Impact of $CD4^+CD25^{high}$ Regulatory T-Cells and FoxP3 Expression in the Peripheral Blood of Patients with Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by various immunological abnormalities. Regulatory T cells (Tregs) $CD4^+CD25^+$ play an important role in maintaining tolerance to self-antigens controlling occurrence of autoimmune diseases. It has been shown that the transcription factor forkhead box P3 (FoxP3) is specifically expressed on $CD4^+CD25^+$T cells. FoxP3 has been described as the master control gene for the development and function of Tregs. A decrease in the number of $CD4^+CD25^{high}FoxP3^+$ regulatory T cells can play a key role in the loss of tolerance to self antigens. The study was designed to assess expression levels of FoxP3 **in peripheral $CD4^+CD25^+$regulatory T cells in patients with SLE and to evaluate the level of some cytokines that are implicated in the extent of the disease activity. The study was carried out on 30 SLE patients, they were 27 females and 3 males, 10 age and sex matched healthy volunteers were studied as a control group. They were divided into two groups: group I: had active disease (12 patients) and group II: had inactive disease (18 patients) according to Systemic Lupus Erythematosus Disease Activity Index. All individual were subjected to CBC, ESR, s.creatinine, RF, CRP, C3, ANA, anti ds-DNA and flowcytometeric assay of $CD4^+CD25^+$ (Tregs) and FoxP3 for patients and controls. Quantitative determination of serum interleukin 10 (IL-10) and transforming growth factor-beta1 (TGF-β_1) concentrations in serum samples by ELISA technique. The results revealed a significant decrease of $CD4^+CD25^{high}$cells in peripheral blood in active lupus patients when compared with patients with inactive lupus and those in healthy controls. Intriguingly, the percentage level of FoxP3 on $CD4+CD25^{high}$cells was significantly decreased in SLE patients with active disease (2.9±1.05) when compared with those with inactive SLE (3.5±0.8) and control groups (4.7±1.2) (*P*<0.05). As regard cytokines levels; the level of IL-10 was significantly increased in patients with active and inactive disease (158.8±50.8, 82.8±14.08 respectively) when compared with the control group (*P*<0.001). While, the level of TGF-β1 was significantly decreased in patients with active and inactive disease (22.5±7.03, 29.07±10.14 respectively), when compared with the control group (*P*<0.05). Our data revealed impaired production of Tregs in SLE patients, which may play a reciprocal role with some cytokines to affect the activity of the disease. Tregs cells should be the target to determine the clinical effectiveness of novel therapy to modulate Tregs *in vivo* besides the conventional treatments.**

Systemic lupus erythematosus (SLE), the prototypical systemic autoimmune disease, is characterized by a wide spectrum of clinical manifestations and abundant production of autoantibodies to nuclear antigens, cell surface molecules, and serum proteins. The diverse presentations of lupus range from rash and arthritis through anemia and thrombocytopenia to serositis, nephritis, seizures, and psychosis. Lupus should be part of the differential diagnosis in virtually any patient presenting with one of these clinical problems, especially in female patients between 15 and 50 years of age (Rahman & Isenberg, 2008).

The ability of the immune system to discriminate between self and nonself is controlled by central and peripheral tolerance mechanisms. The immune system has developed several mechanisms to suppress or regulate immunity in order to protect the body from sustained harmful immune responses (Liu *et al.,* 2004).

One of the key players of immune regulation is the natural $CD4^+CD25^+$ regulatory T cells (Treg). Treg cells show a

potent immunosuppressive function and contribute to immunologic self-tolerance by suppressing potentially auto-reactive T cells (Kuhn *et al.,* 2009).

A number of animal studies showed that $CD4^+CD25^+$ T cells are closely related to the development of autoimmune diseases. Many experimental organ-specific autoimmune diseases, including oophoritis, thyroiditis, gastritis and prostatitis, were induced in susceptible strains of mice by protocols that resulted in removal or delay of the development of $CD4^+CD25^+$ T cells, while the co transfer of $CD4^+CD25^+$T cells prevented the development of autoimmune disease. The data strongly indicate that $CD4^+CD25^+$ T cells play an important role in the pathogenesis of autoimmune diseases (Liu *et al.,* 2004).

