Evaluation of the effects of 1,25VitD3 on Th17 cells and Tregs in HTLV-1 infected cell lines

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Summary. – Human T-lymphotropic virus type 1 (HTLV-1) causes chronic infections of human T lymphocytes. The present study aimed to evaluate the effects of 1,25VitD3 on the proportion of Tregs and Th17 cells, the expression of related transcription factors (ROR-γt and FOXP3) and cytokines (IL-10, TGF-β, IL-6, and IL-17 A) in the HTLV-1infected cell lines MT-2 and MT-4. MT-2 and MT-4 cells and control PBMCs were treated with 1,25VitD3 and percentages of Tregs and Th17 cells was determined by flow cytometry. Gene expression and cytokine levels were analyzed by real-time PCR and ELISA, respectively. Treatment with1,25VitD3 increased the percentage of Tregs in MT-2 and MT-4 cells, while it decreased the percentage of Th17 cells among MT-2 cells. 1,25VitD3 treatment also significantly improved FOXP3 gene expression in MT-2 cells, while reducing ROR-γt-gene expression in MT-2 and MT-4 cells comparing to untreated cells. Treatment with 1,25VitD3 significantly improved IL-10 levels in MT-2 cells, as well as TGF-β levels in both cell lines culture supernatants. 1,25VitD3 treatment diminished IL-6 levels in cell culture supernatants of MT-2 and MT-4 as well as IL-17 A levels in MT-2. Here we showed, that 1,25VitD3 modulated immune responses by enhancing Tregs differentiation and functions as well as inhibiting Th17 differentiation and actions in HTLV-1 infected cell lines. This suggests that VitD3 may have therapeutic effects in HTLV-1-related diseases by suppressing adverse inflammatory responses.

Keywords: Tregs; Th17 cells; HTLV-1; 1, 25VitD3

Introduction

Human T-lymphotropic virus type 1 (HTLV-1) belonging to the *Retroviridae* family causes chronic infection of human T lymphocytes and is endemic in Japan, Caribbean Melanesia, the Middle East, parts of South America, and Africa (Araya *et al.*, 2011; Birmann *et al.*, 2009; Boostani *et al.*, 2021). However, a considerable percentage of affected ca-rriers are asymptomatic (Carpentier *et al.*, 2015; Tarokhian *et al.*, 2018), HTLV-1 infection has been related to multiple human diseases, including neoplastic growth of HTLV-1-infected T cells (such as ATL), and neoplastic inflammatory conditions such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), arthritis Sjögren syndrome, polymyositis uveitis, and bronchoalveolitis (Araya *et al.*, 2011; Carpentier *et al.*, 2015; Nagate *et al.*, 2021).

Heparin, glucose transporter 1, sulfate proteoglycans, and neuropilin 1 are the HTLV-1 receptors. Therefore, HTLV-1 may infect various hematopoietic cells *in vitro* (Higuchi *et al.*, 2020; Koyanagi *et al.*, 1993).

Tregs (regulatory T cells; $CD127^{-}$ $CD4^{+}$ $CD25^{+}$ $Foxp3^{+}$) and Th17 cells (T helper 17; $CD4^{+}$ IL-17 A^{+}) are two subsets

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Abbreviations: ATL = acute T-cell lymphoma; CTL = cytotoxic T lymphocytes; FOXP3 = Forkhead box P3; HAM/TSP = HTLV-1-associated myelopathy/tropical spastic paraparesis; HTLV-1 = human T-lymphotropic virus type 1; IL = interleukin; PBMC = peripheral blood mononuclear cell; TGF-β = tumor growth factor β; Tregs = regulatory T cells

of CD4$^+$ T cells (Mohammadi *et al.*, 2021). Tregs express several cell surface molecules, including CD25, CD45R, CD62L, CD127, CD103, cytotoxic T-lymphocyte antigen-4 (CTLA-4, or CD152), glucocorticoid-induced TNF receptor family-related gene (GITR), and programmed cell death 1 (PD-1) that enable them to be isolated and characterized (Lippi *et al.*, 2020). FOXP3 is a Tregs-specific transcription factor that plays a significant role in Tregs differentiation, development, and function, as well as serving as a specific marker for Tregs characterization (Bhatla *et al.*, 2020). Tregs can regulate innate immune cell proliferation and functions (macrophages, and dendritic cells) as well as suppressing activity of lymphocytes (Th1, Th2, Th17, and B cells) via direct cell interaction and indirectly by producing of potent anti-inflammatory cytokines such as TGF-β and IL-10 (Christoffersson and von Herrath, 2019). This leads to inhibition of excessive immune responses that may contribute to the pathogenesis of viral infections such as HTLV-1 infection (Bouwman *et al.*, 2020; Romano *et al.*, 2019). TGF-β is a multifunctional cytokine secreted by Tregs and other immune and non-immune cells including macrophages, dendritic cells, fibroblasts, keratinocytes and, etc. TGF-β play a regulatory role in maintaining peripheral tolerance (Jin *et al.*, 2014; Sanjabi *et al.*, 2009). IL-10 as a pleiotropic cytokine is secreted by Tregs, activated Th2 cells, B cells, monocytes, and macrophages, and plays a central role in limiting the adverse inflammatory response which could contribute to tissue injury (Jin *et al.*, 2014; Sanjabi *et al.*, 2009). In addition, IL-10 is essential for immune homeostasis (Jin *et al.*, 2014; Sanjabi *et al.*, 2009).

Th17 cells elicit inflammatory reactions by producing cytokines such as IL-22, IL-21, IL-17 A, IL-17 F, and TNF-α as the pro-inflammatory cytokines (Abdollahi *et al.*, 2015; Fujimoto *et al.*, 2020; Konkel *et al.*, 2017; Veldhoen *et al.*, 2006). Th17 cell overreaction can lead to uncontrolled inflammatory reactions that may lead to CNS inflammation and tissue damage in HTLV-1 infection (Martinez *et al.*, 2012). As Tregs regulate the activity of Th17 cells, the imbalance of the Treg/Th17 axis is a key contributor to the pathogenesis of HTLV-1 infection.

