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1,25(OH)₂ vitamin D₃ promotes *FOXP3* expression via binding to vitamin D response elements in its conserved non-coding sequence region

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Abstract

Forkhead box P3 (FOXP3)-positive regulatory T cells (Treg) are a unique subset of T cells with immune regulatory properties. Treg cells can be induced from non-Treg CD4⁺ T cells (induced Treg, iTreg) by T cell receptor (TCR) triggering, IL-2 and TGF- β or retinoic acid. 1,25(OH)₂ vitamin D₃ (VD₃) affects the functions of immune cells including T cells. 1,25(OH)₂VD₃ binds the nuclear vitamin D receptor (VDR) that binds target DNA sequences known as the vitamin D response element (VDRE). Although 1,25(OH)₂VD₃ can promote FOXP3 expression in CD4⁺ T cells with TCR triggering and IL-2, it is unknown whether this effect of 1,25(OH)₂VD₃ is mediated through direct binding of VDR to the *FOXP3* gene without involving other molecules. Also, it is unclear whether FOXP3 expression in 1,25(OH)₂VD₃-induced Treg (VD-iTreg) cells is critical for the inhibitory function of these cells. Here we demonstrated the presence of VDREs in the intronic conserved non-coding sequence (CNS) region +1714 to +2554 of the human *FOXP3* gene and the enhancement of the *FOXP3* promoter activity by such VDREs in response to 1,25(OH)₂VD₃. In addition, VD-iTreg cells suppressed the proliferation of target CD4⁺ T cells and this activity was dependent on FOXP3 expression. These findings suggest that 1,25(OH)₂VD₃ can affect human immune responses by regulating FOXP3 expression in CD4⁺ T cells through

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direct VDR binding to the *FOXP3* gene which is essential for inhibitory function of VD-iTreg cells.

Introduction

CD4⁺ T cells that express the transcriptional factor forkhead box P3 (FOXP3) are regulatory T (Treg) cells with the capacity to regulate immune responses (1). In addition to FOXP3, Treg cells express high levels of CD25 that serves as a cell surface marker for the identification of this cell subset (2, 3). FOXP3 is essential for the immune regulatory function of Treg cells. Transfection of FOXP3 into CD25⁻ CD4⁺ T cells, which do not normally have regulatory function, conferred the immune regulatory property (4–6). Furthermore, mutations in the *foxp3* gene have been found in scurfy mice with X-linked lymphoproliferative disease as well as in humans with immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome (IPEX) (7, 8). While FOXP3⁺ Treg cells are normally generated in the thymus (naturally occurring Treg, nTreg), FOXP3⁺ Treg cells can also be induced from non-Treg CD4⁺ T cells (induced Treg, iTreg) in the presence of anti-CD3/CD28 Abs, IL-2 and TGF-β or retinoic acid (9–18). However, it has been controversial whether iTreg cells have inhibitory function in humans (9–14).

1,25-dihydroxyvitamin D₃ (1,25(OH)₂VD₃), the most physiologically active VD₃ metabolite, exerts an inhibitory effect on immune cells including T cells (15). Of interest, low levels of circulating 25(OH)VD₃ (precursor of 1,25(OH)₂VD₃) are found in patients with autoimmune diseases including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (16–20), suggesting a potential role for this vitamin in autoimmunity. In fact, increased generation of FOXP3⁺CD4⁺ T cells was reported in mice treated with oral or topical 1,25(OH)₂VD₃ (21–23) although the exact mechanism(s) for this finding is yet to be determined. In humans, an increased percentage of FOXP3⁺CD4⁺ T cells was noticed in peripheral blood mononuclear cells (PBMCs) treated with 1,25(OH)₂VD₃ *in vitro* (24). Also, 1,25(OH)₂VD₃ enhanced FOXP3 expression in purified human CD25⁻CD4⁺ T cells in the presence of anti-CD3/CD28 antibodies (Abs) and IL-2 (25).

1,25(OH)₂VD₃ activates the vitamin D receptor (VDR) that heterodimerizes with retinoid X receptor (RXR) (15). The heterodimer then binds to its cognate DNA sequence known as vitamin D response elements (VDREs), leading to the regulation of gene expression. VDREs have two copies of a hexameric DNA sequence, referred to as core binding motif, with the consensus sequence RGKTS_A (R = A or G, K = G or T, and S = C or G) (26). VDREs can be formed by a direct repeat (DR) of two hexameric core binding motifs with three (DR3 type, e.g. 5' AGGTCA-NNN-AGGTCA 3') or four (DR4) intervening nucleotides (26). Also, VDR can bind to everted repeat (ER or inverted palindrome)-type responding elements with 6, 8 or 9 spacing nucleotides (e.g. ER9 AGGTCA-(N)9-TGACCT) (26).

Despite the enhanced FOXP3 expression in CD4⁺ T cells by 1,25(OH)₂VD₃ and the capacity of this vitamin to regulate gene expression via direct binding to the genes, it is unknown whether 1,25(OH)₂VD₃ can directly induce FOXP3 gene expression without involving other molecules. In addition, it is unclear whether FOXP3 expressed in 1,25(OH)₂VD₃-treated CD25⁻ CD4⁺ T cells is critical for the suppression of target T cells in that this vitamin can affect various functions of T cells such as cytokine production (15). Here we demonstrated the presence of VDREs in a conserved non-coding sequence (CNS) region that is analogous to the enhancer 1 of the mouse *foxp3* gene (27). Such VDREs enhanced the *FOXP3* promoter activity in the presence of 1,25(OH)₂VD₃. In addition, FOXP3⁺CD4⁺ T cells generated by a combination of 1,25(OH)₂VD₃, anti-CD3/CD28 Abs and IL-2 suppressed the proliferation of target CD4⁺ T cells dependent on FOXP3 and cell

contact. These findings suggest that $1,25(\text{OH})_2\text{VD}_3$ can affect human immune responses by regulating FOXP3 expression in CD4^+ T cells through direct VDR binding to the *FOXP3* gene which is essential for inhibitory function of $1,25(\text{OH})_2\text{VD}_3$ -induced FOXP3⁺ Treg (VD-iTreg) cells.

