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Research Article

The Promoting Role of Vitamin D3 and B9 on Differentiation of CD4+CD28+ Regulatory T Cells by Triggering FOXP3 Expression

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Abstract

Background: Metabolism plays an important role in determining the fate of immune cells. mTOR is a serine/threonine-protein kinase that plays an important role in the growth, proliferation, survival, and function of immune cells. Lack of mTOR signaling disrupts the differentiation pathways of TH2, TH1, and TH17 cells. Targeting metabolic and signaling pathways (AMPK, HIF-1, mTOR) and controlling TCD4 cell differentiation, could be a way to treating autoimmune diseases. The aim of this study was to investigate the effect of vitamins 1 and 25 dihydroxy vitamin D3 and vitamin B9 on the differentiation of primary $CD4^+$ T-cells and the expression of mTOR *in vitro.*

Materials and Methods: Peripheral blood mononuclear cells isolated from healthy male and divided into five groups include stimulated with Anti-CD3 and Anti-CD28, stimulated with Anti-CD3 and Anti-CD28 and in the presence of IL-2, TGF -β, stimulated with Anti-CD3 and Anti-CD28, without cytokine and in the presence of 1, 25-(OH) 2D3, stimulated with Anti-CD3 and Anti-CD28, without cytokine and in the presence of vitamin B9, and stimulated with Anti-CD3 and Anti-CD28 in the presence of vitamin B9 and 1, 25-(OH) 2D3. After 5-7 days, the differentiation of cells into Treg and the effect of 1, 25-(OH) 2D3 and Vitamin B9 on the expression of T-bet, mTOR, FOXP3, 3-GATA, TGF-β, ROR-γt, and FOXP3 were determined by Real Time PCR and flow cytometry, respectively.

Results: The results of this study showed that in the presence of 1, 25-(OH) 2D3, the expression of FOXP3 transcription factor increased and due to the decreased expression of mTOR gene, the expression of GATA, T-bet and ROR-γt were suppressed and as a result, $TCD4^+$ cells were differentiated into Treg cells. In culture medium containing vitamin B9, an increase in FOXP3 gene expression and no decrease in mTOR expression were seen. Also, in the presence of both vitamins OH 1, 25-(OH) 2D3 and B9, expression of FOXP3 increased in the comparison with the group treated with 1, 25-(OH) 2D3 ($P < 0.05$).

Conclusion: Vitamins 1, 25-(OH) 2D3, and Vitamin B9 increase the differentiation of TCD^+ cells into Treg cells by increasing the expression of TGF-β and FOXP3 genes and suppressing GATA-3, T-bet, mTOR and ROR-γt genes expression.

Keywords: Regulatory T Cell; 1, 25-(OH) 2D3; Vitamin B9; FOXP3

Introduction

Host defense mechanisms via both cellular and humoral weapons of immune system have been evolved to maintain the host cell integrity, balance, and survival [1]. T lymphocytes as an essential part of cell mediated immunity are involved in a broad spectrum of phenotypic and functional characteristics of cellular immunity against infected and malignant cells [2]. $TCD4^+$ lymphocytes as the major components of T cells carry out several substantial functions in both activation and suppression of immune and non-immune cells [3]. Naïve $TCD4^+$ lymphocytes are activated upon binding of T cell receptor (TCR) to antigen loaded major histocompatibility complex (MHC) and differentiate into distinct subsets of T helper cells with various functions according to the polarizing signals derived by certain cytokine milieu and expression of unique transcription factors [4]. T helper subsets including Th1, Th2, Th17, Th9, Th22, follicular helper T cells (TFH), and regulatory T cells (Treg) which exert both inflammatory or immunosuppressive responses [5].

