CD4+CD25-Foxp3+T Cells as a Novel Biomarker for Lupus Nephritis

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Abstract: **Background*:*** Systemic lupus erythematous is a prototypic autoimmune disease that is known to affect virtually every organ system in the body. One of the striking manifestations of SLE is renal affection which may lead to end stage renal disease with marked affection of quality of life of many young patients primarily targeted by such disease. **Objective*:*** The aim of this study is to analyze the presence of CD4+CD25-Foxp3+ T cells in systemic lupus erythematous patients with and without nephritis. **Materials and Methods:** Our case control study aimed at quantitatively analyzing proportions and absolute cell numbers of $CD4^{+}CD25^{-}Foxp3^{+}$Treg cells by flow cytometry and the correlation between results, renal disease and systemic lupus erythematous disease activity index score was documented. Eighty patients were enrolled; of which 30 patients underwent renal biopsy for suspected lupus nephritis and peripheral blood samples were taken at the time of the biopsy and analyzed for proportions and absolute cell numbers of $CD4^{+}CD25^{-}Foxp3^{+}$ Tregcells in a standardized way. The activity of lupus nephritis was classified according to the renal histology. Thirty SLE patients without nephritis and twenty healthy volunteers were investigated for comparative purpose. **Results*:*** Increased proportions of CD4+CD25-Foxp3+ T cells in SLE patients with renal involvement. The sensitivity of proportions of CD4+CD25-Foxp3+ T cells was 100% and the specificity was 60%, and the predictive values for renal disease were 71.43% and 100% for positive and negative predictive values respectively with a predictive accuracy of 80.00%. The proportions of CD4+CD25-Foxp3+ T cells were highest in patients with class IV lupus nephritis. **Conclusions*:*** Comparative analysis of CD4+CD25-Foxp3+ T cells in SLE patients revealed a significant association of this cell population with active nephritis. Therefore CD4+CD25-Foxp3+ T cells may be used to monitor and follow activity of lupus nephritis patients.

[Soha Abdel Fattah Shalaby, Nesreen Ahmed Kotb, Gamal Fathy Elnagar, Hala Mohamed Nagy, Eiman Adel Hasby. **CD4+CD25-Foxp3+T Cells as a Novel Biomarker for Lupus Nephritis.** *Nat Sci* 2019;17(3):92-105]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 14. doi:10.7537/marsnsj170319.14.

Keywords: Biomarker, lupus, nephritis, T cells

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic, autoimmune inflammatory disease that may target any system in the body and is characterized by circulating immune complex, autoantibodies and various clinical manifestations. (1)

Renal affection is one of the manifestations that portend poor prognosis for lupus patients. It may remain silent for years or may manifest as the sole manifestation of the disease with full blown nephritic syndrome and may even progress to end stage renal disease (ESRD). (2)

Immune tolerance relies on a keeping balance between tolerance to self-antigens and generating immune response to invading pathogens. (3)

T regulatory (Treg) cells are a subset of T cells that immunomodulate and prevent the development of uncontrolled immune responses against foreign pathogens or allergens, stabilize normal microflora and have an important role in tumoursurveillance. Treg cells are also implicated in the development of cancer, autoimmunity, allergy and asthma. (4)

Regulatory T cells (Treg) constitute on average 1 to 2% of human peripheral blood mononuclear cells (PBMC). Treg play an important role in T cell homeostasis and are critical regulators of immune tolerance. (5) Genetic or environmental insults can provoke a developmental or functional deficiency in nTreg cells, alter the delicate balance between nTreg/autoreactive T cells, and ultimately provoke autoimmunity. (5)

In the present study we analyzed the presence of CD4+CD25-Foxp3+ T cells in systemic lupus erythematous patients with and without nephritis.

2. Patients and Methods

Our study included 60 patients with newly diagnosed SLE based on four or more American College of Rheumatology criteria for SLE (6) and 20 healthy volunteers as a control. Patients were recruited From Tanta University Hospitals Nephrology and

Rheumatology outpatient clinics and inpatient wards from December 2014 to December 2015. Ethical approval for this study was granted by the local ethics committee of the Tanta University Faculty of Medicine (Approval code: 2883/11/14) and conducted in accordance with the Declaration of Helsinki. Patients gave written informed consent to participate in the study and agreed that the findings of the study will be published in a scientific journal.

Patients were further subdivided into:

Group I: 30 adult SLE patients with a mean age of (26.00±6.23 years) with nephritis who had undergone renal biopsy. Clinical nephritis was suspected if urinalysis showed proteinuria >0.5 g on a 24-hour urine collection and/or hematuria or cellular casts with or without increased serum creatinine. The findings of renal biopsies were classified according to the World Health Organization ISN/RPS (7) and National Institutes of Health (NIH) activity and chronicity indices. (8) So we have two subgroups:

IA: with proliferative lupus nephritis, namely class (III and IV).

IB: with non-proliferative lupus nephritis, namely class (I, II, VI).

Group II: 30 SLE patients without nephritis, with the following selection criteria:

a. Absence of active urine sediment (> 5 erythrocytes per high power field) or casts.

b. Proteinuria of non-nephrotic range (< 0.5 g/24 hours).

c. Creatinine (for male <1.3 mg/dl, for female <1.1 mg/dl).

They were further subdivided according to SLEDAI score into:

IIA: active lupus non nephritis with affection of systems other than kidneys and,

IIB: inactive lupus.

Group III: Age and sex matched 20 healthy volunteers as a control group.

