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ORIGINAL CONTRIBUTION

High-dose cholecalciferol supplementation significantly increases peripheral CD4^{+} Tregs in healthy adults without negatively affecting the frequency of other immune cells

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Abstract
Background Regulatory T cells (Tregs) play a central role in the maintenance of self-tolerance. Animal and in vitro studies suggest that vitamin D is involved in reducing the risk of autoimmunity by modulating Tregs.
Methods In a double-blind, placebo controlled study in 60 healthy volunteers, we assessed the effect of a 12-week high-dose oral cholecalciferol supplementation (140,000 IU/month) on the number and function of $CD4^{pos}CD25^{high}FoxP3^{pos}CD127^{dim}$ Tregs. We also assessed the clinical safety of the supplementation and the effect on the frequency of other immune cells such as monocytes, dendritic cells, natural killer cells, natural killer T cells, B cells and subgroups of T cells. We also tested the in vitro effect of cholecalciferol on Tregs in human cell cultures.
Results By using FACS analysis, ex vivo suppressive co-cultures and apoptosis assays, we were able to show that a cholecalciferol supplementation leads to significantly increased numbers of peripheral Tregs in vivo. Tregs function and the frequency of other immune cells remained unchanged, and no clinically relevant safety concerns were found. The in vitro exposure of human peripheral blood mononuclear cells to cholecalciferol also supported our in vivo findings.
Conclusions Our results indicate a substantial effect of a supplementation with inactive vitamin D on the immune system of healthy humans in vivo and provide a rationale for future studies to investigate the immunomodulatory effects of vitamin D in autoimmune diseases.

Keywords Vitamin D · Regulatory T cells · Immune regulation · Immunotherapy · Tolerance

Introduction

The classical, hormonal actions of vitamin D are related to calcium metabolism and bone health, but 1,25-dihydroxyvitamin D3 synthesized by immune cells has been suggested to have immunomodulatory properties similar to locally active cytokines [1, 2]. Vitamin D deficiency is associated with the development of cardiovascular diseases, various types of cancer and autoimmune disorders such as type 1 diabetes, multiple sclerosis and inflammatory bowel disease [3–5]. An increased incidence of T1D has been associated with reduced vitamin D supplementation [6] or low 25(OH)D levels in epidemiological studies [7]. Many autoimmune diseases are caused by an impairment of immune homeostasis. A balanced immune regulation is dependent on regulatory T cells (Tregs) that have the capacity to actively block immune responses, inflammation and tissue destruction by suppressing immune cell function. Various studies have reported different effects of low Tregs numbers or impaired Tregs function in autoimmune diseases [8–12]. To re-establish immune homeostasis based on Tregs function or number, adoptive transfer of in vitro expanded human Tregs and compounds such as

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rapamycin, anti-CD3 antibodies and IL-2-anti-IL-2 complexes have been used [13–16]. Increasing evidence from in vitro studies and animal models suggests the active form of vitamin D[1] ($1,25(OH)_2D3$—calcitriol) as an alternative immunomodulator that can suppress proinflammatory T helper Th1 and Th17 responses, while promoting the Th2 and regulatory T cell phenotype by enhancing the production of interleukin IL-4, IL-5 and IL-10 [17, 18]. Furthermore, calcitriol has also been shown to influence proliferation, differentiation and various functions such as cytokine production by antigen presenting and adaptive immune cells [19]. The use of calcitriol or analogues is restricted to a lower dosage due to the potential toxic side effects such as hypercalcaemia. Using a high-dose supplementation of the inactive form of vitamin D (cholecalciferol) has been described as safe in humans [20], and a 12-month supplementation recently succeeded in significantly increasing the level of circulating Tregs in newly diagnosed T1D patients [21]. Without active vitamin D supplementation, the percentage of peripheral Tregs was found to be seasonally decreased in healthy humans [22]. In an uncontrolled trial, we have recently found a significant increase in peripheral Tregs after supplementing 50 healthy volunteers with a high-dose cholecalciferol for 8 weeks [23]. Following this promising pilot trial which did not include an placebo supplemented control group, we subsequently designed this randomized, placebo controlled, double-blind trial (RCT) study in healthy humans. A monthly supplementation of either 140,000 IU of cholecalciferol or a placebo was given for a 12-week period to assess the effects of a high-dose cholecalciferol supplementation on immunomodulation and clinical safety parameters. In addition, we assessed the effects of cholecalciferol on Tregs in an in vitro experiment.

