

Letter to the Editor

Vitamin D3 treatment of vitamin D-insufficient asthmatic patients does not alter immune cell function*To the Editor:*

Low vitamin D levels have been associated with increased asthma exacerbations and decreased lung function, as reviewed by Paul et al.¹ Studies have suggested that vitamin D might play a role in modulating the response to steroids in patients with steroid-resistant asthma.^{2,3} T cells, monocytes, and dendritic cells (DCs) express the vitamin D receptor (VDR), as well as 25-hydroxyvitamin-D₃-1 α -hydroxylase, which is required to convert 25(OH)-vitamin D to its active form, 1,25(OH)₂-vitamin D. Addition of exogenous vitamin D to activated T cells and myeloid DCs *in vitro* inhibits the expression of inflammatory cytokines and induces a tolerogenic phenotype.⁴

We performed an ancillary study as part of the Vitamin D Add-on Therapy Enhances Corticosteroid Responsiveness in Asthma (VIDA) trial (NCT01248065).⁵ In this trial vitamin D-insufficient asthmatic patients with persistent symptoms despite low-dose inhaled corticosteroids (ICSs) received vitamin D or placebo with inhaled ciclesonide for 12 weeks, at which time ICSs were tapered and numbers of exacerbations and treatment failures were determined. Peripheral blood was collected at the third visit (V3) before randomization and 12 weeks later at the sixth visit (V6) before the ICS taper.

Intracellular cytokine staining with fluorescently conjugated antibodies against IL-4, IFN- γ , IL-10, or IL-17A was performed on whole blood after stimulation with phorbol 12-myristate

13-acetate and ionomycin. Isolated PBMCs were also stimulated with α -CD3/CD28. Culture supernatants and plasma were assayed for IL-2, IL-4, IL-6, IL-10, IL-17, TNF- α , IFN- γ , and TGF- β . If there was a sufficient sample, CD14⁺ monocytes were isolated and stimulated with LPS, and IL-6, IL-10, and IL-12p40 levels were measured. PBMCs were also stained with mAbs to allow for phenotypic characterization of monocyte and DC populations.

Initial samples were collected at V3 from 100 participants, but samples were not available at the second time point for several participants (see Fig E1 in this article's Online Repository at www.jacionline.org). Only paired V3 and V6 samples were analyzed. The baseline characteristics of participants (see Table E1 in this article's Online Repository at www.jacionline.org) and baseline vitamin D levels were comparable (placebo, 19.4 ± 6.5 ng/mL; vitamin D, 19.3 ± 6.3 ng/mL). At V6, there was no change in vitamin D levels in the placebo group (18.9 ± 8.6 ng/mL), whereas the mean vitamin D level increased to 42.3 ± 10.9 ng/mL in those randomized to vitamin D ($P < .001$ compared with placebo). Ninety percent of the vitamin D-treated participants in the substudy achieved a 25(OH)-vitamin D level of 30 ng/mL or greater.

The fold change in the percentage of CD4⁺ T cells expressing IFN- γ , IL-10, or IL-17 from V3 to V6 was not different between vitamin D- or placebo-treated participants (Fig 1). Although there was a small decrease in the percentage of IL-4-secreting cells in the placebo group from V3 to V6 ($3.2\% \pm 1.6\%$ to $2.6\% \pm 1.2\%$, $P = .03$), there was no significant change in the ratio of T_H1 to T_H2 cells ($P = .55$, Fig 1 and see Fig E2 in this article's Online Repository at www.jacionline.org). Serum cytokine levels were also similar between groups (see Table E2 in this article's Online