$CD4^+$ $CD25^{hi}$ $FOXP3^+$ regulatory Treg cells comprising around 5 - 10% of peripheral blood $CD4^+$ lymphocytes and play some crucial roles in mediating immune responses, maintaining homeostasis as well as regulating peripheral tolerance to self-antigens which prevents autoimmunity along with limiting inflammatory immune responses against commensal pathogens and transplants [6]. At least two subtypes of FOXP3-expressing Treg cells exist termed as thymus derived, naturally occurring Treg (nTreg) defined as antigen-specific regulatory T cells which are involved in the maintenance of peripheral tolerance [7] and another subtype called induced Treg (iTreg) which differentiate in the periphery upon antigen recognition by naive $TCD4^+$ cells through TGF-β signaling [5]. The underlying suppression mechanisms of nTreg and iTreg cells are conducted by contact dependent/independent manner [8]. Treg mediated suppression can be mediated by cytolysis, inhibitory cytokines, maturation of dendritic cell and metabolic or endocrine disruption [7]. Metabolic pathways have a central role in determining the lineage differentiation fate of immune cells, and promote survival of different T cell subsets [9]. Proliferation and differentiation of T cells is dynamically regulated by a serine/threonine protein kinase referred to as Mammalian Target of Rapamycin (mTOR) introduced as a major growth regulator of immune cells [10]. Antigen detection in the absence of mTOR signaling and presence of TGF-β, can induce the differentiation of $FOXP3^+$ regulatory T cell and inhibit the differentiation of Th1, Th2 and Th17 [11]. Hence, targeting the metabolic and signaling pathways particularly signaling of mTOR, hypoxia-inducible factor 1 (HIF-1), AMP-activated protein kinase (AMPK) which are involved in $TCD4^+$ cells differentiation may provide an appropriate therapeutic strategy to prevent the progression of autoimmune diseases [12].

A century ago, a study conducted by Mellanby., *et al*. reported the relevance of vitamin D for securing a balanced immune system [13] and during the last few decades, it has clearly emerged that the importance of vitamin D goes beyond the regulating calcium homeostasis and consequently, bone growth and resorption and focused on immunomodulatory effect of vitamin D which can promote Treg differentiation [14]. Two main forms of vitamin D are cholecalciferol (vitamin D3) with the source of animal foodstuffs and ergocalciferol (vitamin D2) which can be derived from plants. In addition to dietary sources of them, the major source of vitamin D3 can be metabolized in the skin from 7-dehydrocholesterol in the skin through exposure to sunlight. Vitamin D3 is hydroxylated in the liver by Vitamin D 25-hydroxylase also known as Cytochrome P450 Family 2 Subfamily R Member 1 (CYP2R1) to form 25-hydroxyvitamin D3 (calcidiol). Further hydroxylation of vitamin D3 inactive form, is occurred by Cytochrome P450 Family 27 Subfamily B Member 1 (CYP27B1) to produce 1, 25-Dihydroxycholecalciferol (1, 25-(OH) 2D3) or calcitriol as the hormonal form of vitamin D [15]. Previous in vitro studies exhibited profound immunomodulatory features of vitamin D on several components of innate and adaptive immune system. It can prevent the maturation of dendritic cell (DC), thereby modulating T cell proliferation to favor a tolerogenic phenotype. Insufficiency of Vitamin D is associated with an increased risk of developing autoimmune diseases particularly multiple sclerosis (MS) and Type 1 Diabetes (T1D) [16]. It has been indicated that vitamin D as a dietary supplement can suppress the development of experimental autoimmune encephalomyelitis (EAE), a murine model of MS [17].

Folic acid or vitamin B9, has a pivotal role in DNA synthesis, repair and methylation along with protein synthesis [18]. A key enzyme in the folic acid metabolism is methylenetetrahydrofolate reductase (MTHFR) which has an essential function in catalyzing processes of DNA methylation and nucleotide synthesis and it is required for maintenance of homoeostasis in the immune system [19]. It is well recognized that insufficient levels of folic acid can

dramatically effect immune cells function [20]. In a study conducted on patients with megaloblastic anemia, depressed cellular mediated immunity has been observed due to inadequacy of folic acid which in turn can lead to ineffective methylation process that can disrupt homeostasis and finally lead to immune dysfunction [21].

Although several studies have demonstrated the prominent role of vitamin D and B9 in immunomodulation and autoimmunity, further research is still needed to systematically investigate the exact mechanisms involved in balancing the immune system.

The specific objective of current study was to assess the effects of vitamin D3 and B9 on the *in vitro* expression of specific transcription factors for T cells particularly mTOR, ROR-γt, T-bet, GATA-3, TGF-β, and FOXP3 as well as in vitro differentiation of naïve $TCD4^+$ to Treg cells.

Materials and Methods

Sampling

A total of 10 cc peripheral blood divided equally into heparin and EDTA coated tubes were taken from six healthy donors with average age of 29.5 years. Blood samples was diluted with PBS (1:1 ratio) at room temperature. Criteria for excluding the subjects were as follows: individuals on glucocorticoids or immunosuppressants regimen, evidence of active malignancies in the past 5 years or an autoimmune disease, congestive heart failure, alanine aminotransferase (ALT) >3x normal, uncontrolled hyper/hypothyroidism, history of excessive alcohol consumption, drug abuse or smoking. The research was ethically approved by Medical Ethics Committee of Shahid Sadoughi University of Medical Sciences (approval ID: 1393.153) and informed consent was obtained in accordance with the Declaration of Helsinki.

