1,25-Dihydroxyvitamin D3 Regulates T helper and B Lymphocyte Responses Substantially in Drug-Naive Primary Sjögren’s Syndrome Patients’ Mononuclear Cells

Abstract

Background/aim: A correlation between vitamin D deficiency and primary Sjögren’s Syndrome (pSS) has already been described. The limited data have been reported regarding the pathological relevance of vitamin D in primary Sjögren’s syndrome. In this study, the peripheral blood mononuclear cells were cocultured with 1,25-dihydroxyvitaminD3 to determine the modulatory effect of vitamin D3 on T and B lymphocyte phenotypes in pSS.

Materials and methods: Venous blood samples were collected from 11 in treatment phase and 9 drug-naive pSS patients. Peripheral blood mononuclear cells (PBMC) were isolated and separately cultured in the presence and absence of 1,25-dihydroxyvitaminD3 (10mM) for 5 days of culture period. Lymphocyte proliferation was analyzed for CFSE signaling via flow cytometry. CD3+CD4+ cells were analyzed for intracellular IFN-γ and IL-17 expressions. CD19+IgD cells were analyzed for CD38 and CD27 expressions to evaluate naïve and total memory B cell subsets. Culture supernatants were analyzed for the IFN-γ, IL-17 and IL-10 cytokine secretions via flow cytometry.

Results: 1,25-dihydroxyvitaminD3 significantly decreased Th lymphocyte proliferative responses in drug-naïve (p<0.005) and treated pSS patients (p<0.05), and B lymphocyte proliferation in drug-naïve pSS PBMC cultures (p<0.01) compared to mononuclear cell cultures alone. 1,25-dihydroxyvitamin D3 significantly decreased IFN-γ and IL-17
secreting Th cells in both drug naïve (p<0.005 and p<0.01, respectively) and treated subjects (p<0.05 and p<0.05, respectively) by increasing FoxP3 expressing CD4+CD25+ Treg cell frequency. Plasma B lymphocytes significantly reduced in the presence of 1,25-dihydroxyvitaminD3 in drug naïve pSS (p<0.001) and treated patients (p<0.05) mononuclear cell cultures compared to PBMC cultures alone. Total memory B cell subsets significantly increased with 1,25-dihydroxyvitaminD3 in drug naïve pSS when compared with PBMC cultures alone (p<0.005). IFN-γ and IL-17 cytokine levels in culture supernatants significantly reduced (p<0.05 and p<0.01, respectively) in drug naïve pSS patients’ PBMC cultures with 1,25-dihydroxyvitaminD3, and IL-10 levels significantly enhanced in both drug-naïve (p<0.01) and treated pSS patients’ PBMC cultures (p<0.01) in the presence of 1,25-dihydroxyvitaminD3.

**Conclusion:** In conclusion, 1,25-dihydroxyvitaminD3 regulated immune responses in both treated and drug-naïve pSS patients, but have a more pronounced modulatory effect on mononuclear cell responses in drug-naive pSS patients.

**Key words:** Primary sjögren’s syndrome, 1,25-dihydroxyvitamin D3, immunomodulation
Abbreviations

pSS: primary Sjögrens’ Syndrome

PBMC: Peripheral blood mononuclear cells

IL: Interleukin

IFN-γ: Interferon gamma

CFSE: Carboxyfluorescein succinimidyl ester

Th: T helper cell

DC: Dendritic cell
1. Introduction:

Primary Sjögren’s Syndrome (pSS) is an autoimmune inflammatory disease characterized by a reduction in the salivary and lacrimal glands secretory function [1]. Several studies have provided evidence of a significantly increased incidence of lymphoproliferative complications in pSS, and these evidences suggest that the disease may represent a link between autoimmunity and lymphoproliferation [2,3]. At present, it was concluded that low levels of vitamin D in pSS may involve in the development and severity of the disease. Vitamin D deficiency in pSS may be related to several factors such as genetic background or insufficient sun exposure or to dry-eye-related sunlight sensitivity [4].

