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1,25-dihydroxyvitamin D3 regulates t helper and b lymphocyte responses substantially in drug-naive primary Sjögren's syndrome patients' mononuclear cells

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Background/aim: A correlation between vitamin D deficiency and primary Sjögren's syndrome (pSS) has already been described. The limited data has 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-dihydroxyvitamin D3 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 patients in the 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-dihydroxyvitamin D3 (10 mM) 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 naive 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-dihydroxyvitamin D3 significantly decreased Th lymphocyte proliferative responses in drug-naive ($p < 0.005$) and treated pSS patients ($p < 0.05$), and B lymphocyte proliferation in drug-naive 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 naive ($p < 0.005$ 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-dihydroxyvitamin D3 in drug naive 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-dihydroxyvitamin D3 in drug naive 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 naive pSS patients' PBMC cultures with 1,25-dihydroxyvitamin D3, and IL-10 levels significantly enhanced in both drug-naive ($p < 0.01$) and treated pSS patients' PBMC cultures ($p < 0.01$) in the presence of 1,25-dihydroxyvitamin D3.

Conclusion: In conclusion, 1,25-dihydroxyvitamin D3 regulated immune responses in both treated and drug-naive 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

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, 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-dihydroxyvitamin D3 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].

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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 antiinflammatory responses of 1,25-dihydroxyvitamin D3 in pSS.

2. Materials and methods

2.1. Study population

New diagnosed and nontreated nine (age 42.18 ± 9.81) and eleven patients with drug treatment over 12 moths (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, α-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 Table.

2.2. Peripheral blood mononuclear cell (PBMC) isolation from venous blood samples

PBMC were isolated as described previously [5]. Briefly, 10–15 cc 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 (1077 g/mL) (Sigma-Aldrich, Germany) in 15 mL falcon tubes. Samples were centrifuged at 2000 rpm for 20 min, 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 5 x 10^5 cells/well in 500 µL of cRPMI medium (RPMI 1640 medium

Table. Demographic data of pSS patients.

	Drug naive	Treated
Number of patients (Female/Male)	8/1	10/1
Mean age (years)	42.18 ± 9.81	54.27 ± 12.96
Average duration of illness (mean)	2.3 months	89.1 months
Treatment (Drug/no.of patients)	Pilocarpine hydrochloride (n = 1)	Hydroxychloroquine 200 mg (n = 11) Methylprednisolone 4 mg (n = 10) Methotrexate 10 mg (n = 8) Leflunomide 20 mg (n = 2)
Minor salivary gland biopsy focus score (mean)/4 mm^2	3	1
Sicca symptoms % (mean)	100	100
Raynaud % (mean)	19.1	12.6
Arthralgia/arthritis (no. of patients female/male)	7/2	2/9
ANA % (mean)	100	100
Anti Ro % (mean)	67.4	69.6
Anti La % (mean)	43.4	40.9
RF % (mean)	53.7	19.9
CCP % (mean)	11.2	9.8
Serum vitamin D level (ng/mL) (mean)	22.47	27.10

ANA: Antinuclear antibody, RF: Rheumatoid factor, CCP: Cyclic citrullinated peptide.