PBMC Isolation and expansion from adult peripheral blood

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by ficoll gradient centrifugation (GE-HealthCare®, Ficoll-Paque PREMIUM density gradient media) and Lymphocyte Separation Medium (Cellgro®). The mononuclear cells were counted and then, 500000 PBMCs were cultured in each of four separated groups using X-VIVO™ 15 media as follows: 1) PBMCs in the presence of Anti-CD3 and Anti-CD28. 2) PBMCs in the presence of Anti-CD3, Anti-CD28, TGF-β and IL-2 cytokines. 3) PBMCs in the presence of Anti-CD3 and Anti-CD28, without the mentioned cytokines but in the presence of 1.25 (OH) 2D3 metabolite, and 4) PBMCs in the presence of Anti-CD3, Anti-CD28 and simultaneous presence of two metabolites as 1.25 (OH) 2D3 and Vitamin B9 (Table 1).

Groups	Treat
Group 1 (control group)	Anti-CD3 + Anti-CD28
Group 2	Anti-CD3 + Anti-CD28 + TGF-β + IL-2
Group 3	Anti-CD3 + Anti-CD28 + 1.25 (OH) 2D3
Group 4	Anti-CD3 + Anti-CD28 + 1.25 (OH) 2D3 + Vitamin B9

Table 1: Classification of study groups.

Vitamin D metabolite and recombinant human IL-2 treatment (Sigma Aldrich®, hIL2) at the dose of 8.5µl/ml has been used on day 0 and the cells were then harvested at day 5. In addition, vitamin B9 metabolite and IL-2 with the same dose were added on day 2 and the cells were harvested at day 7.

Total RNA extraction and cDNA synthesis

The total RNA extracted using RNX plus solution (Cinnagen, Iran) according to the manufacturer's protocol. RNA was diluted with 20 µl RNase-free water and quantification was determined by spectrophotometry at 260 nm, then stored at -20°C. RNA with a 260/280 nm ratio in the range 1.8 to 2 was considered as high quality. Concisely, in order to generate cDNA, 8µL of extracted RNA and 1 µL oligo dT-adapter primer were added to 1 µl of dNTP mixture, followed by an incubation period of 5 min at 65°C. Further, 0.5 µL reverse transcriptase, 2 µl 10 × RNA PCR buffer, and 7.5µlRNase-free water were mixed together and incubated at 25°C for 10 min, followed by 42°C for 60 min and 85°C for 5 min in ABI Gene Amp PCR System 9700 (Perkin-Elmer Co, USA). Lastly, the cDNA samples were stored at -20°C.

Real-time PCR

The real-time PCR was performed in a Master cycler ep realplex 4S (Eppendorf, Germany) using RealQ Plus 2x Master Mix with Green Dye High ROX (amplicon, Denmark) according to instructions for validation of Real Time PCR. The reactions were carried

out in a total volume of 20 µl, consisting of 10µl of master mix (cyber green), 6 µl of nuclease free water, 1µl of forward primer, 1 µl of reverse primer, and 2 µl of cDNA (50 µg/ml). For each sample, the reactions were performed in duplicate. The β-actin gene was considered as a housekeeping gene, and samples without cDNA were determined as negative control or non-template control (NTC). The primers with certain sequences were utilized as shown in table 2. Quantification of mRNA abundance in samples was measured by the ΔΔCT method (ΔΔCT= ΔCT sample - ΔCT control).

Gene	Forward primers	Reverse primers
Foxp3	5'- GAAACAGCA-CATTCCCAGAGTTC-3'	5'- ATGGCCCAGCGGAT-GAG-3
GATA	5'-TCTTCGCTACCCAGGT-GACCCGA-3'	5'-AAGGGGCGACGACTCT-GCAAT-3'
T-bet	5'-TGTTGTGGTCCAAGTT-TAATCAGCA-3'	5'-CCCGGCCACAGTAAAT-GACAG-3'
TGF-β	5'-CGCGTGCTAATGGTG-GAAAC-3'	5'-CGGAGCTCTGATGT-GTTGAAGA-3'
RORγt	5'-TGCAAAGAAGACCCA-CACCTCACA-3'	5'- ATCGGTTTCGGCTG-GTGCGG-3'
mTOR	5'-TCCGAGAGATGAGT-CAAGAGG-3'	5'-CACCTTCCACTCCTAT-GAGGC-3'
β-actin	5'-CAA GAG ATG GCC ACG GCT GCT-3'	5'-TCC TTC TGC ATC CTG TCG GCA-3'

Table 2: Primers which designed for Real-Time PCR.

