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Full length research paper

The associations among serum levels of vitamin D, TGF-β/IL-6 balance and Treg/Th17 balance in systemic lupus erythematosus patients in Indonesia

Kusworini Handono[1], Dian Hasanah[2], Handono Kalim[3], Herin Mawarti[4]

[1]Department of Clinical Pathology, Faculty of Medicine, Brawijaya University, Malang, Indonesia
[2]Department of Physiology, Faculty of Medicine, Brawijaya University, Malang, Indonesia
[3]Rheumatology Division, Department of Internal Medicine, Faculty of Medicine, Brawijaya University, Malang, Indonesia
[4]Fundamental of Nursing Department, Faculty of Health Science, Pesantren Tinggi Darul Ulum University, Jombang, Indonesia.

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TGF-β1 is required for T lymphocytes differentiation toward Treg and Th17.Differentiationtoward Th17 becomes dominant in high levels of IL-6. Some studies showed that vitamin D decreased IL-6 production and increased TGF-β1production. The aim of this study was to investigate the associations among serum levels of vitamin D, TGF-β1/IL-6 balance and Treg/Th17 balance in SLE.Serum levels of vitamin D, IL-6 and TGF-β1, as well as Treg and Th17 percentage were assessed in 41 SLE patients and 20 healthy controls. Serum levels of vitamin D, TGF-β1 and IL-6 were measured using ELISA. Treg and Th17 percentage were the percentage of CD25+FoxP3+CD4+ and CD3+CD4+IL-17+,respectively, detected by flow cytometry. Vitamin D and TGF-β1 levels were lower in SLE (p<0.001 and p=0.004, respectively). While IL-6 levels, Treg percentage and Th17 percentage were higher in SLE (p=0.006, p=0.007, and p=0.017, respectively). Vitamin D levels were negatively correlated to IL-6 levels (r=-0.325, p=0.019). TGF-β1/IL-6 ratio was negatively correlated to Th17 percentage (r=-0.559, p=0.010). Conclusively, in SLE patients whose vitamin D levels are low, IL-6 levels will increase, resulting in dominance of IL-6 over TGF-β1, which direct the differentiation of T lymphocytes toward Th17. The number of Treg will increase to suppress Th17.

Key words: SLE, vitamin D, Treg, Th17, TGF-β1, IL-6.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that is more frequently encountered lately. In Indonesia, the life expectancies of SLE patients are still low, 70% for 5 years survival rate and 55% for 10 years survival rate (Kalim, 2000). The theory of T helper 1 and T helper 2 cells (Th1 and Th2) imbalance in SLE hasbeen well established. Recently, it has been proposed a new paradigm of imbalance between regulatory T cells (Treg) which are anti-inflammatory and T helper 17 cells (Th17) which are pro-inflammatory (Eisenstein *et al.*, 2009). The balance between these two cells seems to be influenced by cytokines balance; it is the balance between transforming growth factor-β1 (TGF-β1) and interleukin-6 (IL-6). TGF-β1 is required for the differentiation of T lymphocytes toward Treg and Th17, but its effect is more dominant for differentiation toward Treg (Zhou et al., 2008). In high levels of IL-6, the effect of TGF-β1 on differentiation toward Treg is reduced, and differentiation toward Th17 becomes dominant. Therefore, IL-6 acts as a potent pro-inflammatory cytokine for T lymphocytes by increasing differentiation toward Th17 and inhibiting differentiation toward Treg (Bettelli et al., 2006; Mangan et al., 2006). The current standard treatments for SLE are aimed at suppressing the immune response and excessive inflammation by giving immunosuppressive drugs. The use of these drugs has good results in developed countries, but in develo-

