ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
October 2020; 19(5):509-516.
Doi: 10.18502/ijaai.v19i5.4466

Immunomodulatory Effects of Calcitriol through DNA Methylation Alteration of *FOXP3* in the CD4$^+$ T Cells of Mice

Mona Oraei[1], Sama Bitarafan[2], Seyed Alireza Mesbah-Namin[3], Ali Noori-Zadeh[4], Fatemeh Mansouri[1], Karim Parastouei[5], Ali Anissian[6], Mir Saeed Yekaninejad[7], Maryam Hajizadeh[8], and Ali Akbar Saboor-Yaraghi[1,2]

[1] Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[2] Iranian Center of Neurological Research, Neuroscience Institute, Imam Khomeini Hospital Complex, Tehran University of Medical Sciences, Tehran, Iran
[3] Department of Clinical Biochemistry, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[4] Department of Clinical Biochemistry, Faculty of Allied Medical Sciences, Ilam University of Medical Sciences, Ilam, Iran
[5] Department of Cellular and Molecular Nutrition, School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences, Tehran, Iran
[6] Department of Veterinary Pathology, Islamic Azad University, Abhar, Iran
[7] Department of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[8] Department of Parasitology and Mycology, School of Medicine, Iran University of Medical Sciences (IUMS), Tehran, Iran

Received: 8 April 2020; Received in revised form: 20 June 2020; Accepted: 2 July 2020

ABSTRACT

Vitamin D plays a variety of physiological functions, such as regulating mineral homeostasis. More recently, it has emerged as an immunomodulator player, affecting several types of immune cells, such as regulatory T (Treg) cells. It has been reported that vitamin D exerts some mediatory effects through an epigenetic mechanism. In this study, the impacts of calcitriol, the active form of vitamin D, on the methylation of the conserved non-coding sequence 2 (CNS2) region of the forkhead box P3 (*FOXP3*) gene promoter, were evaluated.

Fourteen C57BL/6 mice were recruited in this study and divided into two intervention and control groups. The CD4$^+$ T cells were isolated from mice splenocytes. The expression of *FOXP3*, *IL-10*, and transforming growth factor-beta (*TGF-β1*) genes were relatively quantified by real-time PCR technique, and the DNA methylation percentage of every CpG site in the CNS2 region was measured individually by bisulfite-sequencing PCR.

Vitamin D Intervention could significantly (p<0.05) increase the expression of *FOXP3*, *IL-10*, and *TGF-β1* genes in the CD4$^+$ T cells of mice comparing with the control group. Meanwhile, methylation of the CNS2 region of *FOXP3* promoter was significantly decreased in three of ten CpG sites in the vitamin D group compared to the control group.

The results of this study showed that vitamin D can engage the methylation process to induce *FOXP3* gene expression and probably Treg cytokines profile. Further researches are needed to discover the precise epigenetic mechanisms by which vitamin D modulates the immune system.

Keywords: Calcitriol; *FOXP3* gene; Methylation; Regulatory T-lymphocytes; Vitamin D

Corresponding Author: Ali Akbar Saboor-Yaraghi, PhD; Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. Tel: (+98 21) 4293 3168, Fax: (+98 21) 8895 4913, E-mail: asaboor@tums.ac.ir

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

INTRODUCTION

Vitamin D is a hormone, mostly known for its classical functions include helping to absorb calcium from the intestine, preventing osteoporosis, creating mineral homeostasis, and activating anti-cancer pathways.[1] Moreover, it has been appreciated as a molecule capable of presenting immunoregulatory properties.[2] The overall impacts of Vitamin D on the immune system are to strengthen peripheral tolerance while keeping the innate immunity functions against pathogens. Vitamin D restrains dendritic cells (DCs) maturation and inducing their tolerogenic phenotype by decreasing the expression of HLA-DR, CD86, and CD80 genes. These tolerogenic DCs can establish a regulatory T (Treg) phenotype in $CD4^+$ T cells.[3-5]

Vitamin D receptor (VDR) works as a transcription factor when it engages with the active form of vitamin D, affects many genes expression, and involves in a wide range of biological phenomena.[6,7] Intriguingly, some documents indicated that vitamin D might regulate gene expression independently of the VDR.[8] In addition, vitamin D regulates the expression of histone demethylase gene,[9] which suggests that this vitamin can also modulate gene expression through epigenetic machinery.