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 (10 nM) (Sigma-Aldrich, Germany) at 37 °C 5% CO_2 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., 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 min. Carboxyfluoresce in 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 antihuman-CD3 (PerCp), antihuman-CD4 (PE), antihuman-IFNγ (APC), antihuman-IL17 (APC), and antihuman-IL10 (APC) (BD Biosciences-US). To evaluate B lymphocyte phenotypes cells were stained with antihuman-CD19 (PE), antihuman-CD27 (FITC), antihuman-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 were 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 analyzed 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-naive 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-naive pSS patients (53.1 ± 4.6) compared to conventionally treated patients (38.3 ± 3.2) ($p < 0.05$). Results showed that 1,25-dihydroxyvitamin D3 notably decreased the proliferative response of CD4+T lymphocytes in drug-naive pSS (27.7 ± 5.3) and treated subjects (20.1 ± 5.6) compared with PBMC cultures alone (drug-naive pSS CD3+CD4+ T cells: $p < 0.005$, treated pSS CD3+CD4+ T cells: $p < 0.05$) (see 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-naive patients' cultured mononuclear cells (34.2 ± 4.1) compared to treated patients (25.4 ± 3.8) ($p < 0.05$). 1,25-dihydroxyvitamin D3 significantly suppressed the proliferation of B lymphocytes in drug-naive 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-dihydroxyvitamin D3 (19.7 ± 3.1) compared with mononuclear cell cultures alone (28.6 ± 2.9) ($p > 0.05$) (see 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-dihydroxyvitamin D3 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-dihydroxyvitamin D3 ($pSS_{DrugNaive}$: 1.9 ± 0.2, $pSS_{Treated}$: 1.0 ± 0.4) compared with PBMC cultures alone ($pSS_{DrugNaive}$: 3.5 ± 0.3, $pSS_{Treated}$: 1.6 ± 0.4) (drug-naive: $p < 0.005$ and treated: $p < 0.05$). The frequency of CD3+CD4+T cells producing IL-17 significantly decreased with 1,25-dihydroxyvitamin D3 in drug-naive 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-dihydroxyvitamin D3 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-naive pSS patients in the presence of 1,25-dihydroxyvitamin D3 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 naive 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-naive 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-naive 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 naive patients in the presence of 1,25-dihydroxyvitamin D3 (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-dihydroxyvitamin D3 (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-naive patients' PBMC cultures (16.7 ± 2.3) compared to treated patients (8.6 ± 1.9) ($p < 0.01$). 1,25-dihydroxyvitamin D3 significantly decreased the ratio of plasma B cells in drug-naive 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-dihydroxyvitamin D3 (5.4 ± 1.2) compared with PBMC cultures alone ($p < 0.05$). The decrease in plasma B cell ratio was significant in drug-naive pSS patients cultured with 1,25-dihydroxyvitamin D3 compared to treated subjects' mononuclear cells alone ($p < 0.05$). Figure 3

