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Original article

Regulatory T cells and Disease damage in systemic lupus erythematosus

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Abstract:

Background: Systemic lupus erythematosus (SLE) is an inflammatory, multisystem autoimmune disorder characterized by a multitude of autoantibody production and immune complex deposition, causing damage to multiple organs. Dysfunction of T and B cells are believed to be essential factors involved in the disease pathogenesis. A lack or defect in Treg function is generally considered to support SLE pathology. **Aim**: Our study aimed to assess CD4+ CD25+ Foxp3+ T regulatory cellspercentage in SLE patients and its relation to activity index and damage index. **Methods**: 50 SLE patients and 50 controls were enrolled in the study. Flowcytometric determination of peripheral Treg cells was done for all participants. Disease activity was measured using the Systemic Lupus Erythematosus Disease Activity Index

2000 (SLEDAI -2k) while disease damage was measured using the Systemic Lupus international collaborating clinics American College of Rheumatology Damage Index (SLICC/ACR DI) which were correlated with T regulatory cells percentage in cases only. **Results**: Treg cells percentage was significantly decreased in SLE patients when compared with healthy controls (0.1% to 0.9% vs 0.9% to 2.1%) ($p< 0.001$).As regard SLEDAI -2k, there was a negative correlation between Treg cells percentile and SLEDAI-2k ($p< 0.001$). Also there was a negative correlation between Treg cells percentile and damage index (SLICC/ACR DI) ($p< 0.001$). Regarding the correlation between SLEDAI -2K with damage index (SLICC/ACR DI), we found highly significant positive correlation ($p< 0.001$). **Conclusion**: Our study showed that Tregs percentile were significantly lower in SLE patients when compared with healthy controls.Treg cells % have significant association with SLEDAI and damage index suggesting the value of Tregs as activity biomarker and marker of damage.

1. Introduction:

Systemic lupus erythematosus is a multiorgan autoimmune disease with diverse and varied clinical manifestations and long-term outcomes with exacerbations and remissions. Irreversible organ damage is a primary outcome in SLE. It is occured during the course of SLE caused by both the disease itself and therapies received by patients. Damage to tissues and organs is linked to a higher chance of mortality. **[1]**

CD4+CD25+Foxp3+ Treg cells are a type of CD4+ cell that suppresses the immune response and promotes self-tolerance. Treg cells are mainly produced in the thymus via self-antigen recognition, although they can also be found in the peripheral lymphoid organs. **[2], [3]**Tregs express high levels of IL-2 receptor alpha (CD25) and the transcription factor forkhead box P3 (Foxp3). Alongside IL-2 and CD25, which facilitate

the development, function and stability of Tregs, numerous other cell-surface receptors for Tregs have been identified. IL-2 supports Treg development in the thymus and is also needed for their survival and function in the periphery.**[4]**

The forkhead winged-helix transcription factor Foxp3 (forkhead box p3) discovery as master regulator for Treg cells added a key marker for this T cell subset. Foxp3, in fact, is constitutively expressed at high levels in both natural and adaptive CD4+CD25high Treg cells in human beings and mice. It is required for the natural Treg lineage commitment in the thymus and is essential in stabilizing and amplifying a Treg program induced by interaction between Treg precursors and stromal cells in the thymus. **[5]**The ability of Foxp3+ Tregs to regulate autoimmunity and self-tolerance by inhibiting the proliferation and/or effector functions of CD4+/CD8+ T cells, B cells, NK cells, and Antigen Presenting Cells (APCs) has been demonstrated. **[6]**

Interestingly, it is well accepted that Foxp3, despite being a specific marker for Treg cells, can also be expressed by human effector T cells after activation. But, its expression on these cells is transient and does not reach the expression levels displayed by Treg cells. **[7]**

In SLE, Qualitative and/or quantitative disorders affecting Treg cells may lead to the disequilibrium in peripheral tolerance and may participate in triggering the disease. **[8]**

Adequate evaluation of disease activity, assessment of organ damage using the Systemic Lupus International Collaborating Clinics/American College of Rheumatology (SLICC/ACR) damage index for SLE (SDI) and quality of life assessment among SLE patients help towards better surveillance and treatment, and improved prognosis for the disease. **[9]** So the aim of the study was to assess the concentration of CD4+ CD25+ FoxP3+ natural Treg in SLE patients and study its relation to SLEDAI -2k activity index and damage index (SLICC/ACR DI).

