Treatment of low doses curcumin could modulate Th17/Treg balance specifically on CD4+ T cell cultures of systemic lupus erythematosus patients

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Abstract

Introduction: The balance between T helper 17 (Th17) and regulatory T cells (Treg) is a new paradigm in the pathogenesis of systemic lupus erythematosus (SLE). Currently, there are no drugs that able to modulate Th17/Treg balance specifically in SLE. Curcumin is a bioactive agent that has a specific action against hyperproliferative cells. However, its role in modulating Th17/Treg balance in SLE is still unknown. This research aimed to investigate the role of curcumin in modulating Th17/Treg balance on CD4+ T cell cultures of SLE patients.

Material and methods: CD4+ T cells from SLE 6 untreated patients and 6 healthy subjects were collected, stimulated with Th17 differentiating factors, and curcumin 0.1 and 1 µg/ml was added on cultures. After 72 hours incubation, cells were harvested and measured for Th17 and Treg percentages using flow cytometry and interleukin-17A (IL-17A) and transforming growth factor-β1 (TGF-β1) levels using ELISA.

Results: Administration of low doses of curcumin (0.1 and 1 µg/ml) could decrease Th17 percentages ($p = 0.000$ and $p = 0.000$ compared to control), reduce IL-17A productions ($p = 0.000$ and $p = 0.000$ compared to control), increase Treg percentages ($p = 0.001$ and $p = 0.000$ compared to control), and increase TGF-β1 productions ($p = 0.001$ and $p = 0.000$ compared to control) on CD4+ T cells of SLE patients. Interestingly, these effects were not reproduced on CD4+ T cells cultures of healthy subjects.

Conclusions: These data suggest that curcumin can modulate Th17/Treg balance specifically on CD4+ T cells of SLE patients without affecting healthy subjects.

Key words: systemic lupus erythematosus, curcumin, Th17/Treg balance.

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with multiple organ manifestations [1]. Recently, there is a new paradigm in SLE pathogenesis involving enhancement of Th17 with a parallel reduction of Treg population in SLE patients [2-5]. Other research suggested that improving Th17/Treg balance might reduce disease activity [6]. Therefore, regulating balance between Th17 and Treg has currently become a subject of interest which is intended to improve the clinical outcome and prevent organ damage in SLE.

The expansion of knowledge in SLE pathogenesis has driven the development of many drugs against SLE. Curcumin is one example of bioactive agents that was proven to have immunomodulatory and anti-inflammatory properties in various diseases [7-9]. One previous study also determined that curcumin was able to modulate the cellular response and growth of various cell types of the immune system [10]. However, despite the extent of the role of curcumin on various biologic processes, its role in SLE has rarely been described, especially on Th17/Treg balance.

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Interestingly, curcumin has been reported to have a specific effect on the actively proliferated cells without affecting normal ones [11, 12]. Differences in antioxidant levels [12], transcription factor activity [13], and cell polarities [14] affect the ability of curcumin to enter the cells. Number of studies found that there were abnormalities in T cells from SLE patients, including changes in physiology of T cell receptor (TCR) and post receptor downstream signaling [15, 16], increase of oxidative stress levels [17], epigenetic changes [18], alteration of cytokine productions [19], and susceptibility to differentiate into Th17 [20]. These changes raise a presumption that curcumin may act specifically on T cells from SLE patients, especially in modulating Th17/Treg balance. Thus, this present study was aimed to investigate the role of *in vitro* treatment of curcumin on regulating Th17/Treg balance in both SLE patients and healthy subjects.

Material and methods

Subjects of the study

Six female SLE patients, with a mean age of 24.8 (22-28) years old, diagnosed according to the criteria of Systemic Lupus International Collaborating Clinic (SLICC) 2012 were included in the study. Patients were recruited from the Rheumatology Clinic, Dr Saiful Anwar Hospital, Malang, Indonesia. All participants were newly diagnosed SLE patients who had not been on any treatments of corticosteroids, immunosuppressant, or biological agents. Patients suffered from SLE with mean disease duration 9.8 (8-12) months. All patients had active disease with MEX-SLEDAI score > 3 with mean score 5.5 (4-8). Patients who had severe infection were excluded, as well as pregnant and breastfeeding patients. Six healthy individuals matched in age, gender, ethnicity, and geographical location with a mean age 24.8 (24-25) years were included as controls. This study was approved by the Ethical Committee of Brawijaya University for human research and informed consent was obtained from each subject.

Cells isolation

Ten millilitres of peripheral venous blood were freshly obtained from each subject and collected into heparinized vacutainer. Afterwards, CD4+ T cells were separated using RosetteSep human CD4+ T cell enrichment cocktail (RosetteSep, StemCell Technologies) according to manufacturer's instruction. The purity of CD4+ T cell isolates was confirmed using flow cytometry analysis (FACScalibur) and had high expression of surface marker CD4+ (> 85%).