Full history has been taken particularly for presence of; butterfly rash, discoid rash, photosensitivity, oral ulcers, hair loss, peripheral edema, arthritis, fever, CNS affection and hypertension, Calculation of SLEDAI score: Patients with SLEDAI score less than 6 were considered clinically inactive; patients with score 6- 11 were considered to have mild to moderate disease activity while patients with score 12 or more were considered to have severe disease activity. (9, 10) Lupus rash, discoid lupus or photosensitivity denote active skin involvement; arthritis with synovial swelling denotes active joint involvement; thrombocytopenia, lymphocytopenia, or leukocytopenia or anemia implicate active hematologic involvement. Active renal involvement is defined as nephritis with proteinuria >0.5 g protein/24 h and/or active nephritic sediment. (11)

Calculation of the renal SLEDAI (rSLEDAI) score: consists of the four kidney related items, including hematuria (> 5 RBCs / HPF), pyuria (> 5 WBCs /HPF), proteinuria (> 0.5 gm/24 h) and urinary casts. The presence of each of the parameters is scored as 4 points; the renal activity score can therefore range from 0 to 16. (12) Patients with rSLEDAI ≥ 8 (i.e., at least 2 abnormal results for renal parameters) or when proteinuria was the renal related criterion were considered active. (12)

In addition the candidates were subjected to full blood count, serum albumin, urine analysis to check for protein, pus cells, RBCs, and cellular casts, with midstream urine culture and sensitivity to exclude infection when indicated, urinary 24 hour collection for quantitative assessment of proteinuria, Serum creatinine, blood urea, ESR, ANA, Anti-ds DNA, serum complement c3 & c4 and virology. Radiological investigations included pelvi-abdominal ultrasound for assessment of renal size and echogenicity bilaterally.

Renal Biopsy was done for patients suspected for Lupus nephritis with histopathological examination and grading and determination of activity and chronicity indices after staining by haematoxylin and eosin (H & E) and Masson trichome.

Flowcytometery assessment of CD4+ CD25 – Foxp3+ T- regulatory cells: This kit contains three conjugated antibodies (and a rabbit IgG-PE isotype control) that can be used for the single-step staining of human regulatory T cells.

Detection of FoxP3 in Human Blood Natural Tregs by Flow Cytometry

Human peripheral blood natural regulatory T cells (Tregs) were surface stained with (A) Mouse Anti-Human CD4 Fluorescein-conjugated Monoclonal Antibody (R & D Systems®, Catalog # FAB3791F) and (B) Mouse Anti-Human IL-2 Rα/CD25 APC-conjugated Monoclonal Antibody (R & D Systems®, Catalog # FAB1020A), followed by intracellular staining using Rabbit Anti-Human/ Mouse FoxP3 PE-conjugated Antigen Affinity-purified Monoclonal Antibody (R & D Systems®, Catalog # IC8214P). To facilitate intracellular staining, cells were fixed and permeabilized with FlowX FoxP3 Fixation & Permeabilization Buffer Kit (R & D Systems®, Catalog # FC012). Cells were gated on lymphocytes.

A- Staining Protocol

1. 50µl human blood cells (1 x 106 cells per sample) were washed with 2 mL of Flow Cytometry Staining Buffer (R & D Systems®, Catalog # FC001), by spinning at 300 x g for 5 minutes, using 5 mL flow cytometry tubes.

2. 5 µL of CD4-FITC and 5 µL of CD25-APC antibodies were added to each blood cell tubes.

3. 5 µL of isotype control were added to negative control tube.

4. The mixture was incubated for 30-45 minutes at room temperature **in the dark**.

5. The cells were washed two times with **cold** (1X) PBS. During the washes, fresh (1X) FoxP3 Fixation Buffer were made up by diluting FoxP3 Fixation Concentrate (4X) with FoxP3 Fixation Diluent (*i.e.* 100 µL FoxP3 Fixation Concentrate (4X) + 300 µL FoxP3 Fixation Diluent).

6. The cells were re-suspended in fresh (1X) FoxP3 Fixation Buffer using 0.5 mL/tube, incubated at room temperature for 30 minutes. During this incubation, (1X) FoxP3 Permeabilization and Wash Buffer was made by diluting FoxP3 Permeabilization and Wash Buffer (10X) with distilled water (*i.e.* 100 µL FoxP3 Permeabilization and Wash Buffer (10X) + 900 µL diH_2O) and kept at room temperature.

7. Cells were washed two times with fresh, cold, (1X) FoxP3 Permeabilization and Wash Buffer.

8. 5 µL of FoxP3 antibody or the rabbit IgG-PE isotype control included in this kit were Added to the cells and incubated for 30 minutes at room temperature.

9. The cells were washed once with cold (1X) FoxP3 Permeabilization and Wash Buffer.

10. The cells were resuspend in Flow Cytometry Staining Buffer and acquisition on a flow cytometer was done.

B) Flow Cytometric Analysis

1- fluorescence-activated cell sorter (FACS) caliber flow cytometry from Becton Dickinson was used for analysis.

2-Automated cell quest software was used for data acquisition and analysis.

3- The instrument settings were set by using calibrated beads provided by the manufacturer.

4- Isotopic quality control was used to exclude non-specific binding and auto-fluorescence.

5- 20.000 events were acquired.

6-Lymphocytes were gated according to the forward and the side scattering light properties. In addition gated $CD4^+$ cells were analyzed for the expression of CD25 and Foxp3. Proportions of $CD25^-$ $Foxp3^+$ cells within the gated $CD4^+$ cells are shown. Absolute numbers of cells were calculated from whole white blood counts obtained from routine laboratory testing.

7-Dot blot was set as FL1 and FL2 markers which represents $CD4^+$ (FITC) and CD25- (APC) respectively.

8-Fluorescence markers were set around the negative population that appeared as the cluster of events that were low in both: yellow-green (FITC) and red-orange (PE) fluorescence.

