Original Article

Effects of \(1,25(\text{OH})_2\text{D}_3\) in immune response regulation of systemic lupus erithematosus (SLE) patient with hypovitamin D

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Abstract: Vitamin D deficiency has been associated with pathogenesis of autoimmune diseases including SLE; however, there were still lack of data about the effects of administration of vitamin D in immune regulation in SLE patients. The aim of this study was to investigate the effects of calcitriol/\(1,25(\text{OH})_2\text{D}_3\) on dendritic cells maturation and Th17 and Treg cells activation in SLE patients with hypovitamin D. The monocytes and lymphocytes of five SLE patients with hypovitamin D were divided into 4 groups, P0 (0 nM/control), P1 (1 nM), P2 (10 nM), and P3 (100 nM) as cultured samples. Flowcytometry analysis was used to evaluate dendritic cell maturation (the percentage of CD40, CD86, and HLA-DR expression) and the amount of Th17 and Treg cells (the percentage of Th17 and Treg cells). Cytokines production of IL-12, IL-17A, and TGF-β measured by ELISA. This study showed significant differences in CD40, CD86, and HLA-DR expression and the amount of Th17 and Treg cells (the percentage of Th17 and Treg cells). Cytokines production of IL-12, IL-17A, and TGF-β measured by ELISA. This study showed significant differences in CD40, CD86, HLA-DR expressions, and Th17 percentage in 10 nM of \(1,25(\text{OH})_2\text{D}_3\) compared to that of control. For cytokines secretion, there was also significant difference between IL-12p70 and IL-17A levels in 10 nM of \(1,25(\text{OH})_2\text{D}_3\) compared to that of control. The \(1,25(\text{OH})_2\text{D}_3\) increased Treg cells and TGF-β level but not significant. Our study concluded that \(1,25(\text{OH})_2\text{D}_3\) inhibited dendritic cells maturation and Th17 cells activation in SLE patients. The \(1,25(\text{OH})_2\text{D}_3\) increased Treg cells but not significant.

Keywords: Calcitriol \([1,25(\text{OH})_2\text{D}_3]\), dendritic cell, Th17 cells, Treg cells, systemic lupus erythematosus

Introduction

Systemic Lupus Erythematosus (SLE) is a chronic systemic inflammatory disease with high morbidity and mortality. In Indonesia, life expectancies of SLE patients are still low, 70% for 5 years survival rate and 50% for 10 years survival rate [1]. Pathogenesis and etiology of SLE remains unclear, but evidences suggest that hyperactivity of T helper 17 (Th17) cells associated with alteration of T regulator (Treg) cells function and abnormality of dendritic cells (DC) cause the activation of T and B cells to produce high amount of antibodies and induce toxic inflammatory mediators in tissues that are very important in disease progressivity of SLE patients [2].

A relatively new subset of CD4 Thelper, identified by released of interleukin (IL)-17A, IL-17F, and IL-22, called Th17, has important role in inflammatory process and leads to tissue damage [3, 4]. The increased level of IL-17A that secreted by Th17 cells was detected in patients with autoimmune diseases, such as SLE [5]. In contrast, Treg cells have role in immune system regulation by suppressing autoreactive T cells [6].

One of extraskeletal function of vitamin D is involved in regulation of immune response. Immunologic effects of \(1,25(\text{OH})_2\text{D}_3\) include down-regulating Th1 immune responses, modulating DC maturation, and lowering proliferation of activated B cells, yet in the contrary up-regu-
lating regulatory T cells and preserving innate immune responses [7]. Vitamin D deficiency is associated with an increase of progressivity of autoimmune diseases [8-10]. A decrease of vitamin D level increases IL-6 level and reduces TGF-β level [11]. TGF-β is important for activating transcription factors to induce naïve T Helper (Th0) toward T regulator [12]. Interleukin-6 induces transcription factor STAT3 toward retinoic acid-receptor-related orphan receptor-γt (RORγt) expression. RORγt would inhibit Foxp3 in order that Th0 would not be induced toward Treg, but toward Th17, producing proinflammatory cytokines [13, 14].