ROR-γt plays a role in Th17 differentiation (Castro *et al.*, 2017). IL-17 A is responsible for inducing cell types to produce other pro-inflammatory cytokines, chemokines, and metalloproteinases, resulting in the recruitment of neutrophils to the tissue, thereby contributing to the inflammation process (Neco *et al.*, 2017). IL-6 is a pro-inflammatory cytokine that is a marker of both acute and chronic inflammation (Kany *et al.*, 2019). IL-6 contributes to the Th17 cells differentiation by suppressing FOXP3 and TGF-β gene expression in Tregs (Kany *et al.*, 2019).

MT-2 is a human HTLV-1-infected cell line obtained from leukemic cells from adult T-cell leukemia patients (Hamano *et al.*, 2015) pv7z, and it can be used to analyze molecular and cellular factors are involved in HTLV-1 infection pathogenesis (Meissner *et al.*, 2017). It was found that the majority of MT-2 cells are regulatory T cells (CD4$^+$CD25$^+$FOXP$^+$) implying that HTLV-1 transforms infected CD4$^+$ T cells to Tregs and causes the clonal proliferation (Hamano *et al.*, 2015). Similarly, Tregs have also been suggested to be the most HTLV-1-infected cells in patients with ATL and HAMP/TSP (Tarokhian *et al.*, 2018). However, Tregs proliferation increased in response to HTLV-1 infection and these cells were shown to be functionally impaired *in vivo* and *in vitro*, which might be one of the mechanisms behind the triggering inflammatory responses (Satou *et al.*, 2011; Tarokhian *et al.*, 2018). Th17-mediated pro-inflammatory responses can enhance viral replication. However, the contributions of Tregs and Th17 cells in HTLV-1-associated illnesses (Tarokhian *et al.*, 2018) vary depending on the infection stage and host immunological status (Champs *et al.*, 2019; Kchour *et al.*, 2013; Martinez *et al.*, 2012; Swaims *et al.*, 2010; Tarokhian *et al.*, 2018).

1,25VitD3 (1,25(OH)2D3), as the active form of VitD3, is a multi-target hormone (Heyden and Wimalawansa, 2018) that has a critical role in bone health by controlling calcium and phosphate homeostasis (Veldurthy *et al.*, 2016). Beyond the classical function, it was found that upon binding to vitamin D receptor, 1,25VitD3 regulates function and differentiation of different immune cells such as dendritic cells, T cells, macrophages, and B cells (Nian *et al.*, 2021; Umar *et al.*, 2018).

To the best of our knowledge in HTLV-1 infection the mechanism by which 1,25VitD3 affects the Treg/Th17 axis remains insufficient. In this study, we evaluated the effects of 1,25VitD3 on the proportions of Tregs and Th17 cells as well as the expression of related transcription factors (ROR-γt and FOXP3) and cytokines (IL-10, TGF-β, IL-6, and IL-17 A) in MT-2 cells and pooled PBMCs from healthy donors as control cells.

Materials and Methods

Isolation of PBMCs. 10 ml of blood were collected from each of the 10 healthy donors. PBMCs isolation was carried out by Ficoll, lymphosep (Biosera, UK) from 10 ml of peripheral blood and were pooled. PBMCs were washed twice with PBS (phosphate-buffered saline, Sigma-Aldrich, Israel) and 10^6 cells/ml were cultured in RPMI-1640 media supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml Pen-Strep, 2 mM L-Glutamine. Cell viability, was determined by Trypan Blue dye. Samples were collected only from healthy donors who had given informed consent.

Optimization of 1,25VitD3 concentration. PBMCs were seeded (2×10^6/ml) in 6 well plates and cultivated with different con-

Table 1. Primer sequences used in real-time PCR

Target gene	Sequence 5' to 3'	Purpose	Product length (bp)
'2M	5'-TTGTCTTTCAGCAAGGACTGG-3' 5'-CCACTTAACTATCTTGGGCTGTG-3'	Forward Reverse	127
IL-10	5'-ACTACTTCAAGTTCCACAACATGC-3' 5'GAGTGTCCGCTGCTTCTCTG-3'	Forward Reverse	112
TGF-β	5'-ACTACTTCAAGTTCCACAACATGCR-3' 5'-RGAGTGTCCGCTGCTTCTCTG-3'	Forward Reverse	125
IL-6	5'-GACGAGTTTGTGGACTCCTTTAAG-3' 5'-CCTGCCCAAGTTCCGATCC-3	Forward Reverse	123
IL-17 A	5'-ACTACTTCAAGTTCCACAACATGC-3' 5'-RGAGTGTCCGCTGCTTCTCTG-3'	Forward Reverse	115

centrations of 1,25 VitD3 (10 nM, 30 nM, 50 nM, 100 nM and 0 nM (control)) for 12 h, 24 h, 48 h, and 72 h. Optimization of the concentration of 1,25VitD3 was achieved by assessing the proportion of Tregs and Th17 cells among isolated PBMCs from 3 controls and MT-2 cells, using flow cytometry analysis on FACS Calibur (BD, USA). The concentration of 50 nM 1,25VitD3 treated for 24 h (for real-time PCR and flow cytometry) and 72 h (for ELISA) was selected as the optimal concentration and time of treatment.

Treatments. 2×10^6 PBMCs from each subject were seeded in 6 well plates. PBMCs of each subject (for flow cytometry and real-time PCR) were treated with (1) 50 nM 1,25VitD3 (Sigma, Israel); (2) 10 μM PHA (phytohemagglutinin, Gibco Company, USA); and (3) media only (as the baseline). Treatment of MT-2 and control PBMCs was performed six times. The experiments were performed in triplicates and the values were averaged.