Epigenetic is described as a set of regulatory mechanisms, which have long term effects on gene expression without DNA sequence alternations. The main mechanisms in the epigenetic regulatory systems include DNA methylation and histone modifications. DNA methylation occurs on cytosine in CpG dinucleotide frequently found in CpG islands and short CpG-rich sequences near promoters.[10] Moreover, the DNA methylation contributes to X chromosome inactivation, monoallelic gene expression, genome stability, and development.[11]

There is growing evidence addressing vitamin D as an immunomodulator player, and researchers have broadly studied the various mechanisms by which this vitamin influences immune responses. The VDR has been found on a variety of adaptive and innate immune cells, including T and B cells, monocytes, macrophages, and DCs.[12]

Interestingly, it was found that the VDR has a binding site in the non-coding intronic region of the forkhead box P3 (*FOXP3*) gene.[13] This suggests that vitamin D might regulate gene expression through epigenetic means. Zheng et al discovered that the conserved non-coding sequence 2 (CNS2) region of the *FOXP3* promoter is essential for stable Foxp3 expression in mature Treg cells.[14] This study aims to evaluate the modification effects of calcitriol, the active form of vitamin D, on the methylation status of the CNS2 region of the *FOXP3* promoter accompany with an assessment of expression of this gene and the Treg anti-inflammatory cytokines, IL-10 and transforming growth factor-beta (TGF-β1), in the $CD4^+$ T cells isolated from treated and control mice.

MATERIALS AND METHODS

Experimental Design

National Animal Ethical Guidelines were strictly followed and all animal experiments were approved by the ethical committee of Tehran University of Medical Sciences (ethics committee approval code: IR.TUMS.SPH.REC.1396.3158). Briefly, the number of animals needed for this study was reduced, the risks of pain and suffering that the animals would face were considered, and researchers who have worked with these animals were properly trained. Fourteen C57BL/6 female mice (ten weeks old) were obtained from Pasteur Institute of Iran (Tehran, Iran) and kept in a proper condition described as 12:12-h dark: light cycle, standard defined humidity, and received food and water. The mice were acclimatized for one week before the experiment and then, randomly divided into two experimental groups, including a control group (n=7) and a vitamin D, treated or intervention group (n=7). In the vitamin D group, each mouse received 100 ng calcitriol (Sigma, Germany) through intraperitoneal injection (IP) every other day. The control group mice were received an equal amount of excipient administrated to the vitamin D treated group. All mice in intervention and control groups were sacrificed on day 21.

$CD4^+$ T Cells Isolation

Under aseptic conditions, the mice spleens were resected, and splenocytes were separated by perfusion method, using complete RPMI-1640 (Gibco, USA) medium supplemented with glutamine (Gibco, USA), penicillin (100 U/mL, Gibco, USA), streptomycin (100 µg/mL, Gibco, USA), and 10 % heat-inactivated fetal bovine serum (FBS, Gibco, USA). The $CD4^+$ T cells were isolated from freshly obtained splenocytes using the magnetic-activated cell sorting (MACS) method

(Miltenyi, Germany), a negative selection method, based on manufacturer's instructions. In brief, red blood cells (RBCs) were lysed using an ACK buffer (RBC lysis buffer, Sigma-Aldrich, USA). After washing with the media, the cell suspension was applied on a 70 µm cell strainer to remove any potential tissue debris or cell aggregates. Then the $CD4^+$ T cells were purified using the MACS technique. Finally, isolated $CD4^+$ T cells were stained with FITC anti-mouse CD3 and PE anti-mouse CD4 to assess the purity of these cells. The purity of isolated $CD4^+$ T cells was above 90% in all experiments (Figure 1a).

Quantitative Gene Expression Assessment

The total RNA was extracted from 2.5×10^6 of the isolated $CD4^+$ T cells using an RNA extraction kit (Biobasic, Canada) based on the manufacturer's instructions. Extracted RNA purity was assessed using the evaluation of absorbance ratios at 260/280 and 260/230. The integrity of RNA was examined by separation through a 1% denaturing agarose gel and observation of rRNA density 28S and 18S (Figure 1b). One microgram of the purified total RNA was reverse-transcribed using PrimeScript RT Reagent Kit (Takara, Japan). Real-time quantitative PCR (RT-qPCR) was performed using Power SYBR Green PCR Master Mix (Takara, Japan) according to the supplier's protocol in the StepOne Plus RT-qPCR machine (Applied Biosystems, USA). The mean threshold cycle (Ct) was recorded for each reaction. Expression of IL-10, TGF-β1, and FOXP3 mRNA relative to β-actin mRNA was determined using the $2^{-\Delta\Delta Ct}$ method. All primers used in the RT-qPCR have been listed in Table 1.