In our study, the percentage of CD4+CD25+Foxp3+ Treg cells in SLE patients was decreased when compared with control group. Also there was negative correlation between CD4+CD25+Foxp3+ Treg percentile and SLEDAI and damage index (SLICC/ACR DI). Positive correlation between SLEDAI and damage index (SLICC/ACR DI) was found.

2. Material and Methods:

Our cross-sectional descriptive study included a cohort of fifty SLE adult female patients with age range from 18 to 55 years old who fulfilling the Systemic Lupus International Collaborating Clinics (SLICC) classification criteria [8]. According to the

SLICC criteria for SLE, SLE classification requires: 1) Fulfillment of at least four criteria, with at least one clinical criterion AND one immunologic criterion OR 2) Lupus nephritis as the only clinical criterion in the presence of ANA or anti-dsDNA antibodies. The patients were from the Rheumatology and Rehabilitation Department, Badr hospital Helwan University. In addition Fifty age and sex matched healthy adults were studied as a control group. A written informed consent was taken from each participant. Patient selection done by simple random sampling by convenience through taking odd files number.Pregnancy, end stage renal disease on dialysis, malignancy or other autoimmune disease patients were excluded.

The study was approved by the Ethical Research Committee and Institutional Review Board of Faculty of Medicine, Helwan University, Egypt.

Patients were subjected to routine laboratory investigations including:

Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), Complete blood count (CBC), Complete urine analysis, Liver and kidney function tests, Antinuclear antibodies (ANA) and anti-double-stranded DNA (Anti-ds DNA) antibodies.

Disease activity was assessed with the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI -2k) **[10].** The grades of disease activity were defined as SLEDAI 0= quiescent disease, SLEDAI 1–10 = moderate activity, and SLEDAI >10= high disease activity. Disease damage was recorded according to the Systemic Lupus International Collaborating Clinics/American College of Rheumatology (SLICC/ACR DI) Damage Index **[11].**The cut-off for organ damage was SLICC 0 =no organ damage, SLICC 1–3 =moderate organ damage, and SLICC >3 = severe organ damage.

Following these processes, all participants underwent a comprehensive medical history, clinical examination, and flowcytometric measurement of peripheral Treg cells using anti CD4, anti CD25, and anti FOXP3 monoclonal antibodies.

Identification of CD4+CD25+Foxp3+ Treg cells in PBMCs using flowcytometry analysis:

Human peripheral blood mononuclear cells (PBMCs) were freshly separated by Ficoll density gradient centrifugation then washed with 2.0 mL of flowcytometry staining buffer (R&D Systems®) by spinning at 300 x g for 5 minutes, using 5.0 mL flowcytometry tubes. After that 10 µL of CD4-FITC and 10 µL of CD25-APC antibodies or isotype controls (R&D Systems®) were added which incubated for 30-45 minutes at 2-8 °C in the

dark. The cells were washed two times with cold 1X Phosphate buffer saline (PBS) then resuspended in 0.5 ml of fresh 1X FoxP3/Transcription Factor Fixation Buffer (R&D Systems®) and incubated at 2-8 °C for 30 minutes.During this incubation, 1X FoxP3/Transcription Factor Permeabilization and Wash Buffer was maked up by diluting FoxP3/Transcription Factor Permeabilization and then Buffer (10X) was washed with distilled water (i.e. 100 µL FoxP3/Transcription Factor Permeabilization and the Washed Buffer (10X) + 900 µL diH20) were kept at 2-8 °C.