Vitamin D3 plays an important role in the modulation of the inflammatory responses by regulating the production of inflammatory cytokines secreted by immune cells [5]. The two physiologically active forms of vitamin D are vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) which seems to be more effective than vitamin D2 in humans [6]. Recent clinical studies demonstrated the view that serum vitamin D3 levels of less than 20 ng/mL indicate vitamin D deficiency, while serum levels below 30 ng/mL indicate insufficiency and levels between 30 and 60 ng/mL represent normal values [7]. The studies suggest a contrast relation between serum levels of vitamin D3 and inflammatory markers of CRP and interleukin-6 (IL-6) levels in the bloodstream [8]. Additionally, studies suggest that 1,25-dihydroxyvitaminD3 which is the biological active form of vitamin D3 have been found to decrease the inflammatory cytokine production from T lymphocytes and have a regulatory effect on antigen presenting cells [9-11].
Recent studies show that there is a link between low vitamin D levels and severe complications such as neuropathy and lymphoma in patients with pSS [12,13]. Although studies on pSS patients have provided different data between vitamin D levels and disease prognosis, hypovitaminosis D in genetically susceptible individuals compromises the regulation of dendritic cells, regulatory T-lymphocytes, and T-helper 1 (Th1) and exacerbates CD8+ T cell deficiency and disruption of self-tolerance may cause continuous lymphocyte activation [14].

Several studies have shown that multiple disorders can occur during B cell differentiation and maturation in pSS disease; these disorders include a greater presence of plasma B cells in circulation and a disturbance in B-cell differentiation. B cell hyperactivity and peripheral B cell dysregulation appear to be hallmarks of the disease and may play an important role in lymphoproliferative processes involved in the pathogenesis of pSS.

Although there are data in the literature that pSS patients have low serum vitamin D levels or low vitamin D levels are related to the prognosis of the disease, the effect of 1,25-dihydroxyvitamin D3 the biologically active form of vitamin D, on inflammatory responses in pSS disease has not yet been reported. In this study, we evaluated the regulatory effects of 1,25-dihydroxyvitamin D3 on T and B lymphocytes isolated from peripheral blood samples of pSS patients in vitro. This is the first study demonstrating the anti-inflammatory responses of 1,25-dihydroxyvitamin D3 in pSS.
2. Materials and Method

2.1. Study Population

New diagnosed and non-treated nine (age 42.18±9.81) and eleven patients with drug treatment over 12 months (age 54.27±12.96) were included in the study. According to the G-power analysis, the minimum number of subjects required per group is n=9 and the total sample size is n=18, when the effect size is 0.95, a-error prob is 0.01, power (1-β error prob) is 0.80 calculated by F tests [15]. The study was completed with the number of patients who applied and participated voluntarily as n=11 in the treated group, and n=9 in drug-naive group. Patients included in the study were informed and approval forms were signed by each patient (The present study is the part of the Muğla Sıtkı Koçman University, Clinical Ethical Approval No: 07/08.08.2019). Patients were selected according to 2016 ACR-EULAR Classification Criteria for primary Sjögren’s Syndrome; 1) Oral and ocular symptoms, 2) labial salivary gland (LSG) biopsy to identify focal lymphocytic sialadenitis (FLS) and focus score (FS), 3) Serological assays: including anti-SSA/B(Ro/La), ANA titers, RF. Demographic data were given in the Table.

2.2. Peripheral Blood Mononuclear Cell (PBMC) Isolation from Venous Blood Samples

PBMC were isolated as described previously [5]. Briefly, 10-15cc venous blood samples were collected in heparinized tubes, and immediately diluted 1:1 (v/v) with phosphate
buffer solution (PBS). Diluted blood samples were transferred on the ficoll density
gradient solution (1.077g/mL) (Sigma-Aldrich, Germany) in 15 mL falcon tubes.
Samples were centrifuged at 2000 rpm for 20 minutes, and buffy coat were collected and
two times washed with RPMI 1640 medium (Pan Biotech, Germany). Cell pellet were
finally suspended in 1 mL of RPMI 1640 medium and cell pellet was counted for further
culture stages.

2.3. Culture Conditions

PBMC were cultured in 48 well plates 5x10^5 cells/well in 500μL of cRPMI medium
(RPMI 1640 medium supplemented with %10 Fetal Bovine Serum (FBS) (Pan Biotech,
Germany) and %1 Penicillin/Streptomycin 100 U/mL) (Sigma-Aldrich, Germany) each
well with and without 1,25-dihydroxyvitamin D3 (10nM) (Sigma-Aldrich, Germany) at
37°C 5% CO2 in humidified chamber for 5 days. PBMC were stimulated with anti-CD3
and anti-CD28 (Thermofisher, US) 10 μg/ml and 2 μg/ml, respectively) for T
lymphocytes, and anti-CD40 (BioLegend, San Diego, CA, USA) and recombinant human
IL-2 (Peprotech Inc., Rocky Hill, NJ, USA) for B lymphocytes.

