Study of regulatory T – cells in Systemic Lupus Erythematosus and Rheumatoid Arthritis

M. Nabil Rafat[1], Abdelwahab Lotfy[1], Mamdouh Attia[2], Mahmoud Hadad[1], Farag Khalil[1] and Marwan Sayed[1].

[1]Internal Medicine, El Hussein University Hospital, Faculty of Medicine, Al–Azhar University, Cairo, Egypt.
[2]Clinical Pathology, El Hussein University Hospital, Faculty of Medicine, Al–Azhar University, Cairo, Egypt
marwanahmed@azhar.edu.eg

Abstract: **Aim of the work:** To investigate the frequency of FoxP3$^+$CD4$^+$CD25^{+high} cells (Tregs) in peripheral blood from patients with systemic lupus erythematosus and from patients with rheumatoid arthritis. **Patients and methods:** twenty-five SLE patients (15 patients with active SLE, 10 patients with inactive SLE), twenty- five RA patients (15 patients with active RA,10 patients with inactive RA). and 10 age and sex matched healthy controls were enrolled in the study. Patients underwent clinical and laboratory assessment. The frequency of Tregs was determined by flowcytometry. **Results:** FoxP3$^+$CD4$^+$CD25^{+high}cells (as a percent of total CD4 cells) was significantly lower in SLE and RA patients when compared to healthy controls. Also, active SLE and RA patients showed significant lower Tregs percent when compared to inactive groups.

[M. Nabil Rafat, Abdelwahab Lotfy, Mamdouh Attia, Mahmoud Hadad, Farag Khalil and Marwan Sayed. **Study of regulatory T – cells in Systemic Lupus Erythematosus and Rheumatoid Arthritis.** *Nat Sci* 2018;16(5):38-48]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 7. doi:10.7537/marsnsj160518.07.

Key wards: Tregs, FoxP3, SLE and RA.

1. Introduction

Systemic lupus erythematosus is an autoimmune disease characterized by autoantibody production. The pathogenesis of SLE is not completely understood, with various types of immune cells being involved **(Szmyrka et al., 2014).**

Rheumatoid arthritis (RA) is a common systemic autoimmune disease with chronic relapsing inflammation primarily in peripheral joints. It is characterized by disturbed immune regulation which induces a progressive cartilage and bone destruction. **(Horwitz D., 2010, Adlan et al., 2014).**

Natural Treg cells are CD4$^+$CD25$^+$ T cells generated in the thymus in early years of life with the ability to bind self antigens by their T-cell receptor. They are distinguished from adaptive Treg cells that are induced in the peripheral blood by conversion of CD4$^+$ CD25$^-$ naïve T cells in the presence of a particular microenvironment **(Gerli et al., 2009).**

The importance of Treg cells in the development of autoimmune diseases was recognized by Sakaguchi and colleagues who were the first to show that transfer of CD4$^+$ T-cells depleted of CD25$^+$ T-cells, by specific monoclonal antibodies against CD25, into BALB/c athymic nude mice caused spontaneous development of T-cell dependent autoimmune diseases (such as thyroiditis, gastritis, insulitis, sialoadenitis, adrenalitis, oophoritis, glomerulonephritis (GN), and polyarthritis). When these mice were reconstituted by CD4+CD25+ Tcells within a limited period after CD4$^+$CD25$^-$ T-cell transfer, the autoimmune disease development was successfully prevented **(Sakaguchi et al., 1995).**

The discovery of the forkhead winged-helix transcription factor Foxp3 (forkhead box p3) 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$^+$CD25^{high} 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, inclusive of anergy and defective IL- 2 production, induced by interaction between Treg precursors and stromal cells in the thymus **(Chang et al., 2006).**

Furthermore, it has been shown that persistence of expression of Foxp3 are important for maintaining suppressor function. Interestingly, it is now well accepted that Foxp3, despite being a distinctive marker for Treg cells, can also be expressed by human effector T cells after activation. However, its expression on these cells is transient and never reaches the expression levels displayed by Treg cells. **(Banham., 2006)**

Though the concept of the preventive role of Treg cells in autoimmunity is widely accepted, data regarding SLE and rheumatoid arthritis is inconsistent. The studies on circulating Treg cells characterized phenotypically indicate either a decrease, an increase or no change in their number compared to healthy controls. These discrepancies throughout the literature may be due to the heterogeneity of the disease, studies in patients with different levels of disease activity, the possible impact of immunosuppressive treatment, and other factors **(Wang et al., 2015 and Kleczynska et al., 2011).**

The aim of the present study was to investigate the frequency of $FoxP3^{+}CD4^{+}CD25^{+high}$ cells (Tregs) in peripheral blood from patients with systemic lupus erythematosus and from patients with rheumatoid arthritis.

2. Subjects and Methods

Type of the study: a cross-sectional observational study.

Site and time of the study: Internal Medicine department, Faculty of Medicine, Al-Azhar University. During the period from December 2016 to December 2017.

Subjects: The study was conducted on sixty subjects; 25 patients with SLE; 22 patients were females and 3 were males, 25 patients with RA; 20 patients were females and 5 were males, In addition to 10 age/ sex- matched apparently healthy subjects (M/F = 2/8), served as controls.

The Subjects were divided into three groups:

- **Group 1:** 25 patients with SLE, they were divided according to SLE activity into 2 subgroups;

Group I A: 15 patients with active SLE (SLEDAI > 6).

Group I B: 10 patients with inactive SLE (SLEDAI ≤ 6).

- **Group 2:** 25 patients with RA, they were divided according to RA activity into 2 subgroups;

Group II A: 15 patients with active RA (DAS-28 ≥ 3.2).

Group II B: 10 patients with inactive RA (DAS-28 < 3.2).

- **Group 3:** 10 age/ sex- matched apparently healthy subjects (M/F = 2/8).

Ethical considerations:

a) Before data collection, permission was granted from the ethical committee of Al-Azhar Faculty of Medicine.

b) Informed consent was obtained from every patient to participate in this study.

c) Proper treatment for diseased cases was prescribed.

SLE patients were diagnosed according to SLE International Collaborating Clinics/ American College of Rheumatology (SLICC/ACR) 2012 Criteria **(Petri et al, 2012)**. Rheumatoid arthritis patients were diagnosed on the basis ACR/EULAR (2010) Classification Criteria for RA **(Daniel et al., 2010).**

Any patient suffered from Coronary heart disease, Diabetes, End stage renal disease, Pregnancy, Patient on treatment by immunosuppressive drugs at the time of enrolment and Patient on treatment by high doses glucocorticoids >20 mg/d at the time of enrolment were excluded from the study.

Methods:

All subjects were subjected to

A- Detailed history taking.

B- Full clinical examination.