*Flow cytometry detection of Tregs.*The FOXP3 staining kit (eBioscience, USA) was used for Tregs staining according to the manufacturer's protocol. PBMCs (1×10^6) were stained for the surface markers of Tregs, including CD4 (FITC-conjugate), CD25 (PE-conjugate), and CD127 (APC-conjugate) at 4°C for 30 min and washed by staining buffer. PBMCs were then fixed in a 0.5 ml of fixation buffer after permeabilization with 0.5 ml of permeabilization buffer. FOXP3 (PE-Cy5-conjugate) as the intracellular marker of Tregs was stained at 4°C in dark for 30 min after staining of surface markers. Representative gating strategy for immunophenotyping of T-regs is shown in Fig 2.

Flow cytometry detection of Th17 cells. PBMCs (1×10^6) were treated with 50 ng/ml of PMA (Phorbol 12-myristate 13-acetate, eBioscience, USA) and 1 μg/ml of ionomycin (eBioscience, USA) to stimulate intracellular cytokine production for 5 h in the presence of brefeldin A (eBioscience, USA) at 37°C and 5% CO_2. Cells were stained for surface markers with anti-CD8 conjugated with FITC and anti-CD3 conjugated with PE-Cy5 (BD Biosciences, USA) using the required buffers. The cells were fixed/permeabilized with saponin-containing buffer (eBioscience, USA). Isotype control or anti-IL-17 (PE-conjugated) was used for intracellular staining of Th17 cells (eBiosciences, USA). Representative gating strategy for immunophenotyping of Th17 cells is shown in Fig. 2.

Flow cytometry assessment. FACS Calibur system was used for flow cytometry assessment of 1×10^5 cells. The data were analyzed by the Cell Quest software (Becton Dickinson, USA).

Real-time PCR. RNA extraction kit (Invitek, Germany) was used for total RNA extraction from PBMCs according to the manufacturer's instructions. Reverse transcription was performed by RevertAid™ H primers (Thermofisher, Germany). Primer-BLAST analysis was performed to verify the specificity of primers. RNA quality determination was carried out by agarose gel (2%) electrophoresis, showing 5.8S, 18S, and 28S bands in the UV light transilluminator. The total volume of all PCR reactions was 20 μl containing 10 μl of real-time PCR-SYBR Green master mix (Takara, Japan), 0.3 μl of each primer (Table 1), and 7.4 μl of RNase-free water. Real-time PCR was performed by Rotor-Gene Q cycler (Qiagen, Germany). The following standard PCR reaction conditions were used for all transcripts: 10 minutes at 95°C, 15 s at 95°C (45 cycles), 30 s at 57°C, and 1 min at 60°C. Logarithmic dilution series of the total RNA was used to construct 10-fold dilution standard curves for FOXP3 and ROR-γt. B2M (β-2-microtubulin) was used as the internal control gene to normalize mRNA levels.

ELISA. To measure the levels of cytokines, PBMCs (1×10^6 cells/ml) were cultured in RPMI media with 50 nM 1,25VitD3 or without it. Cell culture supernatants were collected after 72 h and were assayed for concentrations of soluble IL-10, TGF-β, IL-6, and IL-17 A by linked immunosorbent assay using ELISA kits (Biolegend, USA). All samples were analyzed in duplicates. The sensitivity of each assay was as follows: 0.8 pg/ml for IL-17 A, 2.7 pg/ml for IL-10, 1.6 pg/ml for IL-6, and 3.5 pg/ml for TGF-β. Coefficients of variation (CV) were <10% and <5% for inter-assay and intra-assay, respectively.

Analysis of statistics. SPSS 16.0 software was used for statistical analysis. Mean data were compared by ANOVA and

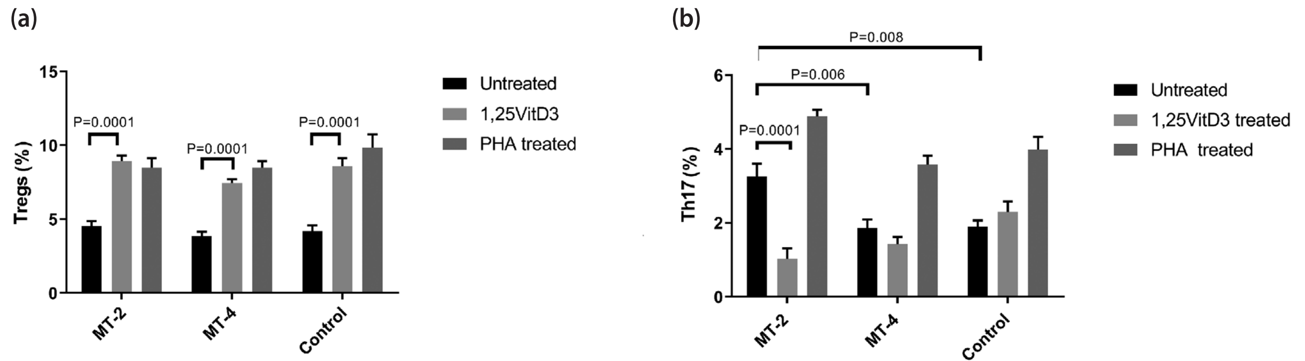

Fig. 1

1,25VitD3 affects the levels of Tregs and Th17 cells

(a) After treatment of MT-2 and MT-4 cells with 1,25VitD3, the percentage of Tregs increased relative to untreated cells or control PBMCs. **(b)** The proportion of Th17 cells in MT-2 cells was significantly higher. In MT-2 cells, the percentage of Th17 cells decreased after treatment with 1,25VitD3 relative to untreated control PBMCs.