The mixture was washed two times with fresh, cold, 1X FoxP3 Permeabilization and Wash Buffer. 10 µL of FoxP3 antibody or the rabbit IgG-PE isotype control included in the kit was added to the cells and incubated for 30 minutes at 2-8 °C. Then the cells were washed one time with cold 1X FoxP3 Permeabilization and Wash Buffer.The cells were resuspended in Flowcytometry Staining Buffer and run on a flowcytometer.

The data was collected and analysed on an EPICS XL flowcytometer with the SYSTEM II version3 software and a standard 3-color filter configuration. Lymphocytes were gated using forward and side scatter properties and CD4+ cells were identified using CD4 expression. CD25high CD4+ Tcells were distinguished from CD25dim CD4+ Tcells after the gated CD4+ Tcells were tested for both CD25 expressions usingPE-anti-Foxp3. Finally, FoxP3 expression was evaluated in CD25highCD4+ Tcells .Treg cells were expressed as a percent of CD4+ T cells as shown in **figure 1.**

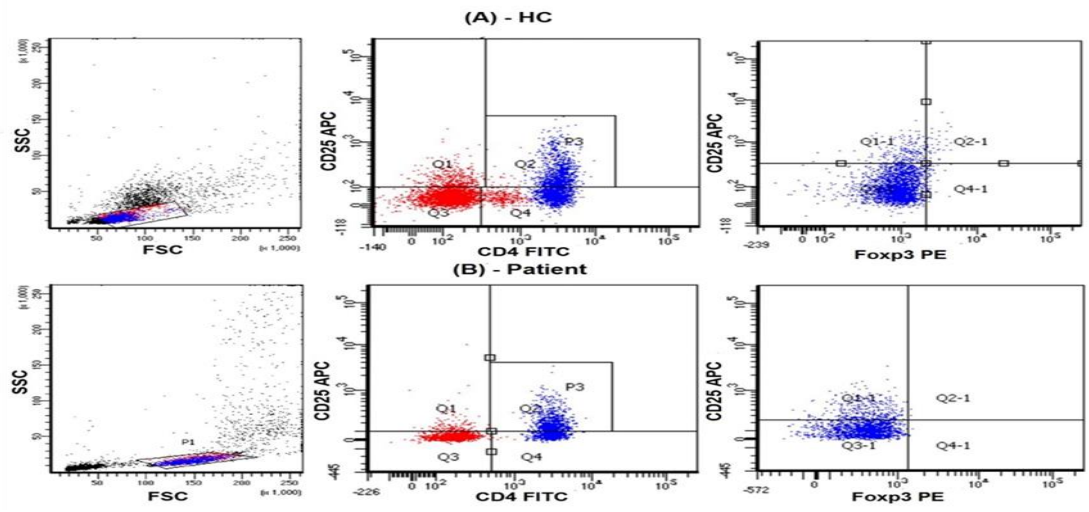

Figure (1): Flowcytometry analysis of CD4+CD25+Foxp3+ Treg in patients and controls

A: shows flowcytometry of CD4+CD25+Foxp3+ Treg % in healthy controls (HC). B: shows flowcytometry of CD4+CD25+Foxp3+ Treg % in SLE patient. In (A) and (B) Peripheral blood mononuclear cells (PBMCs) were gated for lymphocytes via their forward (FSC) and side scatter (SSC) properties. Following the gating of the lymphocyte population, CD4 + cells were acquired. Then CD25 and Foxp3 were expressed following gating of cells based on CD4+ cells.

Statistical analysis:

The statistical package for social sciences, version 20.0, was used to analyse the data (SPSS Inc., Chicago, Illinois, USA). Mean and standard deviation (SD) were used to express quantitative data. Frequency and percentage were used to express qualitative data.Kruskal-Wallis H test was used for multiple-group comparisons in non-parametric data, independent-samples t-test of significance was used when comparing between two means and r-Pearson Correlation Coefficient was used to measure linearcorrelation between two sets of data. The confidence interval in probability (P-value) was set to 95% and the margin of error accepted was set to 5%.So, the p-value was considered significant as the following: Probability (P-value) < 0.05 was considered significant, P < 0.001 was considered as highly significant and P > 0.05 was considered insignificant.