Flow cytometry

Antibodies

Flow cytometry staining of both intracellular and extracellular proteins have been performed by BioLegend antibodies as follows: Human CD28 mAb, Human CD3 mAb, Per CP-Cy 5.5 mouse anti-human CD4, FITC mouse anti-human CD25, Alexa flour 647 anti-human FOXP3 along with Human FOXp3 staining buffer.

Intracellular staining of FOXP3

At the first place, 3µL of anti-CD25-FITC were added to the flow cytometer tube holding permeabilized cells suspension. Following 30 min incubation in the refrigerator, the staining buffer, then, PBS were added and centrifuged at 350xg for 5 minutes. After the secondary incubation in dark room, 3ml of staining buffer were again added and centrifuged. The supernatant was removed and immediately solution B followed by anti-FOXP3 were added to the tube. At the final step, 3ml of staining buffer together with 500µL of cell fix solution were added to the tube. Details about the protocol can be found on the manufacturer's website (https://www.biolegend.com/).

CD25 extracellular staining

To each flow cytometric tube,100 µL staining buffer was added and then 5 µL of anti CD25-FITC for extracellular staining of samples. At the end of the incubation for 30min in refrigerator, 500 ml of staining buffer was added to each tube and after centrifugation, supernatant was removed from the tube. Next, 500 µL of cell fix buffer was added to tubes with cell surface staining for CD25.

Statistical methods

Data analysis was performed withSPSS-V21 software (SPSS Inc., Chicago, Ill., USA 21) and non-parametric Friedman test were applied to detect the differences between groups. The non-parametric Wilcoxon test was utilized as well to calculate the difference between each set of pairs.

Results

We observed expression changes in the mRNA levels of mTOR, ROR-γt, T-bet, GATA-3, TGF-β, and FOXP3 in treated media with D3, D3/B9 and B vitamins. β-actin was considered as housekeeping gene in Real-time PCR assay for normalization of data analyzed by$2^{-\Delta\Delta Ct}$ method.

Vitamin D3 and B9 promoted the FOXP3 gene expression in PBMCs

Real time-PCR results demonstrated that treating with D3, B9 and D3/B9, resulted in 2.7 median fold, 2.6 median fold and 3.1 median fold increases in FOXP3 gene expression respectively compared to untreated group. Additionally, increased expression of FOXP3 gene also exhibited a significant difference between D3, D3/B9 and B9 treated groups (Figure 1).

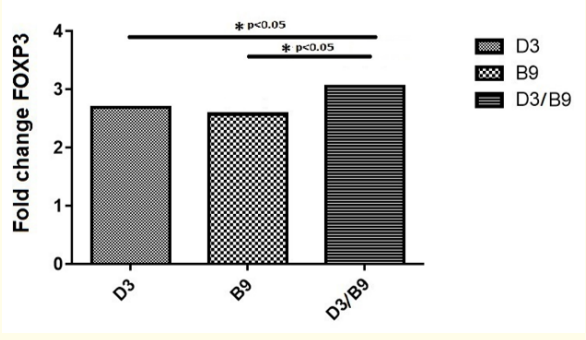

Figure 1: Changes in FOXP3 gene expression in Treg cells treated with vitamins B9, D3/B9 and D3 have been shown. Significant differences between the B9 and D3/B9 treated groups and among D3/B9 and D3 treated groups were displayed ($p \leq 0.05$).

Vitamin D3 and B9 decreased the GATA-3 gene expression in PBMCs

The Real time-PCR results for GATA-3 gene expression in PBMC are shown in figure 2. We found that treating with D3, B9 and D3/B9, resulted in 0.58 median fold, 0.74 median fold and 0.6 median fold decreases in GATA-3 gene respectively compared to untreated group. Furthermore, our results revealed that expression of GATA-3 gene among D3 and D3/B9 groups were significantly different compared to B9 treated group.