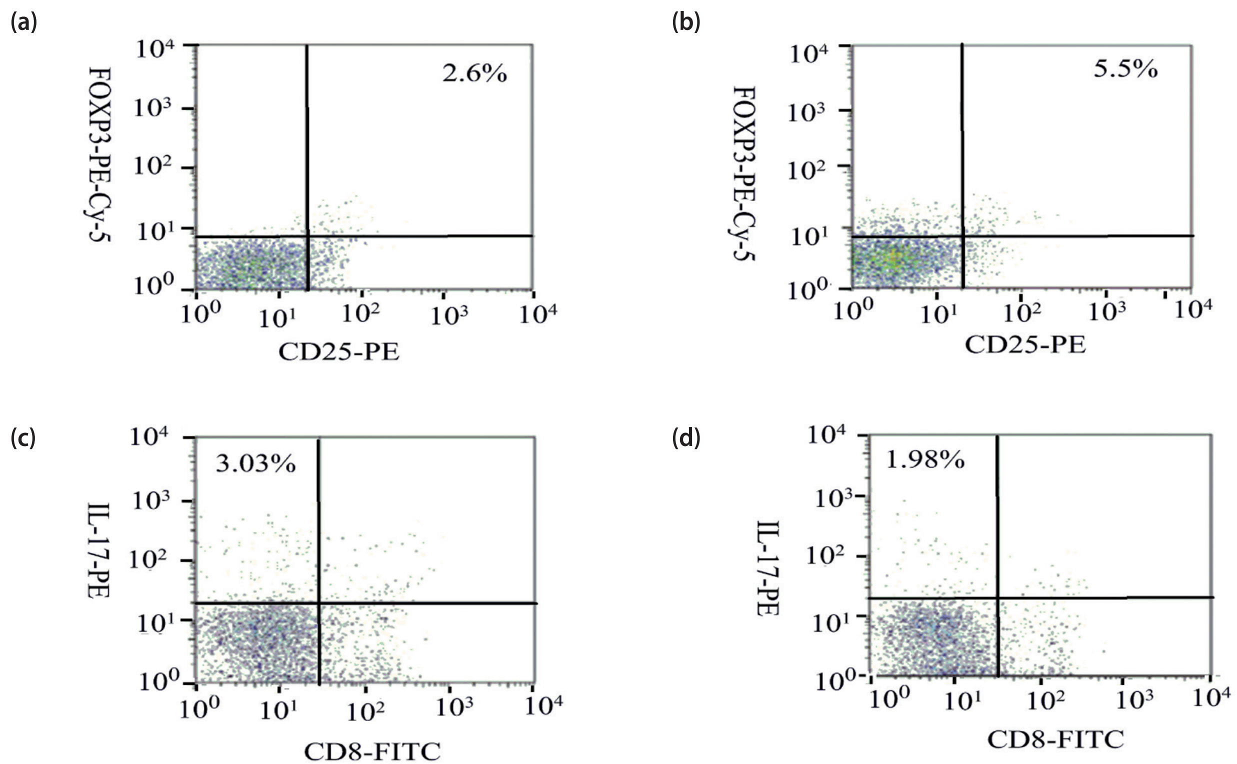

Fig. 2

Flow cytometry detection of Tregs and Th17 cells

Representative histograms of flow cytometric analysis of $CD4^+CD25^{high}$ $FOXP3^+$ Tregs and of $CD8^-IL\text{-}17^+$ Th17 cells among **(a, c)** untreated and **(b, d)** 50 nM 1,25VitD3 treated cells. In HTLV-1 infected cells $CD4^+$ $CD127^-$ cells were gated for Treg analysis, and the percentage of $CD25^{high}$ $FOXP3^+$ cells was determined **(a, b)**. $CD3^+$ cells were gated for Th17 assessment, and then the percentage of CD3+IL-17+ cells was determined **(c, d).**

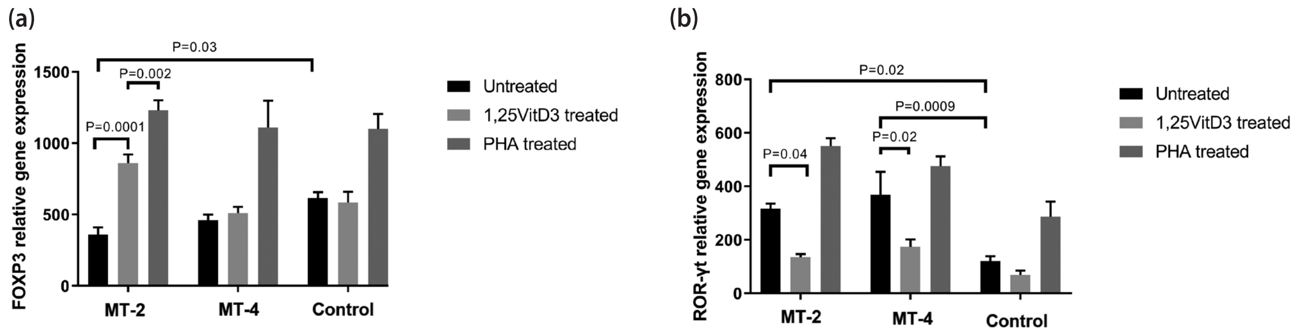

Fig. 3

1,25VitD3 affects FOXP3 and ROR-γt gene expression

(a) FOXP3 gene expression in MT-2 cells was lower than in the control PBMCs. Treatment with 1,25VitD3 increased FOXP3 gene expression at the mRNA level in MT-2 cells. **(b)** ROR-γt-gene expression was higher in MT-2 and MT-4 cells than in the control PBMCs. Treatment with 1,25VitD3 decreased ROR-γt-gene expression at the mRNA level in those cells relative to untreated cells.

parametric T-test. P-values of less than 0.05 were regarded as significant. The data are presented as mean ± SE.