Since their first description in humans, Tregs have been the focus of intense research efforts, notably in the context of autoimmune diseases. It is now clear that the forkhead transcription factor (Foxp3) acts as a critical regulator in the development and function of Tregs. The best evidence for Tregs as being key in the control of self-tolerance in humans therefore comes from the causal association between the rapidly fatal immune dysregulation, polyendocrinopathy, X-linked syndrome, and mutations in FoxP3 (Miyara *et al.,* 2005).

It is firmly established that there are two major categories of Treg cells described to date. The first is the naturally occurring, thymically derived $CD4^+CD25^+$ Treg cells (nTreg cells) that express high level of the transcription factor Foxp3, which is essential for their development and function. The other category is the antigen-specific Treg cells (iTreg cells), that secrete anti-inflammatory cytokines such as IL-10 and/or TGF-β, and regulate immune responses and inflammatory pathologies. However, many questions remain to be answered regarding distinct roles of these (Treg) cell subsets during immune response (Tai-You, 2008).

In the present study, we attempted to study the percentage levels of $CD4^+CD25^+$ T cells Treg and analyze the $FoxP3^+$T cells expression in peripheral blood of SLE patients and to evaluate the levels of IL-10 and TGF-β1 and their implication in the extent of the disease activity.

Subjects and Methods

Subjects

We studied 30 SLE patients who fulfilled at least four criteria of American College of Rheumatology (Arnett *et al.,* 1988) for classification of SLE. They were recruited from the out-patient clinic of Internal Medicine departments in the period from January 2007 to March 2008. They were 27 females and 3 males with a median age of 29 years (range from 18 to 40 years). In addition, 10 age and sex matched healthy volunteers were studied as a control group.

Patients were divided into two groups: group I: had active disease (12 patients) and group II: had inactive disease (18 patients) according to Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score (Bombardier *et al.,* 1992). The mean SLEDAI score in patients with inactive disease was 2.15±0.4, and in patients with active disease was 9.48±2.7. Samples were obtained after informed consent in accordance with procedures approved by the human ethics committee of the Benaroya Research Institute.

Subjects enrolled in the study were subjected to the following:

-Full Medical History Taking and Clinical Examination.

Routine laboratory investigations including, complete blood picture using Sysmex SF 3000 (Roche Diagnostic GmbH, Mannheim, Germany), Erythrocyte sedimentation rate (ESR) using Westergren method, S.creatinine using ADVIA 1650 chemistry autoanalyser (Bayer Diagnostic), Rheumatoid factor (RF) by latex agglutination technique (Dade Behring), Anti nuclear antibody (ANA) and Anti-double stranded DNA autoantibodies (anti-ds DNA) were assessed by indirect immunofluorescent technique (Bio-Rad Laboratories, Redmond, USA), Complement 3 (C3) was measured by immunonephelometric method using Behring

Nepholometer Systems; BN ProSpec (Behring Diagnostics GmbH, Marburg, Germany).

-Specific Investigations Including:

- Flowcytometeric assay of $CD4^+CD25^+$ (Tregs) and FoxP3 expression for patients and controls.
- Quantitative determination of serum interleukin 10 (IL-10) and transforming growth factor-beta1 (TGF-β1) concentrations in serum samples.

Methods

- Sampling

Four ml blood volumes were withdrawn aseptically into a sterile disposable syringe from every patient and control then divided as follow: 2 ml blood were collected in sterile EDTA vacutainer tube for flowcytometry analysis. 2 ml blood were collected in sterile vacutainer tube for cytokines assessment. Sera were separated and stored in -20ºC until used.