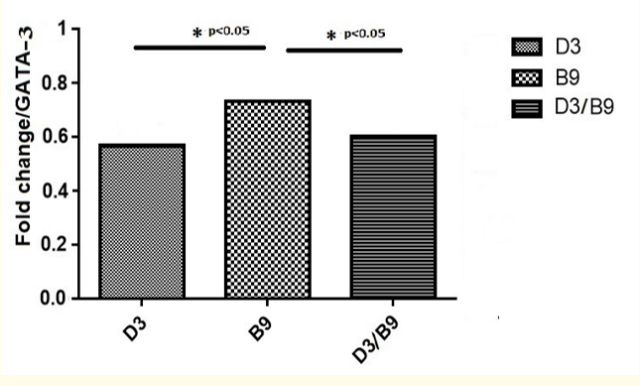

Figure 2: Changes in GATA-3 gene expression have been demonstrated in cells treated with vitamins B9, D3/B9 and D3. These changes showed a significant difference between groups treated with B9 and D3 as well as groups treated with D3/B9 and B9 ($P \leq 0.05$).

Vitamin D3 and B9 had no effect on TGF-β gene expression

The results revealed that TGF-β gene expression had no significant difference between vitamin D3, D3/B9 and B9 treated groups compared to untreated types (Figure 3).

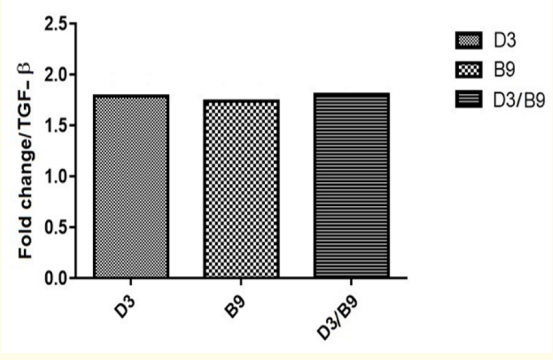

Figure 3: Changes in TGF-β gene expression in different groups treated with vitamins B9, D3/B9 and D3 have been shown. No significant differences were found between groups ($P > 0.05$).

Effects of vitamin D3 and B9 on T-bet gene expression in PBMCs

Changes in T-bet gene expression in PBMCs following treatment with vitamin D3, B9 and D3/B9 are shown in figure 4. Our results revealed that treating with D3, B9 and D3/B9, resulted in 0.42 median fold, 0.64 median fold and 0.45 median fold decreases in T-bet gene expression respectively compared to control group. The alteration levels of T-bet expression were significantly different between the B9 and D3 treated groups, among the D3/B9 and B9 treated groups and also between the D3/B9 and D3 treated groups ($p \leq 0.05$).

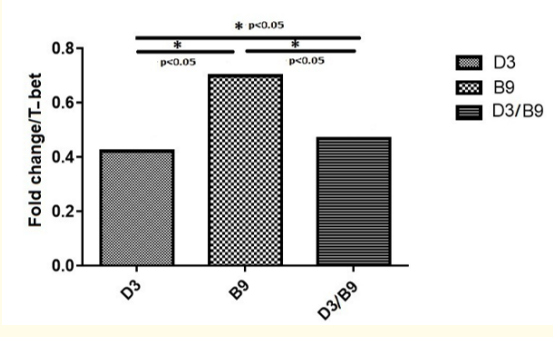

Figure 4: Comparison of T-bet gene expression in groups treated with vitamins B9, D3/B9 and D3.

Vitamin D3 reduced the expression of mTOR in PBMCs

Evaluation of mTOR gene expression showed that treating with D3 and D3/B9, resulted in 0.76 and 0.8 median fold decreases in mTOR gene expression respectively compared to control group while there was no significant difference in the expression of mTOR between vitamin B9 treatment and control group (Figure 5).

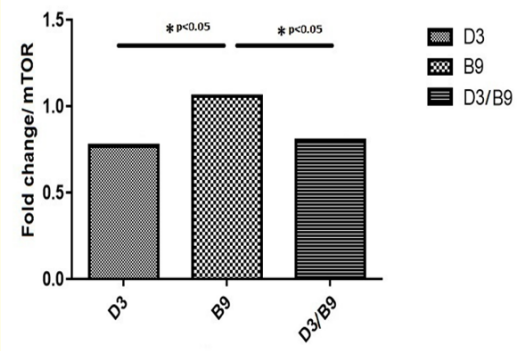

Figure 5: Expression levels of mTOR in the examined groups.