Materials and Methods

Cell preparation, culture and FOXP3 knockdown

This work was approved by the institutional review committee of Yale University. Human peripheral blood was obtained from the N.Y. Blood Center or healthy adult donors with informed consent. $\text{CD25}^- \text{CD4}^+$ T cells (>95% purity) were isolated from peripheral blood mononuclear cells using a cell purification kit (Miltenyi Biotec, Auburn, CA) or a FACSAria[®] (BD Bioscience, San Jose, CA). For induction of FOXP3 and T cell suppression assay, purified $\text{CD25}^- \text{CD4}^+$ T cells were suspended in RPMI 1640 medium with 10% FCS and stimulated for 5 days with $1,25(\text{OH})_2\text{VD}_3$ (10 nM or indicated, Sigma-Aldrich, St. Louis, MO), IL-2 (25 ng/ml, R&D Systems, Minneapolis, MN), anti-CD3 (4 $\mu\text{g}/\text{ml}$, OKT-3, National Cell Culture Center, Minneapolis, MN) and -CD28 Abs (3 $\mu\text{g}/\text{ml}$, BD Pharmingen, San Diego, CA). Cells (VD-iTreg cells) were then rested for 2 days and incubated for 5 additional days with autologous $\text{CD25}^- \text{CD4}^+$ T cells (target cells) that were stained with carboxy fluorescein diacetate succinimide ester (CFSE, Invitrogen, Carlsbad, CA) in a transwell culture system separating the two cell population or a regular culture plate in the presence of anti-CD3/CD28 Abs. To knock down FOXP3 expression, $\text{CD25}^- \text{CD4}^+$ T cells that were stimulated for 3 days with $1,25(\text{OH})_2\text{VD}_3$, anti-CD3/CD28 Abs and IL-2 were transfected with control or FOXP3-specific siRNA using the Amaxa[®] transfection system (Lonza, Walkersville, MD). Transfected cells were co-cultured for 5 days with CFSE-stained autologous $\text{CD25}^- \text{CD4}^+$ T cells in the presence of anti-CD3/CD28 Abs. Some transfected cells were stained with anti-human FOXP3 Abs (BioLegend, San Diego, CA) and analyzed on a flow cytometer. Target T cell proliferation was analyzed by flow cytometry.

Pull-down assay and immunoblotting

Biotinylated DNA fragments containing putative VDRE1 or VDRE2/3 in the intronic CNS region +1714 – +2554 of the *FOXP3* gene were amplified by PCR using sense (5'-biotin CTCCATATGTGGGTCCATGT-3' for VDRE1; 5'-biotin TTTGCTGATTGTTGCTTTGC-3' for VDRE2/3) and anti-sense primers (5'-biotin TAAAGAAGGGCAAGGTGCCA-3'; 5'-biotin CATGTGTGCGAGAGGAGG-3' for VDRE2/3). The sequences in VDRE1, VDRE2 and VDRE3 were mutated using the GENEART Site-Directed Mutagenesis System (Invitrogen). Nuclear extracts from human $\text{CD25}^- \text{CD4}^+$ T cells that were treated for 5 days with $1,25(\text{OH})_2\text{VD}_3$ were obtained using a commercially available kit (Thermo Scientific, Loughborough, UK) and mixed with the biotinylated DNA fragments (27). Beads conjugated with streptoavidin were mixed with the DNA-nuclear extract reaction. VDR and RXR were detected by immunoblotting using anti-VDR and -RXR rabbit polyclonal Abs (Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (27).

Chromatin Immunoprecipitation (ChIP) Assay—As previously described ChIP assay was done using a commercially available kit (Upstate Biotechnology, Inc., Lake Placid, NY). Briefly, human $\text{CD25}^- \text{CD4}^+$ T cells that were treated for 5 days with $1,25(\text{OH})_2\text{VD}_3$. Cells were then treated for 10 min with 1% formaldehyde at 37 °C and washed twice in ice-cold PBS containing protease inhibitors. Cell pellets were lysed in SDS lysis buffer and sonicated under conditions that reduced DNA length to between 200 and 1000 bp. The lysates were incubated overnight at 4 °C with anti-VDR rabbit polyclonal Abs and normal

rabbit IgG antibody (both from Santa Cruz Biotechnology). Immune complexes were precipitated with protein G agarose (Upstate Biotechnology, Lake Placid, NY), and DNA was purified using spin columns. PCR was performed to detect VDRE1 and VDRE2/3 using the following primers: VDRE1, sense (5'-CTCCATATGTGGGTCCATGTCCAA-3') and anti-sense (5'-TAAAGAAGGGCAAGGTGCCAGGGA-3'); and VDRE2/3, sense (5'-TTTGCTGATTGTTGCTTTGCAATA-3') and anti-sense (5'-TGGACACATGCATGGAGAGCCAGA-3'). A 150-bp DNA fragment in the *FOXP3* promoter region (-145 - +5) that did not contain a VDRE was amplified by PCR as a negative control using the following primers: sense (5'-ACCCCGTGATTATCAGCGCACACA-3') and antisense (5'-CCTGGCTTGTGGGAAACTGTCACG-3').

Reporter gene assay

The promoter (-511 - +176) and a highly CNS region (+1714 - +2554) of the *FOXP3* gene were cloned into pGL3 basic vector (Promega, Madison, WI) followed by transfection to primary human CD25⁻CD4⁺ T cells which were stimulated for 5 days with anti-CD3/CD28 Abs and IL-2 in the presence or absence of 1,25(OH)₂VD₃ using the Amaxa transfection system (Lonza, Walkersville, MD) (27, 28). In some experiments, VDRE1, 2 and/or 3 sequences in the CNS region were mutated using the GENEART Site-Directed Mutagenesis System (Invitrogen) and inserted into the vector for *FOXP3* gene reporter assay. Transfected cells were analyzed for luciferase activity and normalized against *Renilla* activity (Promega, Madison, WI).