Culturing and stimulation of CD4+ T cells

CD4+ T cells were cultured in 96 well plates with 5 µg/ml plate bound anti-CD3 (Biolegend). Cells were alliquoted into 5 × 10^5 cells/well with complete RPMI culture me-

dium (Sigma-Aldrich, St. Louis, MO) containing L-Glutamine and 10% fetal bovine serum (Gibco) supplemented with 100 µg/ml streptomycin (Gibco), 100 U/ml penicillin (Gibco) and 5 µg/ml antiCD28 (R&D systems). All cultured CD4+ T cells were stimulated into Th17 by adding 10 ng/ml IL-6 (Biolegend), 5 ng/ml TGF-β1 (Biolegend), 10 µg/ml anti-IFN-γ (Biolegend), and 10 µg/ml anti-IL-4 (Biolegend) in cell cultures. Lastly, curcumin (Sigma-Aldrich, St. Louis, MO) with different concentrations (0, 0.1, 1, 10, and 100 µg/ml) were added in cell cultures. Cells were incubated at 37°C and 5% CO_2 for 72 hours [21].

Apoptosis assay

Cells were harvested and labelled with FITC anti-Annexin V antibody (Biolegend) and propidium iodine (PI) (Biolegend). After staining for 15 minutes in dark, cell death was assessed by flow cytometry (FACScalibur). Early apoptotic cells were positive for Annexin V and negative for PI whereas late apoptotic or early necrotic cells were positive for both Annexin V and PI.

Measurement of Th17 and Treg percentages using flow cytometry

Harvested CD4+ T cells were counted for Th17 and Treg percentages using flow cytometry (FACScalibur). Before detection of Th17 cells, cells were stimulated with 50 ng/ml PMA (Sigma-Aldrich) and 1 µg/ml Ionomycin (Sigma-Aldrich) in the presence of Brefeldin A (BD Pharmingen) at 37°C for 4 hours. Cells were stained with FITC anti-human CD4 antibody (Biolegend). Afterwards, cells were fixed, permeabilized, and labelled with and PerCP/Cy5.5 anti-human IL-17A (Biolegend). Th17 were cells which expressed CD4+ IL-17A+. For detection of Treg, cells were labelled with PerCP anti-human CD4 antibody (Biolegend) and PE anti-human CD25 antibody (Biolegend). FITC anti-human FoxP3 antibody (Biolegend) was added later after cells were fixed and permeabilized. Treg were cells which expressed CD4+ CD25+ FoxP3+.

Enzyme-linked immunoabsorbent assay (ELISA) for cytokines measurement

Cytokines measurements were done to assess Th17 and Treg function by monitoring their cytokine production. Supernatants from CD4+ T cells culture were collected and stored at –80°C for cytokine measurements. Interleukin-17A (IL-17A) (R&D systems) and transforming growth factor-β1 (TGF-β1) (eBioscience) secretion were measured by ELISA kits according to the manufacturer's instructions.

Statistical analysis

Differences between groups were determined using Anova or Kruskal-Wallis while post hoc analysis was performed when necessary. Data were described as mean ± standard

deviation. Statistical analysis and graphs generation was performed using SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered significant.

Results

Comparison of *in vitro* differentiated Th17 and Treg profiles on SLE patients and healthy subjcts before addition of curcumin treatments

First, we compared the percentages and cytokine productions of Th17 and Treg between SLE patients and healthy subjects before curcumin treatment. In culture condition favoring Th17 differentiation, percentages of Th17 derived from SLE patients were statistically significantly higher compared to healthy subjects ($p = 0.000$, Fig. 1A and 1B). Treg percentages from SLE patients were also statistically significantly lower compared to healthy subjects ($p = 0.000$, Fig. 1C and 1D). Similarly, IL-17A levels from SLE patients were statistically significantly higher compared to healthy subjects ($p = 0.000$, Fig. 1E). TGF-β1 levels were also statistically significantly lower on SLE patients compared to healthy subjects ($p = 0.000$, Fig. 1F). These results indicated that there were different responses in CD4+ T cells from SLE patients and healthy subjects. CD4+ T cells from SLE patients were found to be more prone to develop into Th17 subsets than Treg.

Effect of curcumin treatments on apoptosis of CD4+ T cells

We initially determined the toxicity of curcumin treatments on human CD4+ T cells primary culture indicated by rate of apoptosis that occurred. Curcumin was added at different concentration (0, 0.1, 1, 10, and 100 µg/ml) on CD4+ T cells which were given stimulation with Th17 differentiating factors. As seen in Fig. 2A and 2B, we found that viable cells were reduced in dose dependent manner after curcumin treatments. Significant reduction of viable cells was observed after treatment of 10 and 100 µg/ml of curcumin ($p = 0.000$ and $p = 0.000$, respectively compared to 0 µg/ml) while other doses did not significantly reduce the number of viable cells.