Materials and methods

Study design and study population

Ethical approval was obtained from the local Ethics Committee of the Medical University of Graz. Healthy subjects aged at least 18 years gave written informed consent to participate in this single-center, randomized, double-blind, placebo controlled study. Subjects were randomized into two study groups (vitD group and placebo group) using the Randomizer (http://www.randomizer.at) and received 3 monthly doses of either a cholecalciferol supplementation (140,000 IU of Oleovit D3®, Fresenius Kabi, Austria) or equal amounts of placebo (almond oil). The trial was performed in accordance with Good Clinical Practice and the Declaration of Helsinki and registered at ClinicalTrials.gov (number: NCT01248442).

A total of 60 healthy subjects entered the trial. Subjects with hypercalcaemia (serum calcium > 2.65 mmol/l), pregnancy and any disease requiring medical treatment were not recruited. Since BMI has been shown to be inversely associated with serum vitamin D levels, only volunteers with a BMI < 35 kg/m^2 were included [24].

Venous blood was drawn at all study visits after a 12-h overnight fast, and the intake of the study medication was coordinated after confirmation of normal calcium levels. The study was performed from November to March to minimize the effect of extensive sunlight on endogenous vitamin D production.

Safety measurements

Serum calcium levels, urine calcium, urine calcium/creatinine ratio, complete blood cell count, serum phosphorus, serum albumin and PTH were measured regularly during the 12 weeks of supplementation by standard laboratory methods.

Flow cytometry and blood cell quantification

Absolute numbers for different groups of blood leukocytes were determined by using a hematological cell counter (Beckman Coulter, Woerden, The Netherlands). For the quantification of $CD4^{pos}CD25^{high}FoxP3^{pos}CD127^{dim}$ regulatory T cells (Tregs) in peripheral blood, the following antibodies were used: anti-CD4 FITC, anti-CD25 PE-Cy7 and anti-CD127 PE. FoxP3 staining was carried out using FoxP3 fixation/permeabilization buffers and anti-FoxP3 AF-647 antibodies. Finally, cells were analyzed on a FACSCantoII cytometer using the Diva 6.1.3 software.

T cell subpopulations were quantified by staining surface markers with the following monoclonal fluorochrome-conjugated antibodies: anti-CD3 V450, anti-CD4 PE, anti-CD8 PerCP-Cy5.5, anti-CD25 FITC, anti-CD45RO APC, anti-CD45RA PE-Cy7 and anti-CD45 APC-H7. Natural killer cells (NK), natural killer T cells (NKT) and B cells were quantified by using the following monoclonal fluorochrome-conjugated antibodies: anti-CD3 V450, anti-CD45 APC-H7, anti-CD56 PE and anti-CD19 PE-Cy7. Finally, plasmacytoid and myeloid dendritic cells were quantified by using anti-CD123 PE, anti-CD11c APC, anti-HLA-DR PE-Cy7, anti-CD45 APC-H7 antibodies and a ready-to-use lineage marker kit for the exclusion of all other blood cells. After antibody addition and incubation, red blood cells were lysed and fixed by using a Lyse/Fix Buffer. Cells were washed with CellWASH™ and

[1] When the term vitamin D is used without a subscript it always refers to the total of vitamin D2 and vitamin D3. This is also the case for any vitamin D metabolites.

analyzed with a FACSCantoII cytometer. For all assays, isotype controls and the fluorochrome minus one (FMO) method [25] were used to avoid measuring unspecific binding signals and to obtain a more precise definition for cells having fluorescence above the background level. Intra-assay coefficients of variance (CV) for Tregs quantification were 4.5 % ($n = 5$). All material in this section was purchased from BD Biosciences, San Jose, CA, if not stated otherwise.

PBMC isolation and cell sorting

PBMC were isolated from fresh heparinized blood by centrifugation on a Histopaque density gradient (Sigma-Aldrich, Germany), washed and resuspended in X-vivo media completed with 10 % human AB serum (Lonza, USA). For culture requirements, 1×10^6 PBMC were irradiated by gamma ray treatment (10 min/3,000 rads). Remaining PBMC were stained with monoclonal antibodies, and $CD4^{pos}CD25^{high}CD127^{low/neg}$ Tregs and $CD4^{pos}CD25^{neg/pos}CD127^{pos}$ effector cells (Teff) were isolated using a FACSAriaII cell sorter (BD Biosciences).

Apoptosis measurement of Tregs

Within 1 h after sorting, 8×10^3 Tregs were stained with 7AAD and Annexin-V APC (BD) according to manufacturer's instructions and analyzed on a FACSCantoII flow-cytometer for apoptosis quantification.