The collected data were organized, tabulated and statistically analyzed using SPSS software (Statistical Package for the Social Sciences, version 19, SPSS Inc. Chicago, IL, USA). For quantitative data, the range, mean, standard deviation and median were calculated. Boxplots were performed to illustrate lower and upper limits of the range, median, first and third quartiles of the quantitative data. For qualitative data, which describe a categorical set of data by frequency, percentage or proportion of each category, comparison between two groups and more was done using Chi-square test (χ^2 test). For comparison between means of two groups of parametric data of independent samples, student t-test was used. For comparison between means of two groups of non-parametric data of independent samples, Z value of Mann-whitney test was used. For comparison between more than two means of parametric data, F value of ANOVA test was calculated, where scheffe test was performed to compare between each two means if F value was significant. For comparison between more than two means of non-parametric data, Kruskal-Wallis (χ^2 value) was calculated. Correlation between variables was evaluated using Pearson's correlation coefficient (r). (13)

To predict the presence or absence of nephritis among patients with Systemic Lupus Erythematosis (SLE) based on a set of predictor variables, where the dependent variable is dichotomous, Binary logistic Regression was done to estimate Odds ratios (EXP (B)) for each of the independent variables as predictor and risk factor for nephritis. The Receiver Operating Characteristic (ROC) curve: The area under the ROC curve was illustrated to measure how well proportions of CD4+CD25-Foxp3+ T cells marker has sensitivity of diagnosing nephritis among SLE patients. Cut off value of the marker was calculated from the ROC curve. Sensitivity, Specificity, positive and negative predictive values and accuracy of proportions of CD4+CD25-Foxp3+T cells marker were calculated, where the cut off value was calculated from the estimated ROC curve cut off values (3.90). Significance was adopted at $p<0.05$ for interpretation of results of tests of significance (r). (13)

3. Results

Basic demographic data obtained for patients involved in this study are outlined in Table (1).

As shown in figure (1), Anti-dsDNA was higher in patients with lupus nephritis (group I) with mean of (259.33±184.03) (U/ml), in comparison to SLE patients without nephritis (group II) (mean= 140.60±80.49) (U/ml). Regarding ANA values, there

was no statistical significance between the two groups (p= 0.174).

Table (1): Age and sex of the studied patients with systemic lupus erythematosus (SLE) (with and without nephritis) and the control group (n=80).

Variables	The studied subjects (n=80)						χ^2	P
	Patients with SLE (n=60)				Control group			
	With nephritis (n=30)		Without nephritis (n=30)		(n=20)			
	N	%	n	%	n	%		
Sex:								
Males	4	13.3	3	10.0	7	35.0	5.772	0.056
Females	26	86.7	27	90.0	13	65.0		
Age years:								
Range	17-37		16-43		18-42			
Mean±SD	26.00±6.23		26.13±7.12		26.85±7.87			
Median	26.00		25.00		25.00			
F value	0.097							
P	0.908							

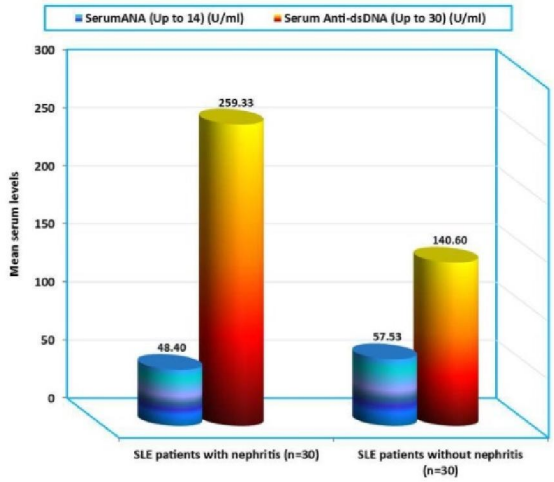

Figure (1): Serum Anti-nuclear-antibody (ANA) and Anti-ds DNA antibodies among the studied patients with systemic lupus erythematous (SLE) (with and without nephritis) (n=60).

As shown in figure (2), C4 show more consumption in patients with lupus nephritis (group I) compared to SLE patients without nephritis (group II), with statistical significance between the two groups (p= 0.008*). Regarding C3 there was no statistical significance between the two groups (P= 0.594).

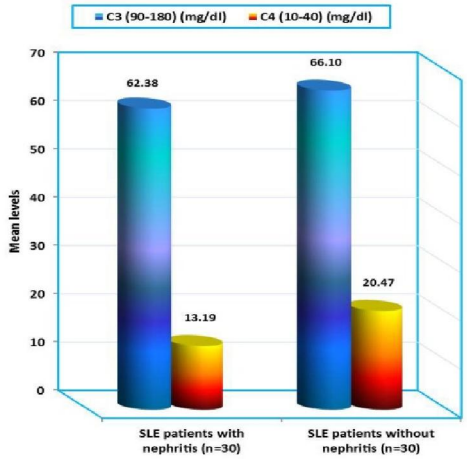

Figure (2): Serum Complement C3 and C4 among the studied patients with systemic lupus erythematosus (SLE) (with and without nephritis) (n=60).

Table (2) illustrate activity of lupus as calculated from SLEDAI (p= 0.0001*) and rSLEDAI (p= 0.0001*), with both scores being higher in patients with lupus nephritis (group I) compared to SLE patients without nephritis (group II). Mean SLEDAI for active lupus nephritis (43.53±13.39) and for active other organ affection (28.25±15.17) with statistical significance between the two groups (P= 0.006*). Also rSLEDAI show statistical significance between (group IA) active lupus nephritis and (group IIA)

active other organ affection (P= 0.0001*) as shown in table (3).

Table (2): SLEDAI and rSLEDAI scores among the studied patients with systemic lupus erythematosus (SLE) (with and without nephritis) (n=60).

Variables	The studied patients with SLE (n=60)				χ^2	P
	With nephritis (n=30)		Without nephritis (n=30)			
	n	%	n	%		
SLEDAI score:						
No or mild activity	0	0	18	60.0	114.286	0.0001*
Active	30	100	12	40.0		
Range	13.00-65.00		4.00-55.00			
Mean±SD	37.37±14.89		16.73±13.45			
Median	37.00		11.00			
t-test	5.631					
P	0.0001*					
rSLEDAI score:						
Inactive (<8)	0	0	30	100	60.000	0.0001*
Active (≥8)	30	100	0	0		
Range	8.00-16.00		0-4.00			
Mean±SD	12.53±2.92		2.67±1.92			
Median	12.00		4.00			
t-test	15.465					
P	0.0001*					

Table (3): SLEDAI and rSLEDAI scores among the studied patients (active nephritis vs active other organ affection) (n=60).