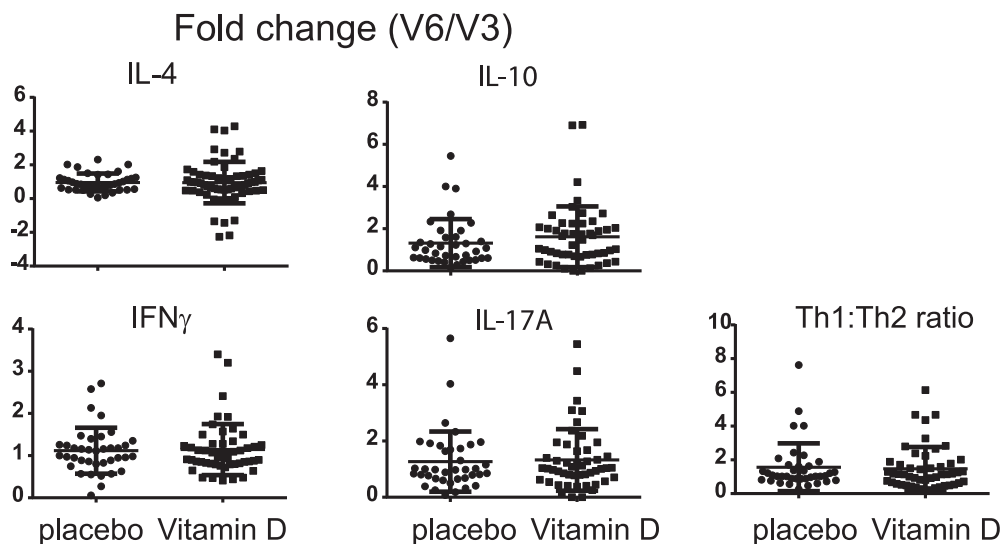


FIG 1. Intracellular cytokine analysis of CD4⁺ T cells. Peripheral blood samples were obtained before (V3) and after (V6) 12 weeks of treatment with placebo or vitamin D. Intracellular cytokine staining of CD4⁺ T cells was performed, and the percentage of CD4⁺ T cells expressing IL-4, IFN- γ , IL-10, or IL-17A was determined. Shown is the fold change from V3 to V6, which was calculated as the ratio of the V6 value divided by the V3 value for each sample pair. There was no difference in this ratio between vitamin D- and placebo-treated participants. The T_H1/T_H2 ratio, which was calculated by dividing the percentage of CD4/IFN- γ -positive by the percentage of CD4/IL-4-positive cells, was also not different. Shown are individual data points and lines representing means $\pm$ SDs.

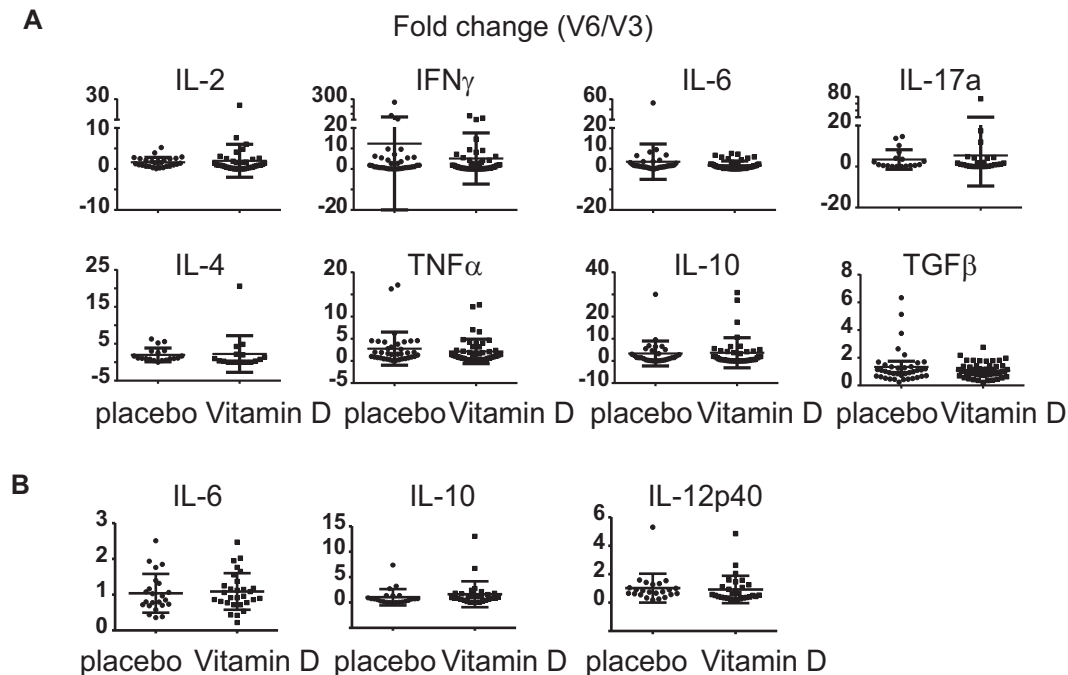


FIG 2. Cytokine secretion by stimulated PBMCs and monocytes. **A**, PBMCs were isolated from samples collected either before (V3) or after (V6) vitamin D supplementation or placebo and stimulated with α -CD3 and α -CD28 for 48 hours. Secreted cytokines were analyzed in the culture supernatant by using a cytokine bead array or ELISA (TGF- β only). The fold change from V3 to V6 was calculated by dividing the value at V6 by the value at V3 for each sample pair. **B**, CD14 $^{+}$ monocytes were isolated from PBMCs from vitamin D-deficient asthmatic patients before (V3) and after (V6) vitamin D supplementation or placebo and stimulated with LPS for 48 hours. IL-6, IL-10, and IL-12p40 production was determined by using an ELISA. The fold change from V6 to V3 (V6/V3) for each sample pair was calculated, and individual data points and lines representing means $\pm$ SDs are shown.

Repository at www.jacionline.org), as was cytokine secretion of PBMCs after *in vitro* stimulation with α -CD3/CD28 (Fig 2, A, and see Fig E3 in this article's Online Repository at www.jacionline.org). No significant differences were observed between groups in the production or fold change of IL-6, IL-10, or IL-12p40 in the culture supernatants of CD14 $^{+}$ monocytes stimulated with LPS (Fig 2, B, and see Fig E4 in this article's Online Repository at www.jacionline.org). Supplementation with vitamin D did not alter the percentage of monocytes or their subsets or expression of the activation marker CD40 or the chemokine receptor CX3CR1, which have been shown to correlate with an inflammatory phenotype (data not shown).^{6,7} In addition, no differences were detected in the percentage or ratio of peripheral blood plasmacytoid or myeloid DCs, cell-surface expression of HLA-DR, or fold change in HLA-DR expression in either treatment group (Figs E5 and E6 and data not shown). All unadjusted comparisons between the placebo- and vitamin D-treated participants were confirmed by using adjusted analysis of covariance results. In addition, analysis comparing only those participants who achieved vitamin D sufficiency with placebo yielded the same conclusions (data not shown).