Moreover, cells undergoing early apoptotic process which stained positive for Annexin V and negative PI increased significantly after treatment of 1, 10, and 100 µg/ml of curcumin ($p = 0.014$, $p = 0.000$, and $p = 0.000$ respectively compared to 0 µg/ml) while addition of 0.1 µg/ml of curcumin did not significantly increase their number. Lastly, cells undergoing late apoptotic process which stained positive for both Annexin V and PI were found enhanced in a dose dependent manner. Significant increase of the number of late apoptotic cells was found after treatment of 10 and 100 µg/ml of curcumin ($p = 0.006$ and $p = 0.000$ respectively compared to 0 µg/ml). There was no statistically significantly difference of the number of late apoptotic

cells in culture treated with 0.1 and 1 µg/ml of curcumin. Based on these results, 10 and 100 µg/ml of curcumin were toxic to CD4+ T cells and these concentrations were not used in subsequent experiments.

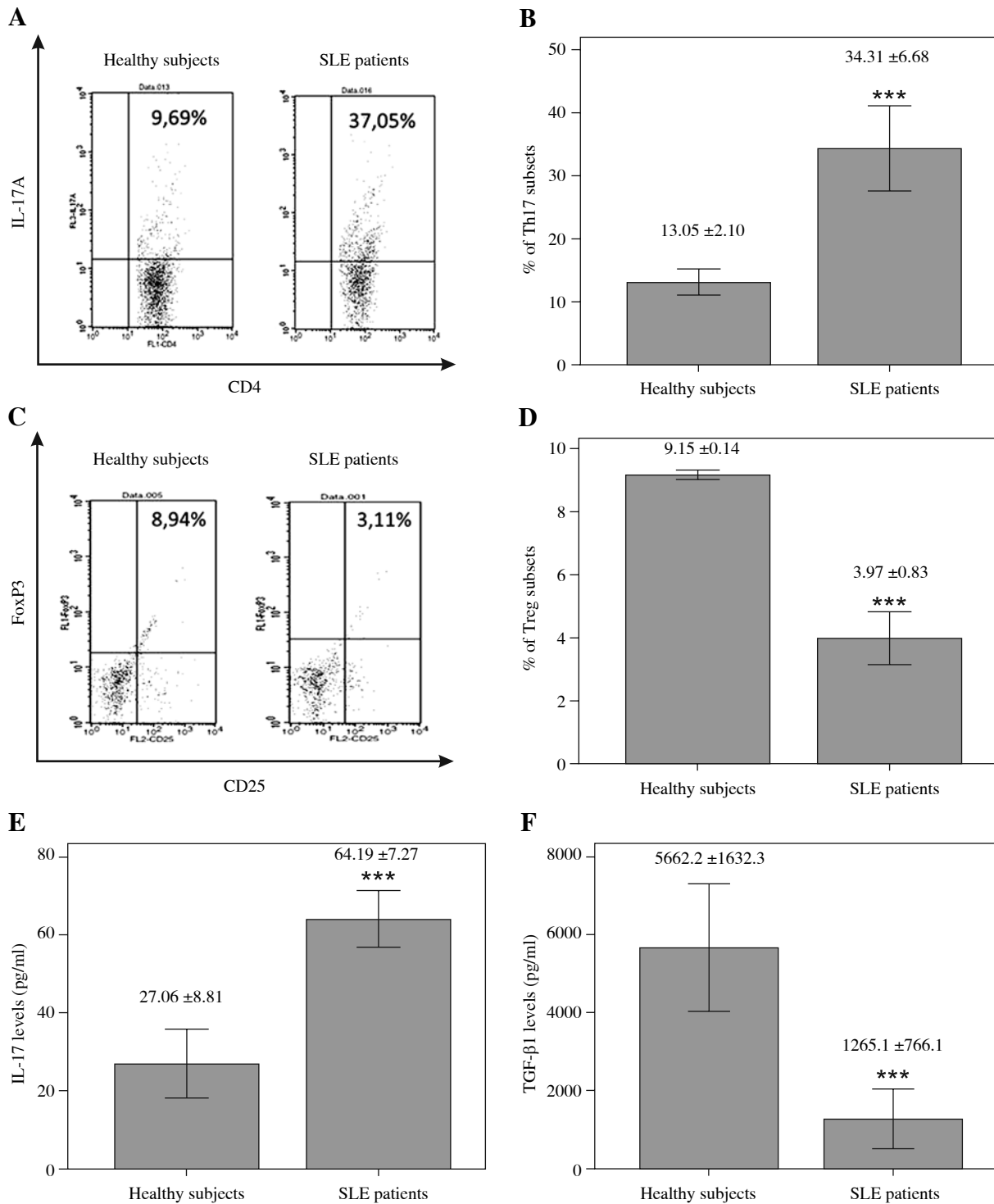### Effect of curcumin treatments on *in vitro* differentiation of Th17