2.4. Flow Cytometry Analysis

Cells were analyzed for CD3+CD4+ T lymphocytes and CD19+ B lymphocytes via flow
cytometry. Cells were collected from culture plates and washed two times with 2 mL of
PBS solution and centrifuged 1500 rpm for 5 minutes. Carboxyfluorescein succinimidyl
ester (CFSE) (Invitrogen, US) labeled cells were separately analyzed for T and B
lymphocyte proliferative responses by gating CD3+CD4+ cells or CD3-CD19+ cells. To
analyze intracellular cytokine secretion cell pellet were stained with anti-human-CD3 (PerCp), anti- human-CD4 (PE), anti-human-IFNγ (APC), anti-human-IL17 (APC), anti-human-IL10 (APC) (BD Biosciences-US). To evaluate B lymphocyte phenotypes cells were stained with anti- human-CD19 (PE), anti-human-CD27 (FITC), anti-human-CD38 (PerCp) and anti-IgD (APC). Gating was done as following; CD3+ lymphocytes were gated to analyze CD4+ T lymphocytes for intracellular cytokines of IFNγ or IL17 or IL10. CD19+CD27+ cells were analyzed for total memory B lymphocytes, and CD19+IgD-CD38+CD27+ cells were analyzed for plasma B cell ratio. Th1/Th17 cytokine bead array kit (BD Biosciences, US) was used to analyze IFN-γ, IL-17 and IL-10 cytokine levels of culture supernatants. All antibodies purchased from BD Biosciences, US. Analysis were performed by BD Accuri C6 plus software (BD Biosciences-US).

2.5. Statistical Analysis

Differences between groups were analyzed using the SPSS program and the GraphPad Prism program (GraphPad Software, Inc., CA, USA). Data were given as mean (Mean) ± standard deviation (SD) (minimum-maximum) values in each group. In detail, the secreted cytokine levels were in pg/mL and given as mean±SD. Flow cytometry analysis for proliferation and cytokine secreting cells were presented as mean fluorescence intensity % (MFI%). Graphical presentation and statistical analysis were calculated using GraphPad Prism 8 software (Graphpad Software Inc., USA). Comparison of the data of more than two groups was done by one-way ANOVA test. Flow cytometry data was analysed by Mann-Whitney U test. An unpaired Student’s t-test was used to compare cytokine levels between groups. P <0.05 values were considered significant [16].
3. Results

3.1. 1,25-Dihydroxyvitamin D3 suppressed both CD4+T and B lymphocyte proliferative responses

Assuming that vitamin D plays a role in suppressing autoimmunity, we investigated the effects of 1,25-dihydroxyvitamin D3 on B lymphocytes, and Th17 and Th1 cell phenotypes in CD4+ T lymphocyte subsets isolated from both drug-naïve pSS and pSS patients under treatment. To compare the effect of 1,25-dihydroxyvitamin D3 on the plasticity of Th1 and Th17 cells, we activated CD4 T cells with anti-CD3/anti-CD28 in the presence and absence of 1,25-dihydroxyvitamin D3. The activation of B lymphocytes was done with anti-CD40.

The 5-day cultured cells were stained with anti-CD3 and anti-CD4 antibodies to gate Th lymphocytes and analyzed for lymphocyte proliferation within the CFSE labeled cells. The lymphocyte proliferation was significantly high in drug-naïve pSS patients (53.1±4.6) compared to conventionally treated patients (38.3±3.2) (p<0.05). Results showed that 1,25-DihydroxyvitaminD3 notably decreased the proliferative response of CD4+T lymphocytes in drug-naïve pSS (27.7±5.3) and treated subjects (20.1±5.6) compared with PBMC cultures alone (Drug-naïve pSS CD3+CD4+ T cells: p<0.005, Treated pSS CD3+CD4+ T cells: p<0.05). Figure 1a.

The B lymphocyte proliferation was evaluated by staining the cells with anti-CD19 antibody after 5 days of culture period. The CFSE labeled cells were analyzed for CD19 expressing cells via flow cytometry. The proliferative response of B lymphocytes was
significantly high in drug-naïve patients’ cultured mononuclear cells (34.2±4.1) compared to treated patients (25.4±3.8) (p<0.05). 1,25-dihydroxyvitaminD3 significantly suppressed the proliferation of B lymphocytes in drug-naïve patients’ PBMC cultures (18.1±2.4) when compared with PBMC cultures alone (p<0.01), but no significant change was observed in treated patients’ PBMC cultures with 1,25-DihydroxyvitaminD3 (19.7±3.1) compared with mononuclear cell cultures alone (28.6±2.9) (p>0.05). Figure 1b.

