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The Effect of Systemic Lupus Erythematosus Over the Physiologic Inhibition of $1,25(OH)_2D$ on Monocyte-Tissue Factor (CD142) Expression

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Authors' contributions

This work was carried out in collaboration between all authors. Author KH designed the study, supervised all the experimental process. Author IS wrote the protocol, managed the literature searches and experimental process. Author TEA helped with the culture process and the flow cytometry analysis. All authors read and approved the final manuscript.

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ABSTRACT

Background and Aims: Systemic Lupus Erythematosus is a systemic autoimmune disease with increased risk of thrombosis through the induction of tissue factor (TF) expression, the principal initiator of coagulation. Despite its correlation with some autoimmune diseases, including SLE; vitamin D also correlates well with thrombotic events in SLE or non SLE patients. Vitamin D is an immunomodulator that might have an anti-thrombotic effect by down-regulating TF expression in an vitro model of healthy subjects. Therefore, we studied the effect of metabolite active form of vitamin D [$1,25(OH)_2D$] on monocyte-TF (CD142) expression of SLE patients.

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Methods: Monocyte culture were obtained from 6 Indonesian SLE patients and 3 age- and sex-matched, healthy Indonesian participants to evaluate the effect of various concentrations of $1,25(OH)_2D$ on monocyte-TF expression in LPS-induced monocytes. The monocyte-TF (CD142) expression was then analyzed by flow cytometry using Monoclonal Anti-human Coagulation Factor III/TF (CD142)-Phycoerythrin and FITC anti-human CD14.
Results: *In vitro* model of monocyte culture from SLE patients revealed that $1,25(OH)_2D$ showed no inhibitory effects of monocyte-TF expression (p = .275). Higher or lower doses of $1,25(OH)_2D$ did not correlate with high or low CD142 expression in monocyte culture of SLE patients(r=.1, p=.322).
Conclusion: *In vitro* study of SLE patients showed that $1,25(OH)_2D$ has no inhibitory effect on TF expression. Ten nM $1,25(OH)_2D$ seemed to be the optimal concentration for suppressing LPS-induced monocyte-TF expression in healthy groups but not in SLE groups.
The different characteristics of monocytes, VDR polymorphism, and the need of higher concentration of vitamin D were the best explanation so far for the different effects in monocyte culture of SLE patients.

Keywords: Systemic lupus erythematosus; thrombosis, tissue factor, vitamin D ($1,25(OH)_2D$).

1. INTRODUCTION

Systemic Lupus Erythematous (SLE) is a chronic systemic autoimmune disease with multi organ involvements and diverse clinical manifestations. Thrombosis is one of the concerning problems in SLE for its high mortality and morbidity rate [1,2]. However, in recent decades, treatments for thrombosis in SLE has shown unsatisfactory outcomes. The use of anticoagulants and antiplatelet aggregation agents for treating and preventing thrombotic events was linked with serious adverse events, reduced quality of life and recurrences. Therefore, novel, safe, and more effective modalities of thrombotic-event prophylaxis for SLE patients are needed [3].

Patients with SLE have an increased risk for thrombosis due to the presence of immune complex and pathogenic autoantibodies. Inflammation is one of the main factors for thrombosis because it can induce the expression of Tissue Factors (TF), the main initiator in coagulation pathways [2]. It has been showed that high TF expression was associated with high thrombosis incidence in SLE patients. Tissue factors's over expression in endothelial cells and circulating monocytes is the essential mechanism for hypercoagulable state and is one of the essential mechanisms for thrombosis events in SLE [3,4].