*Corresponding author. E-mail: dian.hasanah@gmail.com Tel: +6281334933663 / +623412202220

ping countries it is constrained by economic problems. To our best knowledge, the use of supplement in SLE patients has not been comprehensively investigated. A supplement that was recently known as an immune modulator is vitamin D. The associations between vitamin D deficiency and autoimmune diseases had been reported (Irastorza et al., 2008; Ginanjar et al., 2007; Mouyis et al., 2008; Toubi et al., 2010). Although Indonesia is located on the equator with sun exposure throughout the year, vitamin D levels of healthy population in Indonesia were low (Setiati, 2008). Clinical manifestations of SLE patients in Indonesia are different from those reported in the Caucasians. SLE patients in Indonesia have more severe clinical manifestations, such as higher levels of anti-DNA antibody, and experience photosensitivity more often (Kalim, 2000). These facts raise the question of whether the severity of their clinical manifestations is also affected by their vitamin D levels. The concepts mentioned above (concepts about Treg, Th17, TGF-β1, IL-6, vitamin D, and SLE) are related to the following facts. A study by Nonn *et al.,* (2006) showed that vitamin D decreased the production of IL-6. Vitamin D also increased the production of TGF-β1 (Kleinder *et al.*, 2010). If the production of IL-6 can be inhibited, the production of TGF-β1 will increase, and T lymphocytes differentiation toward Treg will be more dominant than differentiation toward Th17. These conditions will be expected to improve autoimmune diseases like SLE. The aim of this study was to determine the associations among serum levels of vitamin D, TGF-β/IL-6 balance and Treg/Th17 balance in SLE patients in Indonesia.

SUBJECTS AND METHODS

Subjects of the Study

Subjects of this study were female SLE patients newly diagnosed based on American College of Rheumatology (ACR) criteria, experienced flare (had systemic lupus erythematosus disease activity index (SLEDAI) score >3), and did not take vitamin D. These patients were outpatients and hospitalized patients in the Department of Internal Medicine Saiful Anwar Hospital Malang. The treatment they received at the time of blood collection was only low dose of methylprednisolone. Controls in this study were healthy women matched in age, gender and ethnicity to patients, and did not take vitamin D. This study met the ethical clearance by Ethics Commission of Faculty of Medicine, Brawijaya University. Informed consents were obtained from all subjects participated in this study. Venous blood was collected from each subjects, 8 ml into heparinized vacutainer and 4 ml into serum separator tube. Serum levels of vitamin D, IL-6 and TGF-β1, as well as Treg percentage were performed on 41 SLE patients and 20 healthy controls. Th17 percentage was performed on 17 SLE patients and 10 healthy controls.

Serum Collection

4 ml of fresh blood from each subject collected in serum separator tube was centrifuged in 4°C for 20 minutes. The supernatant was collected into fresh tubes and stored in -70°C until the next process for enzyme-linked immunosorbent assay (ELISA).

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

8 ml of heparinized blood from each subject was diluted 1:1 with phosphate buffered saline (PBS) in a canonical tube. The mixture was layered over Ficoll® (Amersham Biosciences, cat. 17-1440-03) in the ratio of 2:1 in tube. This tube was centrifuged at 1000xg in 4°C for 30 minutes. PBMCs harvested from the interface between Ficoll® and plasma (the buffy coat) were collected into fresh tube and then washed twice with PBS. These PBMCs were divided into 2 tubes in the ratio of 1:3 for Treg analysis (direct/fresh process) and Th17 analysis (needs activation first), respectively.

Stimulation for Intracellular Staining of IL-17 as A Marker of Th17

Before we analyzed Th17, we performed stimulation for intracellular staining of IL-17, since this cytokine is a marker of Th17. The goal of this procedure was to make IL-17 to be more easily visible. A 10 µg/ml solution of LEAF™ Purified Anti-Human CD3 Antibody Clone UCHT1 (Biolegend, cat. 300413) was prepared in sterile PBS. Cell culture medium (RPMI 1640 with 2mM of ultra-glutamine, 100 U/ml of penicillin, 100 µg/ml of streptomycin) and 35 µl Anti-CD3 were dispensed to each well of the 96-well flat-bottom assay plate. About 500.000 PBMCs were added to each well. Plate was covered and incubated at 37°C in 5% CO_2 and 100% humidity for 3 days. After 3 days, the cells were harvested and collected into fresh tube.