C-Routine laboratory investigations. Erythrocyte sedimentation rate (ESR), C reactive protein (CRP), Fasting and 2hours postprandial blood glucose, Complete blood count (CBC), complete urine analysis, Liver and kidney function test.

D- Measurement of proteins in 24 hour urine (g/24hrs).

E-Antinuclear antibodies **(ANA)** and Anti-double stranded deoxyribonucleic acid antibodies **(anti-dsDNA**. Done by immunofluorescence technique. Titer of 1/40 or more is considered positive. (done for SLE patients only**).**

F-serum complement levels **(C3, C4)**. Doneby nephelometry (Normal level of C3 is 84- 160 mg /dl and for C4 12- 36 mg /dl): (done for SLE patients only**).**

G- Rheumatoid factor **(RF)**, Done by Turbidimetry, normal level is 0-15 IU/Ml**.**: (done for RA patients only).

H -Anti-cyclic citrullinated peptide **(Anti-CCP)**. Done by chemiluminescence, normal level is 0-5 U/ML: (done for RA patients only).

I - The percent of $FoxP3^{+}$ $CD4^{+}$ $CD25^{high}$ Tregcells was carried out by direct immunofluorescence technique using BD FACSCanto Flowcytometer using BD FACSDiva 8.02 system, which was performed on the peripheral blood of both patients and controls:-

1. Two tubes each contains fifty µL of whole anticoagulated blood were set for each subject, one for the tested monoclonal Abs and the other is for isotype controls. Samples in each tube were lysed using 1 mL lysing solution and washed with PBS. Cell counts were adjusted at 5-10x 10^{3}/ul.
2. The cells were stained with combinations of the following antibodies (five µl) of anti-CD25-PE, anti-CD4-FITC cocktail (1st tube) and PE isotype control (2nd tube).
3. Tubes were then incubated in dark for 20 minutes followed by washing with PBS.
4. The cell pellets were resuspended in 0.5 ml of freshly prepared fixation/permealization working solution and incubated for 30 minutes at 4°C in dark.
5. This was followed by washing once with PBS then washing once again with 1ml of 1X permealization buffer.
6. Ten µL of PE- cy5 FoxP3 or PE-Cy5 isotype control were added to respective tubes and incubated for 30 minutes at 4°C in dark.
7. This was followed by washing once with PBS then resuspension in PBS for analysis.
8. Data acquisition and analysis were performed on. BD FACSCanto Flowcytometer using BD FACSDiva 8.02 system.

9. Lymphocytes were gated via their forward and side scatter properties, then $CD4^+$ cells were identified based on their expression of CD4 versus side scatter properties.

10. The gated $CD4^+$ Tcells were then assessed for both CD25 expression where $CD4^+$ $CD25^{high}$Tcells were discriminated from $CD4^+$ $CD25^{dim}$Tcells.

11. Finally, $CD4^+$ $CD25^{high}$Tcells were assessed for FoxP3 expression, figure (1). Treg cells were expressed as a percent of $CD4^+$ T cells.

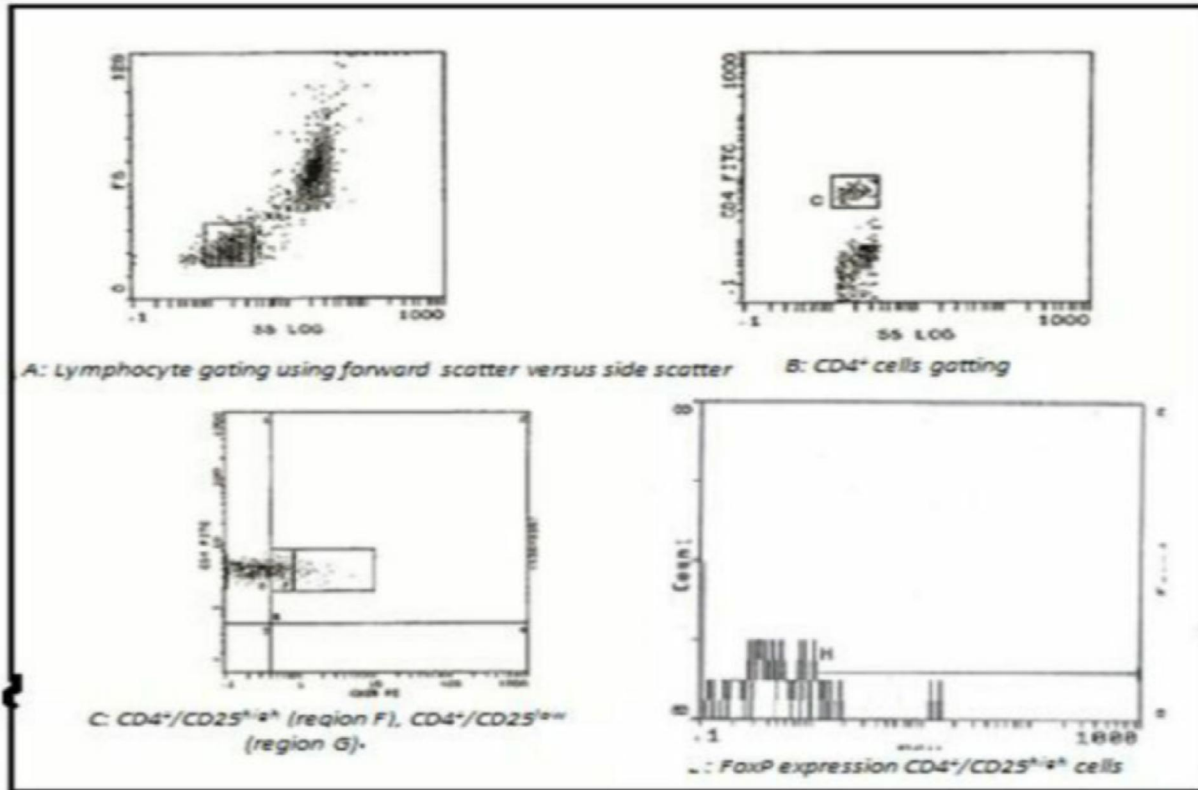

Figure (1): Examples of flowcytometry analysis of peripheral blood cells stained for surface markers of CD4, CD8, and CD25

Statistical Analysis

The data obtained from the history, clinical examination and investigations were tabulated and statistically analyzed with the aid of computer's GraphPad Prism program version 7. Data were statistically described in terms of mean ± standard deviation (± SD), when appropriate. Spearman's test was used for correlation analysis. Student t test was used for comparison of numerical variables between the study groups. P values less than 0.05 were considered statistically significant at the level of 95%.

3. Results

This study included 60 subjects: 25 patients with SLE; 22 patients were females and 3 were males, 25 patients with RA; 20 patients were females and 5 were males, In addition to 10 age/ sex- matched apparently healthy subjects (M/F = 2/8), served as controls.