In vitro data have suggested that CD4 $^{+}$ T cells from patients with steroid-resistant asthma might not augment IL-10 production in response to steroids and that exogenous vitamin D can reverse this defect.² Although we did not directly measure this, we found that *in vivo* treatment of vitamin D-insufficient asthmatic patients with vitamin D had no detectable effect on CD4 $^{+}$ T-cell cytokine profiles or the phenotype of monocytes or DCs.

The samples for this study were obtained as part of a clinical trial that examined whether treatment with vitamin D in symptomatic, vitamin D-insufficient asthmatic patients would provide a steroid-sparing effect.⁵ In that trial vitamin D supplementation did not decrease the rate of first treatment failure or exacerbation, although when only those who achieved vitamin D sufficiency were analyzed, vitamin D supplementation was associated with a decrease in treatment failures and exacerbations. However, analysis of this subgroup in our assays did not alter our findings (data not shown).

In contrast to *in vitro* studies with exogenous vitamin D exposure, we found no detectable effect when vitamin D-insufficient subjects were supplemented *in vivo*. There are several important limitations to these data. Vitamin D3 was used in this trial, which must be converted to its active form, whereas most *in vitro* studies use the active form, 1,25(OH) $_2$ -vitamin D3. Vitamin D levels are affected by the serum concentration of vitamin D-binding protein, which differs in African American and white populations, although vitamin D bioavailable levels do not appear to differ.⁸ Cellular expression of VDR and 1 α -hydroxylase can differ between subjects and influence the cellular level of the active compound. It is possible that other aspects of immune or structural lung cell function, such as the generation of regulatory T cells or of the anti-bacterial cathelicidin might have been altered by vitamin D supplementation.^{4,9} We cannot exclude the possibility that treatment for longer periods of time or with calcitriol or to higher serum vitamin D levels might have yielded different results.

Our data suggest that *in vivo* supplementation with vitamin D in vitamin D-insufficient asthmatic patients has no effect on T-cell,

monocyte, or DC function. The role for vitamin D supplementation as an adjunctive immunomodulatory therapy remains to be established.

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METHODS

Participants

Participants who consented to the parent VIDA trial and who met the eligibility criteria were invited to participate in this substudy. Informed consent was obtained, and peripheral blood was collected at the third parent study visit (V3) before randomization and again 12 weeks later at the sixth parent study visit (V6) before beginning the ICS taper. Samples were shipped overnight in insulated packaging to the research laboratories at Brigham and Women's Hospital (Boston, Mass; Dr Castro) or Washington University in St Louis (St Louis, Mo; Dr Green). Institutional review board approval was obtained from each participating institution. This trial was registered with clinicaltrials.gov as part of the parent trial (NCT01248065).

Analysis of CD4 T-cell function

Intracellular cytokine staining was performed by using the FastImmune protocol for staining of samples of whole blood (BD Biosciences, San Jose, Calif). Briefly, 500- μ L aliquots of whole blood were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 μ g/mL) in the presence of brefeldin A (10 μ g/mL) for 5 hours at 37°C. Samples were subsequently stained with allophycocyanin (APC)-conjugated α -CD4 followed by hypotonic RBC lysis, fixed, and permeabilized and then stained with fluorescently conjugated antibodies against either IL-4, IFN- γ , IL-10, or IL-17A and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, Calif). Data were analyzed with WinList version 7 software (Verity Software Corporation, Topsham, Me).

The gating strategy for CD4⁺ T-cell analysis was as follows. Live cells were forward versus side scatter gated and then gated on the CD4⁺ subset. Percentages of live CD4⁺ cells were then analyzed to determine the percentage of cells expressing each intracellular cytokine. Isotype controls were used to determine the background level, and gates were set to exclude negative cells.

Plasma was collected by means of centrifugation of heparinized blood and stored at -80°C for later analysis and for use in *in vitro* cultures. PBMCs were isolated by means of density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare, Pittsburgh, Pa), and an aliquot was reserved for flow cytometry. PBMCs were cultured at $5 \times 10^5/\text{mL}$ in complete media containing 10% autologous serum, and replicate cultures were either unstimulated or stimulated with α -CD3 (1 μ g/mL) and α -CD28 (1 μ g/mL) for 48 hours. Plasma and culture supernatants were assayed for IL-2, IL-4, IL-6, IL-10, IL-17, TNF- α , and IFN- γ by using a cytokine bead array, according to the manufacturer's instructions (BD Biosciences). TGF- β levels were assayed

by mean of ELISA (eBioscience, San Diego, Calif) after acidification of the sample.

Monocyte isolation and *in vitro* stimulation

PBMCs and autologous serum for culture were isolated, as described above, and an aliquot of cells was reserved for flow cytometry. If there was a sufficient sample, monocytes were isolated from PBMCs by using CD14 magnetic MicroBeads (Miltenyi Biotec, San Diego, Calif) per the manufacturer's instructions. Monocytes were cultured at $5 \times 10^5/\text{mL}$ in RPMI 1640 containing 20% autologous serum, L-glutamine, and penicillin-streptomycin and stimulated with 10 ng/mL LPS (*Escherichia coli* 011:B4; Sigma, St Louis, MO) for 48 hours. Supernatants were stored at -80°C until analysis. IL-6, IL-10, and IL-12p40 levels were determined by means of ELISA (eBioscience).