Flowcytometeric assay of $CD4^+CD25^{high}$ (Tregs) and FoxP3

- Lymphocyte Separation

Peripheral lymphocytes cells were purified from peripheral blood by Ficoll-Hypaque gradients (Biochrom, Berlin, Germany). Separated lymphocytes were washed twice with fluorescence-activated cell sorter (FACS) washing buffer (1% phosphate-buffered saline, 2% fetal calf serum, 0.05% sodium azide, 0.5M ethylenediamine tetra-acetic acid).

- Sample Preparation and Flowcytometry Analysis

Lymphocytes staining was performed using mouse anti-human monoclonal antibodies (mAbs) (anti-CD25) Phycoerythrin (PE) and (anti-CD4) Peridinine chlorophyll protein (Per-CP) conjugate (Dako). The surface staining was done by adding 10 µl of each mAbs to 100 µl of separated lymphocytes in the same tube, incubated for 30 min in the dark at 4ºC, then, washed twice with FACS washing buffer. Finally, 0.5 ml of Phosphate buffer saline (PBS) was added on the washed cells and samples were ready for the measurement of the CD4/CD25 using a FACSCalibur flowcytometry (Becton Dickinson, San Jose, CA).

- FoxP3 Staining

FoxP3 staining was performed according to the manufacturer's protocol {FITC antihuman Foxp3 Staining Set (eBioscience)}. Cells were first stained with surface mAb of interest (anti CD4/antiCD25) followed by FoxP3 intracellular staining using permealizing solution. To avoid nonspecific Fc receptor staining, we used appropriate isotype controls of mouse anti-human mAbs.

With multigated analysis, The percentage of FoxP3 expression were determined on $CD4^+$ T-cells that are very high in $CD25^+$ to avoid contamination with other $CD4^+CD25$ low/intermediate effector T-cells. FACS-acquisition and analysis were performed immediately with FACS CellQuest software (BD Biosciences).

Cytokines Assay

Quantitative determination of sIL-10 and sTGF-β1 were done using enzyme-linked immunosorbent assay (Quantikine; R&D systems, Minneapolis, USA) according to manufacturer's recommendations. The minimum detectable limits of IL-10 and sTGF-β1 were typically less than 3.9 pg/mL and 4.61 pg/mL, respectively.

Statistical Analysis

Data were entered, checked and analyzed using SPSS program package version 10 for windows. Results were expressed as mean± standard deviation (SD), Chi-square and student-t test was used for statistical comparisons between two groups of patients' parametric data. Analysis of variance (ANOVA) and least significant difference (LSD) were done to test the difference between the different studied groups. Correlation analysis was performed with Pearson correlation test. *P*-values below 0.05 were considered significant.

Results

The results of the studied parameters were tabulated and illustrated in the following tables and figures.

The demographic, clinical and laboratory findings of the studied patients revealed that their mean ages was 29.06±5.1 years and their duration of the disease (years) ranged from 0.5-6.8 years with mean of 3.22±1.7. The mean of the SLEDAI score in patients with inactive disease was 2.15±0.4, while in patients with active disease was 9.48±2.7 (table 1).

As regard the laboratory data, ESR estimation revealed a mean value of 78.8±14.9 (mm/hr). The mean value of S. creatinine was 2.6±1.3 mg/dl. Most of the patients were positive for

ANA (28/30 93.3%). Assessments of Anti–ds-DNA Ab by indirect immunoflurescence technique revealed positive results in all patients with active disease whereas only 60% of patients with inactive disease are positive. Complement (C3) assay revealed marked decrease in the level of C3 (40.3±16.35) in SLE patients (table 1).

Table 1. Demographic and laboratory data of the studied patients.

Parameters		Mean ±SD
Age (years)		36.06±5.1
Duration of the disease (years)		3.96±0.99
Disease activity index (SLEDAI)		5.08±4.03
ESR (mm/hr)		78.8±14.9
S. Creatinine (mg/dl)		2.6±1.3
C3 (mg/dl)		40.3 ±16.35
ANA	+ve	28/30 (93.3%)
Anti–ds-DNA Ab	+ve	18/30 (60%)

In this study, T-cells expressing_$CD4^{+}CD25^{+}$ were detected in the three studied groups and by using ANOVA test for statistical analysis, we found a significant decrease in the $CD4^{+}CD25^{+}$ cells in peripheral blood in active lupus patients (mean±SD 14.97±6.6) when compared with patients with inactive lupus(15.5±5.7) and that in healthy controls (21.3±5) (F=3.99, P<0.05) with no significant difference between the active & the inactive diseased patients (P>0.05; Fig 1).