Statistical analysis

A paired *t*-test was used to analyze data with a significance level of $P < 0.05$.

Results

Identification of putative VDREs in the human *FOXP3* CNS region +1714 to +2554 that increase the *FOXP3* promoter activity

Previous studies reported enhanced induction of *FOXP3* in *FOXP3*-negative CD25⁻CD4⁺ T cells by 1,25(OH)₂VD₃ in the presence of anti-CD3/CD28 Abs and IL-2 (25). However, it is unknown whether VDR binds directly to the *FOXP3* gene, leading to such an up-regulation. We thus searched for potential binding sites of the VDR in the human *FOXP3* gene *in silico* using Nubiscan (www.nubiscan.unibas.ch), an algorithm predicting nuclear receptor binding elements (29). We focused our search on the previously reported promoter (-511 ~ +176) and an intronic CNS region of the human *FOXP3* gene (+1714 ~ +2554). This CNS region had been identified as an enhancer in the mouse *foxp3* gene with the binding sites for Smad3 and NFAT that promoted the *foxp3* gene expression (27, 28). Three potential response elements for VDR were identified in the CNS region of the human *FOXP3* gene. These were ER6 types and designated as VDRE1, VDRE2 and VDRE3 (located at +2380 ~ +2397, +2504 ~ +2521 and +2527 ~ +2544 in the human *FOXP3* gene, respectively) (Fig. 1A). We constructed a vector containing the *FOXP3* promoter and the CNS region and then measured the promoter activity in human primary CD25⁻CD4⁺ T cells in the presence or absence of 1,25(OH)₂VD₃ (Fig. 1B). 1,25(OH)₂VD₃ increased the promoter activity in transfected CD25⁻CD4⁺ T cells in response to anti-CD3/CD28 Abs and IL-2. This suggests possible VDR binding to the putative VDREs in the CNS region.

VDR directly binds to VDRE1, 2, and 3 in the CNS region +1714 to +2554 of the FOXP3 gene

We next determined whether VDR and RXR could bind VDRE1, VDRE2 and VDRE3 using a pull-down assay. We constructed biotinylated DNA fragments that contained VDRE1, VDRE2/3, or mutated VDRE1, VDRE2 or VDRE3 (Fig. 2A). The biotinylated fragment for VDRE2/3 had both VDRE2 and 3 since these VDREs are separated by only five nucleotides. The constructed fragments were incubated with nuclear extracts of human CD25⁻CD4⁺ T cells treated with 1,25(OH)₂VD₃ and anti-CD3/CD28 Abs and pulled down with streptavidin. Western blot analysis showed that the pull down with the fragment for VDRE1 or VDRE2/3 had VDR and RXR proteins while the pull down with the fragments containing mutated VDRE1, VDRE2 or VDRE3 had substantially decreased protein binding (Fig. 2B). These findings suggest VDR binding to VDRE 1 and VDRE2/3 in the CNS +1714 ~ +2554 of the human *FOXP3* gene.

To further demonstrate VDR binding to VDRE1, 2, and 3, a chromatin immunoprecipitation (ChIP) assay was done using lysates of 1,25(OH)₂VD₃-treated CD25⁻CD4⁺ T cells. In the sample immunoprecipitated with anti-VDR Abs, we detected VDRE1 and VDRE2/3 by PCR (VDRE1 and VDRE2/3 fragments, respectively, Fig. 3). However, we did not detect a DNA sequence without a VDRE that exists outside the CNS (non-VDRE fragment, -145 ~ +5, negative control) in the same sample. These results of the ChIP assay further support VDR binding to VDRE1 and VDRE2/VDRE3 in the CNS +1714 to +2554 of the human *FOXP3* gene.

VDRE1, 2 and 3 in the human FOXP3 CNS region +1714 to +2554 enhance the FOXP3 promoter activity in the presence of 1,25(OH)₂VD₃.—To determine the role for VDRE1, 2 and 3 in the CNS region in promoting the *FOXP3* promoter activity, we constructed a vector containing the *FOXP3* promoter and the CNS region with mutations in VDRE1, VDRE2 and/or VDRE3. The construct with mutated VDRE1, VDRE2 and/or VDRE3 had decreased *FOXP3* promoter activity compared to the construct with the intact CNS region (Fig. 4). These findings indicate that VDRE1, VDRE2 and VDRE3 in the *FOXP3* CNS region +1714 ~ + 2554 are essentially involved in promoting the *FOXP3* promoter activity in the presence of 1,25(OH)₂VD₃.

Suppression of CD4⁺ T cell proliferation by CD25⁻CD4⁺ T cells treated with 1,25(OH)₂VD₃, TCR triggering and IL-2 is dependent on FOXP3 and cell contact—Although a previous study reported the inhibitory function of FOXP3⁺ iTreg cells induced by the combination of 1,25(OH)₂VD₃, anti-CD3/CD28 Abs and IL-2 (25), it is still unknown whether FOXP3 is essential for this phenomenon. Thus, we induced FOXP3 in purified human CD25⁻CD4⁺ T cells by treating cells with anti-CD3/CD28 Abs, IL-2 and 1,25(OH)₂VD₃. As previously reported (25), FOXP3 expression was higher in the presence of 1,25(OH)₂VD₃ compared to the absence of this molecule (Fig. 5A). A dose-dependent effect of 1,25(OH)₂VD₃ on FOXP3 expression was observed (Fig. 5B). Also, CD25⁻CD4⁺ T cells stimulated with anti-CD3/CD28 Abs, IL-2 and 1,25(OH)₂VD₃ had increased expression of FOXP3 and VDR over time (Supplemental Figure 1). We then co-cultured iTreg cells induced by the combination of anti-CD3/CD28 Abs, IL-2 and 1,25(OH)₂VD₃ (VD-iTreg cells) with target CD25⁻CD4⁺ T cells. The former cells suppressed proliferation of the target cells (Fig. 5C, left panel). Such suppression was largely dependent on cell contact since separating the two cell populations during cell culture reduced the inhibitory effect of VD-iTreg on the target cells (Fig. 5C, right panel). To determine the direct role for FOXP3 in inhibiting the target cell proliferation, we next knocked down the *FOXP3* gene in VD-iTreg cells using siRNA technique (Fig. 5D). VD-iTreg cells treated with FOXP3-specific siRNA had a decreased inhibitory effect on the target cells compared to the same