Monocyte and DC flow cytometry

PBMCs were stained with the following directly conjugated fluorescent mAbs to allow for phenotypic characterization of monocyte and DC populations: anti-CD14-fluorescein isothiocyanate (FITC), anti-CD1a-phycoerythrin (R-PE), anti-CX3CR1-PerCP-eFluor710, anti-CD40-APC, anti-CD16-PE-Cy7, anti-CD11c-PerCP-Cy5.5, anti-CD1c-APC, and anti-CD123-PE-Cy7 and anti-HLA-DR-APC-Cy7, Lin1 (CD3, CD14, CD16, CD19, CD20, and CD56)-FITC, and appropriate fluorescently conjugated isotype controls (eBioscience). Data were acquired on a FACSCanto II (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, Ore). Myeloid (CD11c⁺HLA-DR⁺) and plasmacytoid (CD123⁺HLA-DR⁺) DCs were identified in the lineage (Lin1)[−]HLA-DR⁺ PBMC population after live cell gating.

Statistical analysis

Within-subject comparison of all outcomes from V3 to V6 was performed by using 2-tailed paired *t* tests. Between-subject comparison of the placebo-versus vitamin D-treated participants was performed with the Mann-Whitney *U* test for nonparametric data (GraphPad Prism v6; GraphPad Software, San Diego, Calif). In addition, the change in each study outcome from V3 to V6 was also compared between placebo- and vitamin D-treated participants through an analysis of covariance model with adjustment for center, body mass index, and race (SAS v9.3; SAS Institute, Research Triangle Park, NC). The unadjusted and adjusted analyses were then repeated to compare between those vitamin D-treated participants who reached vitamin D sufficiency (25[OH] vitamin D, ≥ 30 ng/mL) after 12 weeks of treatment versus the placebo group.

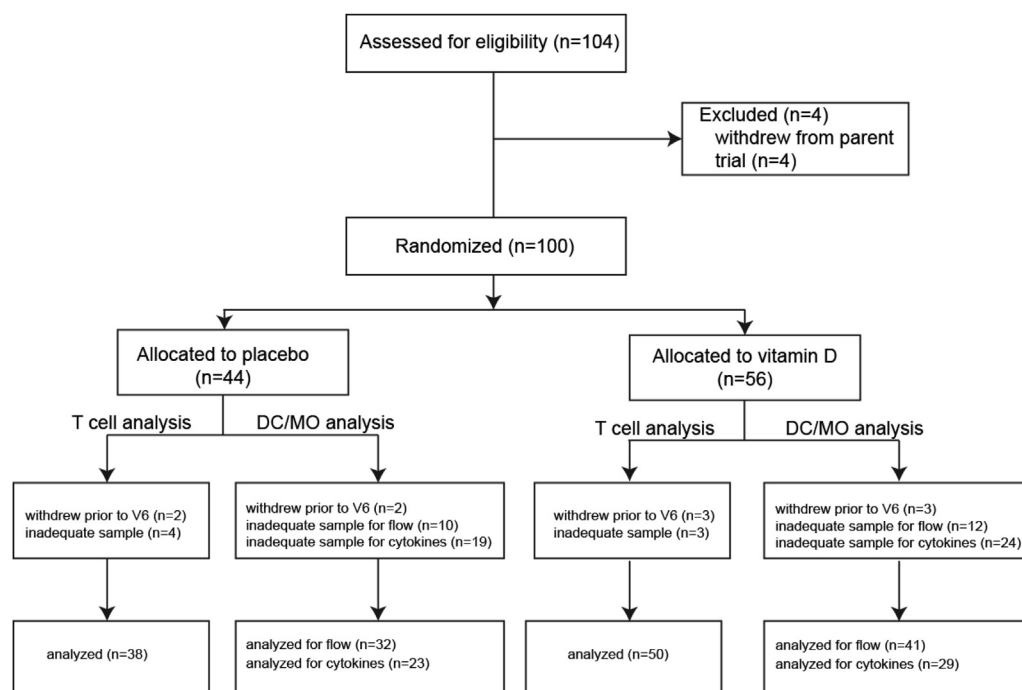


FIG E1. Participant flow. Participants in the VIDA trial were approached for optional enrollment in this substudy. Samples from the first 100 eligible participants consenting to the study were collected at each participating site and shipped to the research laboratories at Washington University (T-cell studies) or Brigham and Women's Hospital (myeloid cell studies). Because the sample requirements were different between laboratories, a sample might have been usable at one site but not the other, accounting for the different numbers available for final analysis.