Results

1,25VitD3 enhanced the percentage of Tregs in MT-2 cells, MT-4 cells and control PBMCs

MT-2 and MT-4 cells treatment with 1,25VitD3 increased the frequency of Tregs. The proportion of Tregs in MT-2 cells and control cells was statistically similar (4.52 ± 0.36 vs. 4.18 ± 0.39, P >0.05; Fig. 1a) as well as the percentage of Tregs in MT-4 cells and control cells (3.85 ±0.29 vs. 4.18 ± 0.39, P >0.05; Fig. 1a). After MT-2 cells treatment with 1,25VitD3, the percentage of Tregs increased relative to untreated MT-2 cells (as the baseline) (8.94 ± 0.37 vs. 4.52 ± 0.36, P = 0.0001; Fig. 1a). 1,25VitD3 treatment also increased the percentage of Tregs relative to untreated MT-4 cells (as the baseline) (7.45 ± 0.25 vs. 3.85 ± 0.29, P = 0.0001; Fig 1a) as well as in the control PBMCs (8.6 ± 0.53 vs. 4.18 ± 0.39, P = 0.0001; Fig. 1a). As a positive control, PHA was used to show that MT-2 cells, MT-4 cells, and control PBMCs respond to a stimulus and the responses does not differ among them.

1,25VitD3 decreased the percentage of Th17 cells in MT-2 cells

1,25vitD3 treatment reduced the percentage of Th17 cells among MT-2 cells. The proportion of Th17 cells in untreated MT-2 cells was significantly higher compared to untreated control PBMCs (3.25 ± 0.35 vs. 1.89 ± 0.18, P = 0.008; Fig. 1b). The percentage of Th17 cells in untreated MT-2 cells was significantly higher compared to untreated MT-4 cells (3.25 ± 0.35 vs. 1.89 ± 0.23, P = 0.006; Fig. 1b). In MT-2 cells, the percentage of Th17 cells decreased after treatment with 1,25VitD3 relative to untreated control PBMCs (1.02 ± 0.28 vs. 3.250 ± 0.35, P = 0.0001; Fig. 1b), while 1,25VitD3 treatment did not significantly change the levels of Th17 cells in MT-4 cells and control PBMCs (1.42 ± 0.19 vs. 1.86 ± 0.23 and 2.3 ± 0.28 vs. 1.89 ± 0.18, P >0.05; Fig. 1b).

1,25VitD3 increased FOXP3 gene expression in MT-2 cells

1,25VitD3 increased the expression of FOXP3 gene in MT2 cells. FOXP3 gene expression was not statically significant between MT-2 cells and control PBMCs (360.6 ± 50.50 vs. 615.14 ± 41.15, P = 0>0.05; Fig. 3a). FOXP3 gene expression was not statically different between untreated MT-4 cells and control PBMCs (460.41 ± 39.40 vs. 615.14 ± 41.15 P = 0>0.05; Fig. 3a). 1,25VitD3 treatment increased FOXP3 gene expression at the mRNA level in MT-2 cells (860.84 ± 60.89 vs. 360.60 ± 50.5; P = 0.002; Fig. 3a) but not in the control PBMCs (585.95 ± 73.41 vs. 615.14 ± 41.15; P >0.05; Fig 3a), and in MT-4 cells (510.4 ± 43.48 vs. 460.41 ± 39.40 P >0.05; Fig. 3a).

1,25VitD3 decreased ROR-γt gene expression in MT-2 cells

1,25VitD3 decreased the expression of ROR-γt in both MT2 and MT-4 cells. ROR-γt gene expression was higher in MT-2 and MT-4 cells than in the control PBMCs (316.41 ± 50.99 vs. 120.54 ± 18.41, P = 0.02 and 368.47 ± 85.59 vs. 120.54 ± 18.41, P = 0.003; Fig. 3b). Treatment with 1,25VitD3 decreased ROR-γt-gene expression at the mRNA level relative to untreated MT-2 cells (140.09 ± 12.01 vs. 316.41 ± 18.99;

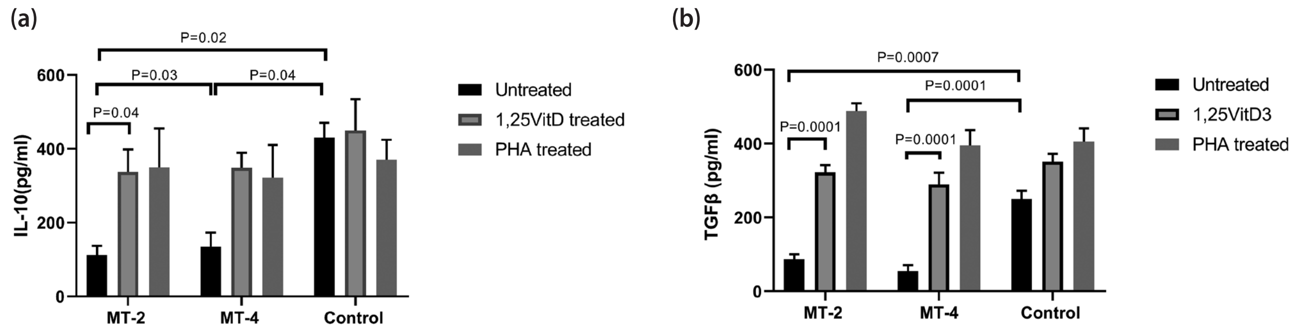

Fig. 4

1,25VitD3 affects the levels of IL-10 and TGF-β in cell culture supernatants

(a) In MT-2 and MT-4 cells, IL-10 levels in cell culture supernatants were lower than in control PBMCs. Treatment with 1,25VitD3 significantly increased IL-10 levels in cell culture supernatants in MT-2 cells compared to untreated MT-2 cells. **(b)** TGF-β levels in the culture supernatants of MT-2 and MT-4 cells were lower than in the control PBMCs. Treatment with 1,25VitD3 significantly increased TGF-β levels in cell culture supernatants in those cells compared to untreated cells.