Our previous study showed that vitamin D level in SLE patients was significantly low compared to that of healthy controls [8]. The low concentration of 25(OH)D serum was related to higher expression of CD11c+/CD86+/CD40+ in patients with SLE compared to that of SLE patients with normovitamin D and also healthy donor (21.08±16.38% and 9.73±12.41%). There was also an increase of IL-17 level in SLE patients. The increase of disease activity in SLE was associated with the increase of IL-17 level which was produced by CD4 T cells [5].

Our study reveals effects of 1,25(OH)$_2$D$_3$ in immune response regulation of Systemic Lupus Erithematosus patients. It is important because even though Indonesia is located on the equator with sun exposure throughout the year, many patients of SLE in Indonesia suffered from hypovitamin D. SLE patients in Indonesia have more severe clinical manifestation [1]. The results of this study will encourage the importance of vitamin D for SLE patients with hypovitamin D.

**Material and methods**

**Sample preparation**

Monocytes and lymphocytes collected from five newly diagnosed female SLE patients (according to the American College of Rheumatology Criteria 1997), with range of age was 16-38 years old (mean=29.00±7.16), in active disease state with Mex-SLEDAI score >5, and hypovitamin D (25(OH)D serum level <30 ng/ml), range of 25(OH)D serum level was 16.5-29.8 ng/ml (mean=23.17±5.33 ng/ml). Subjects divided into 4 groups of 1,25(OH)$_2$D$_3$ doses, P0 (control/0 nM), P1 (1 nM), P2 (10 nM), P3 (100 nM). This study was approved by the Ethics Committee of Faculty Medicine of Brawijaya University Malang and informed consent has been obtained from all of SLE patients. Thirty milliliters of peripheral venous blood was collected and put in vacutainer with EDTA. Two milliliters of serum from Vacutainer was isolated and used to measure the concentration of 25(OH)D serum by ELISA in accordance with the manufacturer’s instructions (NovaTein Bio, USA).

**Cell culture for monocytes and lymphocytes**

Monocytes isolation procedure was in accordance with Xiao et al. [15] with time modification. Briefly, peripheral blood mononuclear cells (PBMC) were obtained from 20 ml of peripheral venous blood by Ficoll-Hypage density centrifugation, then isolated and suspended in 25 ml medium RPMI 1640 (Sigma-Aldrich, USA) containing 2 mM glutamin (Gybco, USA), 10 mM HEPES (Gybco, USA), Penicilin-streptomycin 10.000 U/ml (Gybco, USA), and 10% Fetal Bovine Serum (Gybco, USA), and incubated for 48 hours. 1x10$^6$ cells/well were plated in 96 wells using RPMI 1640 medium supplemented with 10% FBS in the presence of 800 U/ml GM-CSF (Biolegend) and 500 U/ml IL-4 (Biolegend) then incubated in 37°C, 5% CO$_2$ for 6 days. The medium was replaced after 3 days. In day 7, TNF-α 100 U/ml (GenWay) and 1,25(OH)$_2$D$_3$ (CayMan, USA) in different doses were added in immature dendritic cells (iDC) culture. The cell culture was then incubated for 4 days in 37°C, 5% CO$_2$.

Eight millilitres peripheral venous blood from subjects taken for CD4 T cell isolation. RosetteSep Human CD4+ T Cell Enrichment Cocktail at was added to the blood at concentration 50 µl/ml of whole blood, according to the manufacturer’s instruction. The naïve T cells from CD4 T cell isolated were cultured in two TC 96 well plates (1$^{st}$ for Th17, 2$^{nd}$ for Treg) with anti-CD3 plate bound (5 µg/ml, Biolegend), aliquoted into 5x10$^5$ cells/well with medium RPMI 1640 (Sigma-Aldrich, USA) containing L-Glutamine and 10% fetal bovine serum, supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin (Gybco, USA), and 5 µg/ml antiCD28 (R&D). To encourage T naive toward Th17 and Treg, T cells were stimulated by 10 ng/ml IL-6 (Biolegend), 5 ng/ml TGF-β1 (Biolegend), 10 µg/mL anti-IFN-γ (R&D), and 10
µg/mL anti-IL-4 (R&D). In day two, 1,25(OH)$_2$D$_3$ was added with four different doses (0, 1, 10, 100 nM) then incubated for 72 hours at 37°C with 5% CO$_2$.