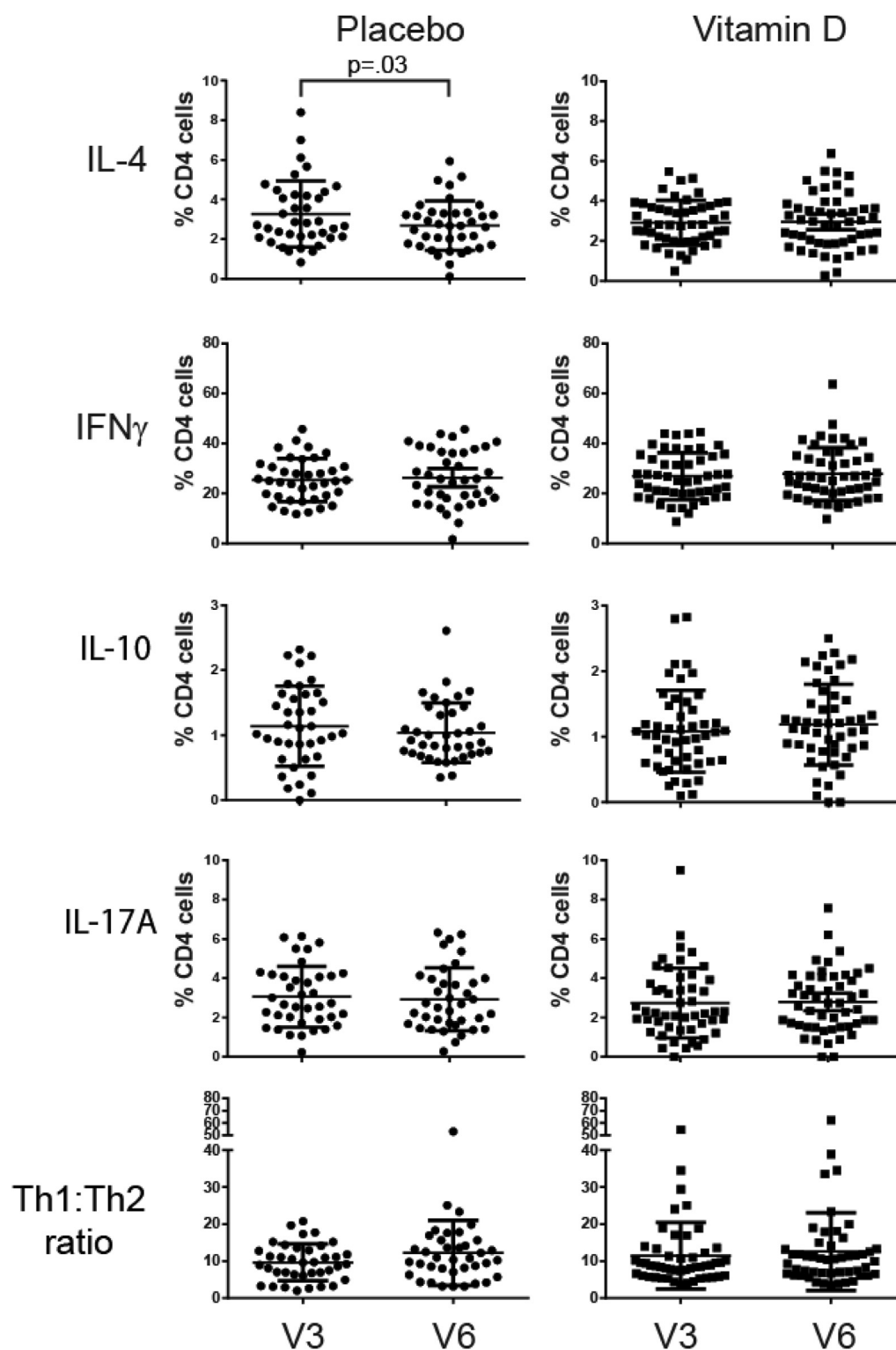


FIG E2. Intracellular cytokine analysis of CD4⁺ T cells. Shown are individual data points for intracellular cytokine staining of CD4⁺ T cells at V3 and V6, which were used to generate the fold change data presented in Fig 1. Samples were obtained before (V3) and after (V6) 12 weeks of treatment with placebo or vitamin D as described for Fig 1 in the body of the article. There was no effect of vitamin D repletion on the percentage of CD4⁺ T cells expressing IL-4, IFN- γ , IL-10, or IL-17A. There was a small but statistically significant decrease in the percentage of IL-4-secreting cells in the placebo-treated group ($P = .03$) but no change in the ratio of Th1 to Th2 cells and no difference in the fold change of IL-4-secreting cells from V6 to V3 (see Fig 1). The Th1/Th2 ratio was calculated by dividing the percentage of CD4/IFN- γ -positive by the percentage of CD4/IL-4-positive cells. Data shown are individual sample values, as well as means $\pm$ SDs for the group.