By using previously obtained dosages of curcumin (0, 0.1, and 1 µg/ml), we examined the effect of curcumin treatments on the differentiation of Th17 from CD4+ T cells cultures from both SLE patients and healthy subjects. As seen in figure 3A, Th17 were cells which expressed both CD4+ and IL-17A+. We found that there were significant reductions of Th17 percentages in dose dependent manner on CD4+ T cell cultures from SLE patients after treatment of 0.1 and 1 µg/ml of curcumin ($p = 0.000$ for each dosage compared to 0 µg/ml, Fig. 3B). Interestingly, as seen in figure 3C and 3D, we found that there were no differences in Th17 percentages measured from CD4+ T cells of healthy subjects after curcumin treatment at concentration of 0.1 and 1 µg/ml ($p = 1.000$ and $p = 0.341$ respectively compared to 0 µg/ml). All of these data indicated that curcumin was able to decrease in vitro differentiation of Th17 on CD4+ T cells from SLE patients specifically without affecting healthy subjects.

Effect of curcumin treatments on *in vitro* differentiation of Treg

We also investigated whether curcumin could induce Treg differentiation specifically on CD4+ T cells from SLE patients in culture condition favoring Th17 differentiation. Treg were cells that expressed CD4+ CD25+ FoxP3+ (Fig. 4A). Treg percentages of CD4+ T cells culture from SLE patients were increased significantly after curcumin treatment at concentration of 0.1 and 1 µg/ml ($p = 0.001$ and $p = 0.000$ respectively compared to 0 µg/ml, Fig. 4B). Interestingly, curcumin ability to induce Treg differentiation was not reproduced on CD4+ T cells from healthy subjects. There were no significant differences in Treg percentages from healthy subjects after curcumin treatments at concentrations of 0.1 and 1 µg/ml (Fig. 4C and 4D). However, we found that Treg percentages on CD4+ T cells culture from healthy subjects were significantly higher after treatment of curcumin at higher concentration using 10 µg/ml of curcumin ($p = 0.004$ compared to 0 µg/ml, data not shown). These results indicated that curcumin could induce *in vitro* differentiation of Treg from CD4+ T cells with different doses response in SLE patients and healthy subjects.
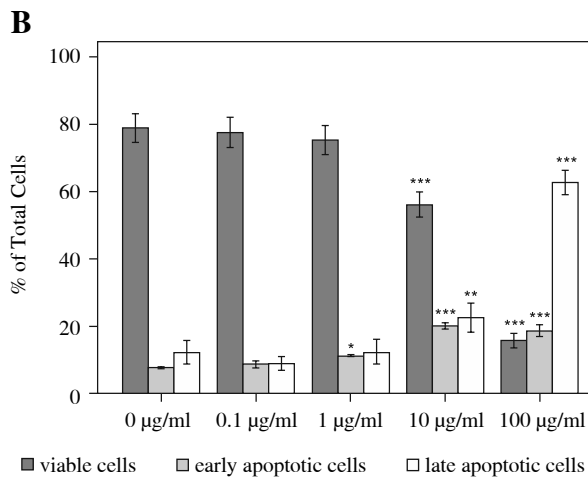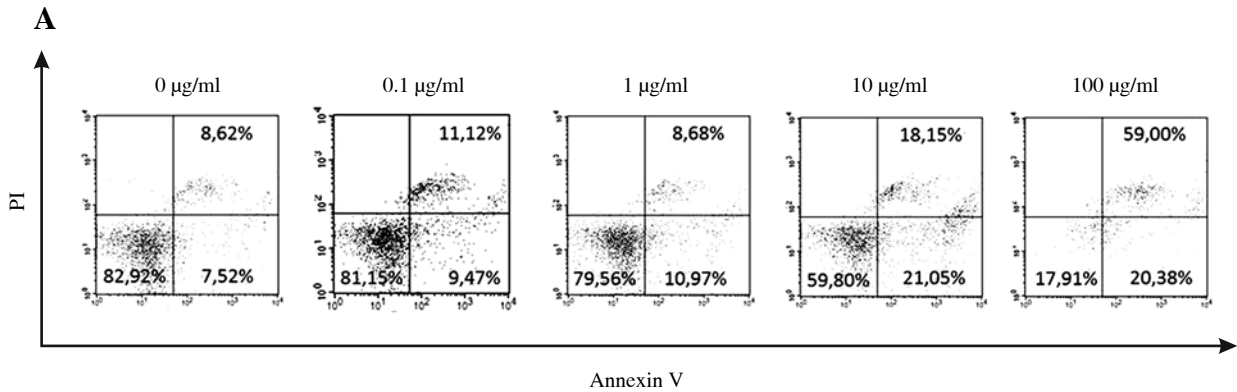
Effect of curcumin treatments on Th17 and Treg functions