The Subjects were divided into three groups:

- **Group 1:** 25 patients with SLE, they divided according to SLE activity into 2 subgroups;

Group IA: 15 patients with active SLE (SLEDAI > 6).

Group I B: 10 patients with inactive SLE (SLEDAI ≤ 6).

The descriptive clinical and laboratory data of SLE patients are shown in Table 1 and Table 2.

- **Group 2:** 25 patients with RA, they divided according to RA activity into 2 subgroups;

Group II A: 15 patients with active RA (DAS-28 ≥ 3.2)

Group II B:10 patients with active RA (DAS-28 < 3.2)

The descriptive clinical and laboratory data of RA patients are shown in Table 3 and Table 4.

- **Group 3:** 10 age/ sex- matched apparently healthy subjects (M/F = 2/8).

Table (1): Comparison between clinical variables in SLE Patients and Control groups

Variable	Group I A *Active SLE*	Group I B *Inactive SLE*	Group III *Control*	P value		
				Group IA & Group III	Group IB & Group III	Group IA & Group IB
Age *(years)*	33 ± 8.6	32.6 ± 5.15	32 ± 6.61	0.6023	0.8235	0.8574
Age of onset *(years)*	28.27 ± 7.6	25.3 ± 8.68				0.3758
Disease Duration *(years)*	6.4 ± 3.69	6.3 ± 4.21				0.5784
Weight *(kg)*	68.13 ± 9.64	68.5 ± 9.51	69.9 ± 4.95	0.5999	0.6847	0.9262
Height *(cm)*	171 ± 8.18	169.1 ± 6.5	172.3 ± 5.7	0.6681	0.2592	0.5449
BMI *(Kg/m^2)*	23.5 ± 3.2	24 ± 3.42	23.58 ± 1.99	0.9448	0.7412	0.7134
Systolic Bl. Pressure *(mmHg)*	149.7 ± 23.03	125.5 ± 16.06	120.5 ± 6.85	**0.0008** ***	0.3772	**0.0085** **
Diastolic Bl. Pressure *(mmHg)*	94.67 ± 15.06	78.5 ± 13.13	76.5 ± 7.09	**0.0017** **	0.6768	**0.0111***
SLEDAI	16.3 ± 5.4	1.6 ± 0.48				**<0.0001******

Table (2): Comparison between Laboratory variables in SLE Patients and Control groups

Variable	Group I A *Active SLE*	Group I B *Inactive SLE*	Group III *Control*	P value		
				Group IA & Group III	Group IB & Group III	Group IA & Group IB
Hemoglobin *(gm/dl)*	9.99 ± 1.39	12.50 ± 0.99	13.43 ± 0.72	**<0.0001** ****	**0.0301** *	**<0.0001** ****
WBC *(x1000/ml)*	8.02 ± 3.34	7.36 ± 1.96	6.9 ± 1.5	0.3347	0.5722	0.5772
Platelets *(x1000/ml)*	182.7 ± 93.35	200.6 ± 104.3	289 ± 88.66	**0.0053** **	**0.0381** *	0.6575
ESR *(mm/h)*	87.93 ± 27.27	48.6 ± 19.59	8 ± 1.6	**<0.0001** ****	**<0.0001** ****	**0.0007** ***
ALT *(IU/ml)*	21.8 ± 8.2	27 ± 10.11	18.7 ± 7.68	0.3531	0.0535	0.1705
AST *(IU/ml)*	23.8 ± 8.6	26.7 ± 7.6	20 ± 7.7	0.2652	0.0676	0.4095
s Creatinine *(mg/dl)*	1.16 ± 0.32	1 ± 0.17	0.85 ± 0.13	**0.0100** **	**0.0469** *	0.1741
BUN	27.3 ± 7.6	19.9 ± 4.9	14.5 ± 1.78	**<0.0001** ****	**0.0045** **	**0.0125** *
e GFR	84.8 ± 27.9	99.6 ± 14.3	115.2 ± 19.1	**0.0064** **	0.0538	0.1375
S. Albumin *(gm/dl)*	2.65 ± 0.43	3.51 ± 0.54	4.39 ± 0.44	**<0.0001** ****	**0.0008** ***	**0.0028** **
24 h. Ur. Proteins *(gm)*	2.85 ± 2.16	0.186 ± 0.08	0.045 ± 0.022	**0.0005** ***	**<0.0001** ****	**0.0008** ***
C3 *(mg/dl)*	51.67 ± 33.61	86.2 ± 21.96	115.9 ± 26.09	**<0.0001** ****	**0.0131** *	**0.0089** **
C4 *(mg/dl)*	14.8 ± 8.5	23.2 ± 9.7	34.4 ± 10.27	**<0.0001** ****	**0.0224** *	**0.0323** *
Anti-dsDNA ab	118.7 ± 67.31	44.3 ± 25.31	11.8 ± 4.59	**<0.0001** ****	**0.0008** ***	**0.0030** **

Frequencies of Treg cells

The distribution of $FoxP3^+CD4^+CD25^{+high}$ cells (Tregs) revealed a highly significant decrease in the frequency of Treg cells in SLE patients compared to healthy controls. **(1.08 ± 0.29 and 2.46 ± 0.7 for active SLE and control respectively, p < 0.0001) (1.54 ± 0.23 and 2.46 ± 0.7 for inactive SLE and control respectively**, p =**0.0003**).

Also, active SLE patients showed significant lower Tregs percent when compared to inactive group.

(1.08 ± 0.29 versus 1.54 ± 0.23, p value =0.0004). (Table 5 and figure 2).

The distribution of $FoxP3^{+}CD4^{+}CD25^{+high}$ cells (Tregs) revealed a highly significant decrease in the frequency of Treg cells in RA patients compared to healthy controls. **(1.01 ± 0.31 and 2.46 ± 0.7 for active RA and control respectively, p < 0.0001) (1.52 ± 0.24 and2.46 ± 0.7 for inactive RA and control respectively**, p =**0.0003**).