3. Results:

Demographic and clinical characteristics of SLE patients and control groups

The present study was conducted on a cohort of fifty female SLE adult patients with age range from 18 to 55 years old mean 36 ±9.5 years, who were diagnosed according to the 2012 SLICC classification criteria. In addition to 50 age and sex matched controls mean of age 34.6 ±10.3 years.

The laboratory results of the patients group revealed: white blood cells (WBCs) range was 2-17.5 mean 6.99 ±3.11 X 10^3/µl including 8 (16 %) patients leucopenic (less than 4 X 10^3/µl), haemoglobin (Hb) range was 8-15.7 mean 11.24 ±1.57 g/dl including 38 (76 %) patients anaemic (less than 12 g/dl), platelets range was 47-468 mean 222.08 ±72.94 X 10^3/µl including 5 patients thrombocytopenic (less than 150 X 10^3/µl), ESR range from 10-100 mean 47.02 ±20.69 mm/1sth, CRP range was 0-48 mean 9.12 ±10.79 mg/dl, alanine aminotransferase (ALT) range was 5-82 mean 23.90 ±13.66 U/L, aspartate transaminase (AST) range was 12-65 mean 27.46 ±11.84 U/L, urea range was 11-106 mean 36.76 ±25.82 mg/dl, creatinine range was 0.5-3.2 mean 0.85 ±0.46 mg/dl, pus cells in urine range was 1-70 mean

11.40 ±13.96 cells /high power field, red blood cells (RBCs) in urine range was 1-40 mean 5.04 ±7.87 cells /high power field, ANA was positive in 100% of patients and Anti-ds DNA was positive in 75% of patients .The cases were classified according to CD4+CD25+Foxp3+ Treg percentage into 3 groups: group 1 (18 patients with CD4+CD25+Foxp3+ Treg percentage ≤ 0.20), group 2 (14 patients with CD4+CD25+Foxp3+ Treg percentage > 0.2-0.5) and group 3 (18 patients with CD4+CD25+Foxp3+ Treg percentage > 0.5 – 0.9).Disease activity of the SLE patients assessed through SLEDAI-2K score range was 4-39 mean 13.54 ±8.59, the damage index SLICC/ACR DI score range was 0-5 mean 1.32 ±0.98 and CD4+CD25+Foxp3+ Treg cells percentage range was 0.1-0.9 mean 0.38± 0.22.The percentage of CD4+CD25+Foxp3+Treg cells controls was significantly lower when compared to healthy (0.1% to 0.9 % vs 0.9 % to 2.1 %, respectively) (p 0.001) as shown in the table (1).

Table (1): Comparison between patients group and control group according to their T regulatory cells%

T regulatory cells%	**Patients Group *(n=50)***	**Control Group *(n=50)***	**z-test**	**p-value**
Mean±SD	0.38±0.22	1.37±0.36	-16.588	<0.001**
Range	0.1-0.9	0.9-2.1		

To assess the relation of CD4+CD25+Foxp3+ Treg percentage with disease activity we tested the CD4+CD25+Foxp3+ Treg percentage against the SLEDAI-2K score. Statistical association between T regulatory cells percentiles and their SLEDAI revealed highly statistically significant association (p <0.001).

In order to determine the relation between CD4+CD25+Foxp3+ Treg cells percentage with organ damage from SLE, we found that T regulatory cell percentiles had a strong statistically significant relationship with Damage index (SLICC/ACR DI) (p 0.001) as shown in table (2).

Table (2): Association between T regulatory cells% percentiles according to their damage index

Damage index	***Percentiles 25th (≤0.20) [n=18]***	***Percentiles 50th (>0.2-0.5) [n=14]***	***Percentiles 75th (>0.5) [n=18]***	**Kruskal Wallis test**	**p-value**
Mean±SD	2.06±1.00A	0.93±0.47B	0.89±0.83B	11.307	<0.001**
Range	1-5	0-2	0-2		