DNA Extraction and Bisulfite-sequencing PCR

DNA was directly extracted from 2.5×10^6 of the $CD4^+$ T cells of each sample using the DNA extraction kit (Biobasic, Canada). Next, bisulfite conversion of non-methylated cytosines to uracils was performed with 1 µg of the genomic DNA using the EpiJET Bisulfite Conversion kit (Thermofisher, USA), according to the manufacturer's instructions. The CpG sites in the promoter region of the *FOXP3* gene were determined by the CLC Drug Discovery Workbench 2 software (Qiagen Bioinformatics). The reference sequence was retrieved from National Center for Biotechnology Information (NCBI) website ranged: 7583899-7584208 on chromosome X of Mus musculus. Then, 1 µL of the bisulfite converted DNA was amplified by hot-start PCR (Table 2).[15]

The PCR products were purified and then bidirectional-sequenced by Bioneer Company (South Korea). The efficiency of DNA bisulfite treatment (cytosine to uracil conversion percentage) was calculated as follows: (number of non-CpG cytosines converted to thymidine after bisulfite treatment/ total non-CpG cytosines before bisulfite treatment) ×100. The calculated efficiency in this study was 100%.

Table1. Information of primers used in RT-qPCR reactions

Gene (Accession number)	Amplicon Size		Sequence	Annealing temperature
FOXP3 (NM_001199347.1)	153	**F**	GTGTCCGACAAGATCTGGTAG	60
		R	GGCACACTCCAACACATAATAG	
TGF-β_1 (NM_011577.2)	194	**F**	AACTATTGCTTCAGCTCCACAGA	58
		R	TTGTGTTGGTTGTAGAGGGCA	
IL-10 (NM_010548.2)	230	**F**	AGTGATTTTAATAAGCTCCAA	58
		R	GAGAGAGGTACAAACGAGGT	
β-Actin (NM_007393.5)	87	**F**	ATGCTCCCCGGGCTGTAT	60
		R	CATAGGAGTCCTTCTGACCCATTC	

F: Forward; R: Reverse

Table 2. Information of the conserved non-coding sequence 2 (CNS2) region primers for amplification and bisulfite-sequencing

The promoter region of forkhead box P3(*FOXP3*)	Amplicon size	Primer sequences (5´ to 3´)	Annealing Temperature (◦C)
CNS2	310		60
		F: TTTATTAAGTATTTAATTTTGGGTTTTTTTGGT	
		R: AAATCTACATCTAAACCCTATTATCACAACCTA	

F: Forward; R: Reverse

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

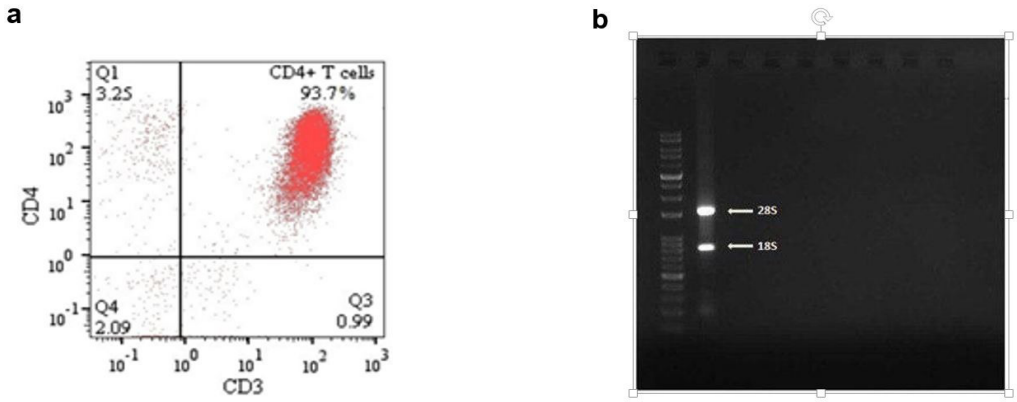

Figure 1. Quality assessment of CD4$^+$ T cells isolation and RNA extraction. CD4$^+$ T cells were purified from mice splenocytes using magnetic-activated cells sorting (MACS) negative selection. The purity of CD4$^+$ T cells was measured through surface staining of the cells with fluorochrome-conjugated anti-mouse CD3 and CD4 (a). Extracted RNA from isolated CD4$^+$ T cells were run on a 1% denaturing agarose gel. The 18S and 28S ribosomal RNA bands are visible in the RNA sample (b).