Suppression co-cultures

Effector T cells (Teff) were seeded in an U-bottom 96-well plate at a concentration of 2.5×10^4/well and cultivated in the presence or absence of autologous Tregs at a 1:1 ratio and the presence of irradiated autologous PBMC (2.5×10^4/well). Cells were stimulated by adding anti-CD3/CD28-coated microbeads at a concentration 1 bead/cell (Invitrogen, Carlsbad, CA) and incubated at 37 °C and 5 % CO_2 for 96 h. During the last 18 h responder, cell proliferation was monitored by analyzing ^{3}H-thymidine uptake (Biotrend, Köln, Germany) measured on a MicroBeta Trilux Counter (Perkin Elmer, Wellesley, USA). Background signals, such as proliferation of pure Tregs and pure irradiated PBMCs, were measured in each single plate to control purity of sorting and irradiation. As pure Tregs are not able to proliferate upon stimulation with anti-CD3/28 beads, only cultures with negative or low proliferation values for pure Tregs were included in the analysis. Finally, all background signals were subtracted from the proliferation values of the effector cells.

Serum vitamin D and CRP measurement

Serum 25(OH)D (calcifediol) was determined with a commercially available ELISA (IDS, Bolden, UK). Serum 1,25(OH)D (calcitriol) was quantified by a combination of immunopurification and an automated chemiluminescent immunoassay (IDS-iSYS; IDS, Germany).

In vitro cell cultures

Isolated PBMC from healthy humans were stained with Violet Proliferation Dye (BD Biosciences, CA), washed and cultivated in cRPMI-10 for 96 h at 37 °C and 5 % CO_2 including a permanent exposure to either solvent or cholecalciferol (Sigma-Aldrich, Germany) at the concentration 50 ng/ml. After 96 h of incubation, cells were stained for FACS analysis of T cell subtypes as described above and percentage of Tregs and $CD8^{pos}$ cytotoxic T cells as well as proliferation was measured using a FACSCantoII cytometer (BD Biosciences).

Statistical analysis

Individual data were analyzed, and mean ± SD or median + interquartile range (IQR) for not normally distributed data was calculated for each group (vitD, placebo). A general linear model (GLM) with repeated measurements was performed to determine changes in serum 25(OH)D levels and immune parameters over 12 weeks of supplementation. To compare vitD and placebo groups, data were tested for normality and compared using a Welch's t test (unequal variances), a Student's t test, a Mann–Whitney U test for unpaired data or a Wilcoxon signed-rank test for paired data. Spearman ordered rank correlations were used for supplementation and effect data. All statistical analysis was performed using SPSS version 19.0 software (SPSS Inc. Chicago, USA). p-values < 0.05 were considered statistically significant and when necessary significance levels were adjusted using Bonferroni corrections for multiple comparisons.

Results

Study population

A total of 57 subjects completed the trial with all 4 visits (baseline, 4, 8 and 12 weeks) excluding 2 volunteers who did not attend the last visit due to personal time constraints and 1 subject who was excluded from the whole data analysis because of additional vitamin D intake during the study. Baseline characteristics of our study participants are

shown in Table 1. Body mass index was similar in both groups and did not change from baseline to 12 weeks ($p > 0.05$, data not shown).

Cholecalciferol supplementation increases serum 25(OH)D levels and calcitriol levels without causing side effects

At baseline, 37 % of our study population had serum 25(OH)D levels between 20–30 ng/ml indicating vitamin D insufficiency and 32 % had a vitamin D deficiency with 25(OH)D levels < 20 ng/ml. Serum 25(OH)D levels increased significantly in the vitD group (25.5 ± 11.4 at baseline vs. 55.1 ± 18.1 ng/ml after 12 weeks, $p \leq 0.001$) and decreased significantly in the placebo group (25.8 ± 10.4 at baseline vs. 21.1 ± 9.8 ng/ml after 12 weeks; $p = 0.003$, Fig. 1a). Serum calcitriol levels were similar between the vitD and placebo group at baseline (74.1 ± 38.1 vs. 75.3 ± 37.1 pmol/ml). After 12 weeks of supplementation, the concentration changed significantly only in the vitD group (100.1 ± 40.7 pmol/ml, $p = 0.016$) but remained stable in the placebo group (78.3 ± 47.5 pmol/ml, $p = 0.7$). In the vitD group, serum 25(OH)D levels increased by 116 % compared to a serum calcitriol increase of 35 % from baseline to 12 weeks.