cells treated with control siRNA (Fig. 5E). These findings indicate that human VD-iTreg cells suppress proliferation of target CD4⁺ T cells and that this suppression is dependent on cell contact and FOXP3 expression.

Discussion

1,25(OH)₂VD₃ can affect the functions of immune cells including T cells (15). 1,25(OH)₂VD₃ binds the nuclear VDR that binds target DNA sequences known as VDRE. Although previous studies reported that 1,25(OH)₂VD₃ promoted FOXP3 expression in CD4⁺ T cells in the presence of anti-CD3/CD28 Abs and IL-2 (21–25), it is unknown whether this effect of 1,25(OH)₂VD₃ is mediated through direct binding of VDR to the *FOXP3* gene without involving other molecules. Also, it is unclear whether FOXP3 expression in VD-iTreg cells is critical for the inhibitory function of these cells. Here we demonstrated the presence of VDREs in the intronic human *FOXP3* CNS region +1714 to +2554 and the enhancement of the *FOXP3* promoter activity by such VDREs in response to 1,25(OH)₂VD₃. Furthermore, VD-iTreg cells suppressed the proliferation of target CD4⁺ T cells, and this effect was dependent on FOXP3 expression and cell contact. These findings suggest that 1,25(OH)₂VD₃ can affect human immune responses by directly binding the *FOXP3* gene and regulating its expression in CD4⁺ T cells and that FOXP3 is essential for the inhibitory function of VD-iTreg cells.

1,25(OH)₂VD₃ is a relatively small lipophilic molecule that can easily penetrate the cell membrane by simple diffusion and complex with VDR (15). The VDR then heterodimerizes with RXR and binds to VDREs in genes. In fact, 1,25(OH)₂VD₃ suppressed the expression of IL-2 and IFN- γ mRNA and protein in T cells by binding of VDR to the VDRE in the promoters of the *IL2* and *IFNG* genes (30, 31). Previous studies reported increased FOXP3 expression in human and mouse T cells by treating with 1,25(OH)₂VD₃ (21–25). This phenomenon appeared to be secondary to the effect of this vitamin on T cells and DCs. 1,25(OH)₂VD₃-treated DCs induced FOXP3⁺ iTreg cells with suppressive activity (22) although the exact mechanism was not elucidated. In humans, increased expression of FOXP3 in CD4⁺ T cells was found in PBMCs treated with 1,25(OH)₂VD₃ (24). This effect was dependent on the production of indoleamine 2,3-dioxygenase (IDO) from DCs that could suppress immune responses and induce FOXP3⁺ Treg cells (32, 33). Similar to our finding, a combination of 1,25(OH)₂VD₃, IL-2 and anti-CD3/CD28 Abs synergistically up-regulated FOXP3 expression in human CD25⁻CD4⁺ T cells in the absence of other immune cells including DCs (25), demonstrating the direct effect of this vitamin on regulating FOXP3 expression in CD4⁺ T cells. Such a direct effect of 1,25(OH)₂VD₃ on CD4⁺ T cells could be secondary to altered expression of molecules that can regulate the *FOXP3* promoter activity in CD4⁺ T cells. Alternatively, but not mutually exclusively, 1,25(OH)₂VD₃ could directly bind VDREs in the *FOXP3* gene, leading to increased FOXP3 expression. Indeed, the results of our studies demonstrate that the intronic CNS region +1714 to +2554 of the *FOXP3* gene have VDREs and that 1,25(OH)₂VD₃ promotes the *FOXP3* gene promoter activity via directly binding to these VDREs.

In our study, we initially identified three putative VDREs in the CNS region +1714 to +2554 of the *FOXP3* gene. These are VDRE1, 2 and 3 that are located at +2380 – +2397, +2504 – +2521 and +2527 – +2544, respectively. The results of our ChIP assay and pull down assay using DNA fragments containing these VDREs showed binding of VDR to VDRE1. Furthermore, mutating the sequence of VDRE1 reduced *FOXP3* promoter activity in the presence of 1,25(OH)₂VD₃. These findings suggest the role for VDRE1 in promoting FOXP3 expression in human CD4⁺ T cells in response to 1,25(OH)₂VD₃. We also showed the binding of VDR to the region covering both VDRE2 and VDRE3. The results of the *FOXP3* promoter assay indicate that both VDRE1 and VDRE2 are involved in enhancing

FOXP3 promoter activity in the presence of $1,25(\text{OH})_2\text{VD}_3$ since mutating individual sequences reduced the promoter activity. These findings suggest that VDRE1, VDRE2 and VDRE3 are essential for the $1,25(\text{OH})_2\text{VD}_3$ -mediated promotion of *FOXP3* in human CD4^+ T cells.