3.2. 1,25-Dihydroxyvitamin D3 downregulated intracellular IFN-γ and IL-17 cytokine secretion in CD4+ T lymphocytes

We evaluated the modulatory effect of 1,25-dihydroxyvitaminD3 on intracellular cytokine production after observing the proliferative response of CD3+ T cells. Results showed a significant decrease in IFN-γ levels in CD3+CD4+ T cells cultured with 1,25-dihydroxyvitaminD3 (pSSDrugNaïve: 1.9±0.2, pSTreated: 1.0±0.4) compared with PBMC cultures alone (pSSDrugNaïve: 3.5±0.3, pSTreated: 1.6±0.4) (DrugNaïve: p<0.005 and Treated: p<0.05). The frequency of CD3+CD4+T cells producing IL-17 significantly decreased with 1,25-dihydroxyvitaminD3 in drug-naïve patients PBMC cultures (0.8±0.2) compared to PBMC cultures alone (p<0.01), and significantly reduced IL-17 levels in CD3+CD4+T cells cultured with 1,25- dihydroxyvitaminD3 in treated pSS patients’ mononuclear cells (1.0±0.4) when compared with PBMC cultures alone (p<0.05). The ratio of IL-17 secreting cells in drug-naïve pSS patients in the presence of 1,25- dihydroxyvitaminD3 was significantly lower compared to treated pSS patients mononuclear cell cultures alone (p<0.05). Figure 2.
3.3. Plasma B cell subsets were decreased in the presence of 1,25-Dihydroxyvitamin D3 in both Drug naïve and Treated pSS patients’ mononuclear cell cultures

Although the results of the studies conducted to date support the evidence of decreased vitamin D levels in pSS in the early stages, the effect of vitamin D deficiency on the pathogenesis of pSS is still debated. Therefore, we evaluated the effect of 1,25-dihydroxyvitamin D3 on B lymphocyte subsets in both drug-naïve and treated pSS patients’ mononuclear cell cultures. PBMCs were stimulated with anti-CD40 and recombinant IL-2 for 5 days in order to activate B lymphocytes. After the culture period CD19+CD27+ cells were analyzed for total memory B lymphocytes, and CD19+IgD-CD38+CD27+ cells were analyzed for plasma B cell ratio. Gating strategy was as following; PBMCs were gated for CD19+IgD- cells to analyze CD38+CD27+ cell ratio to determine the plasma B cell subsets. At the same time CD19+CD27+ cells were gated for the total memory B cells. The total memory B lymphocyte subsets were significantly lower in drug naïve pSS patients (15.6±2.1) compared to treated patients (23.7±2.9) in PBMC cultures (p<0.01). The remarked increase in total memory B lymphocyte ratio was observed in drug naïve patients in the presence of 1,25-dihydroxyvitaminD3 (26.1±3.8) compared with PBMC cultures alone (p<0.005). The total memory B cells tended to increase in treated pSS patients PBMC cultures with 1,25-dihydroxyvitaminD3 (25.4±3.2), but no significant difference was observed when compared to PBMC cultures alone (p>0.05). The plasma B cell subsets were significantly higher in drug naïve patients’ PBMC cultures (16.7±2.3) compared to treated patients (8.6±1.9) (p<0.01). 1,25-dihydroxyvitaminD3 significantly decreased the ratio of plasma B cells in drug naïve patients’ PBMC cultures (4.9±1.4) compared to PBMC cultures alone (p<0.001). Also, a significant decrease in plasma B cell ratio was observed in treated pSS patients’
PBMC cultures with 1.25-dihydroxyvitaminD3 (5.4±1.2) compared with PBMC cultures alone (p<0.05). The decrease in plasma B cell ratio was significant in drug-naïve pSS patients cultured with 1,25-dihydroxyvitaminD3 compared to treated subjects’ mononuclear cells alone (p<0.05). Figure 3

3.4. FoxP3 expression in CD4+CD25+ T cells was increased in drug-naïve pSS patients in the presence of 1,25-Dihydroxyvitamin D3

Regulatory T cells (Tregs) produced in the thymus play an important role in maintaining self-tolerance due to their intrinsic immunosuppressive activity. In addition, Forkhead box protein 3 (FoxP3) expressed by CD4+CD25+ T cells during inflammatory responses play a role in the regulation of immune response and maintenance of self-tolerance. Therefore, we evaluated the effect of 1,25-dihydroxyvitaminD3 on the expression of FoxP3 in CD4+CD25+ T cells.