Comparison between the studied groups using ANOVA test revealed a significant decrease in the $CD4+CD25^{high}$ cells in peripheral blood in active lupus patients (mean±SD 5.9±1.9) when compared with patients with inactive lupus (6.37 ±1.93) and that in healthy controls (8.07±2.04)(F=3.56, P<0.05). No significant difference between control and the inactive diseased patients (P>0.05; Fig 2) was found.

Analysis of variance of the percentages of $CD4+CD25^{high}$ that express FoxP3 in the study groups revealed a significant decrease of these cells in active SLE patients (2.9±1.05) when compared with those with inactive SLE patients (3.5±0.8) and control groups (4.7±1.2) (F=8.26, P<0.001, Fig 3).

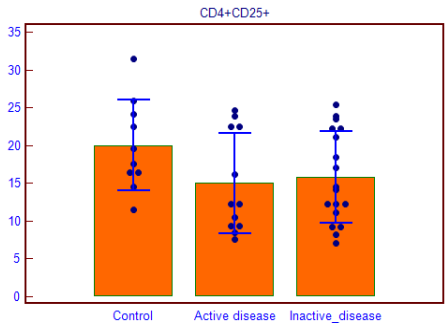

Figure 1. Comparison of the percentages $CD4^{+}CD25^{+}$between the studied groups

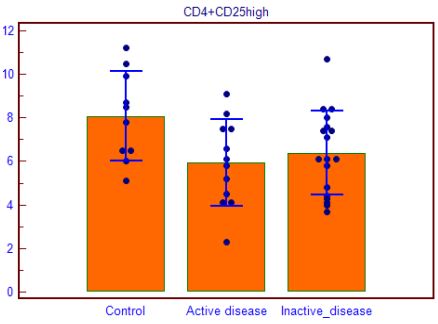

Figure 2. Comparison of the percentages of $CD4^{+}CD25^{high}$ between the studied groups.

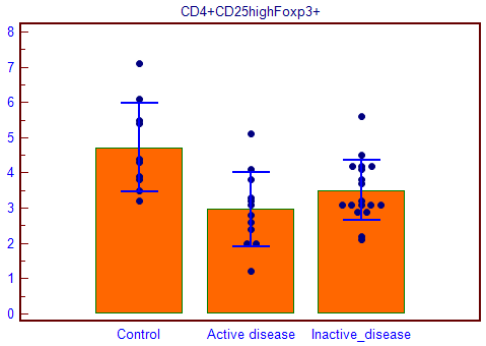

Figure 3. Comparison of the percentages of Foxp3 expression on Tregs in the study groups.

Level of IL-10 was significantly increased in patients with active and inactive disease (158.8±50.8, 82.8±14.08 respectively), when compared with the control group (18.2±7.4) (F=77.96, *P*<0.001) as that raising is enhanced with disease activity, whereas the level of TGF-β was significantly decreased in patients with active and inactive disease (22.5±7.03, 29.07±10.1, respectively), when compared with the control group (32.5±5.7) (F=4.14, *P*<0.05; Table 2)

Table 2. Comparison of the level of cytokines secreted by T-cells in the study groups.