In our study, $\text{CD25}^- \text{CD4}^+$ T cells that were treated with $1,25(\text{OH})_2\text{VD}_3$ in the presence of anti- $\text{CD3}/\text{CD28}$ Abs and IL-2 suppressed proliferation of CD4^+ T cells in a cell number dependent manner. Although a large number of these vitamin-treated CD4^+ T cells expressed *FOXP3*, this molecule might not be necessary for the anti-proliferative effect. Of interest, cell to cell contact was required for suppressing the proliferation of target T cells by VD-iTreg cells, suggesting that soluble factor(s) are not critically involved in this phenomenon. To determine the specific role for *FOXP3* in suppressing the proliferation of target T cells, we knocked down *FOXP3* gene expression in $\text{CD25}^- \text{CD4}^+$ T cells treated with $1,25(\text{OH})_2\text{VD}_3$, anti- $\text{CD3}/\text{CD28}$ Abs and IL-2. Indeed, the *FOXP3* knock down decreased the anti-proliferative function of such $1,25(\text{OH})_2\text{VD}_3$ -treated cells. These findings indicate the indispensable role for *FOXP3* in executing the suppressive function of VD-iTreg cells in humans.

In addition to inducing *FOXP* expression in non-Treg cells, $1,25(\text{OH})_2\text{VD}_3$ appears to have effect on nTreg cells that express high levels of CD25 and *FOXP3*. In fact, $1,25(\text{OH})_2\text{VD}_3$ decreased the proliferation of human nTreg cells in the presence of anti- $\text{CD3}/\text{CD28}$ antibodies and IL-2 (34). In the same study, nTreg cells treated with this vitamin had increased production of IL-10 without any effect on their activation status and inhibitory capacity. It is disputable that $1,25(\text{OH})_2\text{VD}_3$ could affect a small number of non- $\text{CD25}^- \text{CD4}^+$ T cells such as nTreg cells in our study since the purity of $\text{CD25}^- \text{CD4}^+$ T cells may not be 100%. However, we believe this possibility has no significant effect on our findings in that $1,25(\text{OH})_2\text{VD}_3$ suppressed the expansion of nTreg cells (34). A recent mouse study that conditionally targeted VDR in T cells showed no change in the frequency of $\text{Foxp3}^+ \text{CD4}^+$ T cells although intact VDR function in hematopoietic cells was necessary for $1,25(\text{OH})_2\text{VD}_3$ to inhibit experimental allergic encephalitis (EAE), a mouse model of multiple sclerosis (MS) (35). These findings suggest that $1,25(\text{OH})_2\text{VD}_3$ may act directly on pathogenic CD4^+ T cells to suppress EAE.

Decreased blood levels of vitamin D were reported in patients with autoimmune diseases including type I diabetes mellitus (DM), SLE, RA, and MS (15, 20). Given the effects of $1,25(\text{OH})_2\text{VD}_3$ on immune cells, it is conceivable that vitamin D deficiency may have a role in the pathogenesis of autoimmunity. In fact, $1,25(\text{OH})_2\text{VD}_3$ or its analogues have been tried as treatments for autoimmune diseases. For instance, in a murine model of lupus, administration of $1,25(\text{OH})_2\text{VD}_3$ decreased proteinuria and prolonged life span (36, 37). Also, $1,25(\text{OH})_2\text{VD}_3$ prevented disease or reduced disease severity in animal models of MS and type I DM (38–40). Although this therapeutic effect of $1,25(\text{OH})_2\text{VD}_3$ appears to be mediated by several mechanisms, it could be associated with enhanced numbers and/or functions of Treg cells in that topical $1,25(\text{OH})_2\text{VD}_3$ or its analogue enhanced suppressive activity of Treg cells and induced the expansion of this cell subset (21–23). Of interest, decreased numbers and/or function of FOXP3^+ Treg cells were reported in patients with MS, RA and SLE (41–46), raising a possible pathogenic association of vitamin D deficiency with quantitative and qualitative defects of FOXP3^+ Treg cells in these patients. However, in humans, the trial of VD_3 supplement for autoimmune diseases including MS and RA showed conflicting results (20). This controversial issue on the potential therapeutic effect of vitamin D administration in autoimmunity could be clarified with additional clinical studies.

Taken together, the results of our studies demonstrate the presence of VDREs in the intronic CNS region +1714 to +2544 of the human *FOXP3* gene and the enhancement of the *FOXP3*

promoter activity by such VDREs in response to 1,25(OH)₂VD₃. In addition, VD-iTreg cells suppressed the proliferation of target CD4⁺ T cells, and this effect was dependent on FOXP3 expression and cell contact. These findings suggest that 1,25(OH)₂VD₃ can affect human immune responses by regulating FOXP3 expression in CD4⁺ T cells through direct VDR binding to the *FOXP3* gene which is essential for the inhibitory function of VD-iTreg cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article

CNS	conserved non-coding sequence
FOXP3	Forkhead box P3
Treg	regulatory T cells
iTreg	induced Treg
VD	vitamin D
VDR	vitamin D receptor
VDRE	vitamin D response element
nTreg	naturally occurring Treg
SLE	systemic lupus erythematosus
RA	rheumatoid arthritis
RXR	retinoid X receptor
DR	direct repeat
ER	everted repeat
ChIP	chromatin immunoprecipitation
EAE	experimental allergic encephalitis
MS	multiple sclerosis
DM	diabetes mellitus