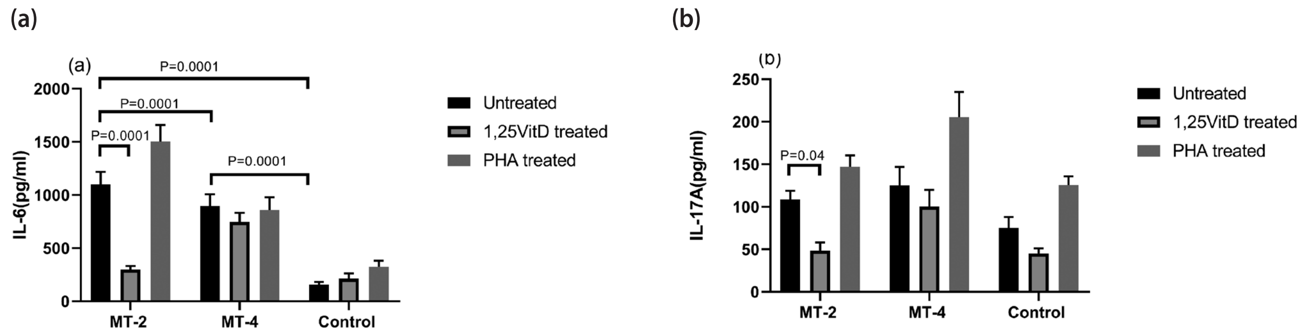

Fig. 5

1,25VitD3 affects the levels of IL-6 and IL-17 A in cell culture supernatants

(a) IL-6 levels in MT-2 and MT-4 cell culture supernatants were higher compared to that in the control PBMCs. Treatment with 1,25VitD3 significantly decreased IL-6 levels in MT-2 cell culture supernatants relative to untreated MT-2 cells. **(b)** In comparison to untreated MT-2 cells, treatment with 1,25VitD3 significantly decreased IL-17 A levels in MT-2 cell culture supernatants.

P = 0.04; Fig. 3b) as well as in MT-4 cells (174.10±27.8 vs. 368.47±85.59; P = 0.02; Fig. 3b) but not in the control PBMCs (68.71±16.09 vs. 120.54±18. 21; P >0.05; Fig. 3b).

1,25VitD3 increased IL-10 levels in MT-2 cell culture supernatants

Treatment with 1,25VitD3 enhanced IL-10 levels in cell culture supernatants only in MT-2 cells. In MT-2 cells, IL-10 levels in cell culture supernatants were lower than in control PBMCs (112.14±25.05 vs. 430.59±40.30, P = 0.02; Fig. 4a). In MT-4 cells, IL-10 levels in cell culture supernatants were also lower than in control PBMCs (135.40±47.46 vs. 430.59±40.30, P = 0.04; Fig. 4a). Treatment with 1,25VitD3 significantly increased IL-10 levels in cell culture supernatants in MT-2 cells (337.82±28.5 vs. 112.14±25.05; P = 0.04; Fig. 4a), but not in MT-4 cells (349.24±40.52 vs. 135.40±47.46; P >0.05; Fig. 4a). 1,25VitD3 treatment also had no significant effect on IL-10 levels in control PBMCs supernatants (450.40±84.5 vs. 430.59±40.30; P >0.05; Fig. 4a).

1,25VitD3 increased TGF-β levels in MT-2 and MT-4 cells culture supernatants

1,25VitD3 increased TGF-β levels in the culture supernatants of both MT2 and MT-4 cells.

TGF-β levels in the culture supernatants of MT-2 and MT-4 cells were lower than in control PBMCs (87.24±12.76 vs. 250.4±22.14, P = 0.0007; 54.58±16.47 vs. 250.4±22.14, P = 0.0001; Fig. 4b). Treatment with 1,25VitD3 significantly increased TGF-β levels in cell culture supernatants in MT-2 cells (322.40±19.44 vs. 87.24±12.76; P = 0.0001; Fig. 4b) and in MT-4 cells (289.75±31.47 vs. 54.58±16.47; P = 0.0001; Fig. 4b) compared to untreated cells, respectively.

1,25VitD3 decreased IL-6 levels in MT-2 cell culture supernatants

Treatment with 1,25VitD3 diminished IL-6 levels in cell culture supernatants only in MT-2 cells. IL-6 levels in MT-2 and MT-4 cell culture supernatants were higher compared to that in the control PBMCs (1099.28±118.67 vs. 158.00±24.40, P = 0.0001; 895.4±110.40 vs. 158.00±24.40, P = 0.0001; Fig. 5a). Treatment with 1,25VitD3 significantly decreased IL-6 levels in MT-2 cell culture supernatants relative to untreated MT-2 cells (299.31±67.56 vs. 1099.28±118.67; P = 0.0001; Fig. 5a), while 1,25VitD3 treatment did not significantly change IL-6 levels in supernatants of MT-4 cells and control PBMCs (745.20±85.50 vs. 895.00±110.40, and 214.01±49.25 vs. 158.01±24.40, respectively; P >0.05; Fig. 5a).

1,25VitD3 decreased IL-17 A levels in MT-2 cell culture supernatants

Treatment with 1,25VitD3 significantly decreased IL-17 A levels in MT-2 cell culture supernatants compared to untreated MT-2 cells (48.60±10.04 vs. 108.29±10.40; P = 0.04; Fig. 5b), while 1,25VitD3 treatment did not significantly alter IL-17 A levels in MT-4 cells and control PBMCs supernatants (100.41±19.40 vs. 125.29±21.40 and, 45.01±6.24 vs. 75.25 ±12.5, respectively; P >0.05; Fig. 5b).

Discussion

The first retrovirus to be identified as a cause of the human disease was HTLV-1, which infects at least 10 million individuals around the world (Afonso *et al.*, 2019; Atabati *et al.*, 2021). HTLV-1 is associated with ATL, which is an intense cancer of $CD4^+$ T cells, as well as other chronic inflammatory disorders like HAM/TSP and uveitis (Poiesz *et al.*, 1980; Yasunaga and Matsuoka, 2011). Given detailed research into HTLV-1 related diseases, the precise mechanism by which HTLV-1 causes the inflammatory conditions is still unknown (Afonso *et al.*, 2019; Tarokhian *et al.*, 2018).