Serum calcium levels were similar in the vitD group and the placebo group at baseline (2.37 ± 0.08 vs. 2.33 ± 0.10 mmol/l) and after 12 weeks of supplementation (2.36 ± 0.08 vs. 2.32 ± 0.08 mmol/l). Other safety determinants (urine calcium, urine calcium/creatinine ratio, complete blood cell count, serum phosphorus, serum albumin and PTH) remained within normal reference ranges during the whole study period.

Table 1 Demographic data of our studied population at time of recruitment

	vitD group	Placebo group	*p* value
Number of subjects	30	29	
Gender (% female)	47	52	0.797
BMI (kg/m^2)	24.5 ± 3.9	23.9 ± 3.4	0.625
Age (years)	27 (24–37)	35 (26–42)	0.192
Serum 25(OH)D (ng/ml)	22.4 (16.8–31.6)	26.0 (17.7–32.0)	0.644
Tregs in CD4 (%)	4.9 ± 0.9	5.3 ± 1.0	0.075

Data presented as mean ± SD when normally distributed or median + (IQR) when not normally distributed

Cholecalciferol supplementation changes the percentage of CD4^{pos} Tregs in the peripheral blood

At baseline, serum 25(OH)D levels correlated significantly with the percentage of peripheral Tregs ($n = 59$, Fig. 1b). The mean percentage of Tregs (%Tregs) within 20,000 CD4^{pos} T cells increased significantly in the vitD group from 4.89 ± 0.93 % at baseline to 5.68 ± 1.18 % after 4 weeks ($p = 0.018$) to 6.14 ± 1.00 % after 8 weeks ($p < 0.001$) and finally reached 6.35 ± 0.78 % after 12 weeks ($p < 0.001$) of cholecalciferol supplementation (Fig. 2a). There was no significant change in %Tregs during the 12-week study period in the placebo group, and the final mean level was 5.46 ± 0.95 % (vs. 5.35 ± 1.01 % at baseline). Between-group comparisons revealed significantly higher percentage of Tregs after 8 and 12 weeks in the vitD group compared to the placebo group (Fig. 2a). Furthermore, after the cholecalciferol supplementation, the increasing percentage of Tregs correlated significantly with increasing serum 25(OH)D levels from baseline to week 12 in the vitD group (Fig. 2b).

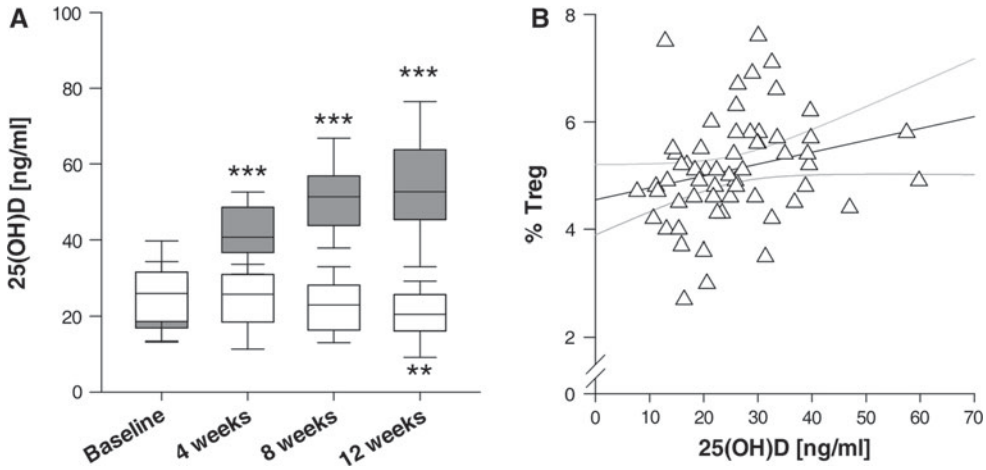

Fig. 1 Correlation between serum vitamin D levels and the percentage of peripheral Tregs. **a** Median serum 25(OH)D concentrations during the 12-week study period in the vitD group (*dark box-plots*, $n = 30$) and the placebo group (*white box-plots*, $n = 29$). $^{**}p < 0.005$, $^{***}p < 0.001$ as compared to baseline. **b** Spearman's rank order correlation of %Tregs and serum 25(OH)D from 59 healthy subjects at baseline. *Lines* indicate regression and 95 % CI ($p = 0.009$, $r = 0.339$)