Treg Immunofluorecent Staining Procedure

All reagents for this procedure were produced by Biolegend. Fresh PBMCs were re-suspended with 0.5 ml of Cell Staining Buffer (cat. 420201). 5 µl of PerCP Anti-Human CD4 Antibody (cat. 317432) and 20 µl of PE Anti-Human CD25 Antibody (cat. 302606) were added to suspension, incubated at room temperature for 15-20 minutes in the dark and then washed twice with 1.5 ml of

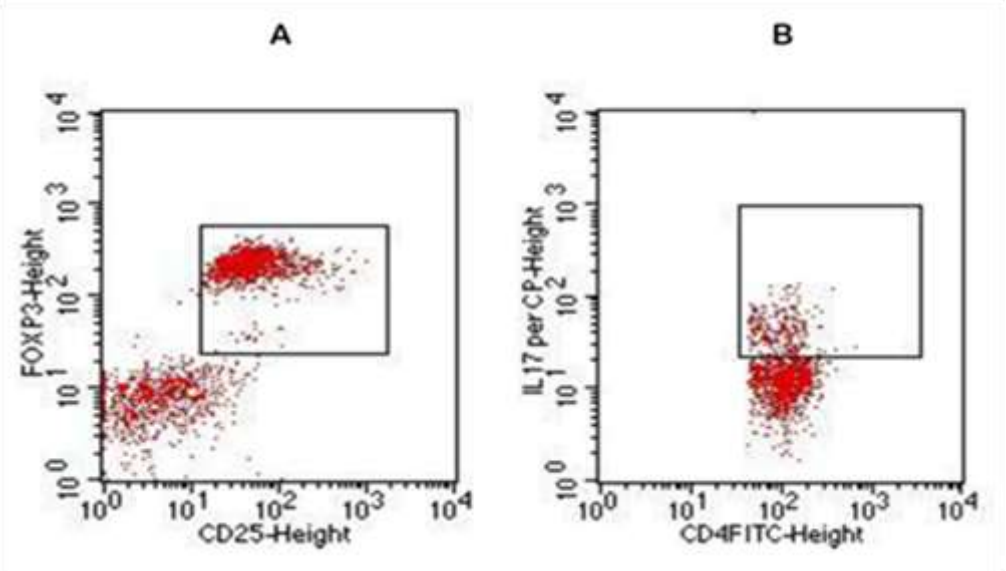

Figure 1

Cell Staining Buffer with a centrifuge at 350xg for 5 minutes. Cells were re-suspended in 0.5 ml of Cell Staining Buffer. The suspension was added by 1 ml of 1x FoxP3 Fix/Perm Solution (cat. 421401), mixed and incubated at room temperature in the dark for 20 minutes, then centrifuged at 350xg for 5 minutes and the supernatant was discarded. Cells were washed with 1.5 ml of Cell Staining Buffer, and then washed with 1 ml of 1x FoxP3 Perm Solution (cat. 421402) by centrifuge at 350xg for 5 minutes and the supernatant was discarded. Cells were re-suspended in 1 ml of 1x FoxP3 Perm Solution, incubated at room temperature in the dark for 15 minutes, centrifuged at 350xg for 5 minutes and the supernatant was discarded, and pellets were re-suspended again in 100 µl of 1x FoxP3 Perm Solution. 20µl of FITC Anti-Human FoxP3 Antibody (cat. 320106) was added and incubated at room temperature in the dark for 30 minutes. Cells were washed twice with 1.5 ml of Cell Staining Buffer, and then re-suspended in 0.5 ml of Cell Staining Buffer and analyzed by flow cytometer.

Th17 Immunofluorecent Staining Procedure

All reagents for this procedure were produced by Biolegend. After 3 days of activation with anti-CD3 antibody, PBMCs were suspended in 1 ml of Cell Staining Buffer, centrifuged, and then re-suspended with 0.5 ml of Cell Staining Buffer. 20 µl of FITC Anti-Human CD4 Antibody (cat. 317408) and 20 µl of PE Anti-Human CD3 Antibody (cat. 300308) were added, incubated at room temperature for 15-20 minutes in the dark, and then washed twice with 1.5 ml of Cell Staining Buffer with centrifuge at 350xg for 5 minutes. Cells were re-suspended in 0.5 ml of Cell Staining Buffer. Cells were fixed in 0.5 ml of Fixation Buffer (cat. 420801) in the dark for 20 minutes at room temperature, and then centrifuged at 350xg for 5 minutes, the supernatant was discarded. Cells were re-suspended in 0.6 ml of 1x Permeabilization Wash Buffer (cat. 421002) with centrifuged at 350xg for 5-10 minutes. Cells were re-suspended in 200 µl of 1x Permeabilization Wash Buffer and added with 5 µl PerCP/Cy5.5 Anti-Human IL-17A Antibody (cat. 512314), incubated for 20 minutes in the dark at room temperature, and then washed twice with 1.5 ml of Permeabilization Wash Buffer with centrifuged at 350xg for 5 minutes. Cells were re-suspended in 0.5 ml of Cell Staining Buffer and analyzed by flowcytometry.