Also, active RA patients showed significant lower Tregs percent when compared to inactive group. **(1.01 ± 0.31 versus 1.52 ± 0.24, p value =0.0002). (Table 5 and figure 2).**

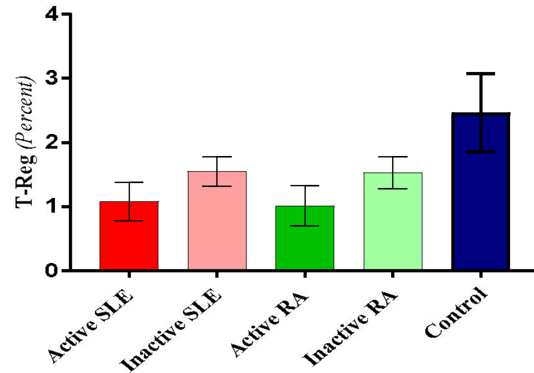

Figure (2): T-Reg percentage in the studied groups

Table (3): Comparison between clinical variables in RA Patients and Control groups

Variable	Group II A *Active RA*	Group II B *Inactive RA*	Group III *Control*	P value		
				Group IIA & Group III	Group IIB & Group III	Group IIA & Group IIB
Age *(years)*	37 ± 8.03	36.1 ± 6.35	32 ± 6.61	0.2135	0.1010	0.9163
Age of onset *(years)*	30.4 ± 7.99	30 ± 7.74				0.9024
Disease Duration *(years)*	6.6 ± 6.97	6.2± 4.1				0.8721
Weight *(kg)*	68.47 ± 6.05	66.6 ± 5.44	69.9 ± 4.95	0.5406	0.1732	0.4404
Height *(cm)*	172.6 ± 5.38	173.7 ± 5.22	172.3 ± 5.7	0.8955	0.5762	0.6175
BMI *(Kg/m^2)*	23.08 ± 3.04	22.15 ± 2.36	23.58 ± 1.99	0.6525	0.1607	0.4239
Systolic Bl. Pressure *(mmHg)*	129.3± 12.37	128 ± 10.59	120.5±6.85	0.0521	0.0764	0.7828
Diastolic Bl. Pressure *(mmHg)*	82.67 ± 7.9	81 ± 6.9	76.5 ± 7.09	0.0604	0.1701	0.5970
DAS-28 score	5.197± 1.19	0.8 ± 0.25				**<0.0001 *****

Table (4): Comparison between Laboratory variables in RA Patients and Control groups

Variable	Group II A *Active RA*	Group II B *Inactive RA*	Group III *Control*	P value		
				Group IIA & Group III	Group IIB & Group III	Group IIA & Group IIB
Hemoglobin *(gm/dl)*	11.27 ± 1.42	12.8 ± 0.87	13.43±0.72	**0.0002 *****	0.0974	0.0062
WBC *(x1000/ml)*	5.93 ± 1.68	6.42 ± 2.32	6.9 ± 1.5	0.1517	0.5827	0.5491
Platelets *(x1000/ml)*	234.5 ± 85.3	229.4 ± 66.6	289 ± 88.6	0.1057	0.0645	0.8742
ESR *(mm/h)*	84.27 ± 22.8	26 ± 9.9	8 ± 1.6	**<0.0001 ******	**<0.0001 ******	**<0.0001 ******
CRP	21.9 ±6.85	8 ± 6.49	0.8 ± 0.4	**< 0.0001 ******	**0.0026 ****	**< 0.0001 ******
ALT *(IU/ml)*	27.1 ± 13.8	23.5 ± 8.39	18.7 ± 7.68	0.0935	0.1991	0.4656
AST *(IU/ml)*	25.13 ± 11.4	22.5 ± 7.6	20 ± 7.7	0.2275	0.4758	0.5294
s Creatinine *(mg/dl)*	1.12 ± 0.3	0.9 ± 0.21	0.85±0.13	**0.0160 ***	0.3590	0.0950
BUN	21.9 ± 10.1	20.3 ± 7.3	14.5±1.78	**0.0315 ***	**0.0265 ***	0.6651
Rheumatoid Factor Posivity	11/15 (73.3%)	7/10 (70%)				NS
ACCP Positivity	12/15 (80%)	7/10 (70%)				NS

Table (5): T-Reg percentage in the studied groups

Variable		Group IA Active SLE N=15	Group I B Inactive SLE N=10	Group II A Active RA N=15	Group II B Inactive RA N=10	Group III Control N=10
T-Reg %	*Mean ±SD*	1.08 ± 0.29	1.54 ± 0.23	1.01 ± 0.31	1.52 ± 0.24	2.46 ± 0.7
	Range	0.66 – 1.62	1.16-1.92	0.63 – 1.72	1.17-1.88	1.8-3.66

P-Value					
Group I A and Group III	**Group I B and Group III**	**Group II A and Group III**	**Group II B and Group III**	**Group I A and Group I B**	**Group IIA and Group II B**
< 0.0001 ****	**0.0003 ******	**< 0.0001 ******	**0.0003 ******	**0.0004 ******	**0.0002 ******

Correlations between T-Reg (Percent) and parameters of lupus activity

In the present study, positive correlations were observed between T-Reg (Percent) and C3 (r = 0.5714 & P = 0.0028) and C4 (r = 0.2870 & P = 0.0185) inSLE patients**,** while negative correlations were observed between T-Reg (Percent) and ESR (r = -0.4933 & P = 0.0122), 24 hr. Urinary protein (r = -

0.4981 & P = 0.0113), Anti-dsDNA ab (r = -0.4325 & p = 0.0308), and SLEDAI (r = -0.5702 & P = 0.0029). **(figure 3-9).**

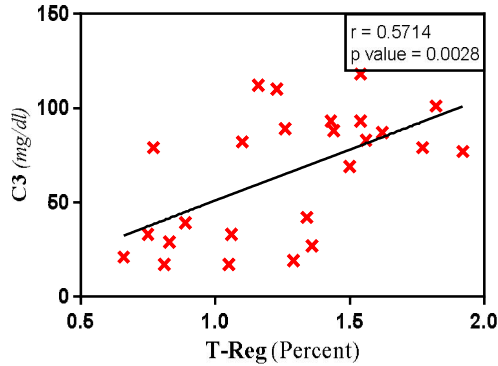

Figure (3): Correlation between T-Reg percent and C3 in SLE patients

Positive correlations is observed between T-Reg (Percent) and C3 (r=0.5714 & P=0.0028) (Both are depleted).

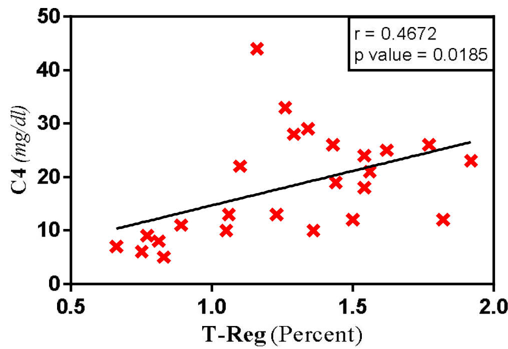

Figure (4): Correlation between T-Reg percent and C4 in SLE patients

Positive correlations were observed between T-Reg (Percent) and C4 (r=0.2870 & P=0.0185).