Variables	The studied patients with SLE (n=60)				t-test P	
	SLE patients With nephritis (n=30)		SLE patients Without nephritis (n=30)			
	Active (n=19)	Inactive (n=11)	Active (n=12)	Inactive (n=18)	Active	Not active
SLEDAI score:						
Range	21-65	13-52	12-55	4-11	2.940	6.647
Mean±SD	43.53±13.39	26.73±11.13	28.25±15.17	9.05±1.95	0.006*	0.0001*
t-test	3.511		5.350			
P	0.002*		0.0001*			
rSLEDAI score:						
Range	8-16	8-16	0-4	0-4	11.850	9.781
Mean±SD	13.47±2.73	10.91±2.59	2.67±1.97	2.67±1.94	0.0001*	0.0001*
t-test	2.522		0.000			
P	0.018*		1.000			

Renal biopsy classes, activity and chronicity indices are shown in figure (3-6).

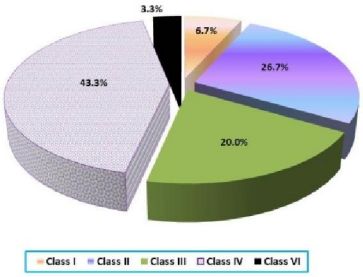

Figure (3): Renal biopsy class among the studied patients with systemic lupus erythematosus (SLE) with nephritis (n=30).

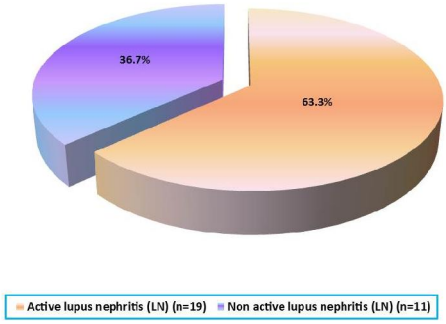

Figure (4): SLE patients with nephritis (active and inactive) ((n=30).

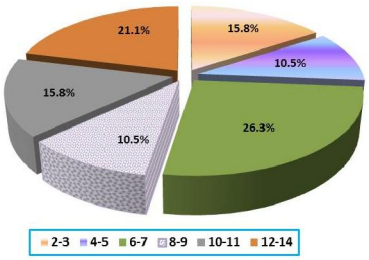

Figure (5): Activity/24 among the studied systemic lupus erythematosus (SLE) patients with active nephritis (n=19).

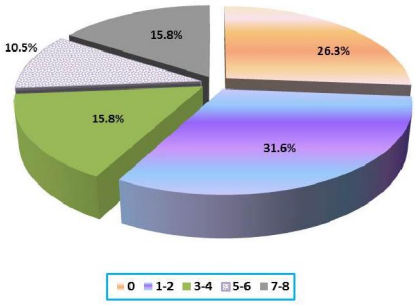

Figure (6 lupus erythematosus (SLE) patients with active nephritis (n=19).

Table (4): Proportions and absolute number of CD4+CD25-FOXp3+T cells among the studied patients with systemic lupus erythematosus (SLE) (with and without nephritis) and the control group (n=80).

Variables	The studied subjects (n=80)		
	Patients with SLE (n=60)		Control group
	With nephritis (n=30) G1	Without nephritis (n=30) GII	(n=20) GIII
Proportions of CD4+CD25-Foxp3+T cells (%):			
Range	4.00-8.80	2.10-7.20	0.70-2.00
Mean±SD	7.06±1.31	4.17±1.10	1.11±0.41
Median	7.45	4.00	0.95
F value	187.966		
P	0.0001*		
Scheffe test	I vs II, P=0.0001*		
P	I vs III, P=0.0001*		
	II vs III, P=0.0001*		
Absolute number of CD4+CD25-Foxp3+T cells :			
Range	16.00-106.00	10.00-65.00	10.05-62.64
Mean±SD	56.00±25.23	36.00±13.16	22.00±12.60
Median	47.00	36.00	19.24
χ^2 value	20.352		
P	0.0001*		
Z test	I vs II, P=0.0001*		
P	I vs III, P=0.0001*		
	II vs III, P=0.054		

Table (4) and illustrates increased proportions of CD4+CD25-Foxp3+ T cells in SLE patients with nephritis (group I) as compared to SLE patients without nephritis (group II) and healthy controls with high statistical significance among groups (P <0.0001).

Analysis of subgroups revealed that proportions of CD25−Foxp3+ within the gated CD4+ cells were significantly increased in (group IA), active lupus nephritis as compared to (group IIA) with active other system affection as shown in table (5). There is no statistically significant difference between active and inactive lupus nephritis as regards absolute CD4+CD25-Foxp3+ T cells count, suggesting that propotions are much more important than absolute cell number as a marker of active kidney involvement.

Table (5): Proportions and absolute number of CD4+CD25-Foxp3+T cells in the studied patients (active lupus nephritis Vs active other organ involvement)

Variables	The studied patients with SLE (n=60)				t-test P	
	SLE patients With nephritis (n=30)		SLE patients Without nephritis (n=30)			
	Active (n=19)	Not active (n=11)	Active (n=12)	Not active (n=18)	Active	Not active
Proportions of CD4+CD25-Foxp3+T cells (%):						
Range	6.60-8.80	4.00-7.50	2.50-7.20	2.10-6.60	9.682	4.509
Mean±SD	7.82±0.68	5.74±1.04	4.40±1.29	4.02±0.96	0.0001*	0.0001*
t-test	6.645		0.919			
P	0.0001*		0.366			
Absolute number of CD4+CD25-Foxp3+T cells:					Z value P	
Range	16.00-106.00	24.33-100.50	10.40-65.00	14.74-63.05	1.916	3.615
Mean±SD	53.00±25.47	59.00±25.56	38.00±15.28	34.00±11.79	0.065	0.001*
Z value	0.615		0.709			
P	0.543		0.484			

***Significant (P<0.05)**
Z value of Mann-Whitney U test

Table (6) shows comparison between patients with active LN (WHO class III and IV) and patients with inactive lupus nephritis (WHO class I, II and VI) regarding all studied parameters demonstrating that antidsDNA (P= 0.0001*), proteinuria (P= 0.0001*) and proportions of CD25−Foxp3+ cells within the gated CD4+ cells (P= 0.0001*) show the highest statistical significance.