To calculate the cytosine methylation percentage, the following formula was used: peak height of cytosine divided by the sum of peak heights of cytosine and thymidine. The peaks were generated by the sequencer and Chromas software (Version 2.6, Technelysium).[16] In this formula, a single thymidine peak (without any trace of cytosine peak) at the related CpG site was considered as non-methylated. Hence, the existence of a single cytosine peak indicating 100% of methylation. The methylation percentage in the overlapping thymidine and cytosine was calculated by the aforementioned formula. The results were presented as a percentage between 0-100%.[15,16]

Transcription Factor Binding Sites Prediction

To identify that the CpG sites within the CNS2 region of the *FOXP3* promoter are candidate places for what transcription factors to bind, an online tool for transcription factor binding site prediction (TFBIND INPUT) (http://tfbind.hgc.jp/) was employed. [17]

Statistical Analysis

Statistical analysis was performed using Graphpad Prism 7 software. All data are presented as means±SEM. The normality of outcome variables evaluated by the Kolmogorov-Smirnov test and normality was rejected for all variables (p>0.20). Therefore, the non-parametric Mann-Whitney test was used to compare the differences between studied groups. p values less than 0.05 were considered statistically significant.

RESULTS

Up-regulation of Treg Signature Cytokines Expression by Vitamin D

Vitamin D was administrated to the intervention group mice to determine whether it impacts on Treg related transcription factor and cytokines induction at the gene levels. Then CD4$^+$ T cells were purified (Figure 1a), and the expression of target genes, including *FOXP3*, *IL-10*, and *TGF-β1*, was evaluated. The results showed that in the intervention group, vitamin D was capable of significantly increasing the expression of *FOXP3* (p=0.021), *IL-10* (p=0.001), and *TGF-β1* (p=0.021) genes compared to the control group (Figure 2).

Reduction Effect of Vitamin D on DNA Methylation of *FOXP3* Promoter

The results showed that the CNS2 region of the promoter of *FOXP3* was hypo-methylated in vitamin D treated mice in comparison with the control group (Figure 3a and b). The sequence graphs of CpG sites demonstrated that methylation of cytosine in first (Figure 3c), fifth, and sixth (Figure 3d) CpG sites on the *FOXP3* promoter was declined in the vitamin D treated group compared with the control group.

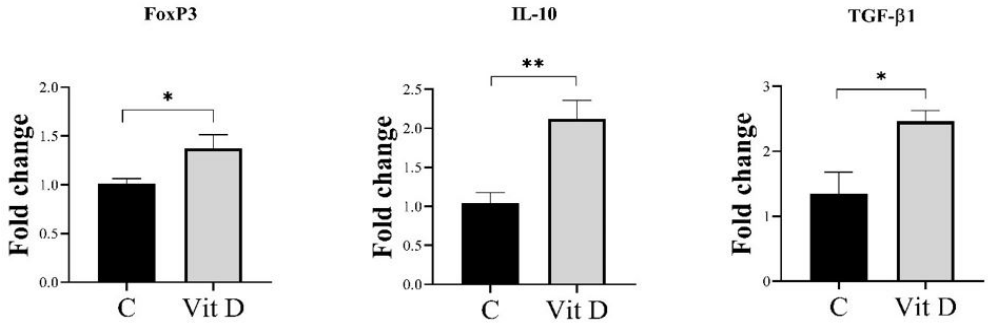

Figure 2. Assessment of vitamin D impacts on the expression of forkhead box P3 (*FOXP3*), transforming growth factor factor-beta (*TGF-β_1*), and *IL-10* genes. The expression of *FOXP3*, *TGF-β_1*, and *IL-10* genes was relatively quantified. Vitamin D induced the expression of all three target genes in the vitamin D treated group (Vit D) comparing with the control (C) group. (n=7); (*: $p<0.05$); (: $p<0.01$).**