In this present study, negative correlations were observed between Treg percentage and ESR (r= -0.397& p =0.025), CRP(r=-0.328 & p = 0.038), Creatinine(r=-0.251& p=0.039), pus in urine(r=-0.410 &P= 0.003), RBCs in urine(r= -0.366 & p= 0.009) and albumin in urine(r = -0.429 & p= 0.002).As regard SLEDAI, there was statistically significant positive correlation with ESR (r= 0.420 & p= 0.019), CRP (r=0.322 & p= 0.034), urea (r=0.298 &p =0.035), creatinine (r= 0.260 & p= 0.037), pus in urine (r=0.323 &p= 0.022), RBCs in urine (r= 0.360 &p =0.010) and albumin in urine (r= 0.482 &p <0.001).Regarding Damage index, there was statistically significant positive correlation with ESR (r=0.330 &p=0.026), urea (r=0.363 & p=0.018),creatinine(r=0.290 &p=0.012) , pus in urine (r=0.387 &p= 0.039) , RBCs in urine (r=0.425 &p=0.036) and albumin in urine (r=0.498 &p=0.003) as shown in table in table (3).

Table (3): Correlation between T regulatory cells%, SLEDAI and Damage index with all parameters, using Pearson Correlation Coefficient in patients group

Parameters	**T regulatory cells%**		**SLEDAI**		**Damage index**	
	r-value	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**
Age (years)	-0.218	0.128	0.174	0.228	0.199	0.135
ESR (mm/1h)	-0.397	0.025*	0.420	0.019*	0.330	0.026*
CRP (mg/dl)	-0.328	0.038*	0.322	0.034*	0.363	0.018*
Wbcs (X 10^3/µl)	-0.075	0.607	0.023	0.876	0.080	0.580
Hb (g/dl)	0.160	0.266	-0.212	0.139	-0.059	0.686
Plt. (X 10^3/µl)	-0.055	0.702	0.106	0.466	0.226	0.114
ALT (U/L)	0.121	0.402	-0.161	0.263	-0.112	0.438
AST (U/L)	0.062	0.669	-0.134	0.354	-0.133	0.358

Urea (mg/dl)	-0.237	0.098	0.298	0.035*	0.141	0.205
Creatinine (mg/dl)	-0.251	0.039*	0.260	0.037*	0.290	0.012*
Pus in Urine (1-4 per field)	-0.410	0.003*	0.323	0.022*	0.387	0.039*
RBCs in Urine (0-1 per field)	-0.366	0.009*	0.360	0.010*	0.425	0.036*
Albumin in Urine (Nil)	-0.429	0.002*	0.482	<0.001**	0.498	0.003*

As shown in table (4), there was a statistically negative correlation between T regulatory cells percent with SLEDAI (r=-0.715 &p0.001) and Damage index (r=-0.457 &p0.001), as well as a significant positive correlation between SLEDAI and Damage index (r= 0.676 &p0.001).Indicating that higher disease activity and organ damage is related to lower CD4+CD25+Foxp3+ Treg cells percentage.

Table (4): Correlation between T regulatory cells% with SLEDAI and Damage index, using Pearson Correlation Coefficient in patients group.

Parameters	**T regulatory cells%**		**SLEDAI**		**Damage index**	
	r-value	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**
T regulatory cells%			-0.715	<0.001**	-0.457	<0.001**
SLEDAI	-0.715	<0.001**			0.676	<0.001**
Damage index	-0.457	<0.001**	0.676	<0.001**		

4. Discussion:

Systemic lupus erythematosus is a complex autoimmune disease that has a chronic relapsing–remitting course with a wide range of symptoms, ranging from moderate to life-threatening sickness **[12].**The current work makes a scientific addition by estimating the value of CD4+ CD25+FoxP3+ Treg cells as a biomarker of activity and damage. Progress in understanding the immunopathogenesis of SLE could also lead to a better knowledge of the disease mechanism and tangible benefits for patients, making it feasible to give the

best treatment for lupus patients and decrease the damage effect of the disease.

