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Oral vitamin D increases the frequencies of CD38⁺ human B cells and ameliorates IL-17 producing T cells

Running title: Vitamin D targeting lymphocytes

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Abstract:

Vitamin D deficiency (serum 25-hydroxyvitamin D <50 nmol/l) has been associated with the onset of immunologic diseases including atopic dermatitis (AD), cutaneous or systemic lupus erythematosus and allergic asthma. In the present study we assessed whether oral vitamin D (cholecalciferol) supplementation leads to a systemic modulation of the phenotype of circulating

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lymphocyte populations and whether a defined serum 25-hydroxyvitamin D (25(OH)D) concentration can be related to the effects on lymphocytes.

Cholecalciferol was administered in a dose-escalation setting to vitamin D deficient individuals from 2000 up to 8000 IU daily for 12 weeks. Individuals without cholecalciferol intake served as controls. Peripheral B cells and T cells were examined by multicolour flow-cytometric analysis.

The mean serum 25(OH)D concentrations increased upon cholecalciferol intake up to 159 ± 28.7 nmol/l, and remained low in the control group 30.0 ± 12.5 nmol/l. Following cholecalciferol intake, the frequencies of circulating CD38 expressing B cells were significantly increased and IFN- γ^+ and/or IL-17 $^+$ CD4 $^+$ T helper cells were decreased. These data were identified to correlate with the serum 25(OH)D levels by applying two different analysis approaches (ROC and a non-linear regression analysis).

Our data indicate that increasing 25(OH)D serum concentrations are associated with an increased expression of CD38 on B cells and a decreased T cell dependent proinflammatory cytokine production. The therapeutical role of our findings in systemic immunological diseases should be explored in the future by further controlled clinical studies.

Keywords: Vitamin D, B cells, T cells, supplementation, cytokines,

Introduction

Immunologic functions of vitamin D are suggested by the association of vitamin D deficiency with the onset of diseases which involve a deviated immune response like atopic dermatitis (AD) (1), cutaneous or systemic lupus erythematosus (2, 3), allergic asthma (4) and multiple sclerosis (5, 6). Vitamin D deficiency is considered when the serum concentration of the main circulating vitamin D form (chem. 25(OH)D) is below 50 nmol/l (7). This is primarily a result of inadequate

UV-exposition, but can be also attributed by an insufficient dietary intake (7, 8). The suggested serum 25(OH)D levels to achieve optimal vitamin D metabolism are ≥ 75 nmol/l (9). The bioactive metabolite of 25(OH)D (calcitriol; chem. 1,25-dihydroxyvitamin D) mediates immune functions by direct actions on immune cells (10, 11). Activated T lymphocytes express vitamin D receptors (VDR) and show in response to calcitriol a reduced proliferation and a decreased production of IL-2, IFN- γ , IL-23 but an increased production of IL-10 (12, 13). In B cells, which also express VDR upon activation, calcitriol induces CD38 and IL-10, but as shown by us previously inhibits IgE class switch recombination and secretion (14-16). Interestingly, certain mutations in the gene locus of the vitamin D receptor affect the VDR activity, Vitamin D status or are associated with diseases (17-19). Activated T - and B lymphocytes express the enzyme CYP27B1. This enzyme catalyzes the synthesis of calcitriol from its precursor. Therefore, upon activation and in the presence of sufficient amounts of 25(OH)D *in vitro* (15, 20) endogenous calcitriol synthesis can occur. However, the 25-hydroxyvitamin D serum concentration which is required to target T and B lymphocytes *in vivo* is not known.

Herein, we investigated circulating populations of B and T lymphocytes upon a defined vitamin D dose escalation during the UV-deprived winter months. Our data provide first evidence that vitamin D supplementation targets peripheral B lymphocytes and promotes a decrease of inflammatory cytokine secreting T lymphocyte frequencies. Based on our data we were able to calculate for the first time vitamin D concentrations in relation to immunomodulatory effects.