Vitamin D3 and B9 in decreased expression of RORγt in PBMCs

The results of RT-PCR analysis for RORγt expression in PBMC showed that the expression level of RORγt gene was significantly reduced in vitamin D3, B9 and D3/B9 treated groups (0.4 fold, 0.57 fold, and 0.42 fold respectively) compared to untreated group. In addition, the expression level of RORγt gene was significantly different in D3 and D3/B9 groups compared to B9 group (Figure 6).

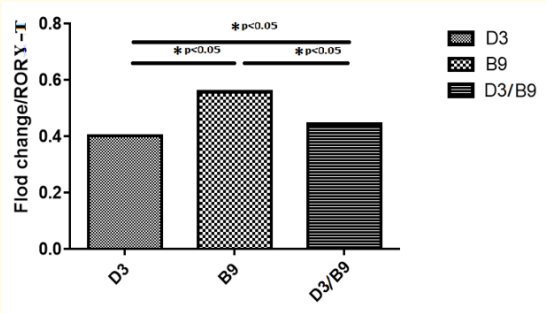

Figure 6: Comparison of the RORγt expression in all treated groups and control group.

Comparison chart for expression of the above-mentioned genes revealed the highest expression of TGF-β, T-bet, mTOR, and RORγt genes in B9-treated group and FOXP3 in D3/B9-treated group. No significant differences were observed in TGF-β expression in the treatment groups (Figure 7).

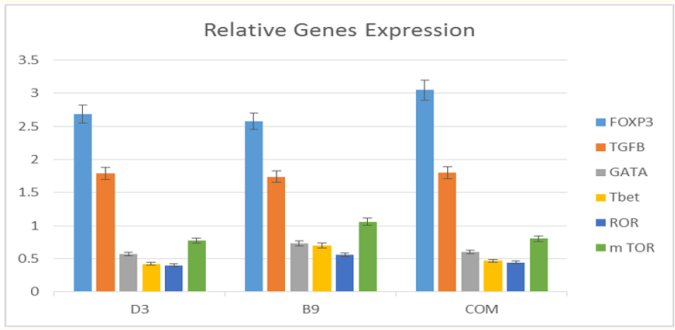

Figure 7: Comparison of expression of FOXP3, GATA-3, TGF-β, T-bet, mTOR, and RORγt genes in all considered groups.

Enhanced expression of Treg surface markers under vitamin D3 and B9 treatment

The number of $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs was analyzed by flow cytometry, using CD4, FoxP3 and CD25 extracellular staining in D3, B9, and D3/B9 treated groups. Lymphocytes were gated by forward and side scatter (Figure 8). Our results exhibited an enhanced expression of Treg surface markers in all treated groups compared to the control group which may suggest a role of vitamins D3 and B9 in the induction of regulatory T cells.

Changes in intracellular FOXP3 expression in vitamin D3 and B9 groups

Considering the increased level of FOXP3 gene expression affected by vitamin B9, D3/B9 and D3 in Real time-PCR, we also evaluated the intracellular FOXP3. Our results showed the increased expression level of intracellular FOXP3 in all treated groups compare to the control group (Figure 9).