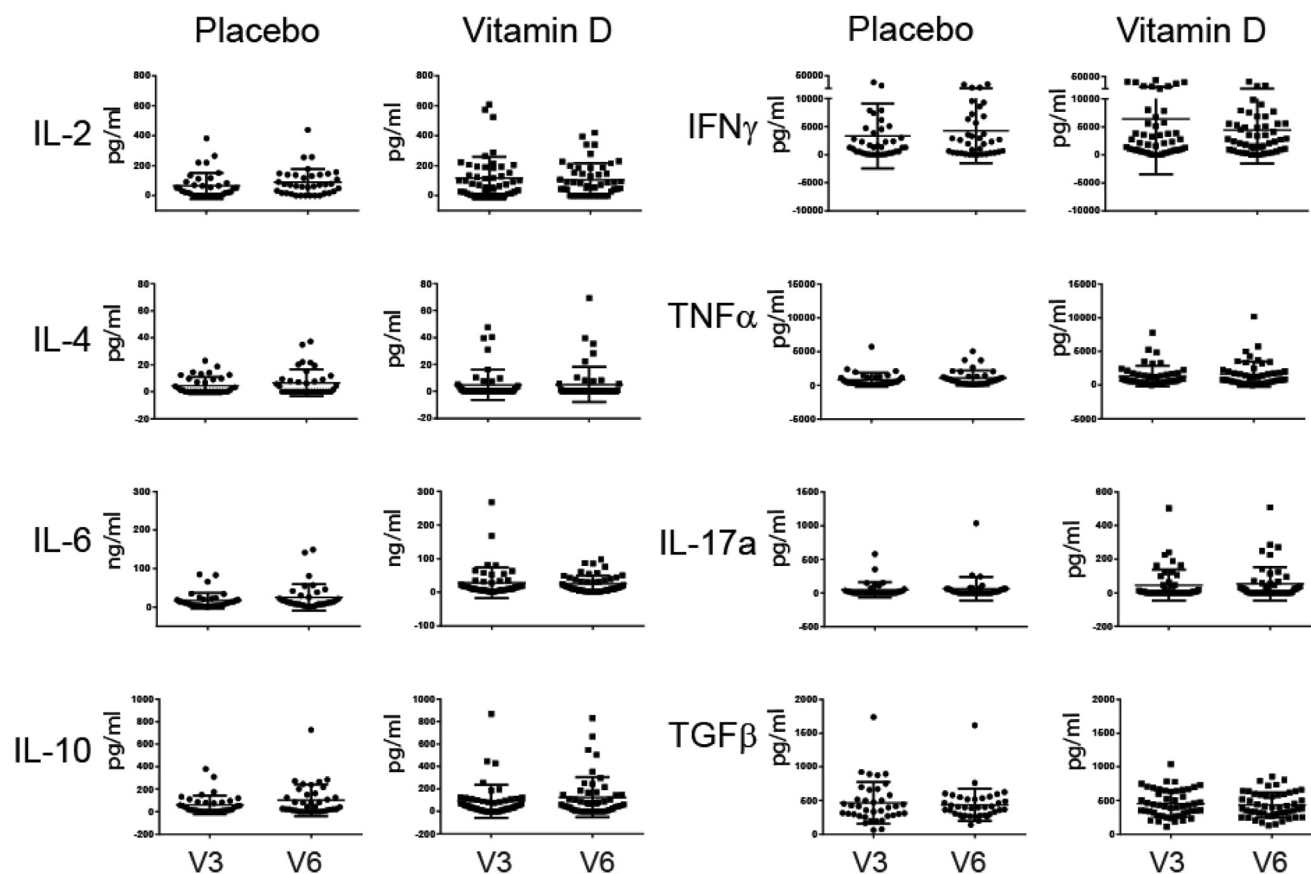


FIG E3. Cytokine secretion of stimulated PBMCs. Shown are individual values used to determine the fold change in cytokine secretion from V3 to V6 in Fig 2, A. PBMCs were isolated from samples collected either before (V3) or after (V6) vitamin D supplementation or placebo and stimulated with α -CD3 and α -CD28 for 48 hours. Secreted cytokines were analyzed in the culture supernatant by using a cytokine bead array or ELISA (TGF- β only). Data shown are individual sample values, as well as means $\pm$ SDs for the group.