Flow Cytometry Analysis

The analysis of the Treg percentage and Th17 percentage were using FACSCalibur (Becton Dickinson). Treg percentage is the percentage of $CD4^+CD25^+FoxP3^+$ cells number from all $CD4^+$ cells number. While Th17 percentage is the percentage of $CD3^+CD4^+IL\text{-}17^+$ cells number from all $CD4^+$ cells number (Figure 1). Samples of flow cytometry analysis in SLE patients. PBMCs were isolated from fresh blood and stained with immunofluorescent for Treg surface markers (CD4 and CD25) and intranuclear marker (FoxP3). PBMCs were also activated with anti-CD3 for three days and stained for Th17 surface markers (CD3 and CD4) and intracellular marker (IL-17). A: Analysis of Treg. Dot in large box show each cell stained for their CD4 and CD25. Dot in small box show each cell stained for their CD4, CD25 and FoxP3; these cells were Treg. B: Analysis of Th17. Dot in large box show each cell stained for their CD3 and CD4. Dot in small box show each cell stained for their CD3, CD4 and FOXP17; these cells were Th17.

Table 1. Vitamin D Levels of SLE Patients and Healthy Controls.

Characteristics	SLE Patients N: 41	Healthy Controls N: 20	p-value
Age (years)	29.35±9.99	32.91±5.92	-
Normal levels of vitamin D	29%	85%	-
Hypovitaminosis D	71%	15%	-
Mean of vitamin D levels (ng/ml)	23.0±11.9	36.0±5.7	<0.001

Table 2. Clinical Manifestations of SLE Patients.

Clinical Manifestations	%
Malar rash	70.73%
Discoid rash	56.10%
Photosensitivity	68.30%
Oral ulceration	58.54%
Arthritis	56.10%
Serositis	19.51%
Nephritis	41.40%
Neurologic manifestations	19.51%
Hematologic manifestations	56.10%
Anti-nuclear antibody (ANA) positive	80.50%
Anti-dsDNA positive	73.20%
Anti-cardiolipin antibody (ACA) positive	19.51%

Serum Level Assay of 25(OH)D_3

Vitamin D assessed in this study was serum 25(OH)D_3, which was quantitatively detected by ELISA in duplicate for each subject according to the manufacturer procedure (Cusabio, cat. CSB-E07900h).

Serum Level Assay of TGF-β1

TGF-β1 was quantitatively detected by ELISA in duplicate for each subject according to the manufacturer procedure (eBioscience, cat. BMS249/3CE).

Serum Level Assay of IL-6

IL-6 was quantitatively detected by ELISA in duplicate for each subject according to the manufacturer procedure (Biolegend, cat. 430507).

TGF-β1/IL-6 Balance and Treg/Th17 Balance

TGF-β1/IL-6 balance was the ratio between TGF-β1 levels and IL-6 levels, and Treg/Th17 balance was the ratio between Treg percentage and Th17 percentage.

Statistical Analysis

All results are expressed as mean ± standard deviation. The comparisons of results between 2 groups were evaluated with independent t-test and the correlations between clinical parameters were evaluated with Spearman's correlation test using SPSS software. Comparisons and correlations were considered as significant if p-value were <0.05.

RESULTS

Subject Characteristics and Vitamin D Levels

The mean of SLE patients age was 29.35±9.99 years old. Most of the clinical manifestations were nephritis, photosensitivity and arthritis (Table 2). Vitamin D levels in SLE patients were significantly lower than those in healthy controls (23.0±11.9 ng/mL versus 36.0±5.7