**Flowcytometry analysis**

In day 10, dendritic cells were harvested. Cells were labeled with PerCP/Cy5.5 anti-human CD40 antibody, FITC anti-human CD86 antibody, PE anti-HLA-DR antibody (Biolegend, USA). Dendritic cell maturation was analyzed by flowcytometry (FACScalibur).

After 3 days, Th17 cells were stimulated with PMA (50 ng/ml; Sigma, St Louis, MO) and ionomycin (1 µg/ml; Sigma) in the presence of GolgiPlug (BD Pharmingen, San Diego, CA) for 4 hours. Cells were labelled with FITC anti-human CD4 (Biolegend). To detect IL-17A cytokines, intracelluller staining was performed with PerCP/Cy 5.5 anti-human IL-17A (Biolegend).

**Enzyme-linked immunoabsorbent assay (ELISA) for cytokines**

Supematans from DC and CD4 T cells were collected and stored at -80°C for cytokines measurements. IL-12 (Biolegend kit), IL-17A (R&D systems), and TGF-β (eBiosciences kit) secretion were measured by ELISA kits according to the manufacturer’s instructions.

**Statistical analyses**

Differences response of 1,25(OH)$_2$D$_3$ treatment between groups were determined by paired t-test. p<0.05 was considered significant. Data were described as mean±SD. Statistical analysis was performed using SPSS 19 version.
1,25(OH)$_2$D$_3$ and systemic lupus eritematosus

Results

Effect of 1,25(OH)$_2$D$_3$ treatment on the dendritic cells maturation

The 1,25(OH)$_2$D$_3$ treatment in DC culture of SLE patients reduced the expression of CD40, CD86, and HLA-DR as seen in Figure 1. There was a significant difference in CD40 expression (Figure 2A) between P1 and P2 compared with P0 (36.87±5.60%; vs. 42.43±6.32%; p=0.006 and 31.66±6.43%; vs. 42.43±6.32%; p=0.009). However there was no significant difference in CD40 expression between P1 and P3 (36.87±5.60% vs. 38.78±9.17%; p=0.570) and also no significant difference in CD40 expression between P2 and P3 (31.66±6.43% vs. 38.78±9.17%; p=0.305).

Expression of CD86 (Figure 2B) showed a reduction in each group versus control, with P2 being the lowest. Significant (p<0.05) compared to control.

Figure 2. Expression of CD40, CD86, and HLA-DR in DC. There were reduced mean of CD40, CD86, and HLA-DR among P1, P2, dan P3 compared with P0. P2 was the lowest. *Significant (p<0.05) compared to control.

A. Treatment of 1,25(OH)$_2$D$_3$ 1 nM and 10 nM showed significant difference in CD40 expression compared to control. B. Treatment of 1,25(OH)$_2$D$_3$ 10 nM showed significant difference in CD86 expression compared to control. C. Treatment of 1,25(OH)$_2$D$_3$ 10 nM dan 100 nM showed significant difference in HLA-DR expression compared to control.

Figure 3. The difference of IL-12p70 secretion level in treatment groups of 1,25(OH)$_2$D$_3$ in DC. There were reduced level of IL-12p70 in groups P1, P2, and P3 compared with P0 (control). Group P2 (10 nM of 1,25(OH)$_2$D$_3$) showed significant difference compared to control.