We also wanted to prove the role of curcumin treatments on Th17 and Treg functions by assessing their ability to secrete cytokines. IL-17A and TGF-β1 were

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A

Healthy subjects   SLE patients

9,69%   37,05%

IL-17A

CD4

B

34.31 ±6.68

***

13.05 ±2.10

% of Th17 subsets

Healthy subjects   SLE patients

C

Healthy subjects   SLE patients

8,94%   3,11%

FoxP3

CD25

D

9.15 ±0.14

3.97 ±0.83

***

% of Treg subsets

Healthy subjects   SLE patients

E

64.19 ±7.27

***

27.06 ±8.81

IL-17 levels (pg/ml)

Healthy subjects   SLE patients

F

5662.2 ±1632.3

1265.1 ±766.1

***

TGF-β1 levels (pg/ml)

Healthy subjects   SLE patients

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to healthy subjects*

Fig. 1. Comparison of Th17 and Treg profiles on CD4+ T cells cultures of healthy subjects and SLE patients before curcumin treatments. A) Representative dot plots that illustrated the expression of CD4 and IL-17A. B) Histogram of Th17 percentages expressing CD4+ IL-17A+. C) Representative dot plots that illustrated the expression of CD25 and FoxP3 which had been gated for CD4 before. D) Histogram of Treg percentages expressing CD4+ CD25+ FoxP3+. E) IL-17A levels on culture supernatant. F) TGF-β1 levels on culture supernatant. Data are presented as the mean ± standard deviation

Fig. 2. Effect of curcumin treatments on apoptosis of CD4+ T cells. A) Representative dot plots that illustrated expression of Annexin V and PI. B) Histogram of viable cells, early apoptotic cells, and late apoptotic cells on each group (0, 0.1, 1, 10, and 100 µg/ml of curcumin). Data are presented as the mean ± standard deviation

measured from culture supernatant from SLE patients and healthy subjects using ELISA. As seen in Fig. 5A, we found that 0.1 and 1 µg/ml of curcumin treatments could reduce IL-17A levels significantly on culture supernatant from SLE patients ($p = 0.000$ and $p = 0.000$ respectively compared to 0 µg/ml). Conversely, TGF-β1 levels decreased significantly after treatment with 0.1 and 1 µg/ml of curcumin as seen in Fig. 5B ($p = 0.001$ and $p = 0.000$ respectively compared to 0 µg/ml). However, both 0.1 and 1 µg/ml of curcumin treatments failed to reduce IL-17A levels or increase TGF-β1 levels on healthy subjects as seen in Fig. 5C and 5D. These results indicated that curcumin could modulate Th17 and Treg functions by decreasing IL-17A and increasing TGF-β1 productions on CD4+ T cells culture from SLE patients without affecting healthy subjects.

Discussion

Our latest understanding of SLE pathogenesis is associated with an imbalance of immune response marked by an alteration from a state of tolerance into non self-tolerance, mediated by imbalance between Treg and Th17 subsets [3]. In our present study, we found that although both CD4+ T cells from SLE patients and healthy subjects

were exposed to similar stimulation with Th17 differentiating factors, Th17 percentages and cytokines from SLE patients were higher while Treg were lower compared to healthy subjects. These results indicate that CD4+ T cells from SLE patients are more susceptible to differentiate into Th17 subsets; meanwhile suppression of differentiation and functions of Treg also occur as compared to healthy subjects. Our results come in agreement with other studies which showed an abnormal increase of Th17 subsets and decreasing of Treg on SLE patients [3-6].