Material and Methods

Study Population

The participants of the study were between 18-60 years and were recruited from the Department of Dermatology and Allergy, Charité – Universitätsmedizin Berlin, Germany. The exclusion criteria included a positive history for sarcoidosis, hypercalcemia, creatinine serum concentration >1 mg/dl, nephrolithiasis, the presence of chronic diseases and a planned exposition to UV-light to countries at lower latitude (<40°). The study was approved by the ethics committee of the Charité - Universitätsmedizin Berlin. Written informed consent was obtained from all participants before the start of the study. The study has been registered at controlledtrials.com (ISRCTN65263333).

Study protocol, schedule of blood sampling and biochemical analysis

Individuals (vitamin D group, n=25) were screened in November 2008 and started in December 2008 with monthly increasing doses of 2.000 I.U., 4.000 I.U and 8.000 I.U cholecalciferol per day. Healthy individuals without vitamin D intake served as a control group (n=18). Compliance was monitored monthly by assessing the study medication and the consecutive measurements of the 25(OH)D serum concentrations. The blood samples for the serum analysis and the peripheral blood cell analysis were collected at the beginning of the study and at the time points as indicated in Fig. 1. Adverse events were documented at each visit. As the 25(OH)D serum concentration is more stable and displays a higher measurement accuracy than calcitriol and it was determined by using the 25(OH)-Vitamin D direct ELISA Kit (IBL, Hamburg, Germany) (21). The results are shown in nmol/l (to obtain ng/ml the multiplication with the conversion factor 2.5 is necessary). The serum immunoglobulin (Ig) M, -A, -G and -E concentrations were measured by ELISA (22) using matched goat anti-human IgM, IgA or IgG (all Jackson

Immunoresearch West Grove, USA) and anti-human IgE antibodies (Southern Biotech Birmingham, USA).

B cell isolation and activation *in vitro*

PBMC were isolated from buffy coats by density gradient separation (lymphocyte separation medium, PAA, Pasching, Austria). B cells were purified to >98% purity using anti-CD19-coupled magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified B cells (1×10^6 /ml) were activated with anti-CD40 mAb (1 µg/ml, clone 626.1, Santa Cruz, CA, USA), recombinant human IL-4 (10 ng/ml, Miltenyi), CpG2006 (3 µg/ml, TibMolbiol, Berlin, Germany) and goat-anti-human Ig-F(ab')₂ fragments (2 µg/ml, Jackson ImmunoResearch, Suffolk, UK). Cells were cultured 4 days in RPMI 1640 in the presence of 50-100 nmol/l 25(OH)D (Biomol, Hamburg, Germany) as described previously (14, 23).

Flow-cytometric analysis

CD19⁺ B cells and their subpopulations were stained from whole blood using fluorochrome-conjugated monoclonal antibodies and characterized according to the surface expression of CD19, CD23, CD27 (all from BD Pharmingen) and CD38 (Beckman Coulter, Krefeld, Germany). CD4⁺ T helper cells were stained accordingly with antibodies against CD45RA (Beckman Coulter) and CD4 (BD Pharmingen). Intracellular T cell cytokine production was analysed as described previously (24). Briefly, 1 ml whole blood was stimulated for 6 h with staphylococcus enterotoxin-B (1 µg/ml SEB; Sigma-Aldrich, St. Louis, MO, USA) and 1 µg/ml anti-CD28 (BD Pharmingen). Brefeldin A 2 µg/ml (Sigma-Aldrich) was added for the last 4 h. After erythrolysis and permeabilization (FACS-lysing solution and Perm 2 solution, BD Pharmingen) the cells were stained with antibodies against CD154, CD4, IL-4, IFN-γ, IL-10 (all

BD Pharmingen) and IL-17 (Ebioscience, San Diego, CA, USA). Flow cytometric analyses were performed using the FACSCalibur cytometer (BD Pharmingen). For *ex vivo* and *in vitro* analysis 35000 B cells and 100000 T cells were acquired. Data were assessed using FlowJo7 software (TreeStar, Ashland, OR, USA).