	Patients with active disease (n=12)	Patients with inactive disease (n=18)	Control (n=10)	*P*
IL-10 pg/ml	158.8±50.8 (65.2-224)	82.8 ± 14.08 (65-103)	18.2±7.4 (6.5-31.4)	<0.001
TGF-β pg/ml	22.5±7.03 (14.4-34.2)	29.07±10.14 (15-46)	32.5±5.7 (25-41)	<0.05

P<0.05 is significant

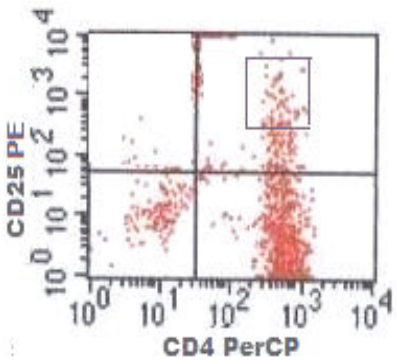

Figure 4. Dotplot analysis of a case of SLE with active disease showing coexpression of CD4+CD25^{high}

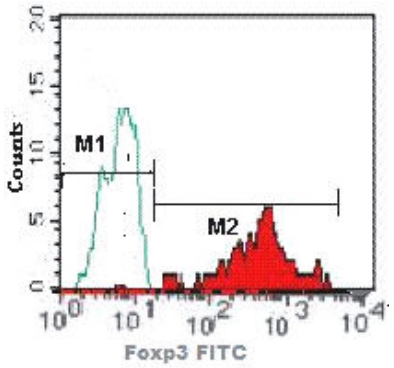

Figure 5. Histogram analysis of FoxP3 positive in a case of SLE with active disease.

Correlation between the percentage of CD4+CD25^{high} FoxP3 Tregs and the studied clinical and laboratory parameters in lupus patients during disease activity showing no significant correlation were found between the Tregs cells expressing Fox in SLE patients and the age, duration of illness, ESR, ANA and ds-DNA. On the other hand, there is a significant positive correlation with C3 (r =0.42, P<0.01; Figures 6 & 7). While, a significant negative correlation between FoxP3 and SLEDAI was found (r =-0.45 P<0.001).

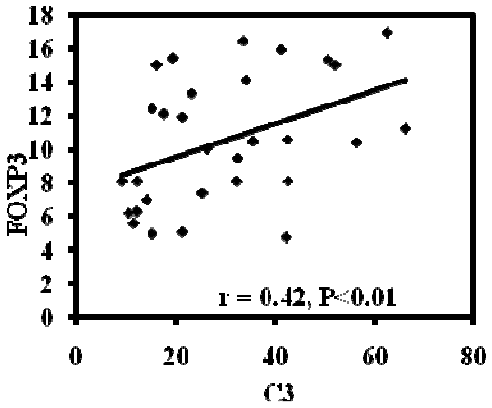

Figure 6. A significant positive correlation between C3and FoxP3. (r= 0.42, P <0.001).

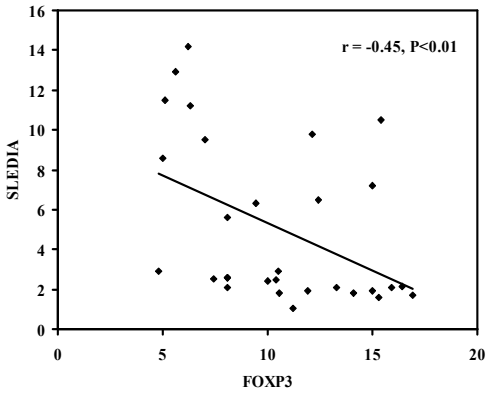

Figure 7. A significant negative correlation between FoxP3 and SLEDIA. (r= 0.45, P <0.001).

Discussion

The immune system is unresponsive to the body's own components and circulating constituents due to self-tolerance (Akiko & Ciriaco, 2006). CD4^{+}CD25^{high}Tregs are the major subset of T-cells which suppress activation of other immune cells and thereby maintain immune system homeostasis, self-tolerance as well as control of the excessive response to foreign antigens (Le & Chao, 2007). Thus, the precise delineations of the behavior of CD4^{+}CD25^{high} Tregs in autoimmune diseases is therefore of great importance for the understanding of the pathogenesis of such diseases. Moreover, the ability to manage such regulatory mechanisms might provide novel therapeutic opportunities in autoimmune diseases (Leipe *et al.,* 2005).