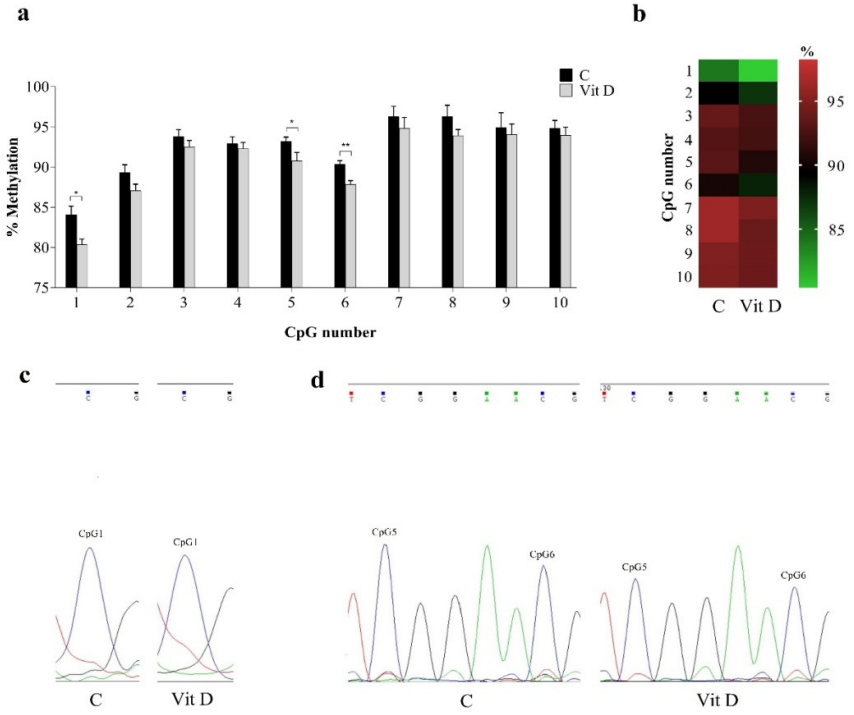

Figure 3. Methylation quantification of cytosines at CpGs occurred in transcription factor binding sites of the conserved non-coding sequence 2 (CNS2). To study the effect of vitamin D on cytosine methylation at CpGs reside on transcription factor binding sites in the CNS2 sequence of forkhead box P3 (*FOXP3*) promoter, this region was bisulfite-treated, amplified and sequenced as described in the methods section. Among 10 CpGs reside in transcription factor binding sites, methylation of cytosine in first, fifth, and sixth CpG sites was significantly reduced in vitamin D treated (Vit D) group compared with the control (C) group (a). The heat map graph of 10 CpG sites and approximate mean percent of methylation on these sites are displayed by colors defined in the legend (b). The sequence graph of the first CpG site (c). The sequence graph of fifth and sixth CpG sites (d). The blue curves represent cytosine, and the red curves represent thymidine. (n=7); (*: $p<0.05$); (:$p<0.01$).**

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

Figure 4. The sequence of the conserved non-coding sequence 2 (CNS2) region of forkhead box P3 (*FOXP3*) promoter and transcription factor binding sites bearing CpGs with a significant reduction in methylation. The sequence of the CNS2 region of *FOXP3* promoter in Mus musculus has been displayed in the 5′ to 3′ direction, containing 310 nucleotides and 10 CpG sites. Using the online TFBIND INPUT database, the binding of transcription factors to the hypomethylated CpGs, the first, fifth, and sixth CpG sites, was predicted. The binding of all transcription factors to the CNS2 region has been proved experimentally (refer to discussion), and they are presented here in the bold format in the picture.

Prediction of Transcription Factors Binding to the CpG Sites

The CNS2 sequence of the *FOXP3* promoter was submitted to the online TFBIND INPUT database for predicting the transcription factor binding sites.[17] Using the online database, the first CpG site of the CNS2 predicted as the binding site for GATA3 and GATA1 transcription factors, and the fifth and the sixth CpG sites bind to STAT1 and STAT3 (Figure 4).

DISCUSSION

In this study, the effect of vitamin D on the expression of *FOXP3*, *TGF-β1*, and *IL-10* genes in the $CD4^+$ T cells of C57BL/6 female mice was assessed. In addition, for the first time, the impact of calcitriol on the DNA methylation level of the CpG sites within the CNS2 region of the *FOXP3* promoter was evaluated. Our data showed that vitamin D raised the expression of *FOXP3*, *TGF-β1*, and *IL-10* genes. In the following, the DNA methylation of cytosine at the CpG binding sites at the CNS2 of *FOXP3* promoter, where transcription factors bind, was examined. Our group previously reported decreased *FOXP3* expression and increased DNA methylation of this region in experimental autoimmune encephalomyelitis (EAE) mice compared to the control group.[15] We found that the DNA methylation of cytosines at CpGs of transcription factor binding sites in the CNS2 region of *FOXP3* promoter was declined in the vitamin D treated mice.