Tissue Factor, also known as factor III/ thromboplastin/ CD142 is a major transmembrane glycoprotein in hemostatic system. TF initiates the extrinsic coagulation and plays an integral role in blood coagulation, thrombin (FIIa) generation and thrombi formation in relation to thrombosis. TF is constitutively expressed by a number of cell types, except those that are in direct contact with blood. Circulating TF is primarily derived from its expression in blood cells (e.g. monocytes, macrophages, granulocytes, and platelets). Monocytes are the only blood cells that express significant amount of TF in circulation following inflammatory conditions. Agents known to induce inflammation, such as bacterial lipopolysaccharide (LPS), interleukin-1, tumor necrosis factor-α (TNF-α), or C-reactive protein (CRP), are among the most potent stimulators of TF expression. Indeed, monocyte-TF expression can be induced by a variety of stimuli including LPS and pro inflammatory cytokine [4,5]. SLE is an autoimmune diseases characterized by a high TF expression in monocytes [6].

It has been reported that SLE patients and animal role model of SLE shows defect or abnormalities in monocyte/ macrophage phenotype and function [7,8]. While the vitamin D receptor (VDR) regulation and tissue factor pathway in SLE monocytes had never been explored previously.

Despite its correlation with autoimmune diseases, such as diabetes mellitus, multiple sclerosis, and SLE; vitamin D also correlates well with thrombosis. It has been shown that both arterial and venous thrombosis events were 50% increased while the synthesis of vitamin D_3 decreased [9]. While approximately 80% of the SLE patients in Indonesia do not have sufficient vitamin D levels and 55.6% of them have vitamin D level < 10 ng/mL [10].

Currently, vitamin D is no longer regarded only as a vitamin, but also as a mediator of many non-

calcemic effects, such as immunomodulation through VDR activation in various immune organs and cells (e.g. monocytes, dendritic cells, and lymphocytes) [11,12]. A hormonally metabolite active form of vitamin D was thought to have antithrombotic effect both in *in vivo* and in vitro studies. In null VDR mice, increased TF expression and decreased thrombomodulin (TM) expression lead to multiple organ thrombosis events. In a LPS-induced disseminated intravascular coagulation (DIC) rat model, administration of $1,25(OH)_2D$ successfully reduced thrombosis events [13]. In cancer patients, vitamin D supplementation reduces thrombosis [14]. In an *in vitro* study, administration of $1,25(OH)_2D_3$ showed both an anticoagulant effect through anticoagulant glycoprotein and TM up-regulation and reduced induced monocyte-TF expression by TNF as well through such mechanisms as inhibition of transcription factors, AP-1 and NF_KB [15].

A previous study showed that specific consentration of vitamin D was capable of down regulating TF expression in healthy subjects. Levin et al. [3] stated that administration of $1,25(OH)_2D$ in various concentrations (10, 50, 100, and 150 nM) is a potent inhibitor for anti-β_2-GP1 antibody-induced TF expression in human umbilical vein endothelial cell (HUVEC) culture with 10 nM as its optimal concentration. Furthermore, 10 nM $1,25(OH)_2D$ can up regulate VDR in an *in vitro* study [3] and administration of 1 nmol/L $1,25(OH)_2D$ can decrease both TNF and ox-LDL induced TF expression in monocyte culture [16].

The successfulness of vitamin D in reducing thrombosis event and inhibiting TF expression in healthy individuals and non-SLE patients encourage this study to understanding the role of *in vitro* $1,25(OH)_2D$ as an inhibitor of *E Coli* LPS induced-TF expression in monocyte culture from SLE patients. Hence, in this study we evaluated the effect of $1,25(OH)_2D$ in various concentrations on monocyte TF expression in SLE patients.

2. MATERIALS AND METHODS

2.1 Subject and Study Design

This study used experimental design with the post test only group design to evaluate the effect of $1,25(OH)_2D$ on *E. Coli* LPS-induced, monocyte TF expression of SLE patients. We used stimulated-monocyte culture from 3 age- and sex- matched healthy participants and 6 SLE patients with 10 ng/mL *E. Coli* LPS for 6 hours. These subjects were divided into 4 groups based on the concentrations of $1,25(OH)_2D$; 1 nM, 10 nM, 100 nM and without $1,25(OH)_2D$ for control group.