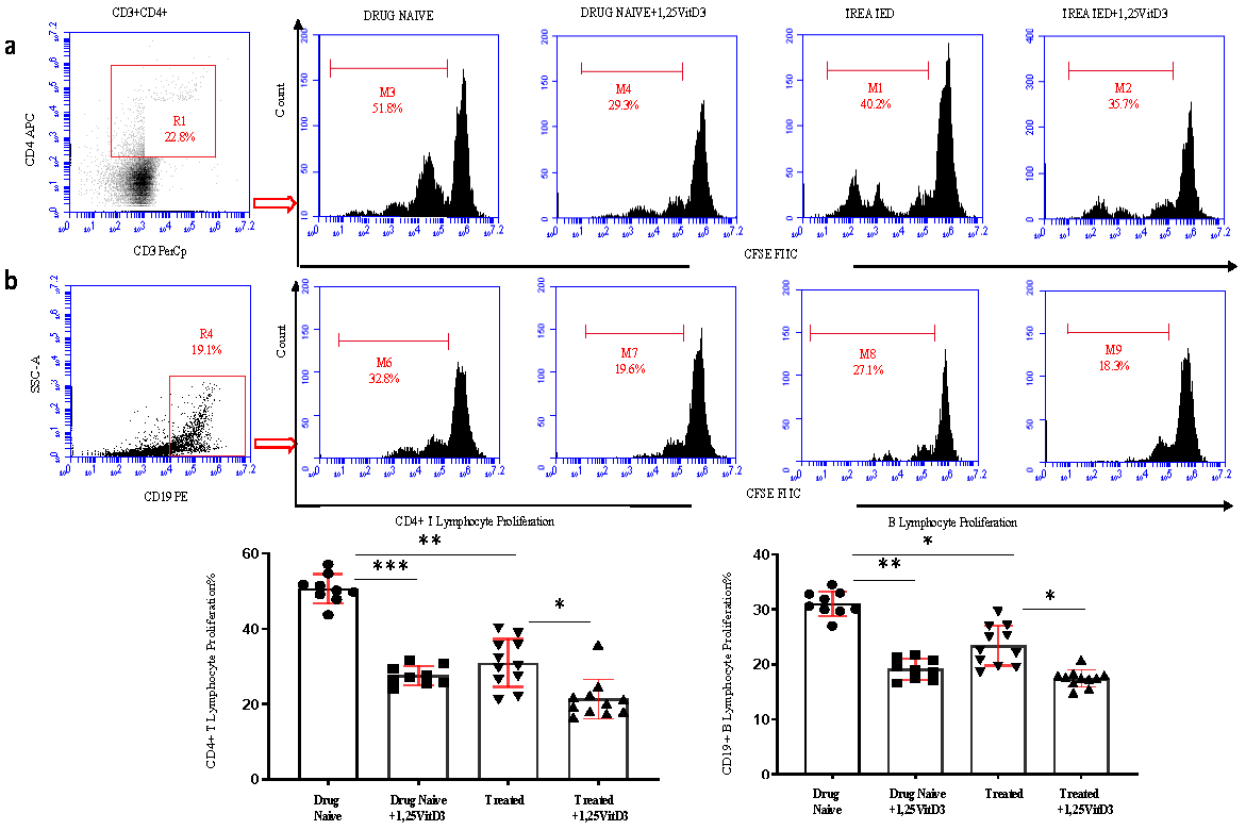

Figure 1. Lymphocyte proliferation. B and Th cell proliferative responses were analyzed in the presence and absence of 1,25-dihydroxyvitamin D3. a) The proliferation ratio of CD3+CD4+ T lymphocytes were significantly high in drug-naive pSS patients' mononuclear cell cultures compared to treated subjects ($p < 0.01$). b) The proliferation ratio of drug-naive 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-naive pSS patients' PBMC cultures in the presence of 1,25-dihydroxyvitamin D3 compared to PBMC cultures alone, but no significant difference was observed in treated pSS patients' PBMC cultures with 1,25-dihydroxyvitamin D3 when compared with PBMC cultures alone (drug-naive pSS CD3+CD4+ T cells: $p < 0.005$, treated pSS CD3+CD4+ T cells: $p < 0.05$) (drug-naive pSS B cells: $p < 0.01$, treated pSS B cells: $p > 0.05$).