In the current study, there was significant decrease in the percentage of CD4^{+}CD25^{high} T cells in patients with active SLE compared with healthy controls. Also, a significant decrease in the percentage of CD4^{+}CD25^{high} T cells expressions was present in patients with active SLE when compared with inactive SLE and healthy controls. These results were in agreement with that reported by (A La Cave, 2008) who found that the number of circulating Treg cells was decreased during active disease, and that the extent of such decrease was positively correlated with severity of the disease. Miyara *et al.* (2005) attributed the decreased percentage of CD4^{+}CD25^{high} Tregs to the increased hypersensitivity of these cells to fas-mediated cell death. They suggested that global

depletion of Tregs occur secondary to the enhanced apoptosis that follows massive exposure to self-antigens.

The latest research suggests that regulatory T cells (Treg) are defined by expression of the forkhead family transcription factor FoxP3 (forkhead box p3). Expression of FoxP3 is required for regulatory T cell development and appears to control a genetic program specifying this cell fate (Lin *et al.,* 2007) and is sufficient to induce a Treg phenotype in conventional $CD4^+CD25^-$T cells (Marson *et a*l., 2007)

In this study, FoxP3 has been shown to be expressed on $CD4^+$ $CD25^{high}$ only, Fontenot *et al.* (2003) reported that FoxP3 is a unique marker of $CD4^+CD25^+$ regulatory T cells, distinguishing them from activated $CD4^+CD25^+$T cells and demonstrated that Foxp3 is specifically required for the thymic development of $CD4^+CD25^+$ regulatory T cells. Miyara *et al.* (2005) found that no $CD4^+CD25^-$cells expressed FoxP3 and they determined by this approach that, on average, 86.5% of the $CD4^+CD25^{high}$ cells were $FoxP3^+$. Interestingly, Real-time quantitative polymerase chain reaction (PCR) analysis revealed that FoxP3 mRNA levels were increased ~40-fold in freshly isolated $CD4^+CD25^+$ T cells when compared with $CD4^+CD25^-$T cells (Fontenot *et al.,* 2003).

In this study, the percent of T cells that expressed $CD4^+CD25^{high}$FoxP3 was found to be decreased in active SLE patients when compared with both the inactive SLE patients and control groups. In agreement with our data, Miyara *et al.* (2005) and Lee *et al.* (2006) reported that the levels of Tregs cells are indeed reduced during SLE flares. Also, Fontenot *et al.* (2003) who found that, the lethal autoimmune syndrome observed in Foxp3-mutant and/or Foxp3-null patients results from a $CD4^+CD25^+$ regulatory T cell deficiency and not from a cell-intrinsic defect of $CD4^+CD25^-$T cells.

Walker *et al.* (2003) demonstrated that the expression of FoxP3 in $CD4^+$ $CD25^+$Tcells correlates with their ability to function as Treg cells. Interestingly, $CD4^+CD25^+$ T cells are generated as a consequence of stimulation of $CD4^+CD25^-$ human T cells, which express FoxP3 and then, acquire Treg function. Furthermore, they suggest that *de novo* generation of Treg cells is a *de novo* natural consequence of immune responses in humans.

As regard the expression of interleukins in SLE patients, our results revealed that there was a significant increase of IL-10 in active SLE patients when compared with inactive and control groups, these results were in agreement with that statement by Dean *et al.* (2000) who reported that serum IL-10 levels are higher in patients with SLE when compared with control groups.

Bach and Francois, (2003) reported that $CD4^+CD25^+$ Tregs potently inhibit T cell proliferation *in vitro* and suppress the activity of autoreactive T cells *in vivo*. The mechanism by which $CD4^+CD25^+$ Tregs suppress other T cell responses is controversial but requires cell-cell contact *in vitro* and may involve cytokines such as IL-10 and TGF-β *in vivo*. Therefore, that raise in IL-10 level is mainly attributable in SLE patients to an increase production by monocytes, a subset of B cells and possibly $CD4^+CD45RO^+$ memory T cells. Gomez *et al.* (2004) found that serum titres of IL-10 in SLE patients are positively correlated with anti-ds DNA antibody titres and the SLEDAI score and negatively correlated with complement C3 levels. IL-10 increases IgG production by B-cells from patients with SLE. Therefore, IL-10 influences the autoimmune response (autoantibody production) (Stichweh *et al.,* 2004).