Samples were collected from healthy participants and SLE patients according to ACR 1997 criteria in Rheumatoid-Immunology Division of Internal Medicine Department, General Hospital of Dr. Saiful Anwar, Malang- Indonesia. The study was approved by the ethics committee of Brawijaya University of Medicine/ Dr. Saiful Anwar Hospital Malang, Indonesia and informed consent was obtained from all participants. Female patients, neither consumed a vitamin D supplementation nor immunosuppressant agents, agreed to participate were included in this study. Patients with recent evidence of vitamin D-related disease, pregnant or breastfeeding were excluded. This study was held in September-December 2013, in Bio-Medical Laboratory of Brawijaya University and Central Laboratory of Dr. Saiful Anwar Hospital, Malang- Indonesia.

2.2 Monocyte Culture and Antibodies for flow Cytometry

Ficoll-Paque™ Premium (GE Healthcare Bio-Sciences AB; Uppsala, Sweden), RPMI Medium 1640 powder (Gibco, life technologies™), Fetal Bovine Serum/ FBS (Gibco), Phosphate Buffered Saline 10x (Sigma-Aldrich, Germany), 1,25 hydroxy vitamin D (Sigma-Aldrich, Germany), Lipopolysaccharides from *Escherichia coli* 0111B4 (Sigma-Aldrich, Germany), PE Anti-human Coagulation Factor III/TF (R&D System, Minneapolis), FITC anti-human CD14 (Biolegend, San Diego).

2.3 Vitamin D Measurement

We used the ORGENTEC 25-OH Vitamin D_3/D_2 Assay (Alegria; Mainz, Deutschland) commercial kit to measure serum concentration of vitamin D. The method for quantitative determination of 25-hydroxyvitamin D is based on ELISA which is measured photo metrically at 650 nm.

2.4 Monocyte Culture

Whole blood was collected in K_3-EDTA lavender tube to prevent coagulation. The peripheral blood mononuclear cell (PBMC) was freshly isolated by separation over Ficoll- Paque. The cellular composition of PBMC preparations was determined by flow cytometry analysis. Then,

PBMCs were further fractionated by adherence to flasks for 48 hours. The isolation of monocytes were washed with RPMI medium without serum. The purified monocyte preparations contained >85% monocytes with contaminating lymphocytes. Monocytes ($1x10^6$ /ml) were cultured in RPMI 1640 medium supplemented with 2mM glutamine, 10 nM HEPES, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin, and incubated at 37°C, 5% CO_2 for 24 hours with and without vitamin D. In the next day, monocytes were incubated in the same medium with addition of 10 ng/mL *E. Coli* LPS in 37°C, 5% CO_2 for 6 hours prior TF measurement. After incubation the cells were harvested by trypsin.

2.5 Measurement of Monocyte-tissue Factor (CD142) Expression

Monocyte-TF (CD142) expression was measured with flow cytometry assay with FITC-anti-human CD14 staining for monocyte marker and PE- anti-human CD142 antibody staining for tissue factor marker. Double positive area (right upper quadrant) represented the monocyte-tissue factor expression.

2.6 Statistical Analysis

We used one way ANOVA assay *(Analyse of Variance)* and a p value <0.05 was considered statistically significant. We use Pearson correlation and linear regression test to show the correlation between the concentrations of vitamin D incubation with TF expression.

3. RESULT

Patient demographics and disease characteristics are shown in Table 1. In this study we use age- and sex- matched healthy participants with similar mean of vitamin D levels for comparison. The means of monocyte-TF (CD142) expression in SLE and healthy participants was showed in Fig. 1 and Table 2. Monocyte-TF (CD142) expression was analyzed using flow cytometry assay and was measured after the LPS induction for 6 hours with and without incubation of $1,25(OH)_2D$ for 24 hours previously. The flow cytometry result could be seen in Fig. 2 with fluorochrom FITC-anti human CD14 for monocyte marker and PE-anti human CD142 for tissue factor marker.