FoxP3 expression in CD4+CD25+ T cells was significantly lower in drug-naïve patients (1.2±0.7) compared to treated pSS patients (2.3±0.6) (p<0.01). FoxP3 ratio was significantly increased in CD4+CD25+ T lymphocytes in drug-naïve pSS patients in the presence of 1,25-dihydroxyvitaminD3 (7.1±0.3) compared with PBMC cultures alone (p<0.001), and also remarked increase was observed in 1,25-dihydroxyvitaminD3 cultured PBMC in treated subjects (4.7±0.7) compared to PBMC cultures alone (p<0.01). In addition, FoxP3 expression was significantly high in drug-naïve pSS patients’ PBMC cultures with 1,25-dihydroxyvitaminD3 compared to those in treated patients (p<0.01). Figure 4.
3.5. The secreted IFN-γ and IL-17 cytokine levels reduced and IL-10 was enhanced in the presence of 1,25-dihydroxyvitaminD3 in drug-naïve and treated pSS patients mononuclear cell cultures

Treg/Th17 balance is important in pSS, as in many other autoimmune inflammatory diseases. In the results obtained in this study, the secreted cytokine profile was evaluated to confirm the decrease in intracellular IFN-γ and IL-17 production and the increase of FoxP3 expression in CD4+CD25+ T cells in the presence of 1,25-dihydroxyvitaminD3. The supernatants of cultured cells in the presence and absence of 1,25-dihydroxyvitaminD3 were analyzed for cytokine profiles via flow cytometry.

After collecting culture supernatants 50 µL of each sample was analyzed by Th1/Th17 CBA cytokine kit (BD Biosciences, US). The secreted IFN-γ levels were significantly high in drug-naïve pSS patients’ mononuclear cell cultures (243.6±71.1) when compared with treated patients (197.4±41.6) (p<0.05). IFN-γ levels significantly decreased in drug-naïve pSS patients (182±32.4) compared to PBMC cultures alone (p<0.05), and tended to decrease in treated subjects’ culture supernatants (165.1±24.6) in the presence of 1,25-dihydroxyvitaminD3 but no remarked change observed compared to PBMC cultures alone (p>0.05).

IL-17 levels were significantly high in drug-naïve pSS patients’ culture supernatants (92.8±11.4) compared to treated pSS patients (47.1±18.3) (p<0.01). The secreted IL-17 levels were significantly reduced in the presence of 1,25-dihydroxyvitaminD3 in the drug-naïve pSS patients’ culture supernatants (23.6±10.1) compared with PBMC cultures...
(p<0.01), and tended to decrease in treated patients culture supernatants (38.2±7.9), but it was not significant (p>0.05).

IL-10 levels were low in drug-naïve pSS patients (6.7±1.1) compared to treated subjects’ PBMC culture supernatants (8.2±1.6), but no significant change observed between two groups (p>0.05). The levels of IL-10 significantly increased in both drug-naïve pSS patients’ cultures with 1,25-dihydroxyvitaminD3 (10.8±2.7), and treated pSS subjects (11.6±3.1) when compared with PBMC cultures alone (p<0.01). IL-10 levels significantly increased in treated pSS patients in the presence of 1,25-dihydroxyvitaminD3 (14.2±2.0) compared to PBMC cultures alone (p<0.01). IL-10 levels were notably high in drug-naïve pSS patients in the presence of 1,25-dihydroxyvitaminD3 compared to treated pSS patients’ mononuclear cell cultures alone (p<0.01). Figure 5

4. Discussion

In addition to its critical function in calcium homeostasis, vitamin D has recently been shown to have a regulatory effect on the immune system. Many studies to date have revealed that vitamin D deficiency contributes to primary Sjögren's Syndrome, suggesting a number of physiological functions of vitamin D. Current findings suggest that low vitamin D levels in patients with Sjogren's Syndrome may be associated with peripheral neuropathy and lymphoma [17]. In the current study, we investigated and compared the immunomodulatory effect of 1,25-dihydroxyvitaminD3, which is the biological active form of vitamin D, on the peripheral blood mononuclear cells of both drug-naïve and pSS patients under treatment. The results demonstrated that 1,25-dihydroxyvitaminD3 have
regulatory effects on Th1 and Th17 lymphocytes in both drug-naïve and treated pSS patients and also can reduce the formation of plasma B cells in drug-naïve subjects.