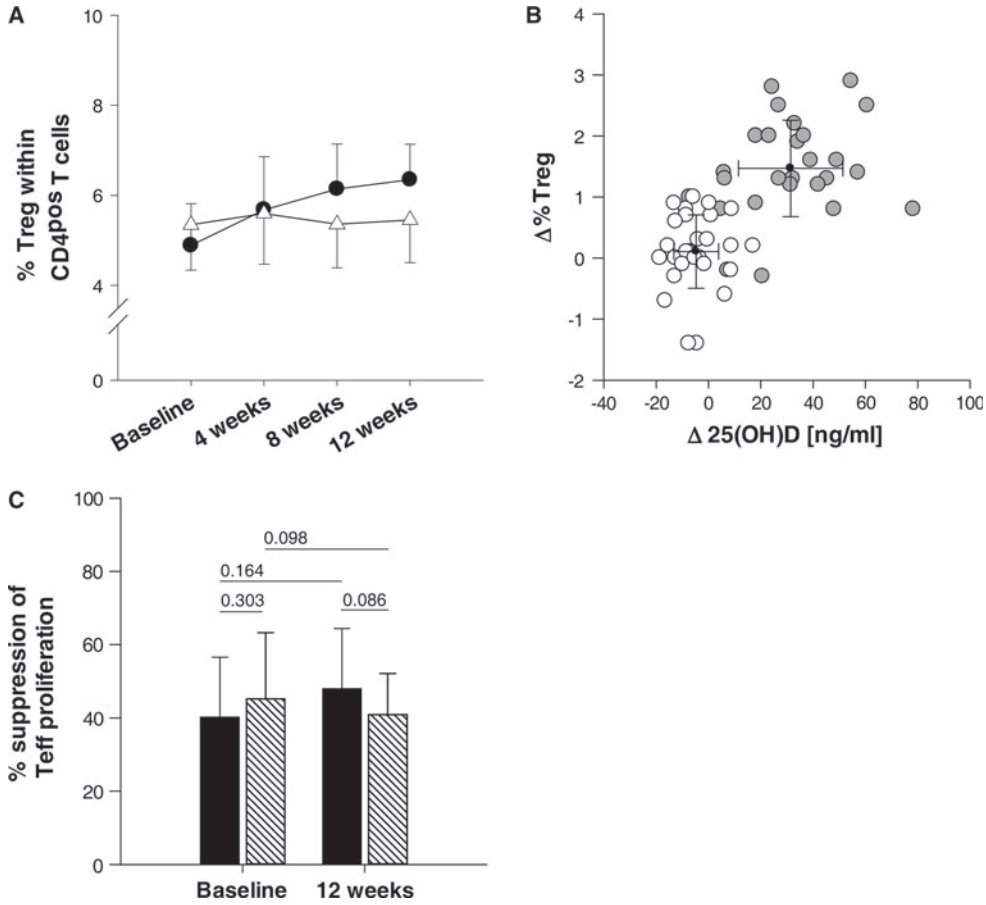

Fig. 2 Elevated percentage of peripheral Tregs after cholecalciferol supplementation. **a** Mean percentage of peripheral Tregs within $CD4^{pos}$ T cells during 12 weeks of cholecalciferol or placebo supplementation in the vitD group (*circles*) and the placebo group (*triangles*). **b** Pearson correlation of delta serum 25(OH)D levels versus delta %Tregs after 12 weeks of supplementation ($p \leq 0.001$, $r = 0.640$). *Circles* represent individual values, bidirectional *bars* indicate standard deviation around the mean for the vitD group (*dark circles*) and the placebo group (*white circles*). **c** Effects of cholecalciferol supplementation on the suppressive potential of FACS sorted Tregs. Suppression analysis was performed in autologous Tregs/Teff co-cultures. Data show results from healthy donors who received cholecalciferol ($n = 26$, *black bars*) or placebo ($n = 25$, *dotted bars*) orally for the whole duration of 12 weeks. Data given as mean ± SD. *p*-values are shown on top of *horizontal lines*

Suppressive capacity and apoptosis of $CD4^{pos}$ Tregs remain unchanged after cholecalciferol supplementation

In order to assess whether cholecalciferol supplementation can influence the suppressive capacity of Tregs, we performed ex vivo co-culture experiments with autologous stimulated effector T cells (Teff). Suppressive capacity, calculated as the reduction in %Teff proliferation in the presence of autologous Tregs, did not vary between the groups at baseline (Fig. 2c). High-dose cholecalciferol supplementation did not significantly increase the suppressive activity of peripheral Tregs significantly in the vitD group (Fig. 2c).