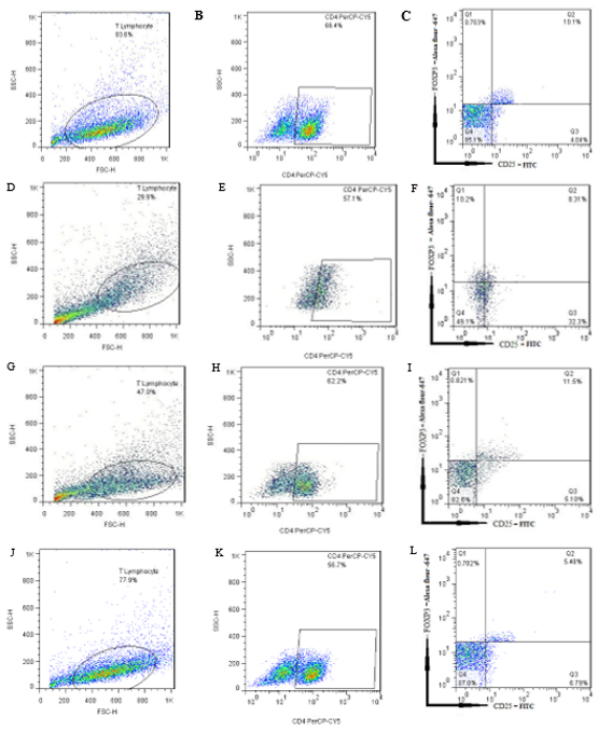

Figure 8: Flow cytometric analysis of CD4+ T cells and CD4+ CD25+ FOXP3+ Tregs population. Cells were gated for lymphocytes based on their side scattering properties. Representative dot plot A- L illustrate details for each condition: (A) the percentage of T lymphocyte in culture media following treatment with vitamin D3, (B) gating strategy for the analysis of CD4+ T cells, (C) the percentage of CD4+CD25+ FOXP3+ regulatory T cells in culture media following treatment with VitaminD3, (D) the percentage of T lymphocyte in culture media treated with Vitamin B9, (E) the gated T CD4+ cells in mentioned media, (F) the percentage of CD4+ CD25+FOXP3+ regulatory T cells in culture media treated with Vitamin B9, (G) the percentage of T lymphocyte in culture media treated with combination of Vitamin B9 and Vitamin D3, (H) the gating strategy for the analysis of CD4+ T cells in mentioned media, (I) the percentage of CD4+ CD25+ FOXP3+regulatory T cells in culture media treated with combination of vitamin B9 and Vitamin D3, (J), (K) and (L) the percentage of T lymphocyte, CD4+ CD25+ FOXP3+ regulatory T cells in control group (vitamin-free medium). As demonstrated, there were remarkable increase in the production of CD4+ CD25+ FOXP3 + Treg cells in three treated groups compare to the control.

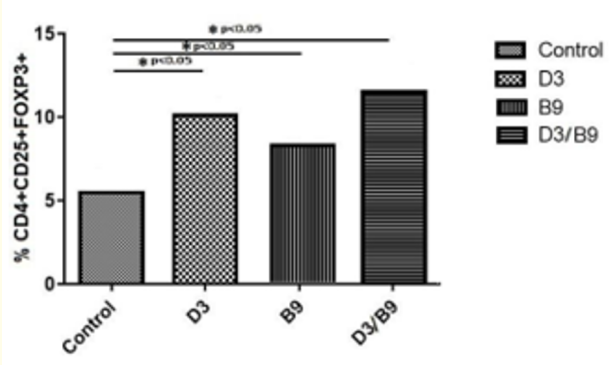

Figure 9: Comparison of intracellular FOXP3 expression in vitamin-treated groups and control group.

Discussion and Conclusion

Immunometabolism highlights the impact of cellular metabolism and nutrients on the functional specificity of immune cells via regulating the activation and proliferation of immune cells by influencing signal transduction and gene expression in both steady and inflammatory states [22]. Moreover, cellular metabolism provides the bioenergetics and biosynthetic demands of the cells for shaping an effective immune response under inflammatory conditions developing in cancers, autoimmune diseases and infections as well [23]. The evidence suggests that immune cells adjust various metabolic pathways for fulfilling their energetic requirements at different situations and according to their functions The transition of T lymphocytes from naïve to highly activated cells and subsequently to memory or regulatory T is paralleled with requires transcriptional reprograming induced by metabolic switch which is tightly regulated by the cell itself [24].

In recent years, the immunomodulatory actions of vitamin D predominated to the historical distinguished role plays in the regulation of calcium-phosphorous and bone metabolism [25,26]. The epidemiological studies have reported that individuals with vitamin D deficiency have increased risk of developing autoimmune diseases such as multiple sclerosis (MS) [27], systemic lupus erythematosus (SLE), and type 1 diabetes (T1D) [28] and supplementing with vitamin D demonstrated considerably deceased risk of MS [29] and T1D.

According to O'Neill., *et al.* during inflammation, the metabolic shift in immune cells is linked by enhanced glucose uptake, stimulated glycolysis and increased activity of the pentose phosphate pathway. Whereas, AMP-activated protein kinase and HIF1β have opposite effects on metabolic shift, the anti-inflammatory cells, such as Tregs induce decreased glycolytic activity and greater levels of oxidative metabolism [30]. A wide spectrum of research, addressed the physiological effects of 1.25 (OH) 2D3 on proliferation, differentiation and, metabolism pathways of $CD4^{+CD25+FOXP3+}$ Tregs in T lymphocytes. This metabolite may differentiate by suppressing the PI3K/AKT/mTOR signaling [31,32]. Likewise, vitamin B9 may increase differentiation of Treg cells with unknown reaction mechanisms [33,34]. Our results demonstrated that Tregs differentiation were remarkably increased in the groups with combined treatment of vitamin D with B9 compared to the either of them separately. It is presumable that in the presence of IL-2 along with anti-CD3 and anti-CD28, vitamin D3 couple to vitamin D receptor (VDR) and then this complex may be capable to bind the Vitamin D response element (VDRE), located in gene enhancer/promoter region and then bind to regulatory sequences of specific transcription factors and subsequently influence the transcription rate of FOXP3 [35,36]. According to our findings, increases of FOXP3 gene expression was seen in $TCD4^{+}$ cells treated with 1,25(OH)2D3 and in the presence of, anti-CD3, anti-CD28 and IL-2. This result can be explained by the production of indolamine 2,3-dioxygenase (IDO) by dendritic cells, which may downregulate the activity of immune cells while induce the differentiation of FOXP3 Tregs [37].

