated with a significant increase in %Tregs among apparently healthy subjects. This finding supports the hypothesis that vitamin D-induced stimulation of Tregs is a possible pathophysiologic mechanism by which vitamin D may prevent autoimmune diseases.

Tregs are critical for controlling immunological tolerance to self-antigens [11-14]. They originate in the thymus but may also derive from peripheral CD4+ T cells following antigen stimulation [11-14]. The current literature supports the concept that Tregs suppress autoreactive effector T cells at the site of inflammation and in the draining lymph nodes [12]. Their immunmodulatory effects involve cytotoxic actions on pathogenic T cells, which are mediated through cell-to-cell contact [12]. In addition, Tregs modulate cytokine profiles at the site of inflammation and secrete cytokines such as transforming growth factor-beta and IL-10, which exert anti-inflammatory actions [12-14]. Mounting evidence linking reduced activity of Tregs to risk of autoimmune diseases and graft-versus-host disease in transplant recipients has already stimulated research work aiming to evaluate and introduce the use of "Tregs therapy" in the treatment of autoimmune diseases and GVHD [13]. In this context, vitamin D may be useful as a therapeutic agent because it exerts immunomodulatory effects that may involve stimulatory actions on Tregs [1-5]. Towards this, previous studies suggest that the impact of vitamin D on dendritic cells results in the development of a tolerogenic dendritic cell type that is able to induce Tregs [5].

In our study we tested the effect of vitamin D supplementation on %Tregs. To the best of our knowledge we are the first to show that vitamin D intake at relatively high doses significantly increases %Tregs in the peripheral circulation. This finding is in line with previous data showing increased Tregs in the draining lymph nodes of mice that were treated with topical application of the active vitamin D analog calcipotriol [19].

Accumulating evidence from experimental studies underlines the importance of vitamin D for Tregs stimulation, but data from clinical studies in humans are sparse [15-18,22]. Previous data on a significant increase of CD4+CD25+ T cells after calcitriol treatment in renal transplant recipients are limited due to the missing determination of FOXP3, which is important to differentiate naturally occurring Tregs from other CD4+CD25+ T cells [22]. Hence, our study, which includes an accurate characterization of Tregs, significantly extends the current knowledge on this topic. Interestingly, %Tregs, which markedly increased after vitamin D intake, were significantly higher at visit 1 than at visit 2. Underlying mechanisms for this difference remain speculative. However, it could be hypothesized that there exists a U-shaped association of vitamin D status and Tregs with optimal stimulatory effects on Tregs at 25(OH)D levels close to those at visit 1 and fewer effects on Tregs at higher and lower 25(OH) D levels. It should also be pointed out that there was no significant correlation of %Tregs and 25(OH)D levels at any of our study visits. There was, however, a significantly positive correlation of %Tregs and 25(OH)D level when the values of %Tregs and 25(OH)D at all study visits were used for a correlation analysis. Importantly, there was no significant change in CRP levels between the study visits, suggesting that infections or other unspecific inflammatory stimuli did not contribute to the observed changes in %Tregs.

Beyond autoimmunity and GVHD, our finding of increased %Tregs after vitamin D supplementation may also have implications for cardiovascular diseases because transfer of Tregs to apolipoprotein E-deficient mice led to reduced atherosclerotic lesion formation and increased plaque stabilization [23]. In humans, patients with

CRP = C-reactive protein

GVHD = graft-vs-host disease

months significantly increased the percentage of CD4+CD25+ T cells of total peripheral lymphocytes [22]. These data suggest that vitamin D supplementation may increase circulating Tregs in humans. This issue has, to our knowledge, not been addressed in previous studies. We therefore conducted a pilot trial to examine whether vitamin D supplementation in apparently healthy individuals increases the percentage of Tregs within 20,000 circulating CD4+ T cells.

PATIENTS AND METHODS

We investigated 50 apparently healthy subjects aged at least 18 years, who were recruited at our outpatient clinic (Department of Internal Medicine, Division of Endocrinology and Nuclear Medicine, Medical University of Graz, Austria). In addition, we invited colleagues of our department as well as their friends and family members to participate in our pilot trial. Exclusion criteria were hypercalcemia (serum calcium > 2.65 mmol/L), pregnancy, participation in other interventional clinical trials, and any disease requiring medical treatment. The study was conducted in the outpatient clinic of our department from February to June 2009. Written informed consent was obtained from all study participants. The study was performed in adherence to the Declaration of Helsinki and we obtained approval from the ethics committee at the Medical University of Graz, Austria.