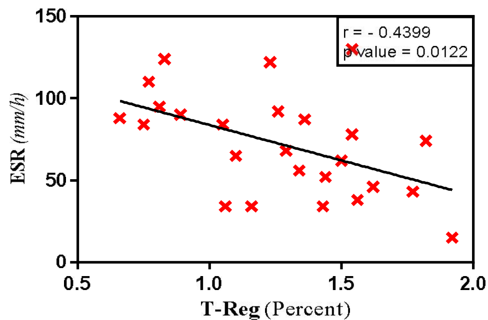

Figure (5): Correlation between T-Reg percent and ESR in SLE patients

Negative correlations is observed between T-Reg (Percent) and ESR (r =0.4933 & P = 0.0122).

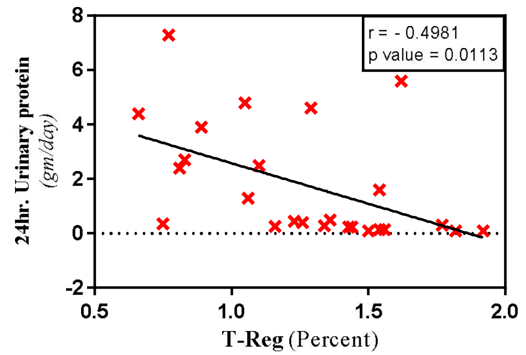

Figure (6): Correlation between T-Reg percent and 24 hr. urinary protein excretion in SLE patients

Negative correlations is observed between T-Reg (Percent) and 24 hr. Urinary protein (r = -0.4981 & P = 0.0113).

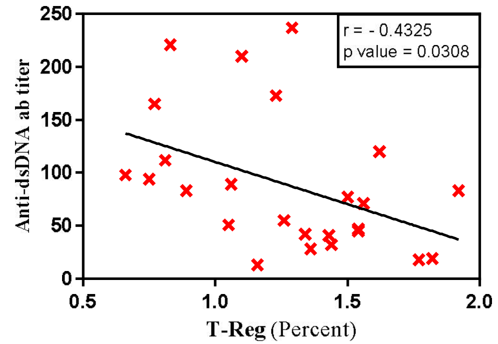

Figure (7): Correlation between T-Reg percent and anti-dsDNA ab titer in SLE patients

Negative correlations is observed between T-Reg (Percent) and Anti-dsDNA ab (r = -0.4325 & p = 0.0308).

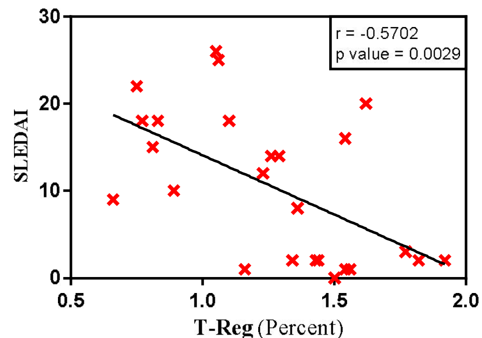

Figure (8): Correlation between T-Reg percent and SLEADI in SLE patients

Negative correlations is observed between T-Reg (Percent) and SLEDAI (r = -0.5702 & P = 0.0029).

Correlations between T-Reg (Percent) andparameters of RA activity

In the current study, negative correlations were observed between T-Reg (Percent) and ESR (r= -0.6018 & P = 0.0015), CRP (r= -0.5931 & P = 0.0018) and DAS-28 score (r= -0.6825 & P = 0.0002) in RA patients.

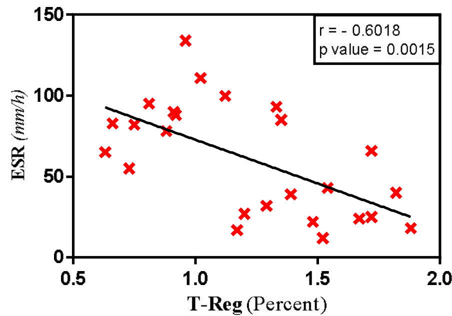

Figure (9): Correlation between T-Reg percent and ESR in RA patients

Negative correlation is observed between T-Reg (Percent) and ESR (r= -0.6018 & P = 0.0015).

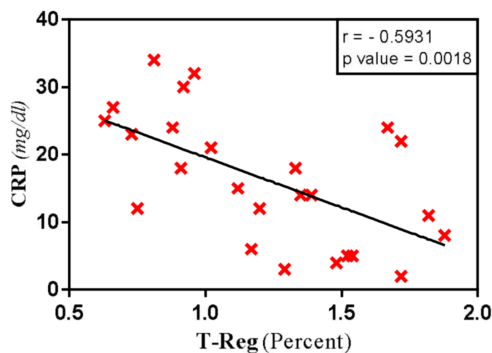

Figure (10): Correlation between T-Reg percent and CRP in RA patients

Negative correlation is observed between T-Reg (Percent) and CRP (r= -0.5931 & P = 0.0018).

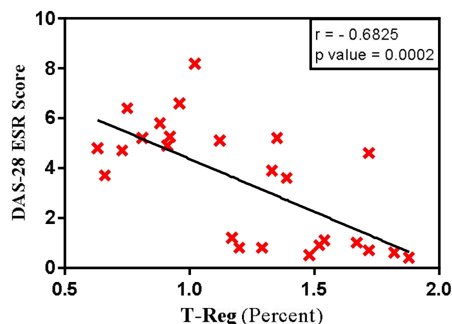

Figure (11): Correlation between T-Reg percent and DAS-28 score in RA patients

Negative correlation is observed between T-Reg (Percent) and DAS-28 score (r= -0.6825 & P = 0.0002).

4. Discussion

The human immune system is equipped with a number of mechanisms involved in maintaining immune tolerance and protection against autoimmunity. T-Reg lymphocytes are the key cells controlling the autoimmunization process. Their role is illustrated by an active and dominant control over the function of effector T cells ***(Leung et al., 2010).***

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that can be fatal. As occurs in other autoimmune diseases, the immune system attacks the body's cells and tissues, resulting in inflammation and tissue damage ***(James et al., 2005).*** It is associated with abnormal immune response including production of autoantibodies; abnormalities of the complement system, T cell–B cell interaction, phagocytosis and T cell hyperactivity represent a central feature of SLE ***(Jacobi and Diamond, 2005)***.

There is a connection between lupus and the disturbance of regulatory T cells which play an important role in maintaining a healthy immune system ***(Horwiz D., 2008).***

RA is a chronic inflammatory autoimmune disease arising from a breakdown in self-tolerance, which leads to aberrant immune responses to autoantigens. T-Regs constitute one of the key mechanisms of self-tolerance and are a major focus of study in RA in order to design new and improved therapies to reinstate self-tolerance ***(Cooles et al., 2013).***

In this study, we investigated the percent of $CD4^+ CD25^+$ FoxP3 T regulatory cells in patients with SLE and RA patients, as well as the correlation with other parameters of disease activity.