Statistical methods

Statistical evaluations were performed with GraphPad Prism 5 (GraphPad Software, Inc., CA, USA). Categorical variables were reported by using the number or percent of observations and data shown as box plots stretching from the interquartile range and the median indicated by a horizontal line. Normal distribution was tested by the Kolmogorov-Smirnov test and the D'Agostino and Pearson omnibus normality test. The data for serum 25(OH)D concentrations, the *ex vivo* CD38 and CD23 expression on B cells were analyzed with the two-way ANOVA and the Bonferroni multiple comparison test. All other parameters including the analysis of cytokine production in T cells were tested by the Student's t-test. P-values ≤ 0.05 were considered to be statistically significant. The association of CD38⁺ B cell, IFN- γ ⁺ or IL-17⁺ T helper cell frequencies and serum 25(OH)D concentrations was analysed by non-linear regression, receiver operating characteristic (ROC) and area under the curve (AUC) using SPSS17.0 (SPSS Inc., IBM, Armonk, NY, USA). The ROC analysis describes the relationship between the true positive (sensitivity) and the false positive (1 – specificity) rate for different cut-off values of serum 25(OH)D. The cut-off value showing the best discriminative performance is associated with highest true positive rate and lowest false positive rate (25). Δ CD38, Δ CD23, Δ IFN- γ ⁺, Δ IL-17⁺ were calculated either from the individual maximal frequency of CD38⁺ B cells ($\geq 80\%$ corresponding to average 75 percentile), IFN- γ and IL-17 producing T helper cells ($\leq 80\%$ and $\leq 55\%$, respectively of average 75 percentile) or in case of Δ CD38 individual baseline frequency

(+40%). Significant 25(OH)D concentrations for B and T lymphocytes targeting were estimated with AUC 0.73, 0.55, 0.680, 0.73 and 0.71 respectively.

Results

Oral vitamin D intake restores serum 25 (OH) vitamin D concentrations during the winter

Vitamin D deficient individuals received increasing dosages of cholecalciferol (Fig. 1) during the winter when solar UV radiation is insufficient for endogenous vitamin D synthesis. Prior to cholecalciferol intake, both study groups were comparable in terms of age, gender or baseline serum 25(OH)D concentrations (Table S1, Fig. 1). Upon a daily intake of 2000 I.U. cholecalciferol (50 µg) for 4 weeks the serum 25(OH)D concentrations increased to 83.4 ± 14.5 nmol/l. The continued daily intake of 4000 I.U. cholecalciferol (100 µg) led to serum 25(OH)D concentrations above 110 nmol/l in 15 individuals (148.3 ± 33.9 nmol/l, $P < 0.001$, Fig. 1) and the vitamin D intake was stopped according to the protocol. In the following 4 weeks 25(OH)D serum concentrations in this subgroup where vitamin D supplementation has stopped reached 96.1 ± 20.1 nmol/l ($P < 0.001$, Fig. 1). The individuals with 25(OH)D serum concentrations below 110 nmol/l (97.0 ± 16.9 , $n = 10$) after 4000 I.U. cholecalciferol intake proceeded with a daily intake of 8000 I. U. cholecalciferol (200 µg) for 4 more weeks. In this subgroup the serum 25(OH)D increased up to 159.7 ± 28.7 nmol/l ($P < 0.001$, Fig. 1). In parallel, the serum 25(OH)D continuously decreased from 49.1 ± 17.3 nmol/l to 30.0 ± 12.5 nmol/l ($P < 0.05$; Fig. 1) in the control group without exogenous vitamin D intake. No adverse event related to the vitamin D intake was reported at any time. The serum calcium levels remained stable throughout the study upon cholecalciferol intake (Fig. S1).