Regardless of the differences in the response of Th17 and Treg subsets from SLE patients and healthy subjects, drugs which act specifically on T cells of SLE patients have not been developed. Our results showed that curcumin at low dose (0.1 and 1 µg/ml) were able to reduce Th17 and IL-17A production and also increased Treg differentiation and TGF-β1 productions on CD4+ T cells cultures of SLE patients without affecting CD4+ T cells of healthy subjects. The ability of curcumin to modulate Th17/Treg balance was also described in other studies. Ma *et al.* [22] reported that curcumin was able to attenuate allergic airway inflammation on mouse models by increasing Treg and reducing Th17 functions. Another study also revealed that curcumin could regulate Th17 differentiation via modulation of STAT3 transcription factor [13, 23]. Curcumin
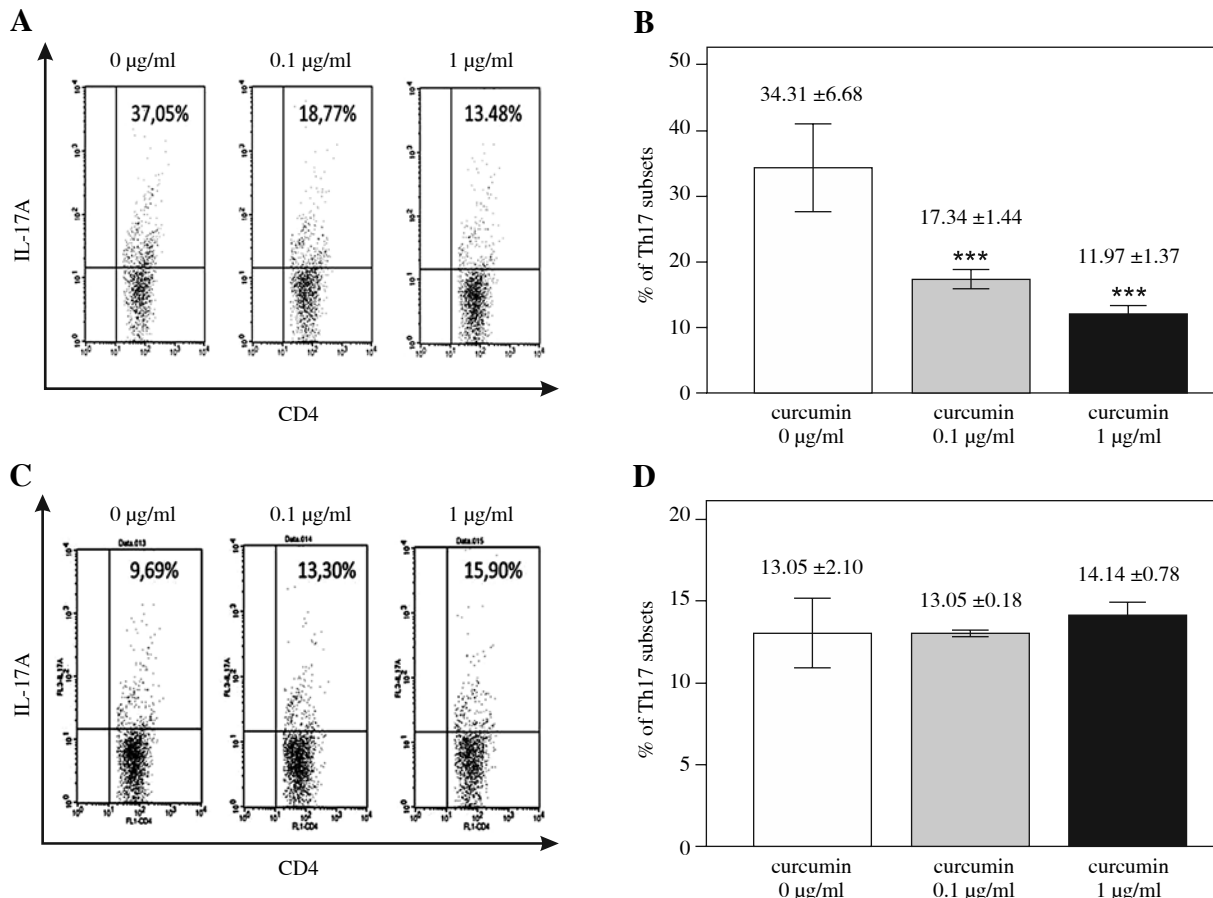
*p < 0.05, **p < 0.01, ***p < 0.001 compared to 0 μg/ml*

Fig. 3. Effect of curcumin treatments on *in vitro* differentiated Th17 from CD4+ T cells cultures. A) Representative dot plots that illustrated the expression of CD4 and IL-17A of SLE patients. B) Histogram of Th17 percentages expressing CD4+ IL-17A+ from CD4+ T cells cultures of SLE patients. C) Representative dot plots that illustrated the expression of CD4 and IL-17A of healthy subjects. D) Histogram of Th17 percentage expressing CD4+ IL-17A+ from CD4+ T cells cultures of healthy subjects. Data are presented as the mean ± standard deviation

was also reported could increase FoxP3 expression in Treg by modulating the expression of mammalian target of rapamycin (mTOR) [24, 25].

Interestingly, we found that the ability of curcumin to modulate Th17/Treg balance is only apparent in CD4+ T cells cultures of SLE patients without affecting healthy subjects. Our results showed that low dose curcumin was not able to modulate Th17/Treg balance on CD4+ T cells of healthy subjects. However, we found that higher doses (10 μg/ml) were able to increase Treg differentiation but not Th17 (data not shown). Another study also found that high doses of curcumin were needed to modulate immune response in normal cells [26]. Ying *et al.* [27] also found that curcumin was able to reduce in vitro differentiation of Th17 from healthy subjects at 5 and 25 μmol/l.