Figure (7) shows Area under Receiver Operation Characteristic (ROC) curve denoting that proportions of CD4+CD25-Foxp3+T cells are more sensitive than absolute number for diagnosis of **nephritis** among SLE patients.

Table (6):): Proportions and absolute number of CD4+CD25-FOXp3+T cells among the studied systemic lupus erythematosus (SLE) patients with nephritis in relation to renal biopsy classes (n=30).

Variables	Renal biopsy classes among the studied SLE patients with nephritis (n=30)	
	With inactive nephritis (Class I & II & VI) (n=11)	With active nephritis (Class III & IV) (n=19)
Proportions of CD4+CD25-Foxp3+T cells (%):		
Range	4.00-7.50	6.60-8.80
Mean±SD	5.74±1.04	7.82±0.68
Median	6.00	7.80
t-test	6.645	
P	0.0001*	
Absolute number of CD4+CD25-Foxp3+T cells :		
Range	24.00-100.00	16.00-106.00
Mean±SD	59.00±25.56	53.00±25.47
Median	63.00	44.00
Z value	0.615	
P	0.543	

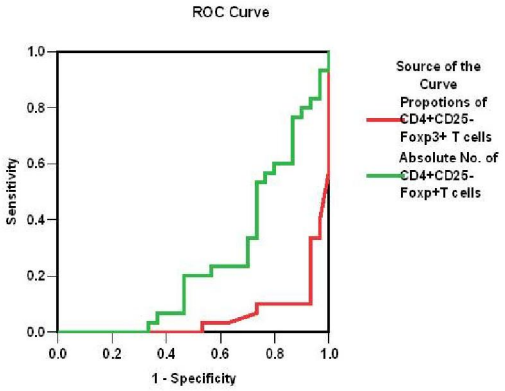

Figure (7): ROC curve denoting sensitivity of proportion and absolute number of CD4+CD25-Foxp3+T cells for diagnosis of nephritis among SLE patients.

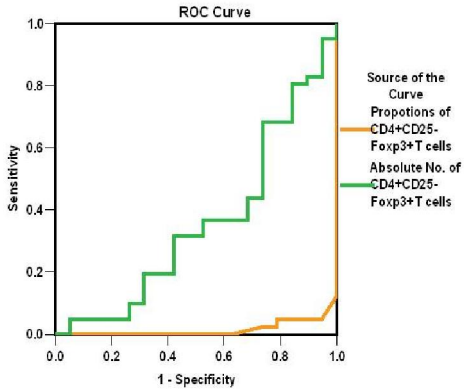

Figure (8): Area under ROC curve denoting sensitivity of proportion and absolute number of CD4+CD25-Foxp3+T cells for diagnosis of active nephritis among SLE patients.

Figure (8) shows Area under Receiver Operation Characteristic (ROC) curve denoting that proportions of CD4+CD25-Foxp3+T cells are more sensitive than absolute number for diagnosis of **activenephritis** among SLE patients.

Table (7) shows sensitivity, specificity of proportions of CD4+CD25-Foxp3+ T as diagnostic of nephritis among SLE patients. Cut off value of the marker was (3.90) as calculated from the ROC curve.

Table (7): Sensitivity, specificity, positive prediction, negative prediction and accuracy of proportions of CD4+CD25-Foxp3+ T cells as diagnostic of nephritis among SLE patients.

Parameter	Sensitivity	Specificity	+ve prediction	-ve prediction	Accuracy	Cut off Value
Proportion of CD4+CD25-Foxp3+T cells	100%	60.00%	71.43%	100%	80.00%	3.90

Flow Cytometry Results

Figures (9-20) illustrate representative FACS-plot of peripheral blood stained for the expression of CD25- Foxp3 + on gated CD4+ cells from sample of the studied population.

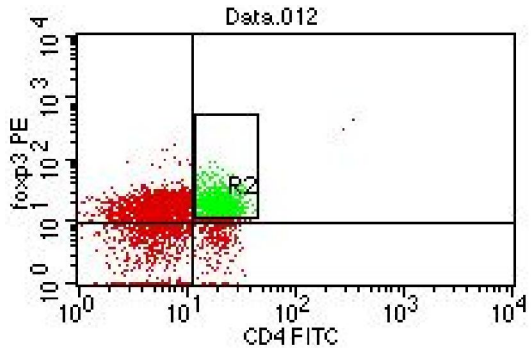

Figure (9): Histogram of a patient with active lupus nephritis (group IA) showing (CD4+Foxp3+) = 8%.

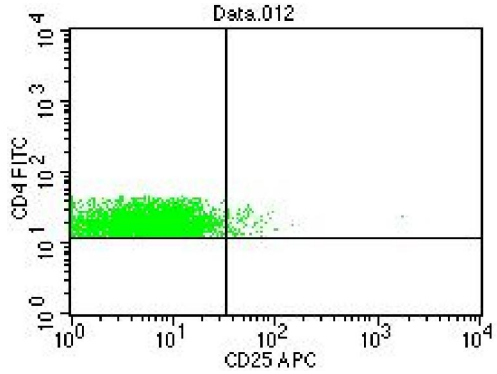

Figure (10): Histogram of a patient with active lupus nephritis (group IA) showing (CD25⁻) of CD4+ FOXP3+ = 8%.