Vitamin D plays an important role in the regulation of pathological immune responses in autoimmune or inflammatory diseases [18]. An increase in the number and function of Tregulatory (Treg) cells is correlated with the higher levels of vitamin D [19]. Recent studies have revealed the presence of the vitamin D receptor in activated T lymphocytes, and it has been understood that vitamin D may play a role in the function of immune cells. Several studies demonstrated low 1,25-dihydroxyvitamin D3 levels correlate with the clinical and serological features of pSS patients. However, the modulatory effects of vitamin D on T and B lymphocyte phenotypes in pSS patients has not defined.

In this study, we first evaluated the lymphoproliferative responses of T and B lymphocytes in the presence of 1,25-dihydroxyvitaminD3. The cultured mononuclear cells showed an increased proliferative response of CD4+ T lymphocytes and B cells especially in drug-naïve pSS patients when compared with treated subjects. 1,25-dihydroxyvitaminD3 significantly decreased the proliferation of both CD4+T and B lymphocytes in drug-naïve pSS patients’ mononuclear cell cultures. There are evidences that 1,25-dihydroxyvitaminD3 regulates lymphoproliferative responses with ligand-activated regulator of gene transcription, and inhibit dendritic cell differentiation and maturation, which in turn leads to down-regulate the expression of major histocompatibility complex II (MHC-II) and reduce costimulation. Therefore, on the basis of previous studies, our results are compatible with previous data [20,21].

Th17 cells are a lymphocyte subclass that plays an important role in mucosal barriers and inflammatory responses to pathogens. In pSS, Th17 cells are also present in salivary gland
lesions of pSS patients and may be elevated in their peripheral blood. Although there is no clear correlation between increased Th17 cell activity and symptoms of the disease in pSS patients, Th17 cells may contribute to the progression of the disease by supporting autoreactive B cell responses [22]. 1,25-dihydroxyvitaminD3 can suppress inflammatory responses in autoimmune inflammatory diseases, such as multiple sclerosis, type 1 diabetes, and IBD. As a result of observations to date, much attention has been drawn to a possible role of vitamin D interaction with the vitamin D receptor (VDR) expressed by Th17 cells. Several studies have shown that 1,25-dihydroxyvitamin D3 can be a promising tool to suppress IL-17A production in patients with multiple sclerosis and rheumatoid arthritis [23-25]. In the present study, high IFN-γ and IL-17 levels were found in both drug-naïve and treated patients. The main findings of the present study were the reduction of IFN-γ and IL-17 secreting T lymphocytes in the presence of 1,25-dihydroxyvitaminD3 both in drug-naïve and treated pSS patients.

IFN-γ is produced by CD4+ and CD8+ T lymphocytes. In this study, we analyzed the secretion of IFN-γ in CD3+CD4+ cell population to determine the effect of 1,25-dihydroxyvitamin D3 on Th1 cells. Studies suggest that an increase in the number of Th1 cells secreting IFN-γ in the exocrine glands may contribute to epithelial cell damage and hence decreased salivary secretion [26,27]. In the previous studies, it was demonstrated that IFN-γ levels in Th1 cells decreased in the presence of 1,25-dihydroxyvitamin D3. This inhibitory effect was occurred with the enhanced expression of CTLA-4, PD1 or PD-L1 on regulatory cells in the immune system, and also with the suppressive effect of IL-10, which is produced by Treg cells [28,29]. Additionally, IFN-γ can synergistically enhance the suppression in proliferative responses with vitamin D3 by expressions of immunoglobulin-like transcript (ILT)-3 and programmed death ligand (PDL)-1 on
tolerogenic DCs [30]. In the present study, we observed reduced number of IFN-γ secreting Th lymphocytes in both drug-naïve and pSS treated patients, while the reduction was striking in drug-naïve patients. The increased suppressive effect of 1,25-dihydroxyvitaminD3 on Th1 cells may be due to high levels of IFN-γ levels in drug-naïve pSS patients’ mononuclear cell cultures, or enhanced IL-10 production by immune cells. Also, cytokine secretion in the supernatants of mononuclear cell cultures correlated with intracellular cytokine production by T lymphocytes. The elevated levels of IFN-γ and IL-17 more remarkable reduced in the presence of of 1,25-dihydroxyvitamin D3 in drug-naïve patients, and IL-10 enhanced with the addition of 1,25-dihydroxyvitamin D3 in the cell cultures both in drug-naïve and treated subjects.