This study used One-Way Anova analysis for evaluating the difference between mean CD142 expression on monocyte culture of SLE patients and of healthy participants, based on various concentrations of vitamin D as treatment factor (p=.275 and p=.197; p<.05 is considered significant). The means CD142 expression showed normal based on Kolmogorov-Smirnov test (p =.422 and p =.922; p >.05 is homogen based on Levene's test (p =.072 and p =.399; p >.05 is considered homogen). This study also used independent t test for evauating the difference of mean CD142 expression on monocyte culture between SLE patients and healthy participants (p =.001 for control group; p =.272 for 1 nM group; p =.736 for 10 nM group and p =.482 for 100 nM group; p<.05 is considered significant).

Table 1. Characteristic of study participants

Characteristics	SLE patients (n=6)	Healthy subjects (n=3)
Sex	Female	Female
Age #	30,3	30
Ethnic	Asian- Indonesia	Asian- Indonesia
Disease features *		-
- Renal involvement	3 (50)	
- Arthritis	1 (16,67)	
- Malar rash/ Oral ulceration	3 (50)	
- Fatigue	3 (50)	
ANA positivity *	6 (100)	-
Anti ds-DNA *	6 (100)	-
Mex-SLEDAI score #	4,83	-
Serum vitamin D (25(OH) level (ng/mL)#	18,25	19,6

** Values are expected as n (%), # Values are expressed as means ( ).*

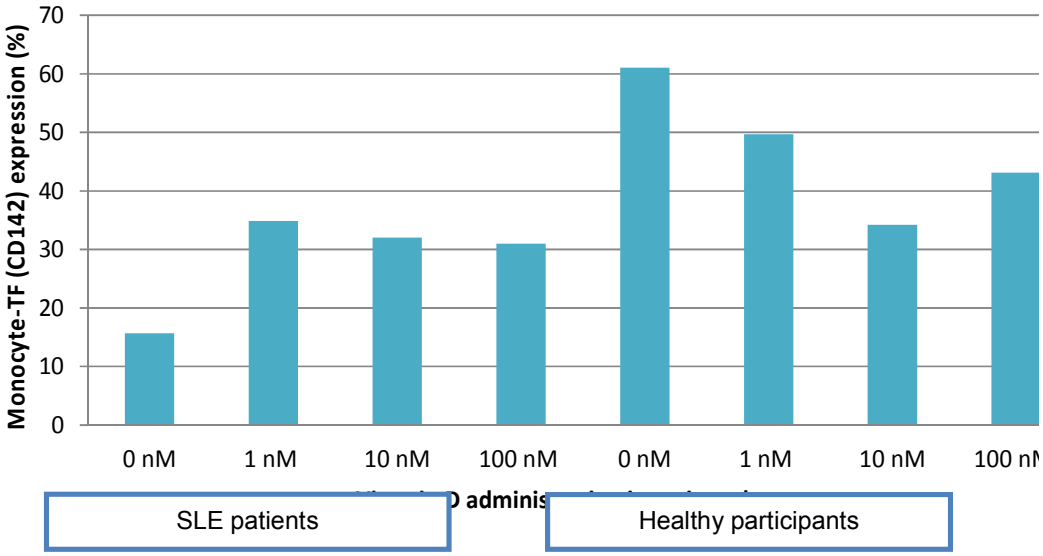

Fig. 1. Graphics of mean monocyte-TF (CD142) expression in LPS-induced and vitamin D pretreatment with various concentrations in SLE (p = .275) and healthy participants

Table 2. The result of CD142 expression on monocytes culture of SLE patients and healthy participants