As an HTLV-1 infected human T-cell line, the MT-2 and MT-4 cells can be useful in finding what-molecular and cellular factors are involved in HTLV-1-related diseases (Meissner *et al.*, 2017). MT-2 cells were found to be mostly T cell-like regulatory cells (Hamano *et al.*, 2015). Furthermore, Tregs have been suggested to be the most HTLV-1-infected cells *in vivo* (Tarokhian *et al.*, 2018). HTLV-1-infected $CD4^+$ T cells from HAM/TSP patients, on the other hand, spontaneously proliferate as a result of oncogene activity, and Tregs are known as a subpopulation of T $CD4^+$ cells (Quaresma *et al.*, 2016). Tregs, which express FOXP3 as the key transcription factor and secrete immunosuppressive cytokines including IL-10 and TGF-β, regulate immune cell functions (Olthof *et al.*, 2015; Romano *et al.*, 2019). Tregs were shown useful for cell-based immunotherapy in autoimmune and inflammatory disorders because they lead to immune homeostasis and the regulation of adverse inflammatory responses (Eggenhuizen *et al.*, 2020; Mohammadi *et al.*, 2021). Th17 cells, which express ROR-γt as a transcription factor and secrete IL-17 A, are one of the most prominent subsets of inflammatory cells (Abdollahi *et al.*, 2016; Guo and Zhang, 2021; Olthof *et al.*, 2015). It was supposed that Tregs impairment may contribute to Th17 cell overreaction leading to uncontrolled inflammatory responses that may exacerbate viral infection since Th17 cell actions are regulated by Tregs (Martinez *et al.*, 2012). HTLV-1 infection disrupts immune homeostasis by altering Treg and Th17 activities, as well as associated cytokines such as IL-17 A, IL-10, and TGF-β resulting in an imbalance of inflammatory and anti-inflammatory responses, as well as a failure of tolerance and exacerbation of inflammation (Araújo *et al.*, 2009; Best *et al.*, 2009; Castro-Costa *et al.*, 2009; de Sá *et al.*, 2016; Quaresma *et al.*, 2016; Sasada *et al.*, 2005).

In the pathogenesis of HTLV-1, an immunosuppressive microenvironment with regulatory T cell contribution may have two opposing roles. On the one hand, regulatory functions of Tregs in suppressing immune responses may have enabled HTLV-1 to escape host immunity, resulting in viral infection progression (Higuchi *et al.*, 2020). In this scenario, Tregs could worsen the HAM/TSP pathogenic process (Yamano *et al.*, 2009). On the other hand, HTLV-1 functionally inhibited Tregs functions thus increasing their proliferation, leading to potentially impairing the suppressive activity of Tregs and ultimately, unregulated inflammation in support of virus survival (Brito-Melo *et al.*, 2007; Oh *et al.*, 2006; Satou and Matsuoka, 2010; Toulza *et al.*, 2008). However, the effects of anti-inflammatory and pro-inflammatory responses on viral infection are assumed to be dependent on the infection phase, and host immune status (Higuchi *et al.*, 2020; Tarokhian *et al.*, 2018).

In this study, we used MT-2 and MT-4 cells as the models for T cells infected with HTLV-1 as these cells were obtained from leukemic cells from ATL patients (Hamano *et al.*, 2015). However, we found that the frequency of Tregs in MT-2 and MT-4 cells were similar to those observed in PBMCs from healthy donors, but FOXP3 gene expression was substantially lower in MT-2 cells. This may relate to an impairment of Tregs function in MT-2 cells. In line with this finding, several studies have shown that low FOXP3 gene expression during HTLV-1 infection may lead to the development of inflammatory disease like HAMP/TSP (Oh *et al.*, 2006). Lower levels of TFG-β and IL-10 as the immunosuppressive secretory cytokines of Tregs in MT-2

and MT-4 cell culture supernatants compared to control cells provide further evidence of Tregs impairment in our research. In this regard, multiple studies in patients with HAM/TSP found a reduction in FOXP3 gene expression as well as the levels of TGF-β and IL-10 (Brito-Melo *et al.*, 2007; Oh *et al.*, 2006; Satou and Matsuoka, 2010; Toulza *et al.*, 2008). Since inflammation was not regulated and the inflammatory process reinforced, this lack of suppressive action could lead to an exacerbation of the HAM/TSP (Quaresma *et al.*, 2016). In contrast, previous research has shown that increased FOXP3 expression in patients with ATL contributes to enhanced Tregs activity, leading to increased secretion of TGF-β and IL-10, which then activates the immunosuppression phenotype shown in HAM/TSP patients (Quaresma *et al.*, 2016). Therefore, we conclude that the role of Tregs in the pathogenesis of HTLV-1 infection varies based on the infection stage and the host immune status (Kchour *et al.*, 2013; Martinez *et al.*, 2012; Swaims *et al.*, 2010; Tarokhian *et al.*, 2018).

We indicated that the percentage of Th17 cells, ROR-γt gene expression as well as IL-17 A and IL-6 levels were higher in HTLV-1 cell lines compared to PBMCs from healthy donors. Therefore, a decrease in impairment in Tregs function (reduction of FOXP3 expression as well as IL-10 and TGF-β levels) may contribute to the enhanced Th17 inflammatory responses. It was demonstrated that excess production of IL-17 A and increased ROR-γt gene expression in the HTLV-1 infected cells may contribute to the pathogenesis of the associated diseases via suppression of Th1 cells activity (Martinez *et al.*, 2012). Mechanistically, inhibition of Th1 cell differentiation resulted in a decrease in the synthesis of IL-2 and IFN-γ, and hence a decrease in cytotoxic T cells (CTLs) functions (Barnowski *et al.*, 2020). This condition is in favor of replication of HTLV-1 as CTLs are the key players in the combat against viral infections (Barnowski *et al.*, 2020; Martinez *et al.*, 2012). ROR-γt expression was also shown to be elevated in ATL patients' skin and other tissues, which was attributed to inflammatory reactions (Martinez *et al.*, 2012). However, it was indicated that Th17 cells may be important to inhibit viral transmission in some cases. As a result, Th17 cell functions can vary depending on the stage of infection, and the host immune background in viral infection, similar to Tregs (Martinez *et al.*, 2012; Tarokhian *et al.*, 2018).