There were reduced level of IL-12p70 in groups P1, P2, and P3 compared with P0 (control). Group P2 (10 nM of 1,25(OH)$_2$D$_3$) showed significant difference compared to control.
was the lowest. There was a significant difference in CD86 expression between P2 and P0 (29.93±7.28% vs. 53.53±13.40%; p=0.011). In contrast, there was no significant difference in CD86 expression between P1 and P3 compared with P0 (38.47±12.97% vs. 53.53±13.40%; p=0.088 and 42.93±14.60%; vs. 53.53±13.40%; p=0.130). This indicated that 10 nM of 1,25(OH)$_2$D$_3$ was able to decrease expression of CD86 SLE patients, whereas 1 nM and 100 nM of 1,25(OH)$_2$D$_3$ were not significantly able to decrease CD86 expression.

Expression of HLA-DR (Figure 2C) showed a reduction in each group versus control, with P2 was the lowest. There was no significant difference in HLA-DR expression between P1 and P0 (34.07±17.45% vs. 39.53±17.74%; p=0.439). In contrast, there was a significant difference in HLA-DR expression between P2 and P3 compared with P0 (27.69±14.77% vs. 39.53±17.74%; p=0.018 and 31.98±17.06% vs. 39.53±17.74%; p=0.038). This indicated that 10 nM of 1,25(OH)$_2$D$_3$ was able to decrease expression of CD86 SLE patients, whereas 1 nM and 100 nM of 1,25(OH)$_2$D$_3$ were not significantly able to decrease CD86 expression.

Effect of 1,25(OH)$_2$D$_3$ treatment on the proportion of Th17 cells

This study showed that there was a reduction of the percentage of Th17 cells in CD4 T lymphocytes by 1,25(OH)$_2$D$_3$ treatments (Figure 4). Expression of the percentage of Th17 cells (Figure 5) showed a decrease in each group versus control in SLE patients. There was a significant difference in the percentage of Th17 cells between P1 and P2 compared with P0 (8.39±3.29% vs. 17.07±2.99%; p=0.043 and 7.17±3.81% vs. 17.07±2.99%; p=0.038). This indicated that 1 nM and 10 nM of 1,25(OH)$_2$D$_3$ were able to reduce the percentage of Th17 cells SLE patients.

In addition, even not significant, the percentage of TH17 cells in P3 tend to be lower than P0 (12.06±4.49% vs. 17.07±2.99%; p=0.153). There was no significant difference in the percentage of Th17 cells between P1 compared with P2 (p=0.738) and P3 (p=0.135); P2 com-
1,25(OH)$_2$D$_3$ and systemic lupus erythematosus

![Graph](image)

Figure 5. Expression of Th17 cells in CD4 T cells. There were reduced percentage of Th17 cells among groups P1, P2, and P3 compared with P0. P2 was the lowest. *Significant (p<0.05) compared to control.

![Graph](image)

Figure 6. The difference of IL-17A secretion level in treatment groups of 1,25(OH)$_2$D$_3$ in CD4 T cells. There was a decrease in IL-17A level in groups of P1, P2, dan P3 compared with P0 (control). Group P2 was the lowest (p=0.047). *Significant (p<0.05) compared with control.

compared with P3 (p=0.305). This indicated that 1 nM, 10 nM, and 100 nM of 1,25(OH)$_2$D$_3$ in CD4 T cell culture SLE patients have ability to reduce the percentage of Th17 cells. The percentage of Th17 cells in P2 was the lowest among others, thus 10 nM was more optimal than others.