It was previously concluded that B cell dysregulation occur in the pSS with the decrease in the percentage of CD27+ memory B cells in peripheral blood and an increase in the CD27+ memory B cells in the affected glands [31]. B lymphocytes, by their nature, generally exhibit increased activation states in pSS. Not surprisingly, most lymphomas in patients with pSS are derived from marginal zone B lymphocytes and are thought to be central players in disease pathogenesis [32]. We therefore investigated the effect of 1,25-dihydroxyvitaminD3 on the B lymphocyte subpopulations. The data showed an increase in the ratio of total memory B lymphocytes both in drug-naïve and treated pSS patients mononuclear cell cultures with 1,25-dihydroxyvitaminD3. In addition, plasma B cell ratio decreased in the presence of 1,25-dihydroxyvitaminD3 in drug-naïve pSS subjects’ mononuclear cell cultures. Vitamin D affects many cells of the immune system. Of these, T cells and antigen-presenting cells are the most extensively studied [33]. In previous studies, the effect of vitamin D on B lymphocytes was investigated, and found to be VDR on IL-10 producing B regulatory cells is a ligand for vitamin D. Active form of vitamin
D has a regulatory effect on B cells due to expression of the metabolizing enzymes CYP27B1 and CYP24A1. B cells, therefore, may participate in vitamin D-mediated immune homeostasis, including plasma cell generation [34-36]. In our study, the increased ratio of total memory B cells may be due to the increase in the number of Breg cells, since a decrease in plasma B cell ratio was observed in the mononuclear cell cultures of drug-naive pSS patients versus the increase in total memory B lymphocytes. The mechanism underlying the increase in total memory B lymphocyte ratio needs to be further investigated.

5. Conclusion

The inhibition of T-cell responses by 1,25-dihydroxyvitaminD3 has previously been described, and studies indicated that 1,25-dihydroxyvitaminD3 modulates the production of cytokines in a time and concentration dependent manner. We attempted to prove this effect of 1,25-dihydroxyvitaminD3 in pSS patients’ mononuclear cell cultures by comparing drug-naïve and treated subjects. We found that Th1 and Th17 responses in both drug-naïve and treated pSS patients’ mononuclear cell cultures reduced in the presence of 1,25-dihydroxyvitaminD3. Additionally, IL-10 production notably increased and lymphoproliferative responses down-regulated with 1,25-dihydroxyvitaminD3. Also, plasma B cell ratio decreased and total memory B lymphocytes increased with 1,25-dihydroxyvitaminD3. These data indicate regulatory effects of 1,25-dihydroxyvitaminD3 on pSS.
Ethics approval and consent to participate

The Ethics for clinical research was approved by the Muğla Sıtıkı Koço mı University with the approval no: 07/08.08.2019). Written consent was obtained from all participants.

Consent for publication

The personal information of the participants is only in our laboratory records and has not been published.

Availability of data and materials

Data obtained in this study and written protocols were given in the text and figures.

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**Table.** Demographic data of pSS patients.

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<td><strong>Anti Ro % (mean)</strong></td>
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<td>69.6</td>
</tr>
<tr>
<td><strong>Anti La % (mean)</strong></td>
<td>43.4</td>
<td>40.9</td>
</tr>
<tr>
<td><strong>RF % (mean)</strong></td>
<td>53.7</td>
<td>19.9</td>
</tr>
<tr>
<td><strong>CCP % (mean)</strong></td>
<td>11.2</td>
<td>9.8</td>
</tr>
<tr>
<td><strong>Serum Vitamin D Level (ng/mL) (mean)</strong></td>
<td>22.47</td>
<td>27.10</td>
</tr>
</tbody>
</table>