Apoptosis of FACS sorted Tregs assessed within 1 h post-sorting by a standard FACS apoptosis assay revealed no significant differences between the vitD and the placebo group after 12 weeks of supplementation (data not shown).

Influence of cholecalciferol supplementation on human peripheral immune competent cells

A multicolor FACS analysis for different types of peripheral blood immune cells was performed to evaluate the effect of cholecalciferol supplementation on the frequency of innate as well as adaptive immune cells (Table 2). The frequency of T cell subtypes, such as $CD4^{pos}$ memory or naive cells, $CD8^{pos}$ memory or naive cells, CD4CD8 double positive cells and $CD8^{pos}CD25^{pos}$ cells, did not change significantly in neither group. Also, cholecalciferol supplementation did not influence the frequency of neutrophilic granulocytes, monocytes, B cells, NK cells, NKT

Table 2 Frequency or absolute numbers of human peripheral immune cells after 12 weeks of supplementation with cholecalciferol or placebo in the vitD and placebo groups

	vitD group	Placebo group	*p* value
% $CD4^{pos}$ in $CD3^{pos}$ Tc	62.8 ± 8.4	61.7 ± 10.6	0.665
% naive $CD4^{pos}$ in $CD3^{pos}$ Tc	33.5 ± 9.9	29.7 ± 8.4	0.134
% memory $CD4^{pos}$ in $CD3^{pos}$ Tc	27.5 ± 6.5	28.8 ± 4.6	0.414
% $CD8^{pos}$ in $CD3^{pos}$ Tc	31.7 ± 7.3	31.4 ± 8.0	0.908
% naive $CD8^{pos}$ in $CD3^{pos}$ Tc	19.5 ± 5.2	17.6 ± 5.6	0.243
% memory $CD8^{pos}$ in $CD3^{pos}$ Tc	10.6 (6.7–12.8)	11.1 (8.5–13.2)	0.332
% $CD19^{pos}$ Bc in lymphocytes	12.3 ± 4.4	12.7 ± 3.7	0.589
% NK cells in lymphocytes	8.7 (6.6–13)	7.6 (5.8–11.7)	0.291
% pDC in lymphocytes*	30.7 ± 9.6	29.2 ± 9	0.561
% mDC in lymphocytes*	47.6 ± 10.4	52.8 ± 8.7	0.046
Monocytes (10^3/μl)	0.44 (0.32–0.54)	0.41 (0.35–0.52)	0.780
Neutrophils (10^3/μl)	2.8 (2.3–4.4)	3.0 (2.4–4.3)	0.936

Data presented as mean ± SD when normally distributed or median + (IQR) when not normally distributed

p-values ≤ 0.025 were considered significant, due to Bonferroni correction of α-values for multiple comparisons

* Blood DC in $Lin^{neg}HLADR^{hi}$ expressing lymphocytes.

cells, myeloid and plasmacytoid dendritic cells (Table 2). The percentage of plasmacytoid dendritic cells was not different between placebo and vitD groups. Although there was a small decrease in the percentage of myeloid dendritic cells in the vitD group compared to the placebo group after 12 weeks, this difference did not remain significant after correcting for multiple comparisons (Table 2). Furthermore, changes in the absolute numbers of myeloid dendritic cells in the peripheral blood revealed no significant changes upon cholecalciferol treatment (supporting information Table S1).

In vitro exposure to cholecalciferol leads to increased percentage of Tregs

Adding 50 ng/ml cholecalciferol to human PBMC primary cultures revealed a trend toward an increase in the percentage of $CD4^{pos}$ Tregs (Fig. 3a) but with a stable proliferation (Fig. 3b). Additionally, the percentage and proliferation of cytotoxic $CD8^{pos}$ T cells was similar in relation to solvent exposure (Fig. 3c). Proliferation controls of cells stimulated by addition of anti-CD3/anti-CD28 beads confirmed vitality and proliferative capability of PBMC used in these experiments; 50 ng/ml is equivalent to a doubled dose relative to the achieved supplementation with cholecalciferol in vivo (140.000 IU/month). Initially, we had also tested 25 ng/ml in our cell cultures (data not shown), but since we did not observe any differences between 25 and 50 ng/ml we decided to use the higher concentration for our experiments to be sure that enough cholecalciferol would be available for the isolated immune cells.

We also tested for an in vitro effect of 25(OH)D (14 and 55 ng/ml) but found no differences compared to solvent exposure (data not shown).