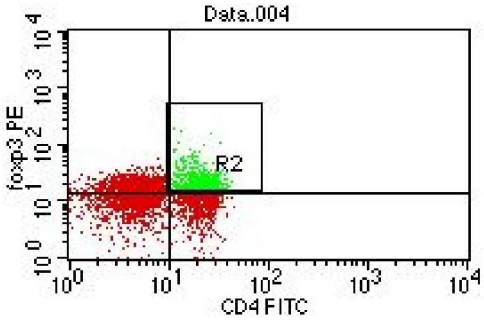

Figure (11): Histogram of a patient with active lupus nephritis (group IA) showing CD4+ FOXP3+ = 7.4%.

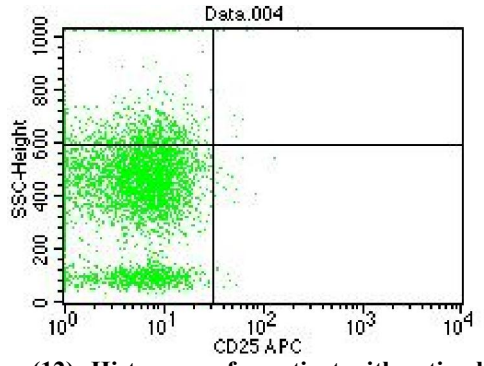

Figure (12): Histogram of a patient with active lupus nephritis (group IA) showing (CD25⁻) of CD4+ FOXP3+ = 7.4%.

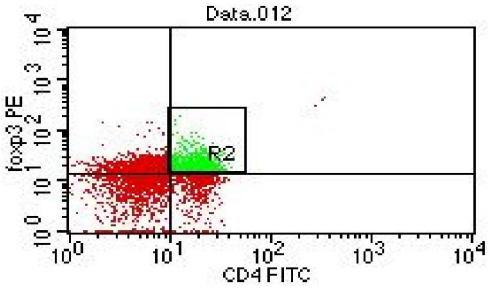

Figure (13): Histogram of a patient with inactive lupus nephritis (group IB) showing CD4+ FOXP3+ = 6.6%.

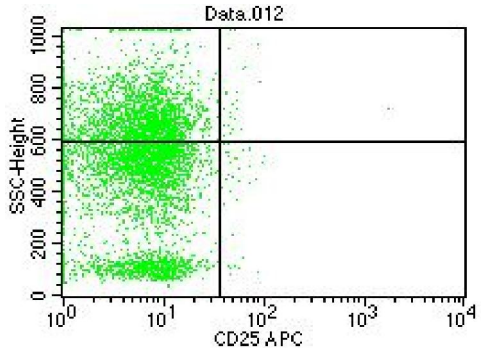

Figure (14): Histogram of a patient with inactive lupus nephritis (group IB) showing (CD25⁻) of CD4+ FOXP3+ = 6.6%.

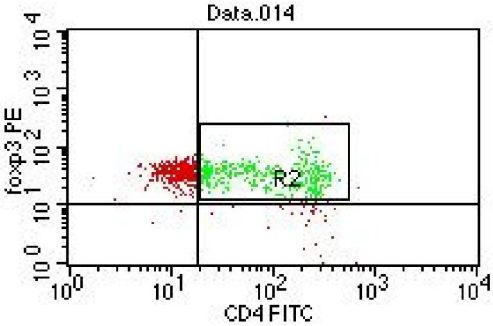

Figure (15): Histogram of a patient without nephritis (group II) showing CD4+ FOXP3+= 4%.

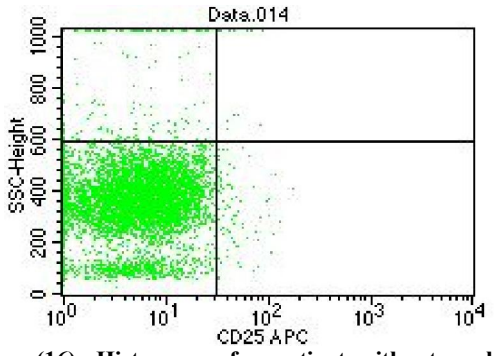

Figure (16): Histogram of a patient without nephritis (group II) showing CD25- of CD4+ FOXP3+= 4%.

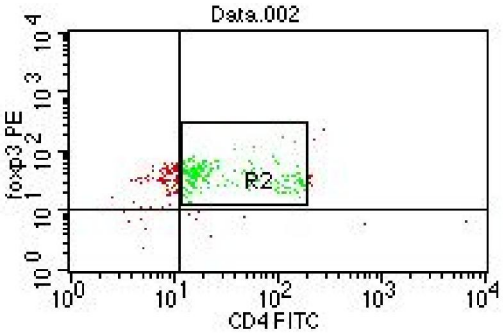

Figure (17): Histogram of a patient with inactive SLE (group II B) showing CD4+ FOXP3+= 2%.
CD25 (2%)

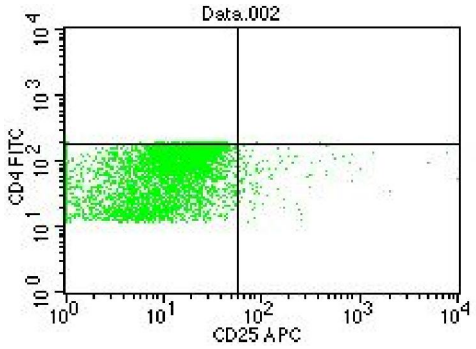

Figure (18): Histogram of a patient inactive SLE (group II B) showing % of CD25−Foxp3+ cells within the gated CD4+ cells= 2%

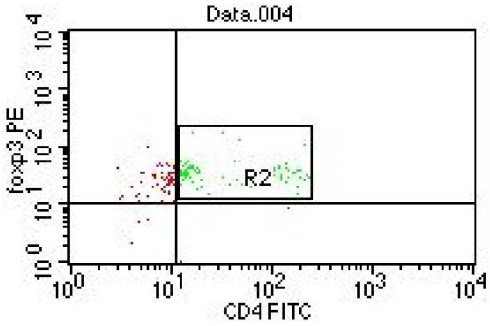

Figure (19): Histogram of a control subject (group III) showing CD4+ FOXP3+= 0.8%.