In this uncontrolled, monocentric pilot trial we performed a baseline visit (visit 0) and two follow-up visits, 4 weeks (visit 1) and 8 weeks (visit 2) after the baseline examination. At each study visit blood was drawn after an overnight fast between 7 and 11 a.m. and a pregnancy test was performed in all female study participants. At baseline (visit 0) and at study visit 1, all subjects with missing exclusion criteria received 140,000 IU vitamin D3 orally (Oleovit D3®, Fresenius Kabi, Austria). Anthropometric measurements (height and weight) were performed with the study participants wearing light clothes and no shoes. Body mass index was calculated as weight (in kilograms) divided by the height squared (in meters).

LABORATORY METHODS

Peripheral blood mononuclear cells were isolated within 24 hours using Histopaque-1077 Hybri Max (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation from sodium-heparinized blood samples and washed twice in Hank's buffered salt solution (Invitrogen, New Zealand). After counting and staining with 0.4% Trypan blue solution (Sigma-Aldrich) to examine the purity and viability, all samples were frozen in fetal bovine serum (Invitrogen) containing 10% DMSO (Sigma-Aldrich) in a controlled-rate automated freezing device to -80°C and then stored in liquid nitrogen. All experiments were performed on thawed cells. An aliquot was counted and stained with 0.4% Trypan blue solution as described above. Cells were stained for surface antigens with the following fluorochrome-conjugated monoclonal antibodies, all purchased from BD Pharmingen (San Diego, CA, USA): anti-CD4 FITC (fluorescein isothiocyanate), anti-CD 25 PE-Cy7 (phycoerythrin cyanin 7) and anti-CD127 PE (phycoerythrin). After surface staining, cells were permeabilized with a special buffer (BD Pharmingen) and intracellular staining for the transcription factor FOXP3 was performed using anti-FOXP3-Alexa Fluor 647 monoclonal antibodies (BD Pharmigen) according to the manufacturer´s instructions. To avoid having to measure signals developed from unspecific binding of FOXP3 antibody, an Alexa Fluor 647 isotype control (BD Pharmigen) was prepared for each sample. At least 20,000 CD4-positive events were acquired from each sample on a BD FACSCanto II and analyzed with FACS-Diva software (Vers 6.1.2). CD4+CD25++FOXP3+ cells with low or absent expression of CD127 were classified as Tregs.

25(OH)D was determined by means of a commercially available enzyme-linked immunosorbent assay (IDS, Bolden, UK) with an intra- and interassay coefficient of variation of 5.6 and 6.4%, respectively. C-reactive protein was measured by Tina quant CRP immunoturbidometric assay (Roche COBAS INTEGRA, Germany). Other laboratory measurements were performed by routine methods.

STATISTICAL ANALYSIS

Baseline characteristics are presented as means ± standard deviation for continuous variables and as percentages for categorical variables. Descriptive statistics and Kolmogorov Smirnov test were used to test for normality of the distribution of the continuous variables. 25(OH)D levels followed a skewed distribution and were thus logarithmically transformed before use in parametric procedures. We performed Pearson correlation analyses of %Tregs and 25(OH)D at each study visit. In addition, we used %Tregs and 25(OH)D values from all study visits for a correlation analysis. Paired Student's *t*-test was used to test for differences in %Tregs, 25(OH)D and serum calcium between the study visits. Statistical analyses were performed by SPSS version 16.0 (SPSS Inc, Chicago) and a *P* value below 0.05 was considered statistically significant.

RESULTS

Of the 50 study participants 46 completed the trial. Follow-up visits were performed 4.4 ± 0.5 (mean ± SD) weeks (visit 1) and 8.8 ± 1.0 weeks (visit 2) after the baseline examination. After the baseline visit, one study subject was excluded because he was also participating in another interventional trial and another participant was excluded due to previously diagnosed type 1 diabetes mellitus. After study visit 1, we excluded a participant due to non-compliance with the study protocol and another one because of mild asymptomatic hypercalcemia (serum calcium 2.67 mmol/L, normal range 2.20–2.65). In this individual, hypercalcemia had resolved spontaneously by the time of the follow-up examination. We observed no clinically significant adverse event during the study.

Clinical and laboratory baseline characteristics of all 50 study participants are shown in Table 1. At baseline, 80% of our study subjects had 25(OH)D levels below 30 ng/ml, which indicates an

FOXP3 = forkhead box P3