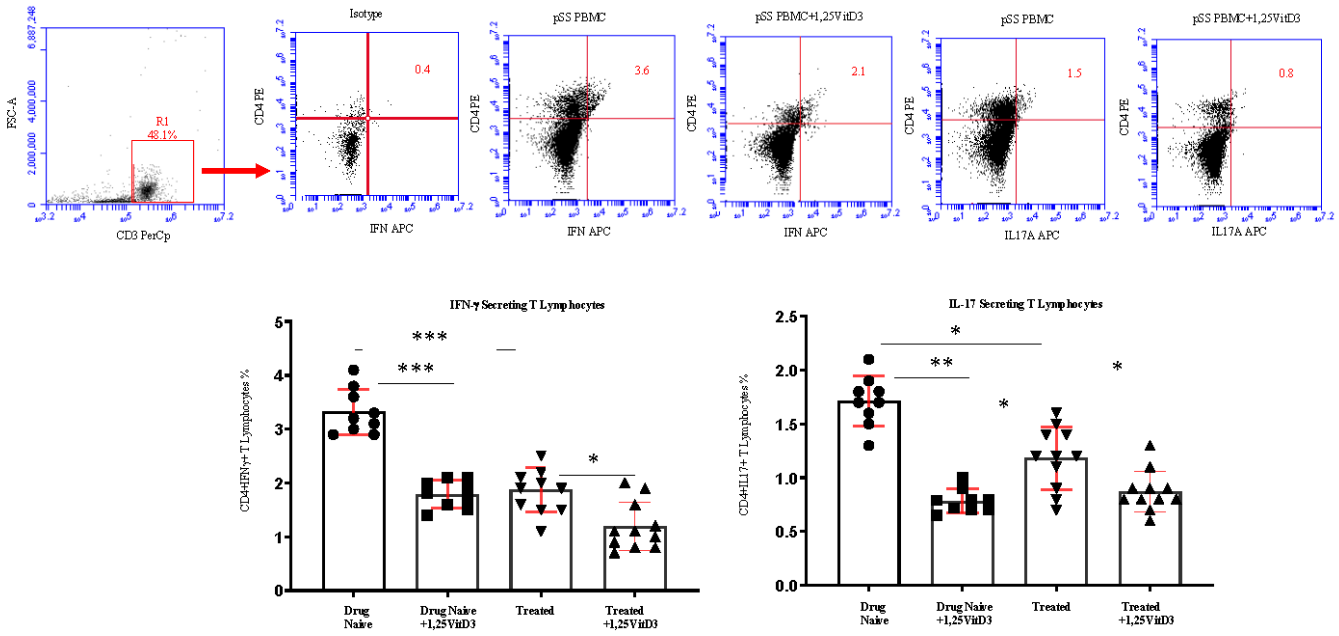

Figure 2. IFN-γ and IL-17 secreting CD4+T cells. Gating was done as following: CD3+ cells were gated from total lymphocytes and analyzed for CD4+IFNγ+ or CD4+IL17+ cell frequency. a) The representative flow cytometry analysis of drug-naive pSS patients' mononuclear cells in the presence and absence of 1,25-dihydroxyvitamin D3. b) Statistical analysis of IFN-γ and IL-17 secreting CD4+T cells in drug-naive and treated pSS patients' PBMC cultures. IFN-γ and IL-17 were significantly high in drug-naive 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-dihydroxyvitamin D3 both in drug-naive and treated subjects compared to mononuclear cell cultures alone (Drug-naive pSS CD4+IFNγ+ T cells: $p < 0.005$, treated pSS CD4+IFNγ+ T cells: $p < 0.05$) (drug-naive pSS CD4+IL17+ T: $p < 0.01$, treated pSS B cells: $p < 0.05$).

3.4. FoxP3 expression in CD4+CD25+ T cells was increased in drug-naive 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-dihydroxyvitamin D3 on the expression of FoxP3 in CD4+CD25+ T cells.

FoxP3 expression in CD4+CD25+ T cells was significantly lower in drug-naive 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-nive pSS patients in the presence of 1,25-dihydroxyvitamin D3 (7.1 ± 0.3) compared with PBMC cultures alone ($p < 0.001$), and also remarked increase was observed in 1,25-dihydroxyvitamin D3 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-naive pSS patients' PBMC cultures with 1,25-dihydroxyvitamin D3 compared to those in treated patients ($p < 0.01$) (see 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-dihydroxyvitamin D3 in drug-naive 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-dihydroxyvitamin D3. The supernatants of cultured cells in the presence and absence of 1,25-dihydroxyvitamin D3 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-naive 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-naive 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-dihydroxyvitamin D3 but no remarked change observed compared to PBMC cultures alone ($p > 0.05$).

IL-17 levels were significantly high in drug-naive pSS patients' culture supernatants (92.8 ± 11.4) compared to