ANA: Anti-nuclear antibody, RF: Rheumatoid factor, CCP: Cyclic citrullinated peptide
**Figure 1. Lymphocyte Proliferation.** B and Th cell proliferative responses were analyzed in the presence and absence of 1,25-dihydroxyvitaminD3. a) The proliferation ratio of CD3+CD4+ T lymphocytes were significantly high in drug-naïve pSS patients’ mononuclear cell cultures compared to treated subjects (p<0.01). b) The proliferation ratio of drug-naïve pSS patients were significantly high in PBMC cultures compared to those in treated subjects (p<0.05). The proliferation of CD3+CD4+ T lymphocytes and CD19+ B lymphocytes were significantly decreased in drug-naïve pSS patients’ PBMC cultures in the presence of 1,25-dihydroxyvitaminD3 compared to PBMC cultures alone, but no significant difference was observed in treated pSS patients’ PBMC cultures with 1,25-dihydroxyvitaminD3 when compared with PBMC cultures alone (Drug-naïve pSS CD3+CD4+ T cells: p<0.005, Treated pSS CD3+CD4+ T cells: p<0.05) (Drug-naïve pSS B cells: p<0.01, Treated pSS B cells: p>0.05).
Figure 2. IFN-γ and IL-17 secreting CD4+ T cells. Gating was done as following: CD3+ cells were gated from total lymphocytes and analyzed for CD4+IFNγ+ or CD4+IL17+ cell frequency. a) The representative flow cytometry analysis of drug-naïve pSS patients’ mononuclear cells in the presence and absence of 1,25-dihydroxyvitaminD3. b) Statistical analysis of IFN-γ and IL-17 secreting CD4+T cells in drug-naïve and treated pSS patients’ PBMC cultures. IFN-γ and IL-17 were significantly high in drug-naïve pSS patients compared to treated pSS patients’ PBMC cultures alone (p<0.005 and p<0.05, respectively). The frequency of IFN-γ and IL-17 secreting CD4+ T cells were significantly decreased with 1,25-dihydroxyvitaminD3 both in drug-naïve and treated subjects compared to mononuclear cell cultures alone (Drug-naïve pSS CD4+IFNγ+ T cells: p<0.005, Treated pSS CD4+IFNγ+ T cells: p<0.05) (Drug-naïve pSS CD4+IL17+ T: p<0.01, Treated pSS B cells: p<0.05).
Figure 3. Total Memory and Plasma B Cell Frequency. Gating was done as following: CD19+IgD- cells were gated from total lymphocyte population, and analyzed for CD27+CD38+ cell ratio for Plasma B cells via flow cytometry. CD19+CD27+ cells were gated from total lymphocyte population and analyzed for total memory B lymphocytes. Flow cytometry analysis of a) Plasma B cell ratio, and b) Total memory B cell ratio. c) Statistical analysis of plasma B cells and total memory B cells. Plasma B cell frequency was significantly high in drug-naïve pSS patients compared to treated patients (p<0.01). Plasma B cell ratio significantly decreased in pSS patients’ PBMC cultures in the presence of 1,25-dihydroxyvitaminD3 (Drug naïve pSS: p<0.001, Treated pSS: p<0.05). Total memory B cell ratio was significantly increased in drug-naïve pSS patients’ mononuclear cell cultures in the presence of 1,25-dihydroxyvitaminD3 (p<0.005).
Figure 4. FoxP3 expressing CD4+CD25+ T regulatory cells. The frequency of CD4+CD25+FoxP3+ Treg cells were significantly low in drug-naïve pSS patients compared to treated subjects ($p<0.01$). Treg cell population significantly increased in PBMC cultures with 1,25-dihydroxyvitaminD3 both in drug-naïve and treated pSS patients compared to PBMC cultures alone (Drug-naïve pSS: $p<0.001$, Treated pSS: $p<0.01$).
Figure 5. Secreted cytokines in culture supernatants. The proinflammatory cytokines (IFN-γ and IL-17) and anti-inflammatory cytokine IL-10 levels were analyzed in the culture supernatants of drug-naïve and treated pSS patients’ mononuclear cell cultures in the presence and absence of 1,25-dihydroxyvitaminD3. IFN-γ and IL-17 levels were significantly decreased in PBMC cultures with 1,25-dihydroxyvitaminD3 in drug-naïve pSS patients, and no significant difference observed in treated subjects when compared with PBMC cultures alone (IFN-γ drug naïve pSS: p<0.05, IL-17 drug naïve pSS: p<0.01, IFN-γ and IL-17 treated pSS: p>0.05 and p>0.05). IL-10 levels significantly increased in both drug-naïve and treated pSS patients’ PBMC cultures with 1,25-dihydroxyvitaminD3 (Drug-naïve pSS: p<0.01, Treated pSS: p<0.01). IL-10 levels were significantly high in drug-naïve pSS patients in the presence of 1,25-dihydroxyvitaminD3 compared to treated patients’ PBMC cultures alone (p<0.01).