Increased frequencies of CD38⁺ peripheral B lymphocytes upon oral vitamin D

To investigate whether B lymphocytes are targeted by exogenous vitamin D peripheral B cell subsets cells were assessed at defined time points by flow cytometry upon oral vitamin D intake. The analysed B cell subsets included CD27⁺ memory B cells and CD27^{neg} naive B cells (26) but also CD38^{high} CD27^{high} antibody secreting cells (27) (Fig. S2a). CD38 surface expression was studied to identify possible vitamin D-responsive B cells as CD38 has been shown to be upregulated by activated VDR *in vitro* (15, 28). Indeed our data show increased frequencies of CD38⁺ B cells in the vitamin D supplemented group after 4 weeks of a daily intake of 2000 I.U. cholecalciferol in both subgroups, with continued intake by a trend (from 3.33% $\pm$ 1.37 to 4.01% $\pm$ 1.32) and also discontinued intake after 8 weeks (from 3.76% $\pm$ 1.54 to 5.29% $\pm$ 1.78, P<0.01). This observation was supported by the finding that an even stronger increase in the frequencies of CD38 expressing B cells occurred after 8 weeks following a daily dose of 4000 I.U. cholecalciferol in a subgroup with continued (to 4.71% $\pm$ 1.52; P<0.05; Fig. 2a) and discontinued intake after 8 weeks (to 4.97% $\pm$ 2.28; P>0.05; Fig. 2a). In the following 4 weeks, the increased CD38⁺ B cell frequencies remained stable until week 12 in a subgroup with continued cholecalciferol intake (to 4.71% $\pm$ 1.38; P>0.05; Fig. 2a) and discontinued (to 5.1% $\pm$ 2.23; P>0.05; Fig. 2a). By contrast, we did not find any changes in the frequencies of CD38⁺ B cells in the control group (mean 4.01% $\pm$ 1.46 to 3.59% $\pm$ 1.06; P>0.05; Fig. 2a). For a better comparison and to exclude an unspecific upregulation of CD38 we assessed in parallel in all samples the expression of CD23 throughout the study. Our data shows that by contrast to CD38, the frequencies of CD23 expressing B cells remained unchanged independent of the vitamin D intake throughout the study in both groups (mean 6.43% $\pm$ 3.36 to 6.27% $\pm$ 3.98; P>0.05; Fig. 2b). No significant differences in the frequencies of naïve, memory B cells as well as antibody

secreting cells and serum immunoglobulin concentrations (IgM, IgA, IgG) were observed upon vitamin D intake (Table S2, S3).

Calculation of vitamin D serum concentrations to target peripheral B lymphocytes

In order to calculate the 25(OH)D concentration in relation to the CD38 induction on B cells we correlated the 25(OH)D serum levels with the frequencies of CD38⁺ B cells at different time points. The serum 25(OH)D levels were plotted on the x-axis and the percentage of circulating CD38⁺ B cells were plotted on the y-axis. These values were normalized to the individual maximal frequency in order to reduce the interindividual variation.

By applying this method we identified a serum 25(OH)D concentration of 69 nmol/l (Fig. 3a) to be required for an increase in circulating CD38⁺ B cell frequencies. Independent from this approach we used additional ROC analysis for the calculation of the vitamin D threshold levels in relation to CD38 expression on B cells. By applying this method we were able to calculate a serum 25(OH)D concentration of 68 nmol/l which is associated with an increase in CD38 expressing B cell frequencies (AUC=0.73, CI=0.62-0.85; sensitivity=0.85, 1-specificity=0.38; Fig. 3b). Moreover, the ROC analysis was performed with Δ CD38 values normalized to the individual baseline frequency of CD38⁺ B cells (68 nmol/l 25(OH)D, AUC=0.71, CI=0.60-0.81; sensitivity=0.97, 1-specificity=0.5; data not shown). Using a similar approach, when the ROC analysis was performed with Δ CD23 values only a random association with the serum 25(OH)D level was detectable (AUC=0.55, CI=0.6-0.81; Fig. 3c) as an AUC of 0.5 specifies no discriminative value

To prove our *in vivo* finding that 25(OH)D enhances CD38 surface expression on B cells we used purified peripheral CD19⁺ B cells and stimulated these cells *in vitro* in the presence of

increasing amounts of 25(OH)D. Indeed our data show that the addition of 25(OH)D to activated B cells resulted in a dose dependent upregulation of CD38 (Fig. S2b, c).