Effect of 1,25(OH)$_2$D$_3$ treatment on the IL-17A production by Th17 cells

Level of IL-17A produced by Th17 cells (CD4+IL17A+) after 1,25(OH)$_2$D$_3$ treatment with different doses showed a decrease compared to control (Figure 6). There was a significant difference in level of IL-17A between P1 and P2 compared with P0 (25.90±11.90 vs. 59.18±26.95; p=0.024 and 15.75±1.22 vs. 59.18±26.95; p=0.047). This indicated that 1 nM and 10 nM of 1,25(OH)$_2$D$_3$ were able to inhibit secretion of IL-17A CD4 T cells patients with SLE. There was no significant difference between P1 and P2 (p=0.185). It indicated that 1 nM and 10 nM of 1,25(OH)$_2$D$_3$ have the same ability to decrease IL-17A level. Interleukin-17 level in P2 was lower than P1 (p=0.02) and P3 (p=0.014). This indicated that 10 nM of 1,25(OH)$_2$D$_3$ treatment was the most optimal dose to reduce IL-17A level CD4 T cells patient with SLE among others.

Effect of 1,25(OH)$_2$D$_3$ treatment on the proportion of Treg cells

This study showed that there was a difference in the percentage of Treg cells in CD4 T cells culture by 1,25(OH)$_2$D$_3$ treatment (Figure 7). Compared with control, Treg cells percentage in CD4 T lymphocytes culture showed an increase in P1, P2, and P3 (Figure 8). The percentage of Treg cells after treatment with different doses of 1,25(OH)$_2$D$_3$ (1, 10, 100 nM) tend to increase versus control, but there was no significant difference in the percentage of Treg cells among P1, P2, and P3 compared with P0 (14.74±9.20% vs. 13.76±8.51%; p=0.886, 8.32±4.13% vs. 13.76±8.51%; p=0.280, and 16.82±7.33% vs. 13.76±8.51%; p=0.536). This indicated that 1,25(OH)$_2$D$_3$ treatment had no response in Treg cells activation.

Effect of 1,25(OH)$_2$D$_3$ treatment on the TGF-β production by Treg cells

This study showed differences mean of TGF-β levels in CD4 T cells culture after 1,25(OH)$_2$D$_3$ treatment compared with control (Figure 9). TGF-β levels in group P1 and P2 tend to be lower than control, whereas in group P3 was higher than control. There were no significant differences in TGF-β levels among P1, P2, and P3 compared with P0 (5810±2024 vs 6571±3359.22; p=0.607, 4192.6±544.39 vs 6571±3359.22; p=0.149, and 6376.80±3247.30 vs 6571±3359.22; p=0.730). It indicated that 1,25(OH)$_2$D$_3$ had no effect on Treg cells function.

Discussions

It has been known that the increase of DC maturation was one of the dysregulation of immune system suffered in SLE patients and lead to the
1,25(OH)\(_2\)D\(_3\) and systemic lupus erithematosus

This study indicated that 1,25(OH)\(_2\)D\(_3\) treatment with different doses (1, 10, 100 nM) has ability to inhibit DC maturation in SLE patients. It can be shown that there were decreased expression of CD40, CD86, HLA-DR, and IL-12p70 level in mature DC of SLE patients compared with control. Study by Zvi et al., also showed that vitamin D supplementation inhibited maturation and activation dendritic cells and showed by the reduced of CD40, CD86, and HLA-DR in DC culture SLE patients [16]. CD40 and CD86 are co-stimulatory molecules, which are expressed in large amounts of mature dendritic cells. They play an important role as second signal in the process of Th cell activation. Similarly, HLA-DR is type II MHC molecule that acts as first signal; and secretion of IL-12p70 that has important role in the regulation of immune system which acts as third signal in Th activation [17]. This study showed that 1,25(OH)\(_2\)D\(_3\) has effect to decrease expression of CD40, CD86, HLA-DR and production of IL-12p70 in DC SLE patients so that it has possibility to inhibit naive Th cells activation to help reduce SLE pathogenity.