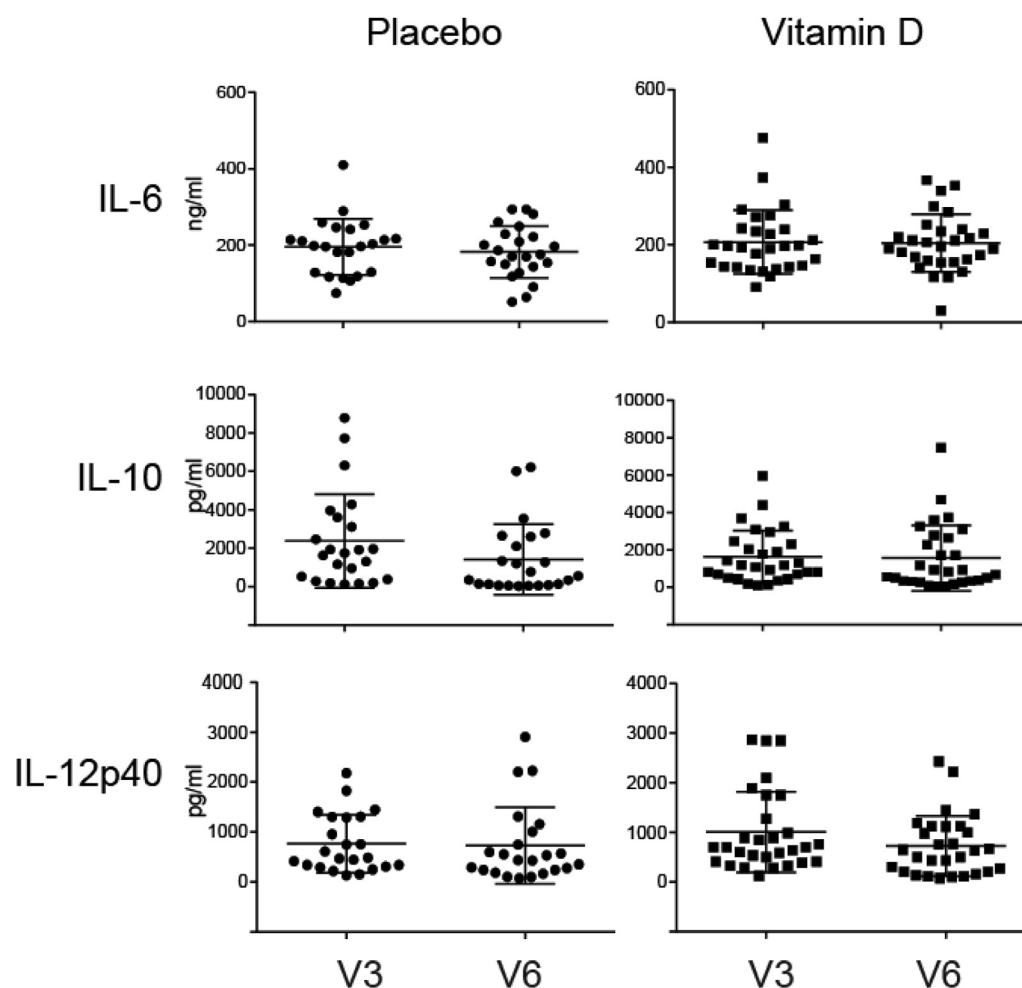


FIG E4. Monocyte cytokine production is not altered by vitamin D supplementation. Shown are individual values used to determine the fold change in cytokine secretion from monocytes shown in [Fig 2, B](#). CD14⁺ monocytes were isolated from PBMCs from vitamin D-deficient asthmatic patients before (V3) and after (V6) vitamin D supplementation or placebo and stimulated with LPS for 48 hours. IL-6, IL-10, and IL-12p40 production was determined by means of ELISA. Data shown are individual sample values, as well as means $\pm$ SDs for the group.