SLE patients (n=6)					
Treatment (doses)	**Median**	**Range**	**Mean ± Std. dev.**	**95% Confidence interval for mean**	
				Lower bound	**Upper bound**
0	12.82	22.17	15.68±7.94	7.35	24.01
1 nM	38.375	47.35	34.86±20.98	12.84	56.87
10 nM	33.285	43.39	32.04±16.60	14.62	49.46
100 nM	29.435	53.92	30.96±22.55	7.30	54.62
Healthy participants (n=3)					
Treatment (doses)	**Median**	**Range**	**Mean ± Std. dev.**	**95% Confidence interval for mean**	
				Lower bound	**Upper bound**
0	52.27	26.97	58.1±14.4	22.34	93.90
1 nM	50.62	10.5	50.0±5.3	36.89	63.11
10 nM	39.04	20.53	35.8±10.6	9.36	62.23
100 nM	39.19	29.82	41.8±15.1	4.36	79.30

To evaluate the magnitude of correlation and influence from vitamin D incubation to mean CD142 expression of monocyte culture of SLE patients, we used parametric test (Pearson correlation test) and linear regression test. We found no significant correlation between vitamin D incubation and CD142 expression of monocyte culture of SLE patients (r =.166; p =.439) and between vitamin D incubation and CD142 expression of monocyte culture of healthy participants (r =-.260; p =.207). The influence of the vitamin D administration for mean CD142 expression of monocyte culture of SLE patients and healthy participants was shown in Fig. 3.

4. DISCUSSION

Tissue factor or CD142 is a major initiator of coagulation. Its expression in circulation was derived from blood cells, primarily monocytes following inflammatory conditions [4]. Previous study showed that incubation with 10 ng/mL LPS for 6 hours was considered an optimal stimulation for up-regulating TF expression on monocyte culture as much as 8.3 times [17]. In this study, we also used 10 ng/mL LPS for 6 hours to induce monocyte-TF expression following the $1,25(OH)_2D$ incubation for 24 hours.