Decrease of IFN- γ and IL-17 secreting T cells upon vitamin D supplementation

Next we assessed whether also T cells were modulated upon cholecalciferol intake. Therefore we measured the overall frequencies of CD45RA⁺ naïve and CD45RA^{neg} memory T helper cells. Their activation was determined by CD154 expression. In addition we assessed their cytokine profile by analyzing IFN- γ , IL-4, IL-10, but also IL-17 secreting T helper cells. We did not observe any changes of the T cell subsets (Table S2) and their activation based on CD154 expression (data not shown). In stimulated CD4⁺ T helper cells we observed a significant decrease of IFN- γ producing T cells in the vitamin D supplementation group (from 20.1% $\pm$ 7.0 to 16.8% $\pm$ 5.2; P<0.001; Fig. 4a), but not in the control group. Furthermore, we detected that the IL-17 producing T helper cells were reduced up to 40% in the vitamin D supplemented group (from 1.64% $\pm$ 0.64 to 1.01% $\pm$ 0.57; P<0.001; Fig. 4b, Fig. S3), but not in the control group. By contrast, neither the frequencies of IL-4 nor of IL-10 secreting T helper cells were changed during the supplementation period and were comparable among the study groups. (Fig. 4c, d). The decrease in the frequencies of IFN- γ and IL-17 producing T cells was comparable between the subgroups with or without discontinuation of the cholecalciferol intake after 8 weeks of supplementation (data not shown).

Again the dose-response analysis performed with percentages of IFN- γ ⁺ and IL-17⁺ T cells normalized to the individual maximal frequencies revealed serum 25(OH)D concentrations of $\geq$ 70 nmol/l to be related to reduced frequencies of IFN- γ (Fig. 4e) and IL-17 (Fig. 4f) producing T cells. Also the ROC analysis of serum 25(OH)D concentrations in relation to the decrease of

IFN- γ ⁺ and IL-17⁺ T cells identified values of 66 nmol/l for IFN- γ (66 nmol/l serum 25(OH)D, (AUC=0.68, CI=0.58-0.78; sensitivity=0.95, 1-specificity=0.53; Fig. 4g) and IL-17, (AUC=73, CI=0.63-0.82; sensitivity=0.96, 1-specificity=0.54 Fig. 4h).

Discussion

The present data from an oral vitamin D dose-escalation study show that human lymphocytes are targeted by the bioactive form of vitamin D *in vivo*. We determined first an increase in the frequencies of peripheral B cells expressing VDR-inducible surface antigen CD38. Moreover we detected lower frequencies of IFN- γ and IL-17 producing memory T cells upon vitamin D supplementation. Based on our findings we were moreover able to calculate 25(OH)D concentrations which are associated with immunological changes of peripheral B and T lymphocytes. These calculations including the application of two independent approaches were supported by *in vitro* data from peripheral B cells. After the activation of the B cells with increasing amounts of 25(OH)D again an induction of CD38 expression on B cells was observed. CD38 is a multifunctional protein with intra- and extracellular activity (29). Signaling via CD38 is suggested to protect B cells from apoptosis (30) and enhance BCR-induced responses (31). Extracellular function include adhesion to cells which express natural ligand (CD31) e.g. endothelial cells (32).

The analysis of the T cell phenotype including the measurements of well established vitamin D-controlled cytokines like IFN- γ and IL-17 indicated that a serum 25(OH)D concentration above 70 nmol/l (using dose-response curve and ROC analysis) is effective to target T cells. Similar vitamin D concentrations (86.5 and 78.0 nmol/l 25(OH)D) have been proposed in the literature previously for optimal VDR function inducing maximal metabolic activity, i.e. intestinal calcium

resorption (33) and parathyroid hormone regulation (34). However, we were not able to detect a linear dose-response of 25(OH)D on B and T cells. Therefore we suppose rather the requirement of a defined 25(OH)D concentration to mediate the biological effects. Such prerequisite for vitamin D action has been suggested by other groups previously as well (20, 34-36). Accordingly, the frequencies of CD38⁺ B cells and the T helper cell cytokine profile remained stable above 75 nmol/l serum 25(OH)D even after discontinuation of the cholecalciferol intake for 4 weeks.

The reduced frequencies of circulating pro-inflammatory IFN- γ and IL-17 secreting T helper cells upon vitamin D supplementation suggest a possible therapeutic potential of 25(OH)D in diseases with a deviated immune response like AD, as circulating IL-17 T helper cell frequencies were associated with severity of atopic dermatitis (37) but also suggested to play a critical role in the pathogenesis of autoimmune diseases (38, 39). Whether VDR binding mediates directly a negative regulation on the *IFN γ* (40) or *IL17* (41) promoter in T cells or whether an indirect modulation of T cells through generation of tolerogenic dendritic cells (42-44) is the underlying mechanism, will need to be determined in more detail in future studies. Here we did not observed any changes in the frequencies of IL-10 producing T helper cells upon vitamin D intake. Probably explained by exclusion of IL-10 from the functional T cell cytokine memory (45). Additionally, serum immunoglobulin concentrations were unaffected by vitamin D intake. This is in line with previous reports that specific antibody titers remain stable over years (46), most likely produced by long-lived plasma cells (47).