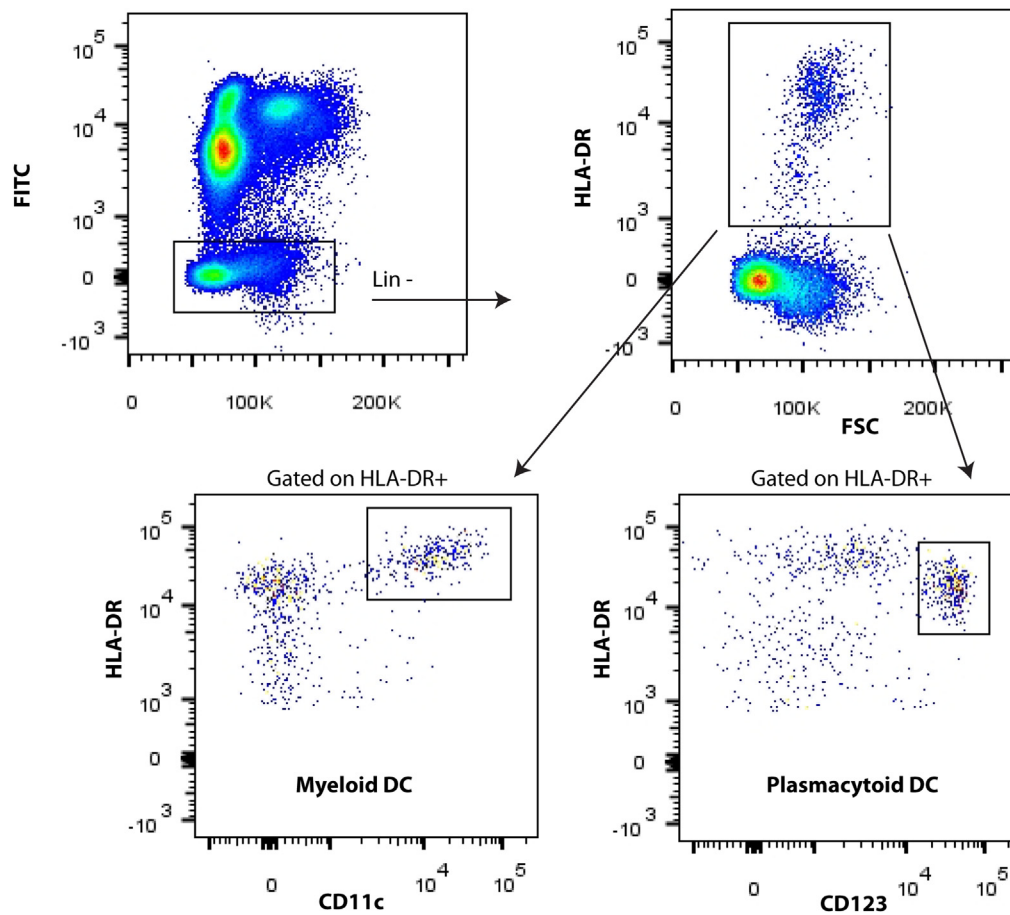


FIG E5. Gating strategy for DC subsets. Live PBMCs were forward versus side scatter gated. We gated on the lineage Lin¹ (CD3, CD14, CD16, CD19, CD20, and CD56)–FITC negative population (Lin⁻). Next, We then gated on the HLA-DR positive population. Myeloid DCs were identified by plotting HLA-DR versus CD11c. The myeloid DC population was identified as the Lin⁻HLA-DR⁺CD11c⁺ population. Plasmacytoid DCs were identified by plotting HLA-DR versus CD123. Plasmacytoid DCs were identified as the Lin⁻HLA-DR⁺CD123⁺ population. Isotype controls were used to determine background levels. *Rectangular gates* indicate selected population. Please see representative plots below.

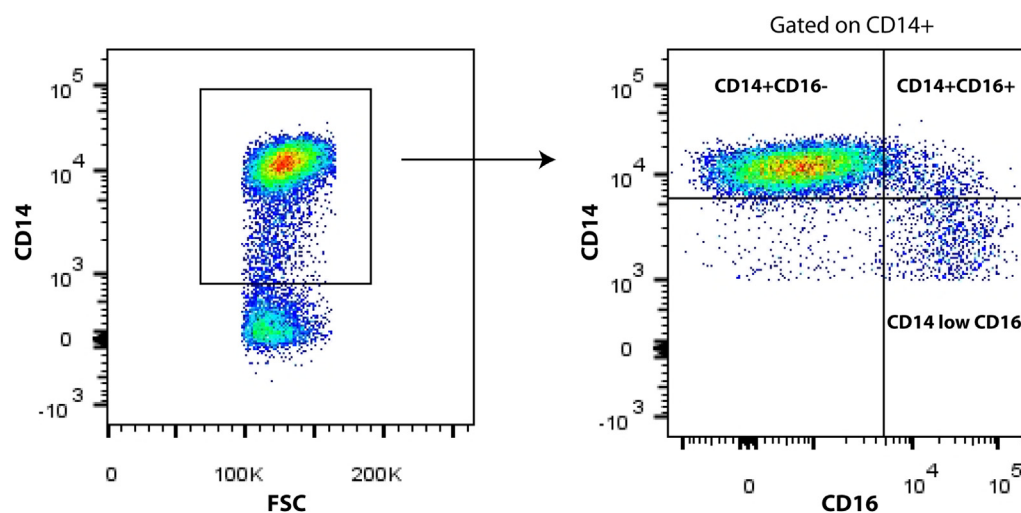


FIG E6. Gating strategy for monocyte subsets. Live PBMCs were gated on forward versus side scatter. We then gated on the CD14⁺ population (monocytes). The 3 human peripheral blood monocyte subsets (CD14⁺CD16⁻, CD14⁺CD16⁺, and CD14^{low}CD16⁺) were identified by quadrants on the CD14 versus CD16 plot. Isotype controls were used to determine background levels. See representative plots below. *Rectangular gates* indicate the selected population.

TABLE E1. Baseline characteristics of randomized participants

	Placebo (n = 44)	Vitamin D (n = 56)
Age (y)	42.1 ± 13.4	44.0 ± 11.6
Sex	15 (33%) male/29 (67%) female	23 (41%) male/33 (59%) female
Race/ethnicity		
White	23 (52%)	28 (50%)
Black	12 (27%)	16 (28%)
AI/AN	0 (0%)	1 (2%)
Asian/PI	2 (4%)	2 (4%)
Hispanic	7 (16%)	7 (12%)
Other	0 (0%)	2 (4%)
Asthma (y)	25.8 ± 13.3	28.1 ± 13.8
Body mass index (kg/m ²)	34 ± 10.9	33.4 ± 7.9
Vitamin D level at entry (ng/mL)	19.4 ± 6.5	19.3 ± 6.3

Where appropriate, values shown are means ± SDs.

TABLE E2. Serum cytokine levels

	Placebo		Vitamin D	
	V3	V6	V3	V6
IL-2	1.42 ± 2.2	1.47 ± 2.2	1.03 ± 2.1	1.06 ± 2.0
IL-4	0.73 ± 1.0	0.77 ± 1.34	0.52 ± 1.0	0.46 ± 0.8
IL-6	1.36 ± 1.9	1.87 ± 4.8	1.36 ± 2.1	0.95 ± 1.5
IL-10	0.67 ± 1.0	0.77 ± 1.26	0.39 ± 0.9	0.43 ± 0.9
TNF-α	0.27 ± 0.7	0.26 ± 0.7	0.19 ± 0.6	0.18 ± 0.6
IFN-γ	0.62 ± 1.2	0.50 ± 1.0	0.33 ± 0.9	0.23 ± 0.68
IL-17A	ND	ND	ND	ND
TGF-β	13,001 ± 4,490	13,175 ± 4,085	13,183 ± 3,829	12,917 ± 4,458

Serum was collected before (V3) and after (V6) 12 weeks of treatment with vitamin D or placebo. Cytokine levels were measured by using a cytokine bead array or ELISA (TGF-β only). Presented are means ± SD. All results shown are in picograms per milliliter.

ND, None detected.