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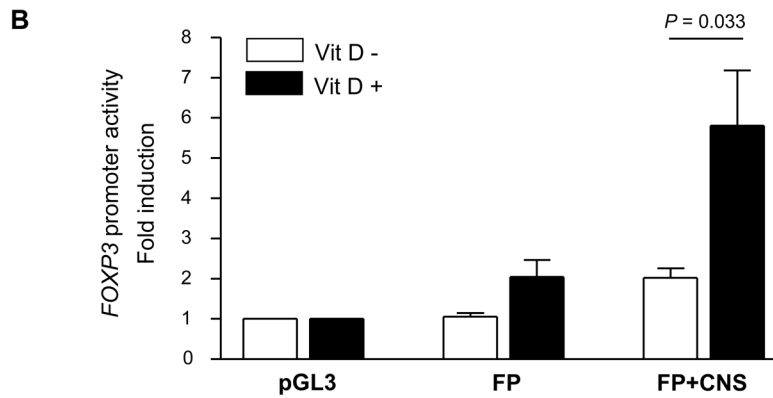
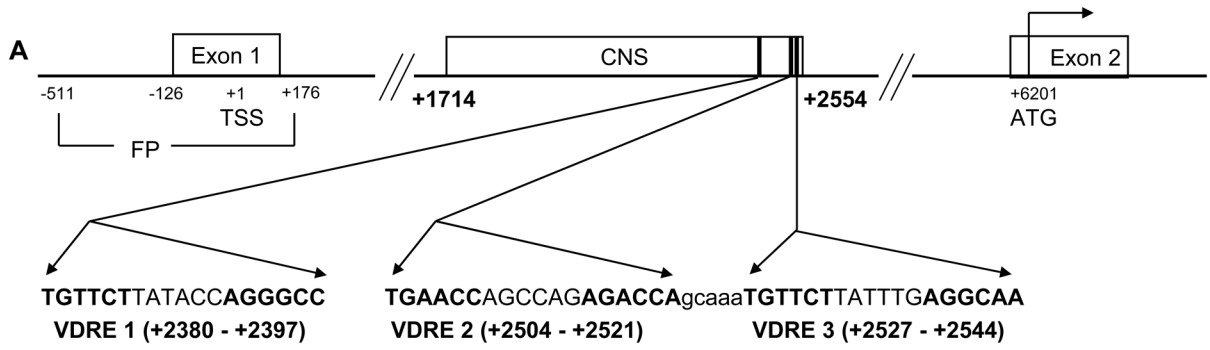


Figure 1. Identification of putative vitamin D response elements (VDREs) in the intronic conserved non-sequencing (CNS) region +1714 to +2554 of the human *FOXP3* gene that increases the *FOXP3* promoter activity in response to 1,25(OH)₂VD₃

(A) The locations and sequences of three putative VDREs in the intronic CNS region +1714 – +2554 of the human *FOXP3* gene. The sequences of the putative VDREs are in bold. (B) The CNS region +1714 – +2554 increases the human *FOXP3* promoter (FP, +151 – +176) activity in the presence of 1,25(OH)₂VD₃, anti-CD3/CD28 Abs and IL-2 as measured by luciferase reporter assay. Human primary CD25⁺CD4⁺ T cells were stimulated for 5 days with anti-CD3/CD28 Abs and IL-2 (25 ng/ml) in the presence or absence of 1,25(OH)₂VD₃ (Vit D, 10 nM) and transfected with pGL3 basic vector (PGL3), vector with an insert containing the *FOXP3* promoter (FP), or vector containing the *FOXP3* promoter and the CNS region +1714 – +2554 (FP+CNS). Cells were additionally incubated for 2 days in the presence of the same stimulation, with or without 1,25(OH)₂VD₃. Luciferase activity was measured and normalized against *Renilla* activity. Data represent the mean + SEM from 6 independent experiments.

A

VDRE 1 : TGTTCTTATACCAGGGCC
VDRE 2/3 : TGAACCAGCCAGAGACCA_{gcaa}TGTTCTTATTTGAGGCAA
VDRE 1 mutant (VDRE1m) : TGTTAGTATACCAGGGCC
VDRE 2 mutant (VDRE2m) : TGAATTAGCCAGAGACCA_{gcaa}TGTTCTTATTTGAGGCAA
VDRE 3 mutant (VDRE3m) : TGAACCAGCCAGAGACCA_{gcaa}TGTTCTTATTTGCTCGAA

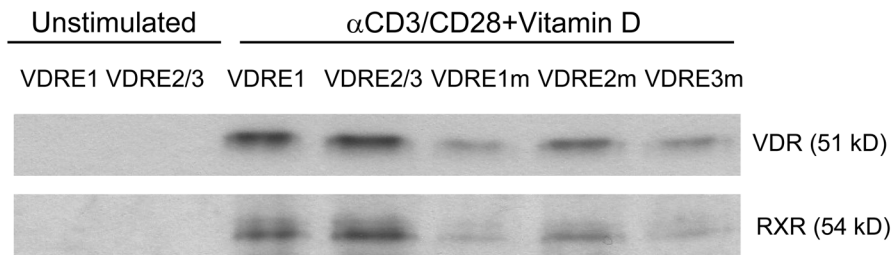
B

Figure 2. Pull-down assay shows the binding of the vitamin D receptor (VDR) to DNA fragments containing VDREs in the CNS region +1714 to +2554 of the human *FOXP3* gene

(A) Biotinylated DNA fragments that contained putative VDRE1 (+2326 – +2476) or VDRE2/3 (+2504 – +2521 and +2527 – +2544, respectively) in the intronic CNS region +1714 – +2554 of the *FOXP3* gene were amplified by PCR. The sequences in VDRE1, VDRE2 and VDRE3 were mutated (VDRE1m, VDRE2m, VDRE3m) using the GENEART Site-Directed Mutagenesis System (Invitrogen). (B) Human CD25⁻CD4⁺ T cells were treated for 5 days with 1,25(OH)₂VD₃ (VD, 10 nM) in the presence of anti-CD3/CD28 Abs and IL-2. Nuclear extracts of the cells were mixed with biotinylated DNA fragments from (A). Beads conjugated with streptoavidin (Dynabeads M-280 Streptavidin Dynal Biotech) were mixed with the DNA-nuclear extracts. VDR and RXR were detected by immunoblotting. Representative data from 2 independent experiments.