Discussion

In this randomized, placebo controlled double-blind study in healthy human adults, we assessed the effects of a high-dose cholecalciferol supplementation on $CD4^{pos}$ regulatory T cells and on the peripheral frequency of different immune competent cells. An increase in serum 25(OH)D levels led to a significant increase in Tregs in the vitD group without changing the functional properties of Tregs or interfering with any other peripheral immune cells. Our in vivo findings were supported by an increase in the percentage of Tregs in vitro after an exposure to the inactive form of vitamin D (cholecalciferol) that did not cause any changes in cell proliferation and did not increase the percentage of cytotoxic T cells.

Recently, Toss et al. [26] reported that a daily supplementation of 40 microgram cholecalciferol (1,600 IU) is sufficient to obtain serum 25(OH)D levels above 50 nmol/l (20 ng/ml) in elderly subjects which is considered to be very safe and sufficient for beneficial effects on bone and mineral metabolism. Since the immunomodulatory effects of vitamin D are dependent on high local concentrations of the bioactive form $1,25(OH)_2D_3$ (calcitriol), we decided to supplement the inactive form of vitamin D (cholecalciferol) at very high doses (140,000 IU/month). We were able to achieve high local concentrations of 25(OH)D that are

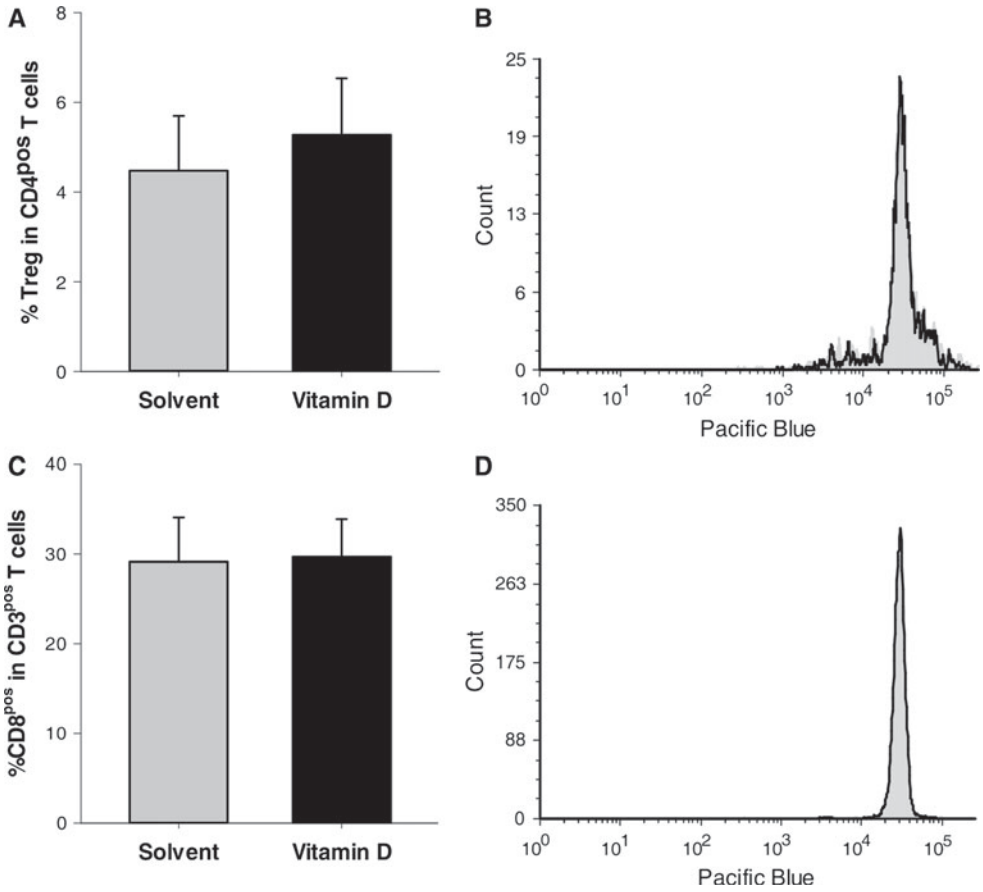

Fig. 3 In vitro exposure of human PBMC to cholecalciferol changes Tregs level. **a** Isolated PBMC from healthy donors were exposed to cholecalciferol (50 ng/ml) during 96 h of incubation and showed a non-significant trend to increased Tregs compared to cells exposed to solvent. **b** Proliferation of Tregs, measured by decrease in Pacific Blue proliferation dye signal, was not influenced by addition of cholecalciferol. **c**, **d** Neither Percentage nor proliferation of cytotoxic $CD8^{pos}$ T cells in a PBMC culture changed upon exposure to cholecalciferol compared to cells treated with solvent. All experiments were done in triplicates with cells from 4 independent healthy donors. Histograms show *curves* for solvent in *gray* and for cholecalciferol in *black*