In the present study, combined presence of IL-2, 1,25(OH)2D3, and anti CD3/CD28 induced the expression of FOXP3 in human $CD25^{+}$ $TCD4^{+}$ cells which can also derived by the presence of dendritic cells in the culture media which can promote the differentiation of $TCD4^{+}$ cells to Tregs. Contrary to the previous studies, Jaehoon Chang., *et al*. documented the inhibitory role of 1,25(OH)2D3 on expression of Foxp3 in myelin oligodendrocyte glycoprotein (MOG)-specific $CD4^{+}$ T cells in the presence of MOG peptide, antigen-presenting cells, and TGF-β They also revealed that 1,25(OH)2D3 can inhibit the generation of IL-17-secreting cells in the presence of IL-6 and TGF-β, however it could not affect the differentiation of IFN-γ-secreting cells while it was associated with decreased levels of Foxp3 and IL-17 expression in 1,25(OH)2D3-treated $CD4^{+}$ T cells [38]. Their results differ from our findings which is possibly as a result of differences in the doses of vitamins and inducing conditions. Kunisawa., *et al*. investigated that vitamin B9- depleted diet led to the reduction in the number of Tregs in the small intestine [39]. Additionally, vitamin B9 is capable to deliver IL-2-independent survival signal for $Foxp3^{+}$ Treg cells [40]. These research findings suggest significant relationships between nutrients and the immune functions, which is mediating in maintaining of the immune intestinal hemostasis.

There are scientific controversies regarding the effect of 1.25 (OH) 2D3 on TH2 cells in culture media containing PBMCs. Some studies notified a decrease in the number of TH2 cells and serum level of IL-4 [41] while the others reported an increased differentiation of TH2 [42] in the presence of 1.25(OH)2D3. Our results exhibited decreased level of GATA-3 derives from reduced differentiation of TH2.

There are some reports on differentiation of $CD4^{+}CD25^{+}FOXP3^{+}$ regulatory T cells in the presence of retinoic acid and TGF-β [43,44]. In the present study the expression levels of ROR-γ, T. bet, and GATA3 were negatively affected by 1,25(OH)2D3.

Our findings suggest a role for 1.25(OH)2D3 with/or vitamin B9 in promoting the FOXP3 and impeding the mTOR genes expression. Moreover, the vitamin B9 influenced the FOXP3 gene expression however there was no significant change in mTOR gene expression. Therefore, it may be inferred that vitamin D3 and vitamin B9 can induce Tregs differentiation through transcriptional regulation Besides, expression in groups treated with vitamin D3 alone or combined with vitamin B9 reduced the expression of T-bet and ROR-γt genes, so it appears that vitamin D3 is able to inhibit the differentiation of TH1 and TH17 cells while there is no role for vitamin B9 in this case. In the present study, the metabolites of vitamins D3 and B9 increased the differentiation of $TCD4^{+}$ cells into Treg they had no effect on TGF-β gene expression.

Several attempts have been highlighted the dysfunction of Tregs in a broad spectrum of inflammatory and autoimmune diseases such as inflammatory bowel disease, type 1 diabetes, multiple sclerosis, and allergic disorders. Defects in the function of regulatory T cells or resistance of effector T cells to suppression by Treg may contribute in the pathogenesis of mentioned diseases. Latest development in immunotherapies proved that balanced contribution of effector and regulatory T cells is pivotal for modulating the autoimmune responses. The isolation, expansion and infusion of regula-

tory T cells to patients suffering from autoimmune diseases or prescription of immunosuppressive agents following transplantation or GVHD are currently under global implementation. An implication of our findings is that both vitamin D3 and B9 should be taken into account for their crucial immunoregulatory role in current treatment strategies for inflammatory and auto immune diseases.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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