When compared to healthy controls, which range from 0.9 % to 2.1 %, Treg cells in the peripheral blood of systemic lupus patients (0.1 percent to 0.9 percent) were significantly lower.These results were in agreement with**Cai et al. (2012); Habibagahi et al. (2011); Henriques et al. (2010); Ma et al. (2013) and Xing et al. (2012)** who investigated the frequencies of circulating Treg in SLE and found a significant decrease in circulating Treg in peripheral blood of diseased patients when compared with controls. The finding of reduced level of Tregs in SLE was explained by the decrease expression of CD25 in Tregs from patients with SLE linked with the production of IL-2 by memory T cells indicating that IL-2 deficiency in SLE patients reflects CD25 reduction in Tregs. Reduced expression of CD25 may impact Treg function in SLE patients because IL-2 receptor-dependent activation of transcription factor STAT5 is required for the suppressive function of Tregs **[18].Yates et al. (2008)** observed that the number of circulating CD4+Tregs expressing CD25 molecules and Foxp3 in lupus patients remained stable. **Azab et al. (2008) and Yan et al. (2008),** on the other hand, have found a larger number of these cells in SLE patients. Differences in the use of specific phenotypic markers for identifying Tregs, as well as the methodology for isolating or stimulating these cells prior to staining, may have caused these inconsistencies.

In contrast to **El-Maraghy et al. (2018)** and **Bonelli et al. (2014),** who found a positive correlation between haematological manifestations including thrombocytopenia and leucopenia, there was no statistically significant correlation between T regulatory cells percent and haematological indices. This could be explained by the overlap of organ manifestations, as most SLE patients suffer from haematological manifestations as well as renal involvement.

In this study, there was a statistically significant negative correlation between the percentage of T regulatory cells and CRP and ESR. These findings are in agreement with those of **M. Nabil et al. (2018) and Lee et al. (2008)**, who look at the relationship between Treg and ESR as an inflammatory marker, as ESR levels are used to assess disease relapse and distinguish infection.However,**Atfy et al. (2009) and Barâth et al. (2007)** discovered no statistically significant link between the percentages of these cells in lupus patients and ESR.

The percentage of T regulatory cells and serum Creatinine had a negative relationship. This finding is consistent with **Maher et al. (2019),** who found that Treg has a role in

lupus nephritis. However, **Atfy et al. (2009) and Barâth et al. (2007)** observed no significant link between the percentages of these cells and creatinine levels in lupus patients.

There was a statistically significant positive correlation between SLEDAI and ESR, which is consistent with **Santhanamet al (2015).Mirzayan et al. (2000) and Chang et al. (2002)** couldn't find any link between ESR and disease activity in their studies. Many complicating factors, such as anaemia, hypoalbuminemia, high cholesterol, or high gammaglobulin levels, might raise ESR. As a result, all of these confounding factors must be eliminated, and it would be ideal if ESR and its relationship with disease activity could be investigated throughout each flare episode.

Damage index and creatinine level showed a positive significant correlation, confirming the findings of **Ghazali et al. (2018),** who investigated the relationship between damage index and other variables affecting lupus patients, demonstrating that renal affection is an irreversible disorder.

SLEDAI and damage index have a significant positive correlation, which is consistent with the findings of **Bruce et al. (2015),** who investigated the relationship between damage index and several factors affecting patients with SLE, including older age at diagnosis, longer duration of SLE, African-Caribbean or Asian ethnicity, high disease activity at diagnosis, and greater overall activity duration. The fact that disease activity and damage have such a strong relationship suggests that they work together to accelerate the onset of permanent organ alterations.

On correlating damage index with Treg %, we found significant negative correlation which to our knowledge has not been discussed before in other studies indicating the role of T regulatory cells in the pathogenesis of SLE and its decrease leads to irreversible damage.

5. Conclusion: Treg cell percentage has a substantial relationship with SLEDAI and damage index, implying that Tregs can be used as an activity biomarker and damage marker. The value of Tregs as activity and damage biomarkers may help in assessing disease status in controversial circumstances and prevent further permanent damage. Further studies on larger scales are required for further assessment of role of Treg in SLE and more correlation with parameters affecting mortality and morbidity of the disease.

Declaration of conflicting interests

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