We excluded the onset of UV-induced endogenous vitamin D synthesis in our study as a source of 25(OH)D by performing this investigation during the winter months (48). Accordingly, the 25(OH)D concentrations declined in the control group, but also in the subgroup where vitamin D

intake has been stopped. These findings are in line with the literature (33, 49) and suggest the need of a UV-dose of 20 mJ/cm² to induce vitamin D production in skin (50) or vitamin D supplementation during the winter to maintain 25(OH)D levels during that time period.

As AD is a chronic disease, a safe long-term treatment is required and thus oral vitamin D, which was well tolerated within this study, may be a possible therapeutical option. Indeed recent data has indicated its potential beneficial role (51, 52). However, right now our study is limited by the group size. Only by larger controlled-prospective clinical studies in healthy, but also e.g. autoimmune patients the exact role of vitamin D on immune functions can be unraveled.

In conclusion, this study indicates that increased frequencies of CD38⁺ B cells and a decreased IFN γ and IL-17 T cell cytokine response is related to serum 25(OH)D concentrations $\geq$ 70 nmol/l. The data provide a molecular basis for future studies on vitamin D functions in humans and patients with a dysbalanced immune response.

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Author contributions

M. Worm and *G. Heine* were involved in the conception and design of the study. *G. Drozdenko* performed generation of data. The results were interpreted and discussed by all authors.

Conflict of Interest

The authors declare that they have no competing interests.

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Figure Legends

Figure 1: Schedule of analysis and 25(OH)D serum concentrations upon cholecalciferol intake. During UV-restricted winter months, increasing oral vitamin D dosages were given daily for 12 weeks. Individuals with daily vitamin D intake up to 8000 I.U. (n=10, dark grey boxes), up to 4000 I.U. (n=15, light gray boxes with vertical pattern) or without (control group, n=18, open boxes). * = $P < 0.05$, *** = $P < 0.001$

Figure 2: Increased frequencies of CD38⁺ cells upon vitamin D supplementation.

CD38 and CD23 expression were monitored *ex vivo* on B cells from both study groups. (a) CD38 expression over time in donors with daily vitamin D intake up to 8000 I.U. (n=10, dark grey boxes), up to 4000 I.U. (n=15, light gray boxes with vertical pattern) or control group (n=18, open boxes). (b) According to (a) but CD23 expressing B cells. Dot plots are gated on B lymphocytes. * indicates $P < 0.05$, ** = $P < 0.01$.

Figure 3: Calculation of 25(OH)D concentration to target B lymphocytes *in vivo*. (a) Serum 25(OH)D concentrations were plotted against the percentage of CD38⁺ B cells. (b) ROC curve analysis with 25(OH)D concentrations and Δ CD38 positive B cell frequencies as classification variable ($\geq 80\%$). (c) According to (b) but Δ CD23 as classification variable. The black arrow indicates the cut-off 25(OH)D concentration associated with lymphocyte modulation. The dashed line at 45° indicates no discriminative ability (AUC=0.5).

Figure 4: Decreased frequencies of INF γ and IL-17 producing T cells upon vitamin D intake. Intracellular cytokine expression was determined in stimulated peripheral CD4⁺ T cells

before and after 12 study weeks *ex vivo*. (a) Frequencies of IFN- γ ⁺ cells within vitamin D intake or control group. (b), (c), (d) According to (a) but frequencies of IL-17⁺, IL-4⁺ and IL-10⁺ cells. All data represent cytokine producing T cell frequencies in percent of CD4⁺CD154⁺ T cells. *** indicates P<0.001. (e) Dose-response curve. Percentage of IFN- γ ⁺ T cells which are normalized to individual maximal cytokine production frequency. The arrow points to the 25(OH)D level which is associated to decreased IFN- γ ⁺ T cells. (f) According to (e) but using the percentage of IL-17⁺ T cells. (g) ROC curve analysis, Δ IFN- γ as classification variable. (h) According to (g) but Δ IL-17 as classification variable. (g), (h) the black arrow indicates the cut-off vitamin D concentration associated with lymphocyte modulation. Dashed line at 45° indicates no discriminative ability (AUC=0.5).