Differences in dose response shown in CD4+ T cells of SLE patients and healthy subjects indicated that cur-

cumin may be more sensitive to enter CD4+ T cells from SLE patients than healthy subjects. Differences in GSH levels [12] or increased reactive oxygen species may be implicated [28]. Another possible mechanism is the higher level of consecutive transcription factors on CD4+ T cells of SLE patients. Higher transcription factors levels in SLE patients, such as STAT3 had been reported to be the one of the causes of higher polarization of Th17 subsets in SLE [20]. Some of these conditions may increase the tendency of curcumin to be absorbed differently by CD4+ T cells of SLE patients and healthy subjects.

This research has shown the possibility of curcumin to be used as a novel therapeutic agent for SLE by alleviating the immune response in SLE specifically through modulating balance between Th17 and Treg. Although curcumin has proven capable to modulate Th17/Treg balance in many other studies, studies that examine its effects on the
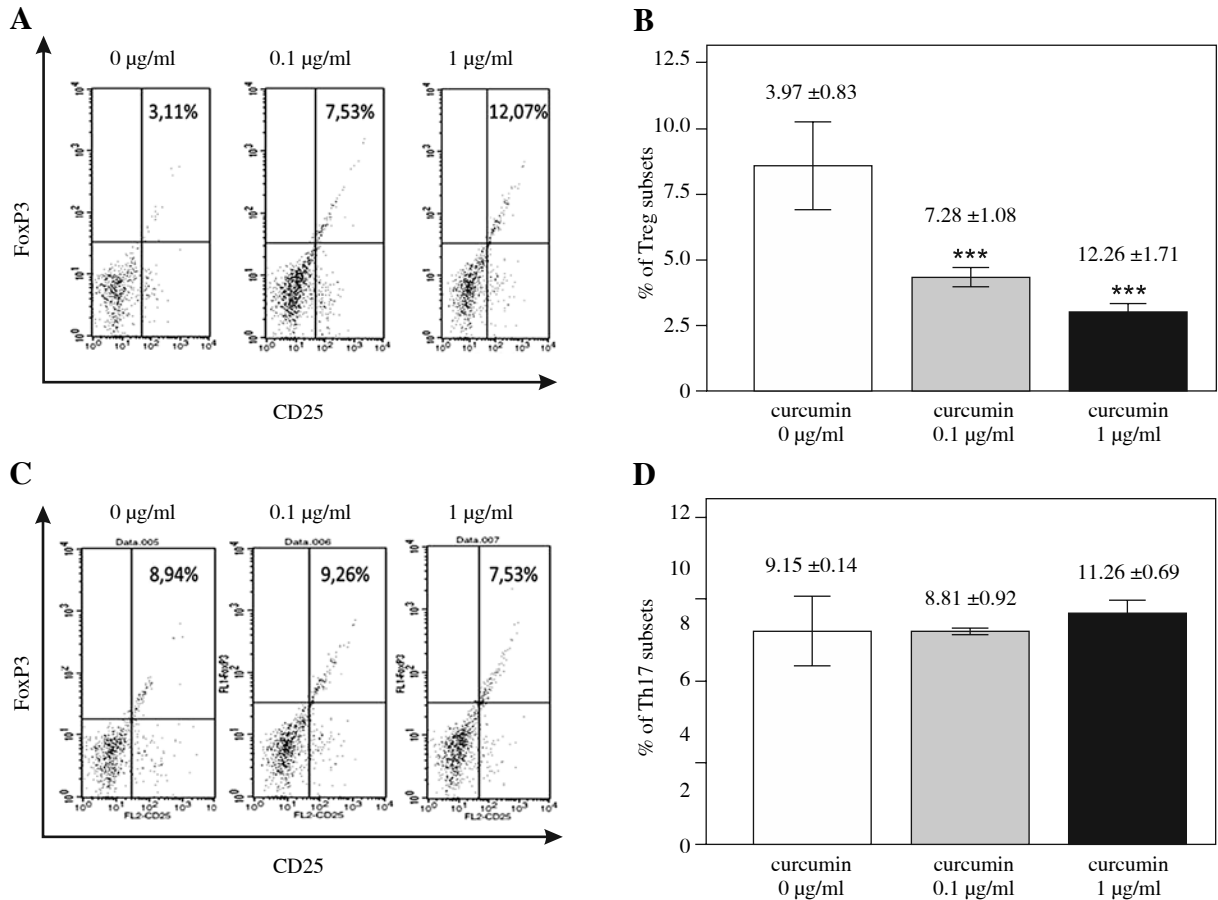
*p < 0.05, **p < 0.01, ***p < 0.001 compared to 0 µg/ml

Fig. 4. Effect of curcumin treatments on *in vitro* differentiated Treg from CD4$^+$ T cells cultures. A) Representative dot plots that illustrated the expression of CD25 and FoxP3 which had been gated for CD4 before of SLE patients. B) Histogram of Treg percentages expressing CD4$^+$ CD25$^+$ FoxP3$^+$ from CD4$^+$ T cells cultures of SLE patients. C) Representative dot plots that illustrated the expression of CD25 and FoxP3 which had been gated for CD4 before of healthy subjects. D) Histogram of Treg percentages expressing CD4$^+$ CD25$^+$ FoxP3$^+$ from CD4$^+$ T cells cultures of healthy subjects. Data are presented as the mean ± standard deviation

Th17/Treg balance in SLE have not been conducted yet. Limitations of the present study include the lack of experiments for the assessment of the precise pathway regulated by curcumin in order to modulate Th17/Treg balance in SLE patients. Moreover, we did not compare the uptake of curcumin between CD4$^+$ T cells from SLE patients and healthy subjects. Therefore, this does not rule out the possibility of opening further research in the future to cover the limitations in this study.