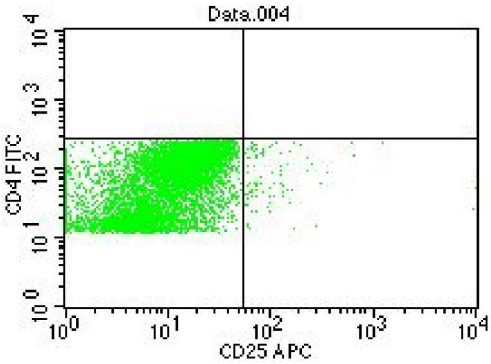

Figure (20): Histogram of a control subject (group III) showing % of CD25−Foxp3+ cells within the gated CD4+ cells= 0.8%

4. Discussion

T regulatory cells (Treg) play an important role in the maintenance of immune cell homeostasis, as it has been reported that CD4+CD25+ T cells suppress the auto-reactive responses in autoimmune diseases such as systemic lupus erythematosus (SLE). Recent studies have suggested dysregulation of the regulatory T cells (Tregs), especially natural Tregs, as a major risk factorfor the expression of human autoimmune diseases, such as SLE. Until now the association of a certain organ manifestation with the presence of different T cell subsets has not been investigated so far. (14)

The main goal of the study was to investigate whether the quantitative changes of Treg subpopulations are related to clinical status of patients with lupus nephritis (LN). We investigated the population of CD4+CD25-Foxp3+ T cells in a group of SLE patients and correlated disease activity and peripheral blood CD4+CD25-Foxp3+ T cells. We showed that CD4+CD25-Foxp3+ T cells increases and is associated with a specific organ manifestation, mainly renal involvement. We further correlated $CD4^{+}CD25^{-}Foxp3^{+}$ cells with levels of proteinuria, disease activity using SLEDAI score and rSLEDAI and complement levels. Our results showed that T cells from patients with SLE contain a sizeable proportion of $CD4^{+}$ cells expressing the Treg-associated transcription factor Foxp3, but lacking another important marker of Treg, CD25 ($CD4^{+}CD25^{-}Foxp3^{+}$). Their proportion in SLE patients is much higher than healthy controls. We observed a significant increase in proportions of $CD4^{+}CD25^{-}Foxp3^{+}$ T cells in patients with renal involvement (7.06±1.31%) as compared to patients with no renal involvement (4.17±1.10%; P= 0.0001*) and the healthy control (1.11±0.41), also in patients with active nephritis (7.82±0.68%) as compared to SLE patients with active other organ manifestations (4.40±1.29%).

We found a strong positive correlation between $CD4^{+}CD25^{-}Foxp3^{+}$ T cells and the occurrence of active proliferative lupus nephritis. High proportions of peripheral blood CD4+CD25-Foxp3+ T cells correlated with more severe forms of lupus nephritis (class IV, III). Regarding absolute values of CD4+CD25-Foxp3+ T cells, we also found higher numbers in lupus nephritis patients (mean= 56.00±25.23) when compared with lupus without nephritis (36.00±13.16) and the control (22.00±12.60). A similar result was observed when we compared absolute numbers of CD4+CD25-Foxp3+ T cells in active and inactive SLE (P= 0.039).

We found that the sensitivity of proportions of $CD4^{+}CD25^{-}Foxp3^{+}$ T cells was 100% and the specificity was 60%, and the predictive values as diagnostic tool of nephritis among SLE patients were 71.43% % and 100% for positive and negative predictive values respectively with a predictive accuracy of 80%.

Bonelli, et al. (2014); Zhang, et al. (2008); Bonelli, et al. (2011); Lin, et al. (2007); Lyssuk, et al. (2007) also described increased proportions of CD4+Foxp3+ T cells that are CD25-negative or express only low levels of CD25 in SLE patients. (11, 15-18) Zhang, et al. (2008) analyzed percentages of CD4+CD25−FoxP3+ T cells in patients with a recent onset of SLE and found a significant increase in this cell population compared with those in healthy individuals. Furthermore, the percentages correlated with anti-dsDNA titers and most of them were CD127low. (15)

Increased percentages of CD4+CD25−FoxP3+ T cells in SLE patients compared to HCs was reported by Bonelli, et al. (2014) and colleagues (11); however, the marker combination of CD4+CD25−CD127− to substitute for intracellular FoxP3 was used.

In line with our study results, El-Maraghy, et al (2016) concluded that level of CD4+CD25-Foxp3+ T cells was significantly increased in SLE patients (15.57 ±4.32%) as compared with the control group (2.46 ±0.65%). It was also positively correlated with renal impairment and hematological involvement. (19)

In contrast, Miyara, et al. (2005) showed that phenotypic and functional characteristics of Tregs isolated from lupus patients are the same as those found in healthy controls. A decrease in the proportion of circulating Tregs is was never evidenced in active patients compared with healthy controls or with inactive patients. (20)

Suen, et al. (2009) also described increased proportions of CD25lowTreg and CD25-Treg in patients with active and inactive SLE as compared to healthy control. (21)

Zhang, et al (2008); Scheinecker, et al (2008); Crispin, et al (2003); Vargas-Rojas, et al (2008) have observed decreased proportions of CD4+CD25high Tregin SLE patients during disease activity with an inverse correlation of Treg numbers with clinical disease activity. (15, 22, 23)