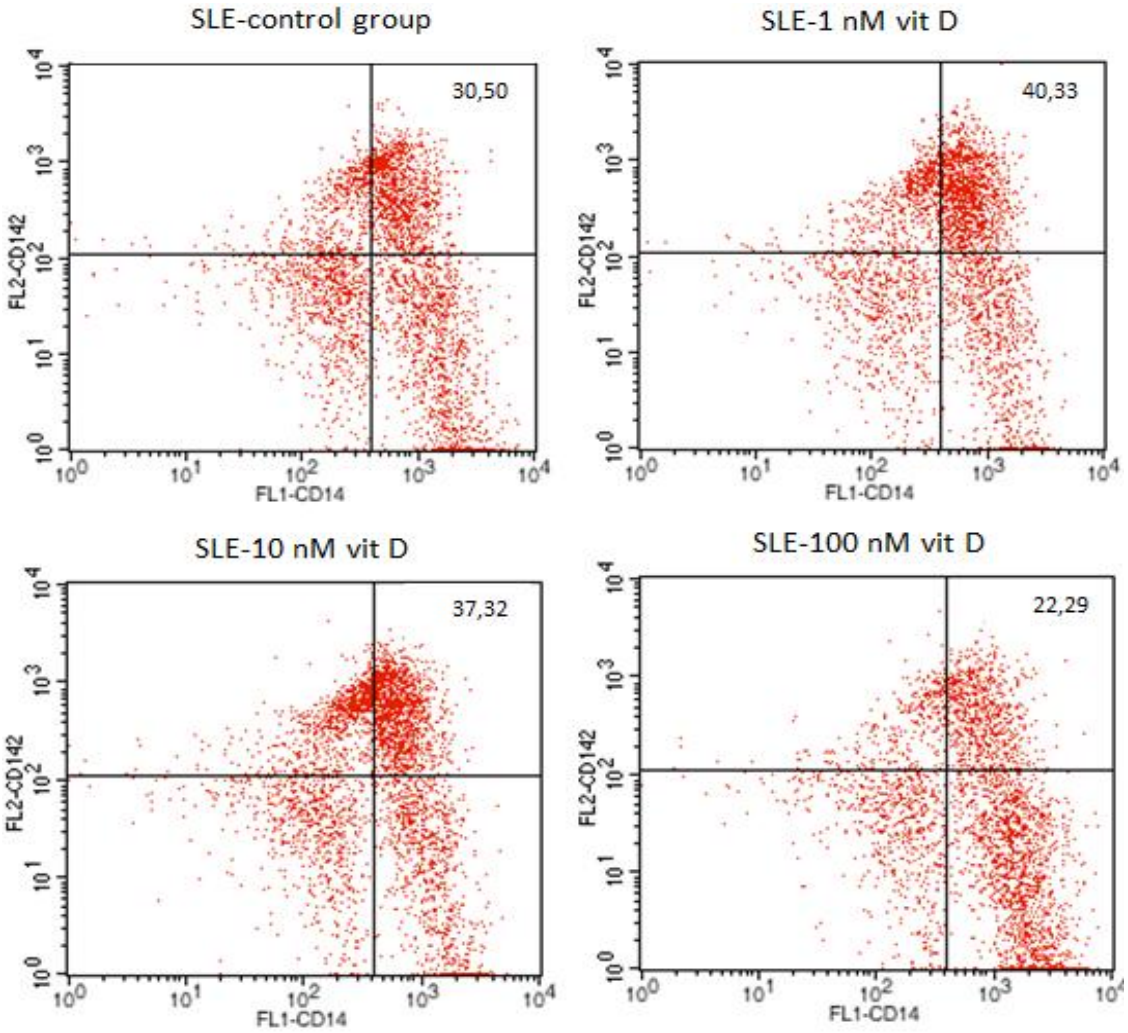

Fig. 2. Monocyte-TF (CD142) expression according flow cytometry with fluorochrom FITC-anti human CD14 and PE-anti human CD142 of monocyte culture from SLE patients. This figure was a representative flow cytometry result from 1 SLE patients. Monocyte-TF (CD142) expression was shown in upper right (double positive) area and shown as percentage. There were 4 different treatment, with 3 groups with different concentrations of vitamin D (1 nM, 10 nM, and 100 nM) and control group without vitamin D. The same treatments and analysis were done for healthy group (data not shown)

In this study, monocyte-TF was measured with flow cytometry assay with the used of FITC-anti-human CD14 staining for monocyte marker and PE- anti-human CD142 antibody for TF marker. Adams et al. [18] showed that TF levels of 40 SLE patients were not significantly different with non-SLE control group, even though those SLE patients have elevated autoantibody levels. However, in this study, there are various monocyte-CD142 expression among SLE patients and healthy participants after LPS induction which were significantly different (p =.001). Unfortunately, the limitations of this study were we did not have any data regarding TF expression prior to LPS induction and circulating TF levels for evaluating if the monocyte-CD142 expression followed the TF level's circulating blood characteristics and limited numbers of patients. To the best of our knowledge, this study was the first study for evaluating the effect of 1,25(OH)2D incubation on TF expression of monocyte culture from SLE patients. Previous studies used PBMC or monocyte or other cell culture from either cell line or healthy individuals that had different characteristics with SLE patients. The other