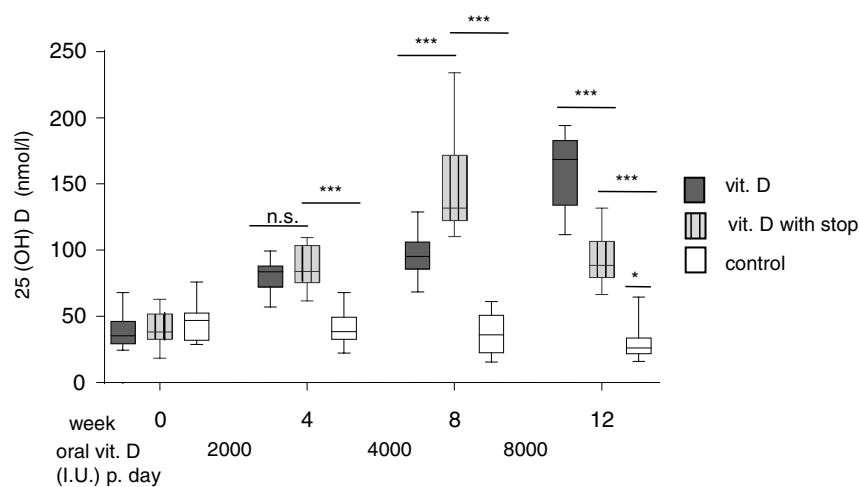


Figure 1

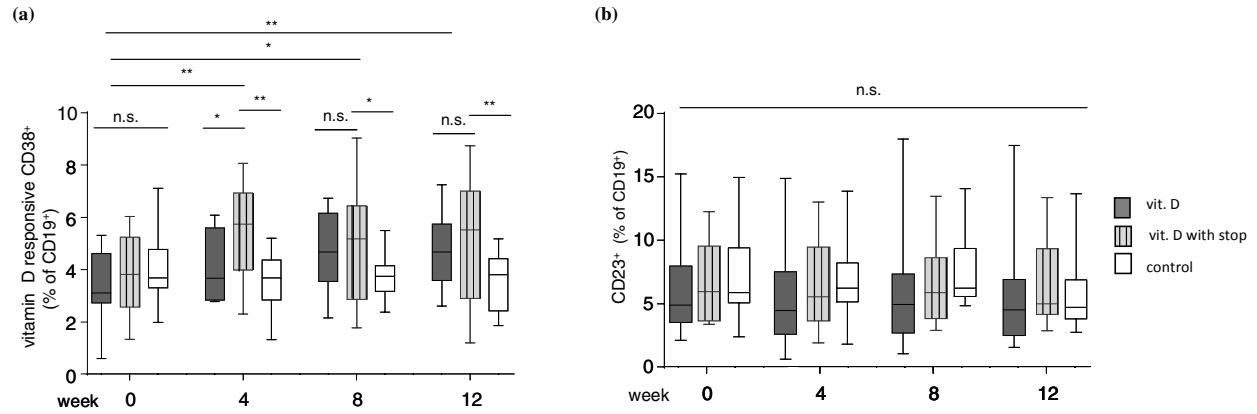


Figure 2