The present study included 50 paients divided into group Ia (15 patients with active SLE), group Ib (10 patients with inactive SLE), group IIa (15 patients with active RA) and group IIb (10 patients with inactive RA). $CD4^+ CD25^+$ FoxP3 T regulatory cells were estimated for all patients and their results were compared to those of 10 control subjects (group III).

In the present cross sectional, observational study on SLE and RA patients, results revealed that $CD4^+CD25^+$FoxP3 T regulatory cells (as a percent of total CD4 cells) was significantly lower in SLE and RA patients when compared to healthy controls. Also, active SLE and RA patients showed significant difference when compared to inactive groups.

In the present study, there was a significant correlation between T-Reg percent and C3, C4 levels, ESR and SLEDAI in SLE patients, and a significant

correlation between T-Reg percent and ESR, CRP and DAS-28 in RA patients.

Our results of decrease T-Reg in SLE patients when compared to healthy controls are in agreement with **(Lyssuk et al., 2007; Valencia et al., 2007, Bonelli et al., 2008 and Miyara et *al*., 2005)**, who investigated the frequencies of circulating Treg cells in SLE and found a significant decrease in circulating Treg cells in peripheral blood of diseased subjects when compared to controls.

In accordance to these results, were ***liu et al. (2004)*** and ***Yang et al. (2005)*** who found that proportion of peripheral blood T-reg cells in SLE group was significantly lower than that in healthy control group. This finding was explained by many studies on the role of Treg cells in autoimmune diseases especially in SLE, which showed that the pathogenesis of SLE involves breakdown of immunologic self-tolerance resulting in the development of autoantibodies. Many T-cell and B-cell abnormalities have been described, and these include defects in regulatory T cells that normally prevent pathologic self-reactivity ***liu et al. (2004)*** and ***Yang et al. (2005)***

Iikuni et al. (2009) adds supportive evidence to this explanation, they stated that in human SLE, the Treg cells can directly suppress autoantibody-producing B cells, including those that belong to pathogenic subtypes that are found expanded in active SLE. The cell-to-cell dependent mechanisms of suppression of autoreactive B cells by Treg cells in SLE, that could represent an attempt to directly control humoralautoimmunity, involve the release of perforin and granzyme by activated Treg cells and the induction of apoptosis in these autoreactive B cells.

In an attempt to understand the role of Treg cells in pathogeneses of SLE ***(Scalapino and Daikh 2009)*** stated that, In addition to the B cells, Treg cells also suppress lupus $CD4^+$ T cells that provide help to autoantibody-producing B cells**.** This intermediate suppression of humoral immune responses by Treg cells in SLE involves the induction of a state of hyporesponsiveness to stimulation (anergy) in the $CD4^+$ Th cell and might represent a modality for the host to more effectively reduce the production of pathogenic autoantibodies, since both hyperactive Th cells and B cells would be rendered inactive by suppressor Treg cells.

Contrary to these finding, some studies report increased levels of T-Reg cells in SLE compared with those in healthy controls ***(Lin et al., 2007)***, or a resistance of lupus effector T cells to T-Reg cell suppression instead of defects of SLE T-Reg cells ***(Venigallaet al., 2008).***

It is evident from the analysis of the different studies that the data arising from the evaluation of the global $CD4^+CD25^+$ cell population are inconclusive, probably because of the extreme heterogeneity of this T-cell subset. Indeed, since the percentage of circulating T-Reg in humans should be less than 2–3%, the very high numbers and the wide variability of the reported percentages of the cell subpopulations considered in these studies, ranging from 6.5% to 31.3% in normal controls and from 6% to 37.8% in SLE patients, appear to support this hypothesis **(Gerli et al., 2009).**

Most of the earlier studies focused only on phenotypic characterization of circulating T cells, but they were limited by difficulties in distinguishing T-Reg cells from simply activated T cells bearing the CD25 surface molecule, as T-Reg cells in humans are more represented in $CD25^{high}$ cell fraction, that, however, is difficult to be unequivocally defined. It has been demonstrated, in fact, that the higher is CD25 surface expression the higher is suppressor activity. In order to more accurately discriminate between T-Reg and activated T cells, additional evaluations of Foxp3 mRNA expression within the $CD4^+CD25^+$ cell population have been performed in some studies in SLE patients. Recently, a more extensive analysis of Foxp3 expression has been possible with the availability of new tools for Foxp3 detection by flowcytometry that have improved data reliability. **(Gerli et al., 2009).**

Analysis of the results about Foxp3 cell expression in SLE is more complex, since the evaluation of its expression has been carried out with different methods and it has been analyzed within different cell subsets. However, it is of interest the observation that Foxp3 expression, when evaluated in both $CD25^+$ and $CD25^-$ cell subsets, seems to be reduced in the cell population bearing the CD25 and increased in the T-cell subset lacking this surface molecule. **(Zhang et al., 2008).** Interestingly, similar results have been reported in a cohort of new-onset SLE patients **(Cepika et al., 2007 and Bonelli et al., 2008).**

Pathogenic basis of SLE may result also by an imbalance between effector and regulatory T cells and not only by T-Reg cell impairment only. There is some evidence that impaired suppressor function is demonstrable when Treg cells are co-cultured with autologous effector T cells but not with effector T cells obtained from healthy donors, thereby suggesting a possible resistance of effector T cells to T-Reg inhibition **(Mellor-Pitaet al., 2006 and Lee et al., 2008)**. Also these data, however, have not been confirmed in subsequent studies **(Vargas-Rojas et al., 2008)**.

It is conceivable that many discrepancies about Treg cell findings in SLE may be dependent on both different phases of disease activity and

immunosuppressive treatments that may affect viability and function of T cells. These findings are in agreement with the general observation that numerically decreased and/or functionally defective Treg cells seems to be usually associated with active phases of the disease, whereas a phenotypic and functional picture similar to controls is the most frequent finding in inactive SLE **(Valencia et al., 2007, Cepika et al., 2007 and Yates et al., 2008)**.

In the present study, there was a significant correlation between T-Reg percent and C3, C4 levels, ESR and SLEDAI in SLE patients. These results were discordant with ***(lee et al 2008),*** who found a non-significant correlation with either of C3 and C4 levels. However, ***(lee et al 2008),*** found a significant correlation with ESR levels.

Also, a significant negative correlation between T-Reg and anti-dsDNA ab in SLE patients, which is in agreement with ***L. Ma et al., 2013,*** who found that the numbers of $CD4^+CD25^+FoxP3^+$ in the anti-dsDNA+ patients were significantly fewer than those in the anti-dsDNA- patients.

Our result of decrease T-reg cells percent in active SLE group compared to inactive group suggest an important role of these cells in pathogenesis of lupus activity. Also, significant correlation of T-regwith marker of disease activity is another evidence which can support this suggestion.