Remarkably, our results showed that TGF-β1 levels were significantly decreased in SLE patients when compared to normal controls. Wang *et al.* (2007) have reported that TGF-β1

is a crucial factor during the clearance process of apoptotic cells by macrophages and has suggested that deficiency of TGF-β1 may contributes to a reduced rate of phagocytosis of macrophages, as well as the development of auto antibodies. So, TGF-β1 can be a relevant factor in the pathogenesis of SLE and other systemic rheumatic diseases.

In agreement with our results, Ohtsuka *et al.* (1999) reported that the levels of TGF-β1 are lower in patients with SLE and this is probably because of the high levels of IL-10 that suppress TGF-β1 production by NK cells.

In addition, TGF-β1 plays a role in the conversion of a similar subset of $CD4^+CD25^-$ T cells to the regulatory $CD4^+CD25^+$T cells (Yamagiwa *et al.,* 2001). The point of interest here is that TGF-β, a factor known to be a negative regulator of $CD4^+CD25^-$ T cells, is conversely, a positive regulator of $CD4^+CD25^+$ regulatory T cell pool *in vivo*. Usually, TGF-β is best known for its ability to inhibit rather than promote T cell proliferation. One exception comes from the work of Peng *et al.* (2004), indicating that in the presence of sufficient IL-2 to counteract its suppressive effects on T cell proliferation, TGF-β1 showed positive effects on the proliferation and survival of Treg cells that develop potent suppressive activity.

In this study, there was no impact of the percentage of $CD4^+CD25^{high}$ Tregs in active SLE patients and other parameters as age, duration of illness, creatinine and ESR. This was evidenced by the non significant correlation found between the percentages of these cells in the lupus patients compare with these parameters when studied during disease activity. Similar findings were reported in pediatric (Lee *et al.,* 2006) and adult lupus patients (Barâth *et al.,* 2007).

Significant negative Correlations were found between $CD4^+CD25^{high}$FoxP3 and SLEDAI, this goes hand in hand with Lin *et al.* (2007) who found that FoxP3 expression in circulating $CD4^+CD25^+$Treg cells derived from SLE patients inversely correlates with disease activity. This implies functional defects in regulatory T cells that may potentially contribute to SLE immunopathogenesis.

In our study, C3 shows positive correlation with $CD4^+CD25^{high}$FoxP3 cells, a similar result was detected by Bijl *et al.* (2006) who reported that decreased Treg subpopulations were associated with lower plasma levels of complement C3 and C4 in patients with SLE.

Recently, Treg cells are isolated and can be expanded *in vitro* and *in vivo*, and their role is the subject of intensive investigation, particularly on the possible Treg cell therapy for various immune-mediated diseases. A growing body of evidence has demonstrated that Treg cells can prevent or even cure a wide range of diseases, including tumor, allergic, autoimmune diseases, transplant rejection and graft-versus-host disease (Tai-You HA, 2008).

In conclusion, decreased frequency of $CD4^+CD25^{high}$ in $CD4^+$ cells and decreased expression of FoxP3 on Treg cells may be related to the immunodysregulation of SLE. We highlight on the reduced level of Treg cells in patients with SLE, which may be related to the suppression of TGF-β1 production and the enhanced production of IL-10 and its effect on the Foxp3 expression.

The presence of documented results show deficient $CD4^+D25^{high}$Tregs can be used in determined the disease status, whether active or inactive, and to determine the clinical effectiveness of novel therapy to modulate Treg *in vivo*.

A greater effort should focus on studying the influence of current and new drugs used in SLE on the expression of FoxP3. This could lead to improved targeted therapies and to the discovery of several new compounds to be used for Treg function modulation *in vivo*.

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