necessary for immunomodulatory effects by 1α(OH)ase induction directly in immune cells without any clinical side effects and at the same time avoiding any limiting factors such as the short half-life of calcitriol (4–6 h). Safety parameter measurements throughout our trial are in line with previous studies that have reported no clinically relevant side effects such as hypercalcaemia or hypercalciuria after a high-dose cholecalciferol supplementation in patients with relapsing remitting multiple sclerosis [20].

Calcitriol has been shown to induce regulatory immune cells by modulating the maturation and tolerogenic properties of APC in vitro [17]. In cell cultures lacking the presence of APC, calcitriol also directly affects Tregs causing decreased proliferation while preserving the suppressive function and slightly increasing IL-10 production [27]. Furthermore, treatment of naive human T cells with calcitriol and IL-2 directly suppresses the production of proinflammatory cytokines while promoting high expression of FoxP3, CTLA-4 and IL-10 in cells with a regulatory phenotype [18]. In our in vitro experiments, we assessed the capacity of isolated healthy PBMC to use cholecalciferol for Tregs induction.

In vivo studies in animal models have already demonstrated that a vitamin D treatment can be associated with an increased number and a modified function of Tregs as well as a shift toward anti-inflammatory signaling [28–31], but our study is first to show a significant influence of a cholecalciferol supplementation on the percentage of peripheral Tregs in healthy humans. During 12 weeks of a high-dose oral cholecalciferol supplementation, serum 25(OH)D increased significantly to a level considered to reduce the incidence rates of type 1 diabetes, multiple sclerosis and rheumatoid arthritis [6, 32–34]. Large epidemiological studies have also shown clear links between insufficient 25(OH)D levels and a wide range of human autoimmune disorders, cardiovascular diseases and cancer [3]. In our study, the level of serum 25(OH)D was significantly correlated with the percentage of Tregs at baseline, and during the following 12-week trial, the observed increase in serum 25(OH)D was paralleled by a significant increase in the percentage of Tregs in the supplementation group. Similar to our results, an increase in the percentage of Tregs after a cholecalciferol supplementation has been recently reported in newly diagnosed type 1 diabetes patients, although the percentage of Tregs did not differ between treatment and placebo group after 12 months of supplementation [21]. Since both studies have used peripheral Tregs, it remains unclear whether changes in the percentage of Tregs from peripheral blood cells reflect respective changes in tissue cells.

In contrast to these findings, another study reports decreased peripheral Tregs levels associated with cholecalciferol treatment in HIV patients [27]. But Tregs have been reported to be a reservoir for viral replication, and vitamin D enhances the production of cathelicidin which exhibits anti-viral properties in human immune cells [35, 36]. Seasonally elevated serum 25(OH)D levels in the summer have been found to correlate with a decreased percentages of peripheral Tregs in healthy humans [22]. But the same study also found stable absolute numbers of Tregs and an increased expression of the transcription factor FoxP3 in the Tregs population. In patients with multiple sclerosis, serum 25(OH)D levels but not 1,25(OH)2D3 were positively correlated with Tregs suppressive functionality [37], indicating a role for the inactive form of vitamin D in immune regulation. In vivo treatment of healthy subjects and steroid resistant asthma patients with calcitriol showed an increase in responsiveness to dexamethasone treatment and IL-10 synthesis which is strongly connected to Tregs [38].

Inadequate numbers or a decreased function of Tregs have been shown to lead to autoimmunity in humans [39]. Cellular therapies in patients using Tregs have been suggested for the treatment of autoimmune diseases, transplantation and graft versus host diseases [13, 14], and in animal models, a high number of Tregs have already been shown to decrease the rejection of transplanted organs and to reduce the progression of autoimmune diseases [40].

Our study provides significant new support to show that sufficient 25(OH)D serum levels influence Tregs numbers which are particularly important during the treatment of autoimmune diseases. The capacity of a high-dose cholecalciferol supplementation to increase the number of peripheral $CD4^{pos}$ regulatory T cells without causing a loss of their suppressive functionality is relevant to clinical practice and indicates the potential of a vitamin D supplementation to support disease modulating therapies without safety concerns.

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Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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