There is growing evidence that vitamin D signaling gives rise to some epigenetic alternations in genes and plays a role in pro-inflammatory or anti-inflammatory networks, resulting in immunomodulation.[12,13] Epigenetic strategies to augment immunomodulation, specifically by Treg cells, have shown promising results. To achieve this end, some researchers followed a pharmaceutical approach to perform acetylation experiments on the *FOXP3* gene. By this, they enhance the immunosuppressive function of Treg in some inflammatory conditions such as arthritis, colitis, and transplant rejection models.[18-20] Similarly, vitamin D was found to engage the epigenetic mechanisms to enhance Treg phenotype and function.[21] Consistent with these findings, we demonstrated that vitamin D

lessen methylation level in the CNS2 region of the *FOXP3* promoter, and this finding supports the upregulation of the *FOXP3* expression in response to vitamin D treatment. Among ten CpG sites that occur in the CNS2 transcription factors binding sites, three CpG sites indicated a significant reduction of DNA methylation level. The First CpG site is where that GATA binding protein 1 (GATA1) and GATA binding protein 3 (GATA3) bind to the CNS2, and it was uncovered that the GATA3 supports the FOXP3 activity[21] and the GATA1 in accompanied with other transcription factors such as the special AT-rich sequence-binding protein 1 (SATB1) contributed to preserving Treg phenotype.[23] On the fifth and sixth CpG sites of the CNS2 region, both signal transducer and activator of transcription 1 (STAT1) and signal transducer and activator of transcription 3 (STAT3) were able to bind. These two transcription factors showed contradictory roles. Ouaked et al showed that the binding of the STAT1 to the *FOXP3* promoter enhanced histone modifications and promoted *FOXP3* expression,[24] which is consistent with our findings regarding the upregulation of the *FOXP3* expression probably result from a reduction in CpG DNA methylation at the STAT1 binding site.

On the contrary, STAT3 destabilizes Treg and limits its functions,[25] which is expected to happen in the inflammatory conditions. So, which one is allowed to gain this CpG site and switch on the interest path? Vitamin D probably orchestrates this event by reducing STAT3 expression.[26,27] Thus, the STAT1 would be the probable winner here and one of the downstream players in the vitamin D immunomodulatory effects.

It has been shown that vitamin D is capable of upregulating anti-inflammatory cytokines.[28] Vitamin D was found to induce IL-10 gene expression in human $CD4^+$ T cells, simultaneously expressing the *FOXP3* gene.[29] In line with these findings, we showed that vitamin D provokes *TGF-β1* and *IL-10* gene expression in $CD4^+$ T cells. Kang et al found that VDR binds to the CNS region in *FOXP3* promoter and caused upregulation in *FOXP3* expression[13] which is in accordance with our findings regarding the upregulation of FOXP3 upon Vitamin D treatment.

Given these findings, we conclude that vitamin D immunomodulatory roles probably benefit from DNA methylation alterations of the CNS2 region of the *FOXP3* gene promoter, which leads to modification of the transcription factor's expression. We can also conclude that by upregulating the Treg anti-inflammatory cytokines in $CD4^+$ T cells, besides FOXP3 upregulation, vitamin D can modulate the adverse immune responses in certain conditions like autoimmune diseases. This is a preliminary study, and more researches are needed to define what inflammatory pathways could be epigenetically affected by vitamin D. In this regard, it is worthy to explore that in what inflammatory circumstances such as multiple sclerosis, graft versus host disease, or rheumatoid arthritis vitamin D assistance can be applied to ameliorate the disease conditions.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

This study has been financially supported by a grant of Tehran University of Medical Sciences, grant number, 96-02-27-35436, and registration code: 20653. The authors are very grateful for this assistance.

REFERENCES

1. Wimalawansa SJ. Non-musculoskeletal benefits of vitamin D. J Steroid Biochem Mol Biol 2018;175:60-81.
2. Wei R, Christakos S. Mechanisms underlying the regulation of innate and adaptive immunity by vitamin D. Nutrients 2015;7(10):8251-60.
3. Penna G, Roncari A, Amuchastegui S, Daniel KC, Berti E, Colonna M, et al. Expression of the inhibitory receptor ILT3 on dendritic cells is dispensable for induction of CD4+ Foxp3+ regulatory T cells by 1, 25-dihydroxyvitamin D3. Blood 2005;106(10):3490-7.
4. Barragan M, Good M, Kolls JK. Regulation of dendritic cell function by vitamin D. Nutrients 2015;7(9):8127-51.
5. Vanherwegen A-S, Eelen G, Ferreira GB, Ghesquière B, Cook DP, Nikolic T, et al. Vitamin D controls the capacity of human dendritic cells to induce functional regulatory T cells by regulation of glucose metabolism. J Steroid Biochem Mol Biol 2019;187:134-45.
6. Pike JW, Meyer MB, Benkusky NA, Lee SM, John HS, Carlson A, et al. Genomic determinants of vitamin D-regulated gene expression. Vitam Horm 100: Elsevier; 2016. p. 21-44.
7. Carlberg C, Molnár F. Vitamin D receptor signaling and its therapeutic implications: Genome-wide and structural