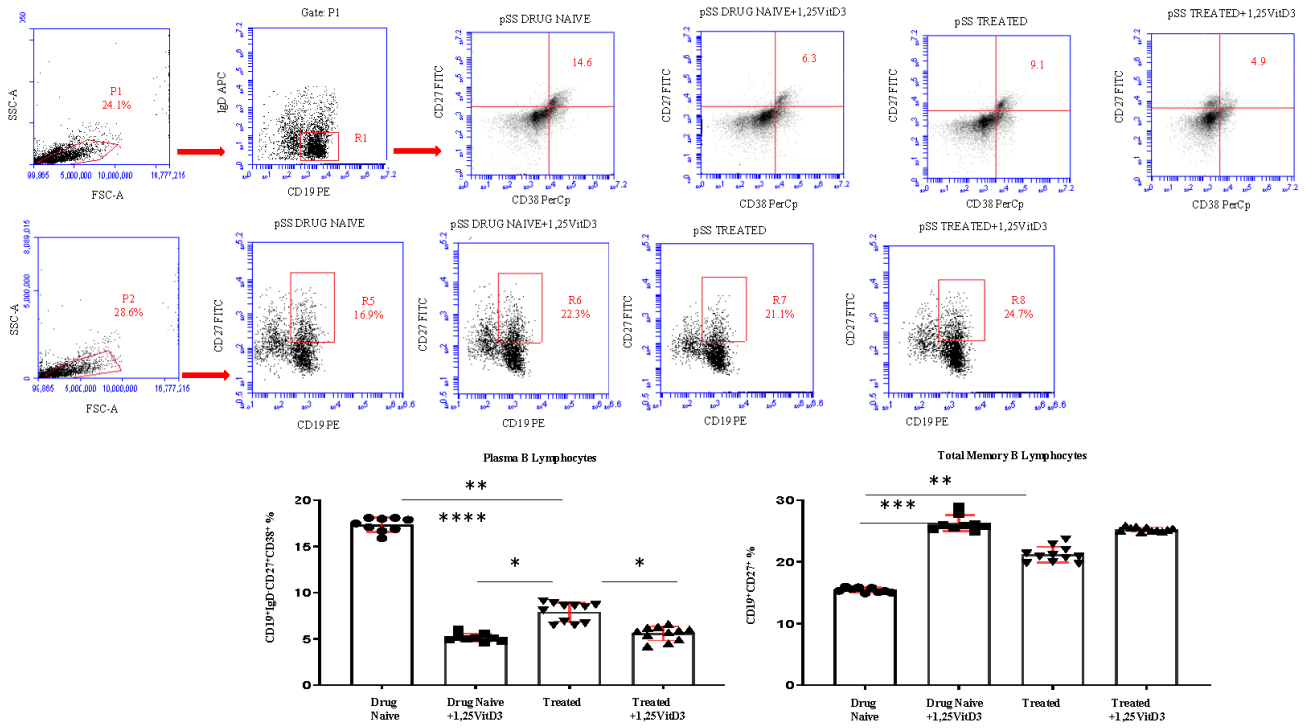

Figure 3. Total memory and plasma b cell frequency. Gating was done as following: CD19+IgD-cells were gated from total lymphocyte population, and analyzed for CD27+CD38+ cell ratio for plasma B cells via flow cytometry. CD19+CD27+ cells were gated from total lymphocyte population and analyzed for total 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-naive 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-dihydroxyvitamin D3 (drug naive pSS: $p < 0.001$, treated pSS: $p < 0.05$). Total memory B cell ratio was significantly increased in drug-naive pSS patients' mononuclear cell cultures in the presence of 1,25-dihydroxyvitamin D3 ($p < 0.005$).

treated pSS patients (47.1 ± 18.3) ($p < 0.01$). The secreted IL-17 levels were significantly reduced in the presence of 1,25-dihydroxyvitamin D3 in the drug-naive 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-naive 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-naive pSS patients' cultures with 1,25-dihydroxyvitamin D3 (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-dihydroxyvitamin D3 (14.2 ± 2.0) compared to PBMC cultures alone ($p < 0.01$). IL-10 levels were notably high in drug-naive pSS patients in the presence of 1,25-dihydroxyvitamin D3 compared to treated pSS patients' mononuclear cell cultures alone ($p < 0.01$) (see 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-dihydroxyvitamin D3, which is the biological active form of vitamin D, on the peripheral blood mononuclear cells of both drug-naive and pSS patients under treatment. The results demonstrated that 1,25-dihydroxyvitamin D3 have regulatory effects on Th1 and Th17 lymphocytes in both drug-naive and treated pSS patients and also can reduce the formation of plasma B cells in drug-naive 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