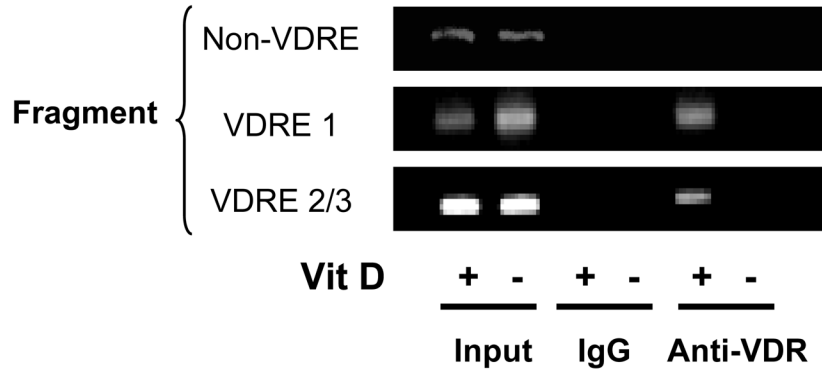


Figure 3. Detection of VDR binding to VDRE1 and VDRE2/3 in the CNS region +1714 to +2554 of the human *FOXP3* gene using chromatin immunoprecipitation (ChIP) Assay
Human CD25⁻CD4⁺ T cells were treated for 5 days with or without 1,25(OH)₂VD₃ (Vit D, 10 nM) in the presence of anti-CD3/CD28 Abs and IL-2. Cell lysates were immunoprecipitated with anti-VDR Abs or control IgG. Immune complexes were collected, and PCR analysis was performed to determine the presence of VDRE1, VDRE2/3 and non-VDRE (-145 – +5, control) regions against input DNA. Representative data from 3 independent experiments.

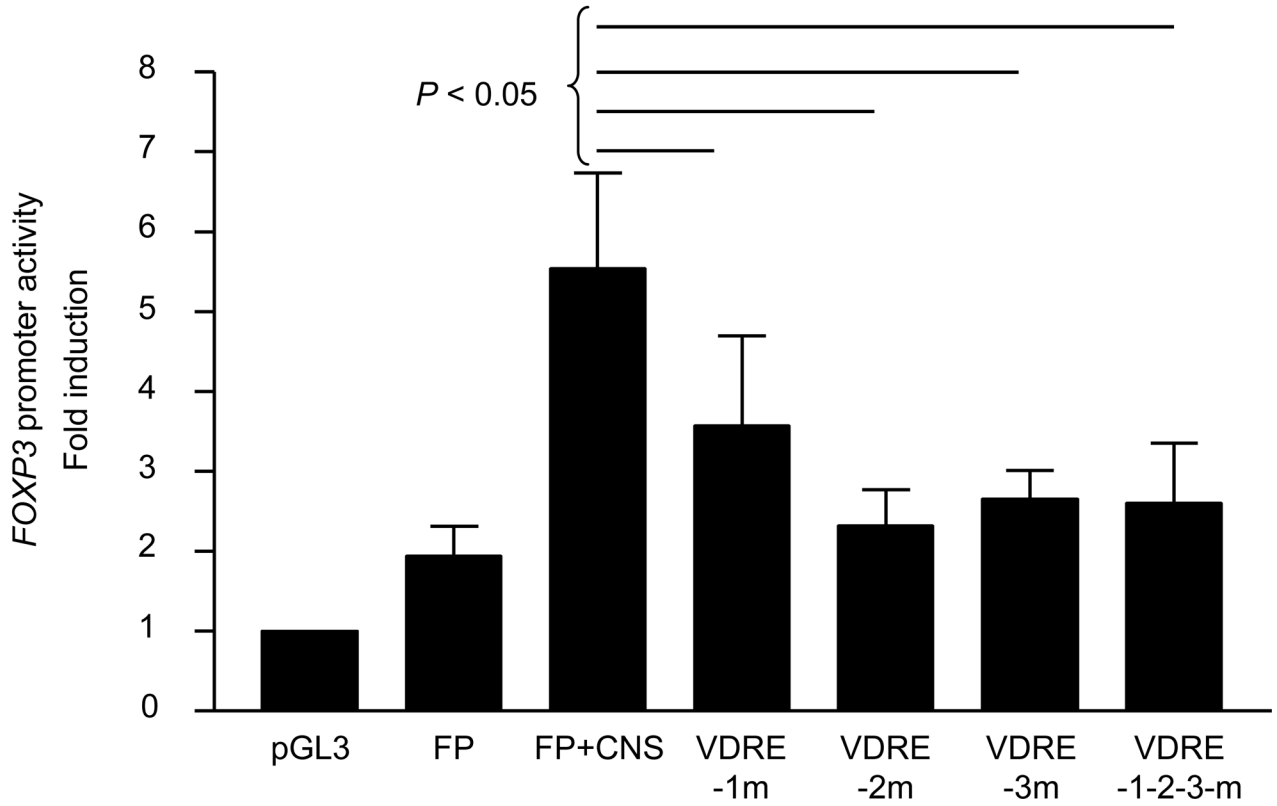


Figure 4. VDRE1, 2 and 3 in the CNS region +1714 to +2554 of the *FOXP3* gene are required for enhancing the *FOXP3* promoter activity in the presence of 1,25(OH)₂VD₃
 Human primary CD25⁻CD4⁺ T cells were incubated for 5 days with anti-CD3/CD28 Abs and IL- 2 (25 ng/ml) in the presence of 1,25(OH)₂VD₃ (Vit D, 10 nM) and transfected with a pGL3 basic vector (PGL3), a vector with an insert containing the *FOXP3* promoter (FP), or a vector containing the *FOXP3* promoter and the CNS region +1714 – +2554 (FP+CNS) with intact or mutated VDRE1, 2 and/or 3 (VDRE1m, 2m, 3m). Cells were incubated for an additional 2 days in the presence of the same stimulation. Luciferase activity was measured and normalized against *Renilla* activity. Data represent the mean + SEM from 7 independent experiments.