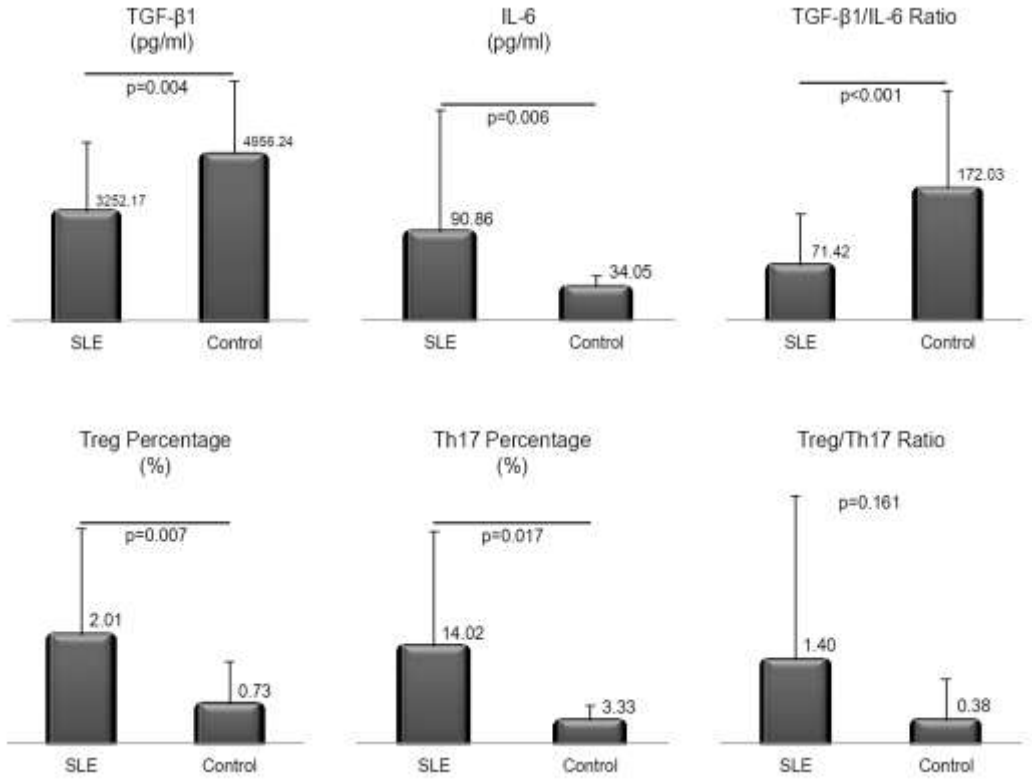

Figure 2

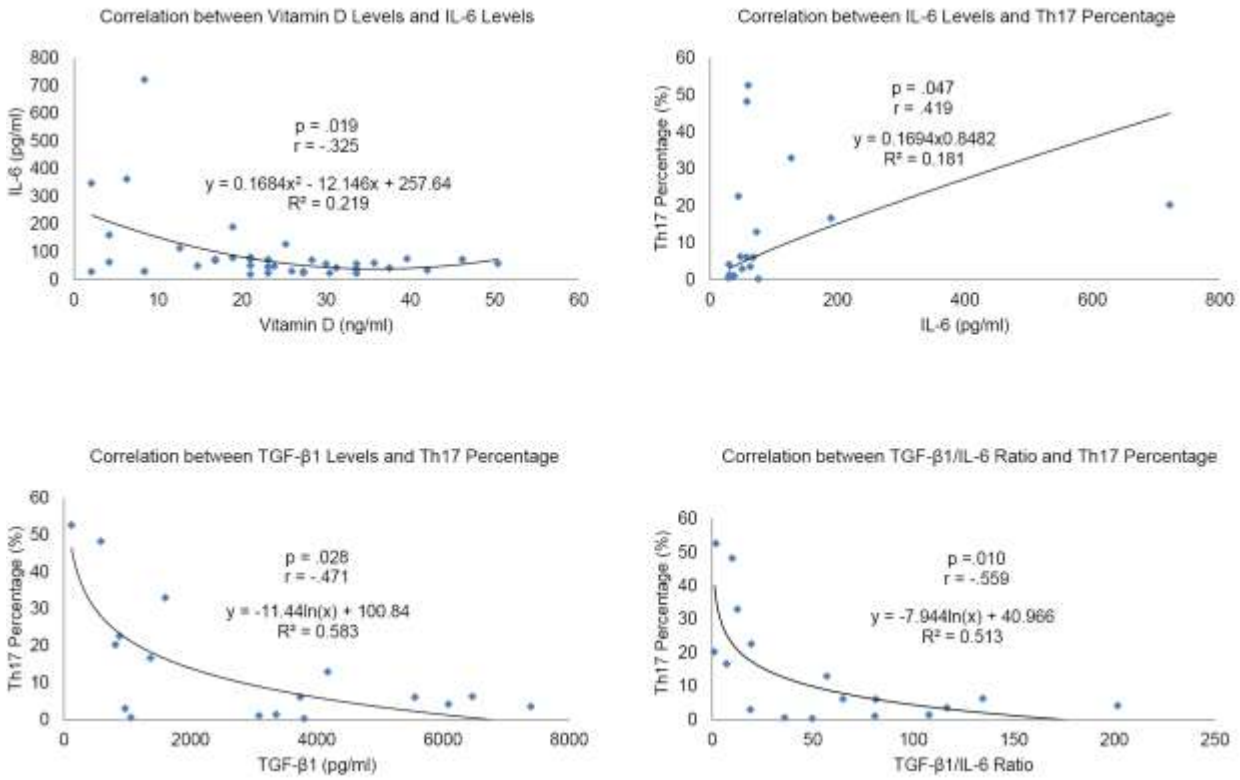

Figure 3. Spearman's correlation and regression tests between variables in SLE patients. Only significant results are shown in this figure.

ng/mL, p<0.001). Hipovitaminosis D (levels <30 ng/ml) were observed in 29 SLE patients (71%) and 3 healthy controls (15%), whereas normal levels of vitamin D (levels ≥30 ng/ml) were observed in 12 SLE patients (29%) and 17 healthy controls (85%) (Table 1).

TGF-β1 Levels, IL-6 Levels, Treg Percentage, Th17 Percentage, TGF-β1/IL-6 Ratio and Treg/Th17 Ratio

TGF-β1 levels were lower in SLE patients compared to healthy controls (3252.17±2057.16 versus 4956.24±2181.94, p=0.004), while IL-6 levels were significantly higher in SLE patients controls (90.86±124,98 versus 34.05±11.02, p=0.006), so that TGF-β1/IL-6 ratio was lower in SLE patients (71.42±66.60 versus 172.03±126.49, p<0.001).

Treg percentage was higher in SLE patients (2.10±1.96 versus 0.73±0.78, p=0.007), and so was Th17 percentage (14.02±16.50 versus 3.33±2.11, p=0.017), while Treg/Th17 ratio was not significantly different (Figure 2).Figure 2. Comparison of variables between SLE patients and healthy controls using independent t-test. P values are shown for each comparison.