Most researchers [Lin, et al. (2007); Miyara. et al. (2005); Suen et al. (2009); Crispin, et al. (2005); Golding, et al. (2013); Ma, et al. (2013); Cai, et al. (2012); Xing et al. (2012); Habibagahi, et al. (2011), Henriques et al. (2010), Barreto, et al. (2009), Suarez A, Barath et al. (2007a, 2007b)], indicate a reduced number of circulating CD4+CD25high/CD4+CD25+Foxp3+ during disease activity. (17,20,21,24-34) The decrease negatively correlates with disease activity and/or levels of anti-dsDNA antibodies in the patients' sera (Miyara. et al. (2005); Bonelli, et al. (2008); Ma. et al. (2013); Mellor-Pita. et al. (2006)). (20,22,35, 36)

Others have reported unchanged number of circulating regulatory T $CD4^{+}$ cells which express the CD25 molecules and/or Foxp3 (Zhang, et al. (2008); Vargas-Rojas, et al. (2008); Yates, et al. (2008)) (15,26,37), or on the contrary—higher number of these cells in patients with SLE (Lin et al. (2007); Azab et al. (2008) compared to the control group. (17,38)

Concerning the influence of the disease activity, our study was able to describe a correlation between proportions of CD4+CD25-Foxp3+ T cells and disease activity using SLEDAI score and rSLEDAI and found significant correlations with both scores: SLEDAI (r= 0.5; P <0.001), The dependence of the variability of CD4+ Foxp3+CD25- regulatory cell number with SLEDAI and rSLEDAI indices seems to be a promising exponent of disease activity, particularly renal involvement.

In addition we observed a significant correlation with anti-dsDNA antibody levels (r= 0.6; P <0.0001) and a significant inverse correlation between the levels of complement factor C4 (r= -0.523; P= 0.003*) and proportions of CD4+CD25-Foxp3+ T cells. These correlations were not significant for absolute cell numbers of CD4+CD25-Foxp3+ T cells.

El-Maraghy, et al (2016) observed a significant correlation for the percentage of CD4+CD25-Foxp3+ T cells with clinical disease activity scores and disease duration ($r = 0.6$, $p < 0.001$; $r = 0.3$, $p = 0.02$ respectively). (19)

Yang, et al. (2009) studied subjects with untreated new-onset SLE and found increased percentages of CD4+CD25-Foxp3+ cells that were also CD127low. However, this group also reported that the prevalence of these cells correlated positively with the anti-dsDNA antibodiestitre. (39)

Lin, et al. (2007) reported a significant increase of CD4+Foxp3+ T cells in patients with active SLE, determined by the SLEDAI score, as compared to

patients with inactive SLE or healthy controls. In contrast, however, they observed no correlation between CD4+CD25-Foxp3+ T cells and disease activity, the complement levels and concentrations of anti-dsDNA antibodies. (17)

Zhang, et al. (2008) also reported direct correlation between proportions of CD4+CD25-Foxp3+ T cells and anti-dsDNA titre, but there was no correlation with C3 levels or the SLEDAI score. (15)

Ma et al. (2013); Cai et al. (2012); Xing et al. (2012), Żabińska, et al. (2016) reported that the absolute number of CD4+CD25+Foxp3+ regulatory cells negatively correlated with disease activity measured by the SLEDAI scale. (26-28, 40)

Miyara; et al (2005) also observed that CD4+CD25high T-cell depletion in lupus patients was associated with the clinical severity of the flare. (20)

Suen, et al (2009) showed first that a significant decrease in the frequency of CD4+ $CD25^{high}$ FoxP3+ T cells and significant increase in frequencies of $CD25^{low}$ FoxP3+ and CD25− FoxP3+ CD4+ T cells was present in patients with active SLE compared with healthy controls. The elevation of these two Treg subpopulations was associated with lower plasma levels of complement C3 and C4 in patients with SLE. In addition, the ratios of the three subsets of CD4+ FoxP3+ Tregs were inversely correlated with the titer of anti-double-stranded DNA IgG in patients with inactive, but not active, SLE. (21)

Regarding proteinuria, we observed a significant correlation between proportions of CD4+CD25-Foxp3+ T cells and the extent of proteinuria (r= 0.559; P= 0.001*) in patients with renal involvement reflecting the disease activity in patients with active nephritis. This is in agreement with studies of (Zhang, et al. (2008); Lin, et al. (2007); Suen, et al. (2009. (15, 17, 21) The correlation with the extent of proteinuria suggests that the assessment of $CD4^{+}CD25^{-}Foxp3^{+}$ cells can be used as a tool to recognize and monitor SLE patients with renal involvement.

Fathy A, et al. (2005) also found significant decrease of CD4+ CD25+ T cells in SLE patients compared to healthy controls. No correlation was seen between percentage of CD4+ CD25+ T cells and SLE disease activity. (41)

These conflicting results may be due to difficulty to determine the boundary between $CD25^{high}$ and $CD25^{low}$ cells on the fluorescence plots. For better distinguishing subsets of T lymphocytes, it is therefore recommended that simultaneous analysis of CD25 and Foxp3 expression on $CD4^{+}$ T cells be done. If the CD25 molecule is used as the sole marker for regulatory cells, $CD25^{high}$ cells can be contaminated by $CD25^{low}$ effector T cells, while too strict gating of $CD4^{+}$ T cells with high expression of CD25 molecule may lead to false reduced number of regulatory cells in human peripheral blood mononuclear cells. (21)

Conclusion

Clear increase in the percentage of CD4+CD25-Foxp3+ T cells in patients with renal involvement and in patients with more proliferative lupus nephritis support the hypothesis that propotions of CD4+CD25-Foxp3+ T cells may have a pathogenic role associated with this particular organ manifestation and may serve as a tool to avoid unnecessary renal biopsies and/or treatment intensifications.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Acknowledgement

We acknowledge the college affairs for postgraduate studies in the faculty of medicine in Tanta University for funding this research study. We acknowledge colleagues for their support and cooperation in the recruitment of patients and collection of blood samples.

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