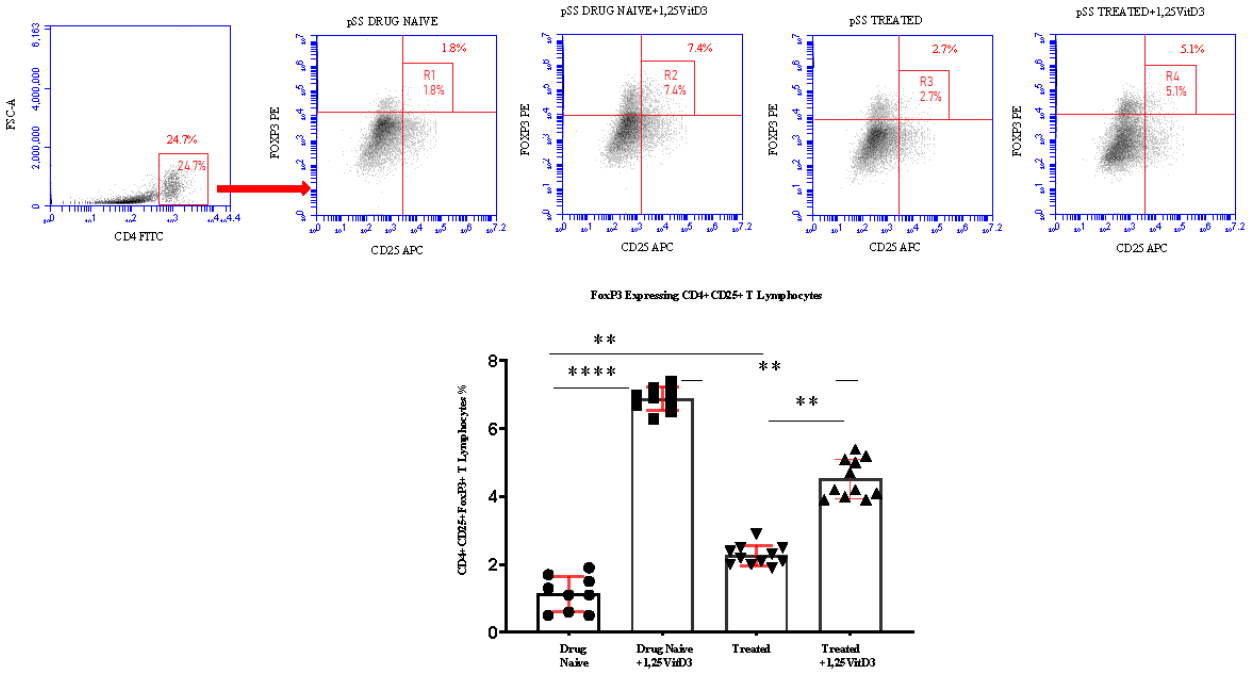

Figure 4. FoxP3 expressing CD4+CD25+ T regulatory cells. The frequency of CD4+CD25+FoxP3+Treg cells were significantly low in drug-naive pSS patients compared to treated subjects ($p < 0.01$). Treg cell population significantly increased in PBMC cultures with 1,25-dihydroxyvitamin D3 both in drug-naive and treated pSS patients compared to PBMC cultures alone (drug-naive pSS: $p < 0.001$, treated pSS: $p < 0.01$).

of vitamin D on T and B lymphocyte phenotypes in pSS patients have not not defined.

In this study, we first evaluated the lymphoproliferative responses of T and B lymphocytes in the presence of 1,25-dihydroxyvitamin D3. The cultured mononuclear cells showed an increased proliferative response of CD4+ T lymphocytes and B cells especially in drug-naive pSS patients when compared with treated subjects. 1,25-dihydroxyvitamin D3 significantly decreased the proliferation of both CD4+T and B lymphocytes in drug-naive pSS patients' mononuclear cell cultures. There are evidences that 1,25-dihydroxyvitamin D3 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-dihydroxyvitamin D3 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-naive 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-dihydroxyvitamin D3 both in drug-naive 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