Only Treg/Th17 ratio was not significantly different between SLE patients and healthy controls.By Spearman's correlation test and regression test, there were negative correlation between vitamin D levels and IL-6 levels (p=0.019), positive correlation between IL-6 levels and Th17 percentage (p=0.047), negative correlation between TGF-β1 levels and Th17 percentage (p=0.028), and negative correlation between TGF-β1/IL-6 ratio and Th17 percentage (p=0.010).

There was no correlation between Th17 percentage and Treg percentage (Figure 3).Figure 3. Spearman's correlation and regression tests between variables in SLE patients. Only significant results are shown in this figure.

DISCUSSION

Low Vitamin D Levels in SLE Patients in Indonesia

The majority of SLE patients (71%) had hypovitaminosis D, whereas in healthy controls, only 15% had hypovitaminosis D (Table 1).

This finding is in line with continued to experience hypovitaminosis D after receiving vitamin D 400-800 IU/day (Toloza *et al.*, 2010). Low levels of vitamin D can be either a cause or result of SLE, and it cannot be inferred.

Low levels of vitamin D were suspected to lead to autoimmunity due to dendritic cells hyperactivity, T cells and B cells hyperactivity, reduced Treg suppression and increased inflammatory cytokines (Irastorza *et al.*, 2008; Ginanjar *et al.*, 2007; Mouyis *et al.*, 2008; Toubi *et al.*, 2010).