view. Can J Physiol Pharmacol 2015;93(5):311-8.
8. Heikkinen S, Väisänen S, Pehkonen P, Seuter S, Benes V, Carlberg C. Nuclear hormone 1α, 25-dihydroxyvitamin D3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. Nucleic Acids Res 2011;39(21):9181-93.
9. Pereira F, Barbáchano A, Singh PK, Campbell MJ, Muñoz A, Larriba MJ. Vitamin D has wide regulatory effects on histone demethylase genes. Cell Cycle 2012;11(6):1081-9.
10. Ambrosi C, Manzo M, Baubec T. Dynamics and context-dependent roles of DNA methylation. J Mol Biol 2017;429(10):1459-75.
11. Bestor TH, Edwards JR, Boulard M. Notes on the role of dynamic DNA methylation in mammalian development. Proc Natl Acad Sci 2015;112(22):6796-9.
12. Sassi F, Tamone C, D'Amelio P. Vitamin D: nutrient, hormone, and immunomodulator. Nutrients 2018;10(11):1656.
13. Kang SW, Kim SH, Lee N, Lee W-W, Hwang K-A, Shin MS, et al. 1, 25-Dihyroxyvitamin D3 promotes FOXP3 expression via binding to vitamin D response elements in its conserved noncoding sequence region. J Immunol 2012;188(11):5276-82.
14. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. Nature 2010;463(7282):808-12.
15. Noori-Zadeh A, Mesbah-Namin SA, Saboor-Yaraghi AA. Epigenetic and gene expression alterations of FOXP3 in the T cells of EAE mouse model of multiple sclerosis. J Neurol Sci 2017;375:203-8.
16. Jiang M, Zhang Y, Fei J, Chang X, Fan W, Qian X, et al. Rapid quantification of DNA methylation by measuring relative peak heights in direct bisulfite-PCR sequencing traces. Lab Invest 2010;90(2):282-90.
17. 1. Wimalawansa SJ. Non-musculoskeletal benefits of vitamin D. J Steroid Biochem Mol Biol 2018;175:60-81.
2. Wei R, Christakos S. Mechanisms underlying the regulation of innate and adaptive immunity by vitamin D. Nutrients 2015;7(10):8251-60.
3. Penna G, Roncari A, Amuchastegui S, Daniel KC, Berti E, Colonna M, et al. Expression of the inhibitory receptor ILT3 on dendritic cells is dispensable for induction of CD4+ Foxp3+ regulatory T cells by 1, 25-dihydroxyvitamin D3. Blood 2005;106(10):3490-7.
4. Barragan M, Good M, Kolls JK. Regulation of dendritic cell function by vitamin D. Nutrients 2015;7(9):8127-51.
5. Vanherwegen A-S, Eelen G, Ferreira GB, Ghesquière B, Cook DP, Nikolic T, et al. Vitamin D controls the capacity of human dendritic cells to induce functional regulatory T cells by regulation of glucose metabolism. J Steroid Biochem Mol Biol 2019;187:134-45.
6. Pike JW, Meyer MB, Benkusky NA, Lee SM, John HS, Carlson A, et al. Genomic determinants of vitamin D-regulated gene expression. Vitam Horm 100: Elsevier; 2016. p. 21-44.
7. Carlberg C, Molnár F. Vitamin D receptor signaling and its therapeutic implications: Genome-wide and structural view. Can J Physiol Pharmacol 2015;93(5):311-8.
8. Heikkinen S, Väisänen S, Pehkonen P, Seuter S, Benes V, Carlberg C. Nuclear hormone 1α, 25-dihydroxyvitamin D3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. Nucleic Acids Res 2011;39(21):9181-93.
9. Pereira F, Barbáchano A, Singh PK, Campbell MJ, Muñoz A, Larriba MJ. Vitamin D has wide regulatory effects on histone demethylase genes. Cell Cycle 2012;11(6):1081-9.
10. Ambrosi C, Manzo M, Baubec T. Dynamics and context-dependent roles of DNA methylation. J Mol Biol 2017;429(10):1459-75.
11. Bestor TH, Edwards JR, Boulard M. Notes on the role of dynamic DNA methylation in mammalian development. Proc Natl Acad Sci 2015;112(22):6796-9.
12. Sassi F, Tamone C, D'Amelio P. Vitamin D: nutrient, hormone, and immunomodulator. Nutrients 2018;10(11):1656.
13. Kang SW, Kim SH, Lee N, Lee W-W, Hwang K-A, Shin MS, et al. 1, 25-Dihyroxyvitamin D3 promotes FOXP3 expression via binding to vitamin D response elements in its conserved noncoding sequence region. J Immunol 2012;188(11):5276-82.
14. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. Nature 2010;463(7282):808-12.
15. Noori-Zadeh A, Mesbah-Namin SA, Saboor-Yaraghi AA. Epigenetic and gene expression alterations of FOXP3 in the T cells of EAE mouse model of multiple sclerosis. J Neurol Sci 2017;375:203-8.
16. Jiang M, Zhang Y, Fei J, Chang X, Fan W, Qian X, et al. Rapid quantification of DNA methylation by measuring relative peak heights in direct bisulfite-PCR sequencing traces. Lab Invest 2010;90(2):282-90.
17. Tsunoda T, Takagi T.Estimating transcription factorbindability on DNA. Bioinformatics 1999;15(7-8):622-30.