VitD3 has an essential role in bone metabolism and calcium homeostasis (Veldurthy *et al.*, 2016). Additionally, VitD3 also has other essential physiological roles, including involvement in the modulation of immune responses (Martens *et al.*, 2020; Poles *et al.*, 2021). These findings are known as 'non-classical actions' and were revealed 30 years ago when 1,25VitD3 receptor (VDR) was identified in multiple cell lines (Eisman *et al.*, 1979; Makishima *et al.*, 2002). Upon binding to VDR, VitD3 regulates immune cell proliferation, differentiation, and functions, including T cells, dendritic cells, and macrophages that are an important part of the non-classical actions of VitD3 (Chun *et al.*, 2014; Khorasanizadeh *et al.*, 2019). Modulation of Tregs and Th17 cell functions in diseases with inflammatory etiologies, such as autoimmune diseases (most notably in multiple sclerosis) and recurrent abortion, is one of the most important activities of VitD3 in modulating immune responses (Abdollahi *et al.*, 2020a,b; Fletcher *et al.*, 2019; Miclea *et al.*, 2020).

In this study, we found that the active form of VitD3, 1,25VitD3 (50 nM), promotes anti-inflammatory status in MT-2 and MT-4 cells. Mechanistically, 1,25VitD3 increased the frequency of Tregs in MT-2 cells, MT-4 cells, and the control PBMCs, while it decreased parentage of Th17 cells at the same dose just in MT-2 cells as compared to PBMCs from healthy donors. 1,25VitD3 increased the expression of FOXP3, the transcription factor and internal marker of Tregs, while decreased the expression of ROR-γt as the key transcription factor of Th17 cells. In cell culture supernatants of HTLV-1 infected cell lines, increasing levels of immunosuppressive cytokines (TGF-β and IL-10) and reducing levels of IL-6 and IL-17 A, provided more evidence of the anti-inflammatory properties of 1,25VitD3 in this research.

VitD3 has a beneficial effect on antiviral functions. During viral infections, vitamin D can cause the development of cathelicidin antimicrobial peptides such as LL-37 and human β-defensins (Bergman *et al.*, 2007; Liu *et al.*, 2007). VitD3 also showed the immunomodulatory effects in several viral infections including, EBV, influenza, HIV, HBV, and RSV (Hamano *et al.*, 2015; Hansdottir *et al.*, 2010; Pender, 2012). It was shown that in EBV infection, low vitamin D levels may suppress the activation of CTLs leading to invading of infected B cells (Pender, 2012). Furthermore, the immunoregulatory activity of VitD3 seems to be mediated by IL-10-secreting Tregs, as it has been proposed that a deficiency in IL-10 expression is caused to limit the anti-inflammatory effect of VitD3 (Hayes and Acheson, 2008; Spach *et al.*, 2006). Mechanistically, VitD3 could decrease the inflammatory chemokines and cytokines such as IL-8, TNF-α, and IL-6 in HIV and influenza infection (Derakhshan *et al.*, 2020; Khare *et al.*, 2013).

It was found that VitD3 deficiency (25(OH)2D3) levels less than 30 ng/ml were attributed to an increased risk of respiratory infections (Derakhshan *et al.*, 2020). VitD3 deficiency was also implicated in the HAMP/TSP patients compared to the healthy controls (Derakhshan *et al.*, 2020). Since HAM/TSP is an inflammatory disease caused by HTLV-1 infection (hypersensitivity type IV), VitD3 anti-inflammatory effects in HAM/TSP could improve the disease (Derakhshan *et al.*, 2020). VitD3, for example in Multiple Sclerosis patients, can play a role in HAM/TSP

pathology by regulating the immune responses to HTLV-1 (Ratner and Poiesz, 1988).

In summary, we assume that by promoting anti-inflammatory responses, 1,25VitD3 modulated Treg/Th17 axis in HTLV-1 infected cell lines.

Conclusion

Our findings showed that Tregs were impaired in HTLV-1 infected cell lines, while Th17 cell differentiation and function was enhanced compared to the control PBMCs, reflecting the inflammatory state in HTLV-1 infection and may contribute to the pathogenesis of HTLV-1 related diseases such as ATL and HAMP-TSP. 1,25VitD3 served as a modulator of immune responses, balancing the Treg/Th17 axis in HTLV-1 infected cell lines. According to our findings, 1,25VitD3 altered Treg and Th17 cell differentiation, as it elevated FOXP3 expression in Tregs while decreasing RORγ-t expression in Th17 cells. 1,25VitD3 also inhibited the production of IL-6 in MT-2 cell culture supernatants which are involved in Th17 cell differentiation. Mechanistically, 1,25VitD3 increased IL-10 and TGF-β levels, two anti-inflammatory cytokines released by Tregs. On the other hand, 1,25VitD3 reduced the levels of IL-17 A secreted by Th17 cells.

Collectively, according to the findings of this study, 1,25VitD3 can act as both immunoregulatory (improving Tregs functions) and the immunosuppressive agent (decreasing the functions of Th17 cells), illustrating the role of VitD3 as an immunomodulator in HTLV-1 infection. This highlights the importance of sufficient vitamin D level and also taking VitD3 supplementation in patients with HTLV-1. In this regard potential, clinical trials in the field of beneficial use of VitD3 as a complementary therapy in patients with HTLV-1 infection are recommended.

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