Our result of decrease T-reg cells percent in RA compared to control group was in agreement with other groups ***(Lawson et al., 2006, Sempere-Ortells et al., 2009, Samson et al.,2012, Chen et al., 2012).*** The exact mechanisms that reduce the level of T-Regs in RA are not clear. It was suggested that regulatory cells are recruited to sites of inflammation in an attempt to suppress disease, resulting in a relative reduction in the peripheral blood population ***(Lawson et al., 2006).***

Reports of a higher number of T-Regs present in synovial fluid than that in the peripheral blood in RA patients support this explanation. T-Regs accumulated in inflamed joints express high levels of surface and intracellular CTLA-4, GITR, OX-40, and FoxP3 ***(Mottonen et al., 2005)***.

Moreover, T-Regs were found to display increased tendency to undergo spontaneous apoptosis in active RA ***(Toubi et al., 2005, Li et al., 2014).*** Chavele and Ehrenstein proposed that in an inflammatory condition like RA, it is quite possible that Tregs in the presence of the different proinflammatory cytokines will become unstable and convert to pathogenic T-cells ***(Chavele and Ehrenstein, 2011).*** This decline in regulatory T-cell numbers may pre-dispose to persistent auto-immune diseases including RA ***(Lawson et al., 2006)***.

Contrary to our results there are reports of increased Tregs in RA peripheral blood ***(van Amelsfort et al., 2004 and Han et al., 2008),*** but in their research they assessed different $CD4^+CD25^+$ T cell subtypes (including $CD4^+$ T-cells expressing low levels of CD25 and those expressing FoxP3 and those not expressing it) which could account for the discrepancy in the results.

We found a significant negative correlation between DAS score and the decrease in frequency of Treg cells. The lower the frequency of Tregs, the higher the DAS score reflecting higher disease activity, also, ESR and CRP. This may indicate the importance of T- reg cells in pathogenesis of RA activity, but the precise role of their deficiency in pathogenesis of activity need to be defined by further studies. Others didn't report any correlation between Treg cell frequencies or cytokines with DAS score ***(Al-Zifzaf et al., 2015).***

Conclusion

1. $CD4^+CD25^+$FoxP3 T regulatory cells (as a percent of total CD4 cells) was significantly lower in SLE and RA patients when compared to healthy controls.
2. Also, active SLE and RA patients showed significant lower T- reg cells percent than inactive groups.
3. Moreover, $CD4^+CD25^+$ FoxP3 T regulatory cells percentage of CD4 cells was significantly correlated with ESR, C3, C4 levels and SLEDAI score in SLE patients, Also, it was significantly correlated with ESR, CRP and DAS-28 score in RA patients which is the main marker of disease activity, indicating a power to detect patients with active disease.

Recommendations

1- Measurement of CD4+CD25+ FoxP3 T regulatory cells percentage of CD4 cells in SLE and RA patients for evaluation of disease activity.

2- Further evaluation of T-Reg in a large prospective longitudinal study to confirm its potential application as a superior or additional biomarker for non-invasive monitoring of disease activity and for the detection flares, and to evaluate the therapeutic role of T-Reg and their released cytokines in SLE and RA.

3- Incorporating other new biomarkers together with T-Reg frequency and function, may augment its value as an index of disease activity.

References

1. *Adlan AM, Lip GY, Paton JF, Kitas GD, Fisher JP.* Autonomic function and rheumatoid arthritis-A systematic review. Semin Arthritis Rheum. 2014; 44:283-304.

2. *Al-Zifzaf D, El Bakry S, Mamdouh R, Shawarbyc L, Abdel A, et al. (2015)* FoxP3+T regulatory cells in Rheumatoid arthritis and the imbalance of the of the Treg/TH17 cytokine axis. e Egyptian Rheumatologist 37: 7-15.
3. *Banham AH.* Cell-surface IL-7 receptor expression facilitates the purification of FOXP3(+) regulatory T cells. Trends Immunol 2006;27:541–4.
4. *Bonelli, M., Savitskaya, A., von Dalwigk, K., Steiner, C. W., Aletaha, D., Smolen, J. S. and Scheinecker, C.,* Quantitative and qualitative deficiencies of regulatory T cells in patients with systemic lupus erythematosus (SLE). Int. Immunol. 2008. 20: 861–868.
5. *Cepika AM, Marinic I, Morovic-Vergles J, Soldo-Juresa D, Gagro A.* Effect of steroids on the frequency of regulatory Tcells and expression of FOXP3 in a patient with systemic lupus erythematosus: a two year follow-up. Lupus 2007;16:374–7.
6. *Chang X, Zheng P, Liu Y. FoxP3:* a genetic link between immunodeficiency and autoimmune diseases. Autoimmun Rev 2006;5:399–402.
7. *Chavele K., Ehrenstein M.* Regulatory T-cells in systemic lupus erythematosus and rheumatoid arthritis. FEBS Letters. 2011;585(23):3603–3610.
8. *Chen R, Tao Y, Qiu K, Huang W, Huang C, Li J.* Association of circulating Treg cells with disease activity in patients with rheumatoid arthritis. Nan Fang Yi Ke Da Xue Xue Bao 2012;32(6):886–9.
9. *Cooles FA, Isaacs JD, Anderson AE.* Treg cells in rheumatoid arthritis: an update. Curr Rheumatol Rep 2013;15(9):352.
10. *Daniel Aletaha, Neogi T, Silman AJ, Funovits J, Felson DT, et al., 2010* rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Ann Rheum Dis. 2010 Sep;69(9):1580-8. doi: 10.1136/ard.2010.138461.
11. *Gerli R, Nocentini G, Alunno A, Bocci EB, Bianchini R, Bistoni O, et al.* Identification of regulatory T cells in systemic lupus erythematosus. Autoimmun Rev. 2009;8:426–430.
12. *Han GM, O'Neil-Andersen NJ, Zurier RB, Lawrence DA*. CD4+CD25high T cell numbers are enriched in the peripheral blood of patients with rheumatoid arthritis. Cell Immunol 2008;253(1–2):92–101.
13. *Horwitz, D. A.,* Identity of mysterious CD4+CD25-Foxp3+ cells in SLE. Arthritis Res. Ther. 2010. 12: 101.
14. *Horwiz DA.* Regulatory T cells in systemic lupus erythematosus: past, present and future. Arthritis Res Ther 2008; 10:227.
15. *Iikuni N, Lourenço EV, Hahn BH, La Cava A.* Cutting edge: regulatory T cells directly suppress B cells in systemic lupus erythematosus. J Immunol 2009; 183:1518–1522.
16. *Jacobi AM1, Diamond B (2005):* Balancing diversity and tolerance: lessons from patients with systemic lupus erythematosus. J Exp Med. 2005 Aug 1;202(3):341-4.
17. *James William, Berger Timothy, Elston Dirk (2005).* Andrews' Diseases of the Skin: Clinical Dermatology. (10th ed.). Saunders. ISBN 0-7216-2921-0.
18. *Kleczynska W, Jakiela B, Plutecka H, et al.* Imbalance between Th17 and regulatory T-cells in systemic lupus erythematosus. Folia Histochem Cytobiol. 2011;49:646–653.
19. *L. Ma, P. Zhao, Z. Jiang, Y. Shan and Y. Jiang;* Imbalance of different types of CD4+forkhead box protein 3 (FoxP3)+ T cells in patients with new-onset systemic lupus erythematosus; Clinical and Experimental Immunology; 2013; 174: 345–355.
20. *Lawson CA, Brown AK, Bejarano V, Douglas SH, Burgoyne CH, et al. (2006)* Early rheumatoid arthritis is associated with a de cit in the CD4+CD25high regulatory T cell population in peripheral blood. Rheumatology (Oxford); 45: 1210-1217.
21. *Lee HY, Yun HJ, Kim YM, et al.* Altered frequency and migration capacity of CD4+CD25+ regulatory T cells in systemic lupus erythematosus. Rheumatology (Oxford) 2008; 47:789–794.
22. *Leung N, Mabarrack N, Barbour A, Cummins A, Barry S.* Foxp3+ regulatory T cells, Th17 effector cells, and cytokine environment in inflammatory bowel disease. J Clin Immunol. 2010;30:80–89.
23. *Li N, Ma T, Han J, Zhou J, Wang J, Zhang J, et al.* Increased apoptosis induction in CD4+ CD25+ Foxp3+ T cells contributes to enhanced disease activity in patients with rheumatoid arthritis through IL-10 regulation. Eur Rev Med Pharmacol Sci. 2014; 18:78-85.
24. *Liu MF, Wang CR, Fung LL, Wu CR.* Decreased CD4+CD25+ T cells inperipheral blood of patients with systemic lupus erythematosus. Scand J Immunol 2004; 59:198–202.
25. *Lyssuk, E. Y., Torgashina, A. V., Soloviev, S. K., Nassonov, E. L. and Bykovskaia, S. N.,* Reduced number and function of CD4+CD25highFoxP3+ regulatory T cells in patients with systemic lupus