Associations among Vitamin D Levels, TGF-β1/IL-6 Balance and Treg/Th17 Balance in SLE Patients

In this study, there was negative correlation between vitamin D levels and IL-6 levels in SLE patients. Various studies showed that excessive production of IL-6 contributed to the pathogenesis of various autoimmune and inflammatory diseases. Many signaling pathways involved IL-6 in the pathogenesis of experimentally or naturally occurring autoimmune disease (Kimura *et al.*, 2007). Vitamin D may inhibit IL-6 synthesis via mitogen-activated protein kinase phosphatase 5 (MKP5). Vitamin D receptor (VDR) is known to associate with the VDRE in the MKP5 promoter. MKP5 mediates p38 inactivation by $1,25(OH)_2D$. IL-6 is the downstream pathway after the activation of p38. (Nonn *et al.*, 2006).Regulator of nuclear orphan receptor (RORγt) is a major regulator for T lymphocyte differentiation toward Th17, while for Treg is forkhead box P3 (FoxP3). It has been suggested that differentiation into Th17 or Treg depends on the balance between RORγt and FoxP3-regulated cytokines. TGF-β1 at low levels causes differentiation toward Th17, while in high levels causes to differentiation toward Treg (Eisenstein *et al.*, 2009; Jianxin *et al.*, 2009). TGF-β1 can induce the expression of FoxP3 and RORγt; but the effect is more dominant on Treg differentiation because FoxP3 can associate with RORγt and inhibit transcription of RORγt (Zhou *et al.*, 2008). In high levels of IL-6, this inhibition is reduced, and Th17 differentiation become dominant (Bettelli *et al.*, 2006; Mangan *et al.*, 2006). The administration of $1,25(OH)_2D$ in vitro led to increased expression of TGF-β1 in Treg (Kleinder *et al.*, 2010). This finding is supported by the result of this study that SLE patients (who mostly had hypovitaminosis D) had lower TGB-β1 levels than healthy controls. Mechanism of how vitamin D can increase TGB-β1 levels is unknown yet.In this study, there was negative correlation between TGF-β1/IL-6 ratio and Th17 percentage, but no correlation between TGF-β1/IL-6 ratio with Treg percentage. When TGF-β1 dominance over IL-6 decreases, Th17 percentage will increase as the above mechanisms. Treg seems to just compensate for these adverse conditions by increasing their number to cope with increased Th17 number to maintain the homeostasis.

The Percentage of FoxP3$^+$ Treg in SLE Patients

In this study, FoxP3$^+$ Treg percentage increased in SLE patients. Although most of the experts stated that SLE is caused by the low number of Treg, one study revealed that Treg actually increased in SLE patients and positively correlated to disease activity (Yan *et al.*, 2009). Some possible causes of this condition are as follows.Increased peripheral FoxP3$^+$ Treg percentage in SLE patients may be aimed at increasing the pool to overcome the expansion of effector cells (autoreactive T cells and B cells). Increased peripheral FoxP3$^+$ Treg per-

centage in SLE patients may also be caused by defective suppressive function of Treg. The evidence that support the hypothesis of suppressive function of Treg in SLE patients related to low levels of vitamin D are as follow. Vitamin D plays a role in the synthesis of TGF-β1. One of the sources of TGF-β1 is Treg which has a direct effect to suppress effector cells. TGF-β1 levels in SLE patients in this study were low. This situation will cause reduction in suppressive function (Wahl *et al.*, 2004; Fahlen *et al.*, 2005; Andersson *et al.*, 2008; Takaki, 2008; Wong, 2008; Chen *et al.*, 2010). TGF-β1 also alters the production of IgE antibodies which are not inflammatory to IgG and IgA which are inflammatory. If the levels of TGF-β1 were low, the antibodies will be more inflammatory (Taylor *et al.*, 2006). Several factors may contribute to the increase of Treg percentage. Autoimmunity allows the conversion of naive T cells into iTreg. TGF-β1 had been predicted to facilitate the conversion of iTreg from $CD4^+$ T cell precursors (Juang *et al.*, 2005). However, in this study, TGF-β1 levels in patients were lower than those of healthy controls. There may be other factors that can induce this conversion, which is more potent than TGF-β1.

CONCLUSION

In SLE patients, vitamin D levels are negatively correlated to IL-6 levels. When vitamin D levels are low, the levels of IL-6 will increase, resulting in the dominance of IL-6 over TGF-β1. This domination will direct the differentiation of T lymphocytes toward Th17 rather than toward Treg. When the number of Th17 increases, the number of Treg will increase to suppress Th17. This seems to be an effort to restore the homeostasis.

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