studies used autoantibody or inflammatory cytokine from SLE patients for induction of TF expression. In this study we can observe the response differences among SLE patients and healthy individuals after 1,25(OH)2D administration. The One-Way Anova test showed no significant differences between CD142 expression in each SLE group (p =.275). The result of Tukey's Test showed no significant differences between each SLE group (p>.05). Whereas in healthy participants, eventhough our descriptive data showed similar results with previous studies which showed that vitamin D administration down regulated TF expression [3,15,16], ANOVA test showed no significant differences due to limited numbers of subjects. In this study, the different outcomes in SLE groups showed that there were some differences in SLE patients such as monocyte characteristics (either in monocyte response to LPS or vitamin D administration), polymorphism of VDR gene and also the regulation of monocyte-TF. There are known abnormalities among monocyte/ macrophage of SLE patients, including surface protein expression or antigen presentation, cytokine production, and phagocytosis capability [7,8]. Other possible defects such as immunomodulatory abnormalities need further investigation. Another factor that might contribute to this different result was VDR polymorphisms in SLE patients. It is known that the deficiency of active vitamin D and polymorphism of VDR are associated with increased incidence of several autoimmune diseases, including SLE. An association between VDR gene polymorphisms and SLE in Asian patients has been reported [19]. All those characteristic differences both in monocyte and VDR lead to another thought that in SLE patients, we need larger concentration of 1,25(OH)2D to suppress the LPS-induction of monocyte-TF. Although Levin et al. [3] stated that the optimal dose of vitamin D for inhibition of the TF expression in HUVEC is 10 nM, that optimal dose was for endothelial-TF of healthy participants, not SLE patients. However, there is no significant correlation between 1,25(OH)2D incubation and mean CD142 expression in this study (r=.1, p=.322). This means that higher or lower doses of 1,25(OH)2D did not correlate with high or low CD142 expression in monocyte culture of SLE patients. In the other hand, in healthy groups it seemed that the optimal dose for suppressing LPS induced-TF monocyte expression was also 10 nM (Fig. 1), eventhough the differences were insignificant. This findings showed that monocyte of SLE patients and healthy participants were different in their response to LPS (p =.001) but not to vitamin D apparently. Although previous study also mentioned that 10 nM 1,25(OH)2D was the optimal concentration for immunoregulatory in SLE patients [20], it seemed that the antithrombotic effect of vitamin D by inhibiting monocyte-TF expression demand a larger concentration. Therefore, further researches with more study subjects are needed as well as researches in the TF pathway of monocyte from SLE patients and vitamin D-TF expression linked in monocyte culture from SLE patients with larger concentrations of 1,25(OH)2D.

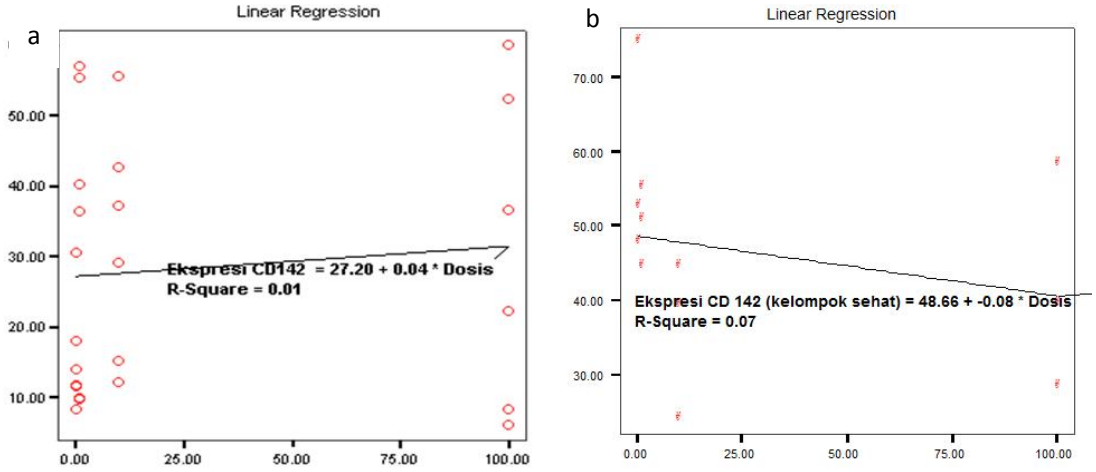

Fig. 3. Linearity graphic between vitamin D administration and monocyte-TF (CD142) expression in monocyte culture of SLE patients (a) and healthy participants (b). The influence of vitamin D was only 1% in SLE patients and 6.8% in healthy participants (*p* >.05)

5. CONCLUSION

There were differences of LPS induced-monocyte-TF expressions among SLE patients and healthy participants. *In vitro* study of SLE patients showed that $1,25(OH)_2D$ has no inhibitory effect on TF expression. The mechanism for this is unknown. Ten nM $1,25(OH)_2D$ seemed to be the optimal concentration for suppressing LPS-induced monocyte-TF expression in healthy groups but not in SLE groups.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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