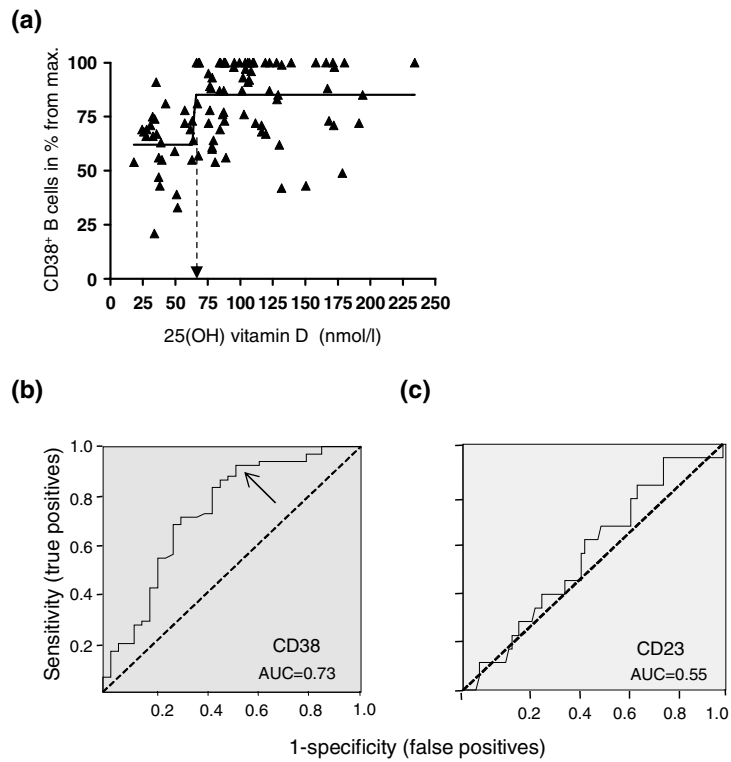


Figure 3

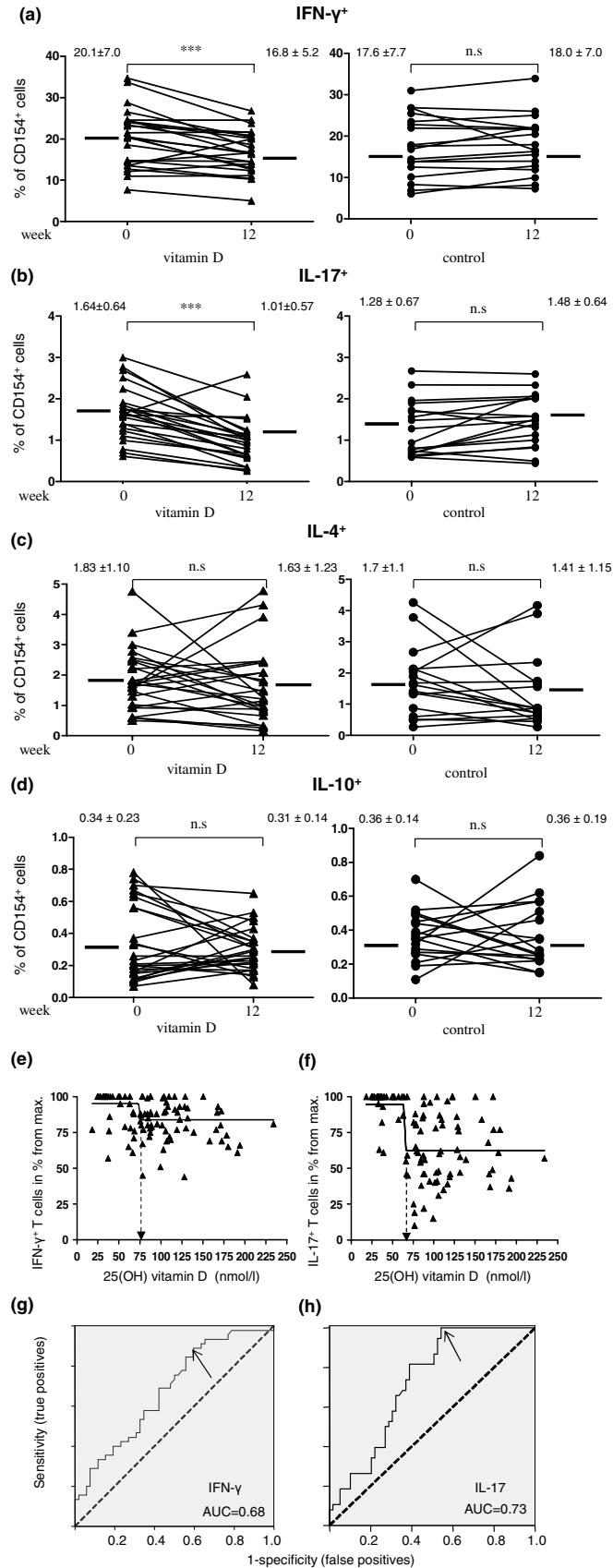


Figure 4