18. Tao R, De Zoeten EF, Özkaynak E, Chen C, Wang L, Porrett PM, et al. Deacetylase inhibition promotes the generation and function of regulatory T cells. Nat Med 2007;13(11):1299-307.
19. Reilly CM, Thomas M, Gogal Jr R, Olgun S, Santo A, Sodhi R, et al. The histone deacetylase inhibitor trichostatin A upregulates regulatory T cells and modulates autoimmunity in NZB/W F1 mice. J Autoimmun 2008;31(2):123-30.
20. Saouaf SJ, Li B, Zhang G, Shen Y, Furuuchi N, Hancock WW, et al. Deacetylase inhibition increases regulatory T cell function and decreases incidence and severity of collagen-induced arthritis. Exp Mol Pathol 2009;87(2):99-104.
21. Spanier JA, Nashold FE, Mayne CG, Nelson CD, Hayes CE. Vitamin D and estrogen synergy in Vdr-expressing CD4+ T cells is essential to induce Helios+ FoxP3+ T cells and prevent autoimmune demyelinating disease. J Neuroimmunol 2015;286:48-58.
22. Wang Y, Su MA, Wan YY. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. Immunity 2011;35(3):337-48.
23. Fu W, Ergun A, Lu T, Hill JA, Haxhinasto S, Fassett MS, et al. A multiply redundant genetic switch'locks in'the transcriptional signature of regulatory T cells. Nat Immunol 2012;13(10):972.
24. Ouaked N, Mantel P-Y, Bassin C, Burgler S, Siegmund K, Akdis CA, et al. Regulation of the foxp3 gene by the Th1 cytokines: the role of IL-27-induced STAT1. J Immunol 2009;182(2):1041-9.
25. Laurence A, Amarnath S, Mariotti J, Kim YC, Foley J, Eckhaus M, et al. STAT3 transcription factor promotes instability of nTreg cells and limits generation of iTreg cells during acute murine graft-versus-host disease. Immunity 2012;37(2):209-22.
26. Bansal AS, Henriquez F, Sumar N, Patel S. T helper cell subsets in arthritis and the benefits of immunomodulation by 1, 25 (OH) 2 vitamin D. Rheumatol Int 2012;32(4):845-52.
27. Lam E, Choi SH, Pareek TK, Kim B-G, Letterio JJ. Cyclin-dependent kinase 5 represses Foxp3 gene expression and Treg development through specific phosphorylation of Stat3 at Serine 727. Mol Immunol 2015;67(2):317-24.
28. Skrobot A, Demkow U, Wachowska M. Immunomodulatory role of vitamin D: a review. Current Trends in Immunity and Respiratory Infections: Springer; 2018. p. 13-23.
29. Urry Z, Chambers ES, Xystrakis E, Dimeloe S, Richards DF, Gabryšová L, et al. The role of 1α, 25-dihydroxyvitamin D 3 and cytokines in the promotion of distinct Foxp3+ and IL-10+ CD 4+ T cells. Eur J Immunol 2012;42(10):2697-708.