This study showed that 1,25(OH)\(_2\)D\(_3\) was able to reduce the percentage of Th17 and IL-17A levels in CD4 T cells SLE patients. Study by Tian et al. reported that vitamin D3 inhibited differentiation of Th1 and Th17 cells in Behcet disease in vitro. Vitamin D also inhibited the expression of Th17 cells effector molecules such as RORC, IL-17A, IL-23R, and CCR6 [18] and stimulated the secretion of regulatory IL-10 by naive CD4+ T cells. Co-cultured CD4 T cells with DC also showed suppression effect of
vitamin D3 on IL-17A and IFN-γ levels in the supernatants cell culture. Colin et al. showed that 1,25(OH)\textsubscript{2}D\textsubscript{3} decreased IL-17A and IFN-γ levels and also increased IL-4 level from PBMC patient with rheumatoid arthritis (RA) [19]. Other study [20] showed that 1,25(OH)\textsubscript{2}D\textsubscript{3} inhibited human IL-17A CD4 T cells in healthy donor and IL-17A in murine model of multiple sclerosis.

Our study showed that percentage of Th17 was decreased by 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment in 1 nM and 10 nM. Chang et al. reported that 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment in mice ameliorated experimental autoimmure encephalomyelitis that accompanied with reduced of IL-17A expression. In vitro, treatment of CD4+ T cells with the physiological dose of 1,25(OH)\textsubscript{2}D\textsubscript{3} preferentially inhibited cytokines production by Th17 cells, in a VDR-dependent manner [21]. Interleukin-6 and TGF-β were important in development of Th17 by activating STAT3 and inducing transcription factors, RORγt and RORα [22, 23].

This study showed there was an increase in the percentage of Treg cells in CD4 T cells culture SLE patients after 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment but not significant. This condition supported by previous study that there was a significant increase of Treg after vitamin D supplementation in vivo [24-26]. Development of Th0 cells toward Treg cells needs other stimulations, not only TGF-β. There are other mechanisms in Treg activation and differentiation. Chen et al. found that TNF was able to increase Treg. Interaction between TNF-α and its receptor (TNFR) at Th0 will increase TGF-β [27, 28], thus the amount and activity of Treg will increase, too.

The optimal vitamin D level in serum was >75 nM (>30 ng/ml) [29]. In this study, 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment with 100 nM did not show significant decrease in expression of CD40, CD86, HLA-DR, percentage of Th17 cell, and level of IL-12p70 and IL-17A, compared with control. This condition indicated that after 1,25(OH)\textsubscript{2}D\textsubscript{3} reached the optimal dose, even if 1,25(OH)\textsubscript{2}D\textsubscript{3} was given in higher dose, it wouldn’t give an optimal result; 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment skewed back in result as seen in control. Dose (1 nM) equals with 0.4 ng/ml [29], so 10^{-7} M (100 nM) equals with 40 ng/ml. If this dose (100 nM) is given to treat culture, it probably would give a toxic/opposite effect. Sudden ‘stressor’ in cell culture will make cell give another respond to maintain its homeostasis. Metabolite of 25(OH)\textsubscript{D} in human should change to 1,25(OH)\textsubscript{2}D\textsubscript{3} to become active. The active metabolite, 1,25(OH)\textsubscript{2}D\textsubscript{3} will bound to VDR, nuclear receptor that regulate trancription of vitamin D targets gen. Similarly in DC, 1,25(OH)\textsubscript{2}D\textsubscript{3} with 10 nM was more potential to decrease percentage of Th17 cell and IL-17A level than other doses. Dose 100 nM of 1,25(OH)\textsubscript{2}D\textsubscript{3} gave inverse effect by increasing percentage of Th17 cells and IL-17A level. However, dose 1 nM of 1,25(OH)\textsubscript{2}D\textsubscript{3} has inhibition effect but this dose is included in physiology doses [30-32], so it is less potential.

In conclusion, it was found that 1,25(OH)\textsubscript{2}D\textsubscript{3} especially dose of 10 nM, significantly inhibited dendritic cells maturation, as seen in the decrease of CD40, CD86, and HLA-DR expressions and cytokines secretions of IL-12p70, also in the decreased percentage of Th17 cells and IL-17A levels in CD4 T cells culture of SLE patients, but not Treg cells and TGF-β.

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Disclosure of conflict of interest

None.

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1,25(OH)₂D₃ and systemic lupus eritematosus