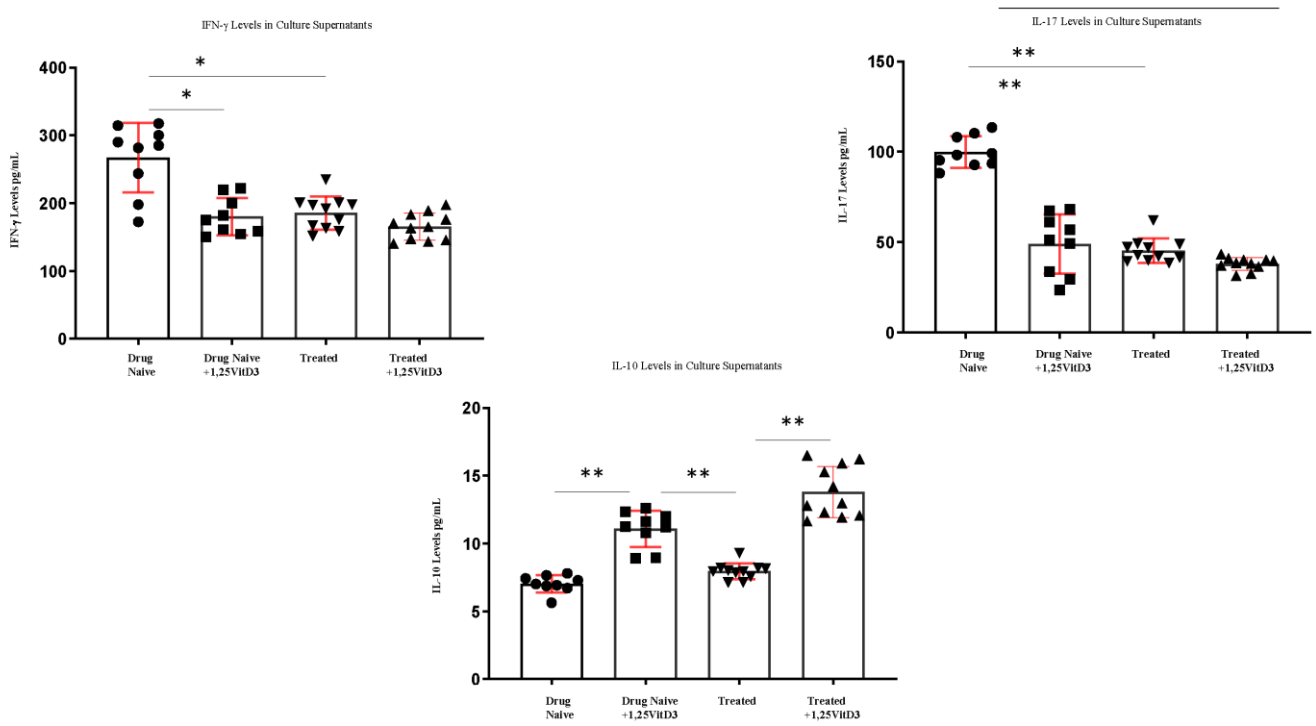

Figure 5. Secreted cytokines in culture supernatants. The proinflammatory cytokines (IFN-γ and IL-17) and antiinflammatory cytokine IL-10 levels were analyzed in the culture supernatants of drug-naive and treated pSS patients' mononuclear cell cultures in the presence and absence of 1,25-dihydroxyvitamin D3. IFN-γ and IL-17 levels were significantly decreased in PBMC cultures with 1,25-dihydroxyvitamin D3 in drug-naive pSS patients, and no significant difference observed in treated subjects when compared with PBMC cultures alone (IFN-γ drug naive pSS: $p < 0.05$, IL-17 drug naive 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-naive and treated pSS patients' PBMC cultures with 1,25-dihydroxyvitamin D3 (drug-naive pSS: $p < 0.01$, treated pSS: $p < 0.01$). IL-10 levels were significantly high in drug-naive pSS patients in the presence of 1,25-dihydroxyvitamin D3 compared to treated patients' PBMC cultures alone ($p < 0.01$).

both drug-naive and pSS treated patients, while the reduction was striking in drug-naive patients. The increased suppressive effect of 1,25-dihydroxyvitamin D3 on Th1 cells may be due to high levels of IFN-γ levels in drug-naive 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 1,25-dihydroxyvitamin D3 in drug-naive patients, and IL-10 enhanced with the addition of 1,25-dihydroxyvitamin D3 in the cell cultures both in drug-naive 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-dihydroxyvitami nD3 on the B lymphocyte subpopulations. The data showed an increase in the ratio of total memory B lymphocytes both in drug-naive and treated pSS patients mononuclear cell cultures with 1,25-dihydroxyvitamin D3. In addition, plasma B cell ratio decreased in the presence of 1,25-dihydroxyvitamin D3 in drug-naive 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-dihydroxyvitamin D3 has previously been described, and

studies indicated that 1,25-dihydroxyvitamin D3 modulates the production of cytokines in a time and concentration dependent manner. We attempted to prove this effect of 1,25-dihydroxyvitamin D3 in pSS patients' mononuclear cell cultures by comparing drug-naive and treated subjects. We found that Th1 and Th17 responses in both drug-naive and treated pSS patients' mononuclear cell cultures reduced in the presence of 1,25-dihydroxyvitamin D3. Additionally, IL-10 production notably increased and lymphoproliferative responses down-regulated with 1,25-dihydroxyvitamin D3. Also, plasma B cell ratio decreased and total memory B lymphocytes increased with 1,25-dihydroxyvitamin D3. These data indicate regulatory effects of 1,25-dihydroxyvitamin D3 on pSS.

Ethics approval and consent to participate

The ethics for clinical research was approved by the Muğla Sıtkı Koçman 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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Conflict of interest

The authors declare no conflict of interest.

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