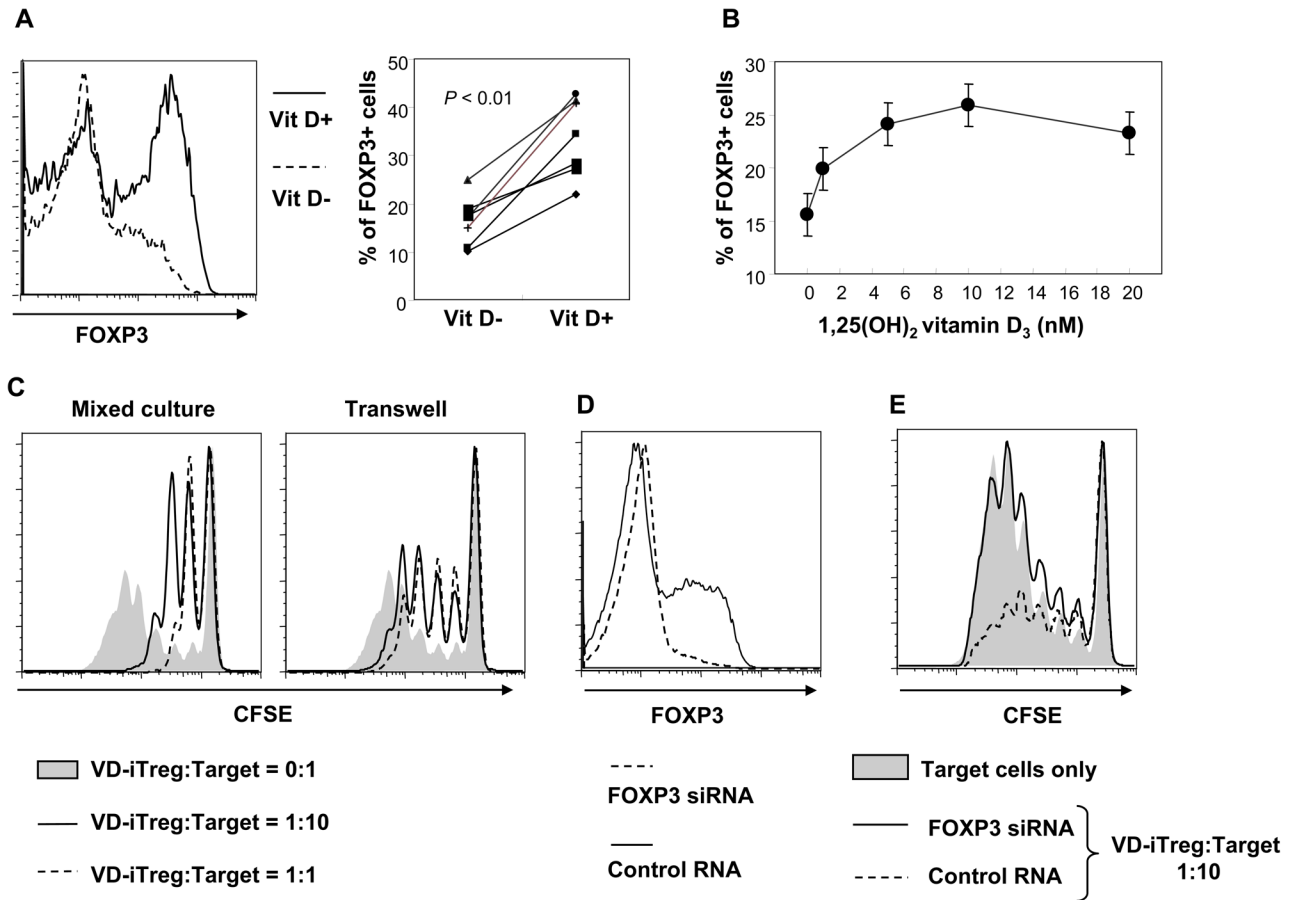


Figure 5. 1,25(OH)₂VD₃ promotes FOXP3 expression in human CD25⁻CD4⁺ T cells in the presence of anti-CD3/CD28 Abs and IL-2, and these vitamin D-treated cells (VD-iTreg cells) suppress target T cell proliferation dependently of cell contact and FOXP3

Human CD25⁻CD4⁺ T cells were purified from peripheral blood of healthy individuals. (A–B) Cells were treated for 5 days with 1,25(OH)₂VD₃ (10 nM or indicated), anti-CD3/CD28 Abs and IL-2 to generate 1,25(OH)₂VD₃-induced Treg (VD-iTreg) cells. Intracellular FOXP3 expression was determined by flow cytometry. (C) VD-iTreg cells were generated as in (A), rested for 2 days and then co-cultured for 5 days with CFSE-stained autologous CD25⁻CD4⁺ T cells (target) at different ratios in a transwell culture system separating the two cell populations or a regular culture plate in the presence of anti-CD3/CD28 Abs. (D–E) Cells were stimulated for 3 days with 1,25(OH)₂VD₃ (10 nM), anti-CD3/CD28 Abs and IL-2. Cells were then transfected with control or FOXP3-specific siRNA (Invitrogen, Stealth Select RN, HSS 121458) using electroporation (Amaxa Biosystems). (D) Cells were fixed, permeabilized and stained with Abs to FOXP3 or isotype controls and analyzed on a flow cytometer. (E) CD25⁻CD4⁺ T cells that were transfected with control or FOXP3-specific siRNA and treated with 1,25(OH)₂VD₃, anti-CD3/CD28 Abs and IL-2 were incubated for 5 days with CFSE-stained CD25⁻CD4⁺ T cells (target cells) in the presence of anti-CD3/CD28 Abs. Cells were analyzed on a flow cytometer. Representative data from 7 (A) or 3 (B–E) independent experiments (one experiment per donor).