Conclusions

The results of this present study indicate that curcumin at low doses (0.1 and 1 µg/ml) can modulate Th17/Treg balance on CD4$^+$ T cells culture of SLE patients by reducing Th17 percentages and IL-17A production, increasing Treg percentages and also TGF-β1 productions in dose dependent manner. This ability to modulate Th17/Treg balance is specifically present only on CD4$^+$ T cells of SLE patients without affecting CD4$^+$ T cells of healthy subjects.
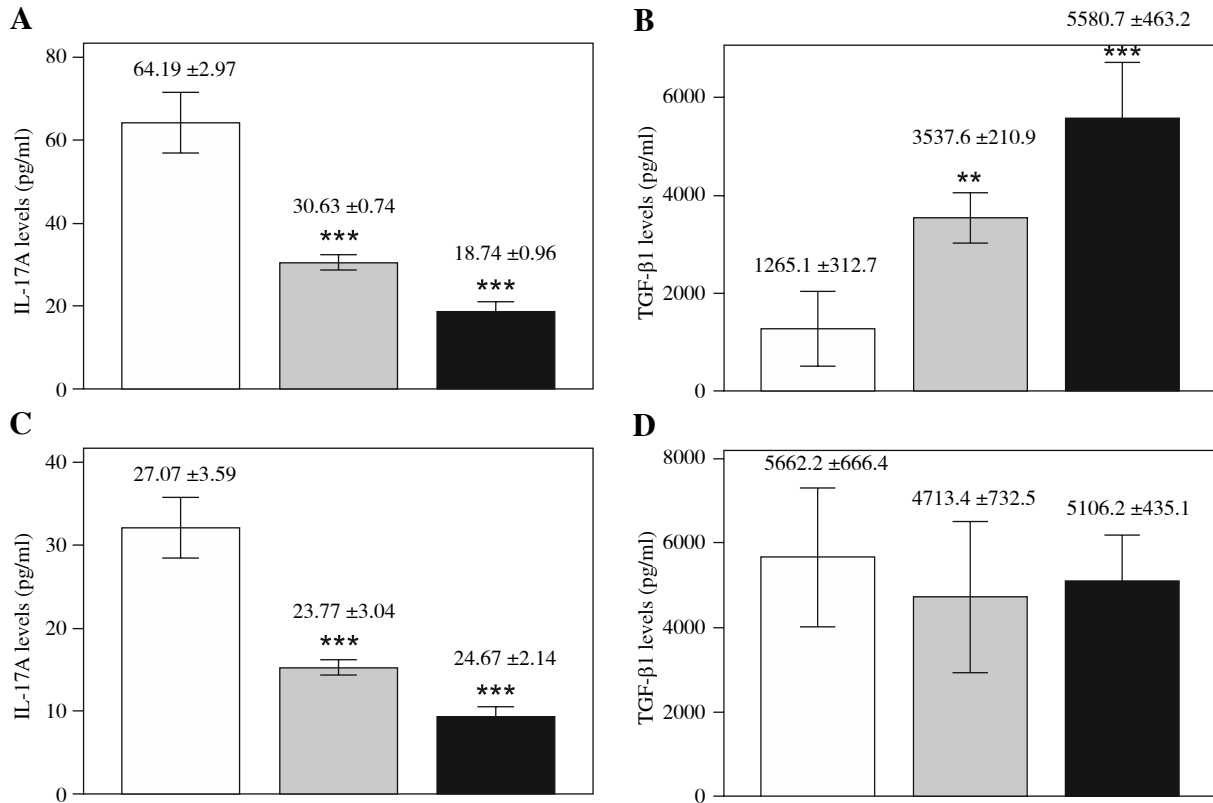
*p < 0.05, **p < 0.01, ***p < 0.001 compared to 0 µg/ml*

Fig. 5. Effect of curcumin treatments on IL-17A and TGF-β1 productions. A) Histogram of IL-17A levels from culture supernatant of SLE patients. B) Histogram of TGF-β1 levels from culture supernatant of SLE patients. C) Histogram of IL-17A levels from culture supernatant of healthy subjects. D) Histogram of TGF-β1 levels from culture supernatant of healthy subjects. Data are presented as the mean ± standard deviation

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