erythematosus. Adv. Exp. Med. Biol. 2007. 601: 113–119.
26. *Mellor-Pita S, Citores MJ, Castejon R, Tutor-Ureta P, Yebra-Bango M, Andreu JL, et al.* Decrease of regulatory T cells in patients with systemic lupus erythematosus. Ann Rheum Dis 2006;65:553–4.
27. *Miyara, M., Amoura, Z., Parizot, C., Badoual, C., Dorgham, K., Trad, S., Nochy, D. et al.,* Global natural regulatory T cell depletion in active systemic lupus erythematosus. J. Immunol. 2005. 175: 8392–8400.
28. *Mottonen M, Heikkinen J, Mustonen L, et al.* CD4þCD25þ T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis. ClinExp Immunol 2005;140:360e7.
29. *Petri M, Orbai AM, Alarcón GS, Gordon C, Merrill JT et al.,* Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. Arthritis Rheum. 2012 Aug;64(8):2677-86. doi: 10.1002/art.34473.
30. *Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M.* Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995;155:1151–64.
31. *Samson M, Audia S, Janikashvili N, Ciudad M, Trad M, Fraszczak J, et al.* Brief report: inhibition of interleukin-6 function corrects Th17/Treg cell imbalance in patients with rheumatoid arthritis. Arthritis Rheum 2012;64(8):2499–503.
32. *Scalapino KJ, Daikh DI.* Suppression of glomerulonephritis in NZB/NZW lupus-prone mice by adoptive transfer of ex vivo expanded regulatory T cells. PLoS One. 2009;4:e6031.
33. *Sempere-Ortells JM, Pe ́rez-Garcia V, Marin-Alberca G, Peris- Pertusa A, Benito JM, et al. (2009)* Quanti cation and phenotype of regulatory T cells in rheumatoid arthritis according to disease activity score-28. Autoimmunity 42: 636-645.
34. *Szmyrka-Kaczmarek M., Kosmaczewska A., Ciszak L., Szteblich A., Wiland P.* Peripheral blood Th17/Treg imbalance in patients with low-active systemic lupus erythematosus. Postepy Hig. Med. Dosw. 2014;68:893–898.
35. *Toubi E, Kessel A, Mahmudov Z, Hallas K, Rozenbaum M, Rosner I.* Increased spontaneous apoptosis of CD4+CD25+ T cells in patients with active rheumatoid arthritis is reduced by infliximab. Ann N Y Acad Sci 2005;1051:506–14.
36. *Valencia X, Yarboro C, Illei G, Lipsky PE.* Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus. J Immunol 2007; 4:2579–88.
37. *van Amelsfort JM, Jacobs KM, Bijlsma JW, et al.* CD4(þ)CD25(þ) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis Rheum 2004;50:2775e85.
38. *Vargas-Rojas MI, Crispín JC, Richaud-Patin Y, Alcocer-Varela J.* Quantitative and qualitative normal regulatory T cells are not capable of inducing suppression in SLE patients due to T-cell resistance. Lupus 2008;17:289–94.
39. *Venigalla, R. K., Tretter, T., Krienke, S., Max, R., Eckstein, V., Blank, N., Fiehn, C. et al.,* Reduced CD4+, CD25- T cell sensitivity to the suppressive function of CD4+, CD25high, CD127 -/low regulatory T cells in patients with active systemic lupus erythematosus. Arthritis Rheum. 2008. 58: 2120–2130.
40. *Wang T, Sun X, Zhao J, Zhang J, Zhu H, Li C, et al.* Regulatory T cells in rheumatoid arthritis showed increased plasticity toward Th17 but retained suppressive function in peripheral blood. Ann Rheum Dis. 2015;74:1293–1301.
41. *Yang XY, Lu XY, Xu DH, Lu QH, Wang QH, Wu HX.* Clinical significance of CD4+CD25+ T cells in peripheral blood of patients in systemic lupus erythematosus. Zhonghua Nei Ke Za Zhi 2005; 44:570–572.
42. *Yates J, Whittington A, Mitchell P, Lechler RI, Lightstone L, Lombardi G.* Natural regulatory Tcells: number and function are normal in themajority of patients with lupus nephritis. ClinExp Immunol 2008;153: 44–55.
43. *Zhang B, Zhang X, Tang F, Zhu L, Liu Y.* Reduction of forkhead box P3levels in CD4+CD25 high T cells in patients with new onset systemic lupus erythematosus. ClinExp Immunol 2008;153:182–7.

3/24/2018