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Circulating CD19 CD24^{hi} CD38^{hi} Regulatory B Cells Percentage in Lupus Nephritis Patients: Does It Differ?

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ABSTRACT

Background: There is a debate about the functional role of regulatory B cells in the lupus nephritis (LN) pathogenesis.

Objective: The aim of the present study was to analyze total B lymphocytes and their subtype regulatory B lymphocytes (Breg) % in systemic lupus erythematous (SLE) patients, in addition to assess their association with LN activity.

Patients and Methods: The present study included 19 lupus nephritis (LN) patients, 11 SLE patients without lupus nephritis (non-LN). They were matched with 20 healthy individuals as a control group. LN activity was evaluated by Renal SLEDAI (rSLEDAI), nephritis patients were divided into active lupus nephritis (renal SLEDAI≥4) and non-active lupus nephritis patients (renal SLEDAI = 0). Full history taking, clinical examination and baseline laboratory investigations were done for all participants. Total B lymphocytes and their subtypes Breg were measured by flow cytometry.

Results: SLE diseased patients have significantly higher total B lymphocytes compared to matched controls; also, SLE diseased patients (LN and non-LN) have significantly lower CD19+ CD24^{hi} CD38^{hi} compared to matched controls, regardless of LN activity.

Conclusion: The present study supports B cell and Breg role in aetio-pathogenesis of SLE and also contributes to the onset of LN which indicates a dysfunctional regulatory mechanism.

Keywords: Systemic lupus erythematous, Lupus nephritis, B lymphocytes and regulatory B lymphocytes.

INTRODUCTION

Systemic lupus erythematous is a chronic autoimmune disease associated with multiple organ manifestations [1]. Patients with SLE frequently exhibit renal involvement, and despite modern available therapeutic options, the development of LN greatly raised the risk of renal failure and patient mortality [2]. However; the possible aetio-pathogenesis of LN is not well understood but B cells play an essential role [1].

B cells play a pathogenic role in LN which could be contributed to the production of cytokine and autoantibodies, their ability to activate T cells through co-stimulatory molecules, and their function as antigen-presenting cells [3].

Bregs are a sub-set of B cell lymphocyte which have the ability to adversely inhibit the immune response [4], primarily by secreting transforming growth factor (TGF)–β, regulatory cytokines like interleukin (IL)-10 and by expressing inhibitory antibodies that block T cells and auto-reactive B cells, which are ultimately connected to the SLE pathogenesis and disease activity [5]. Also there is a proof that Bregs are crucial in numerous animal models of autoimmune disease and inflammation. Bregs are an undeniably significant part of the immune system, even if their definition and mechanism of action are still being recently investigated [4, 6].

Human Bregs are primarily divided into memory (CD24^{high}CD27^{+}) and transitional (CD^{19+}CD24^{high}CD38^{high}) types [7, 8]. Bregs are functionally compromised in SLE patients, according to earlier research, which raises the possibility that they are involved in the pathophysiology of lupus [7, 9]. However, there is a debate on how Bregs function in SLE, the Bregs percentage in SLE diseased patients is either increased or decreased [1, 10-13]. Therefore, the aim of the current study was to evaluate the role of B lymphocytes and Breg in patients with SLE, especially in patients with LN.

PATIENTS AND METHODS

Study design:

The present study was an observational cross-sectional study conducted on 30 SLE diseased patients (LN patients, n=19), (non-LN patients, n=11). They were matched with 20 healthy individuals as a control group.

Inclusion criteria:

All recruited patients were diagnosed with the revised and validated classification criteria of the Systemic Lupus Collaborating Clinics (SLICC) and the American College of Rheumatology (ACR) SLE [14]. Patients recruited from outpatient clinics and inpatient of Rheumatology, Rehabilitation and Physical Medicine Department, Assiut University Hospitals, Egypt in the period from 1st of May 2018 up to the end of May 2019. The control group included volunteers with similar ages and sexes.

Exclusion criteria:

Patients, who were pregnant, aged less than 18 years old or those with other systemic autoimmune disorders were excluded.

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Ethical aspects:

The study design was approved by the Scientific Ethics Committee of the Faculty of Medicine, Assiut University (17200028), and all patients gave written informed consent. The WHO and Declaration of Helsinki recommendations regarding the protection of the rights and well-being of the people studied were also followed.

Data collection:

Sociodemographic details, a thorough clinical examination, a complete medical history, and a history of treatment were all recorded. Laboratory results (proteinuria > 500 mg/day and/or cellular casts [red blood cells, granular, tubular, or mixed]) were used to determine the diagnosis of LN, and the diagnosis was further supported by renal biopsies obtained from patient records. LN activity was evaluated by Renal SLEDAI (rSLEDAI). Hematuria, pyuria, proteinuria, and urinary casts are the four renal-related parameters of the used score. The renal SLEDAI has a maximum score of 16 and a range of 0 (inactive renal disease). When the renal SLEDAI score was 4, it was assumed that the patient had active lupus nephritis [15, 16].

All patients were subjected to venous blood sample collection under strict aseptic conditions for investigations (such as complete blood picture, erythrocyte sedimentation rate, liver function tests, kidney function tests, lactate dehydrogenase and creatine phosphokinase), the immunological markers namely; anti-nuclear antibody and anti-double stranded deoxyribonucleic acid. Twenty-four hours proteins in urine and creatinine clearance were estimated. Complete urine analysis for: pyuria (>5 WBCs/HPF), hematuria (>5 RBCs/HPF) and urinary casts was done.

Evaluation of Breg cells frequencies: Breg cell frequencies were measured by FACSCalibur flow cytometry using FITC-conjugated-CD38, PE-conjugated-CD24 and PerCP-conjugated CD19 (All from BD Bioscience, USA). CellQuest software (Becton Dickinson Biosciences, USA) was used for analysis. The lymphocytes were identified using a forward and side scatter histogram. CD19+ B cells were then gated. Then the CD38 and CD24 expression on the CD19+B cells were discovered. Regulatory B cells were identified as CD19+CD24+hiCD38+hi cells.

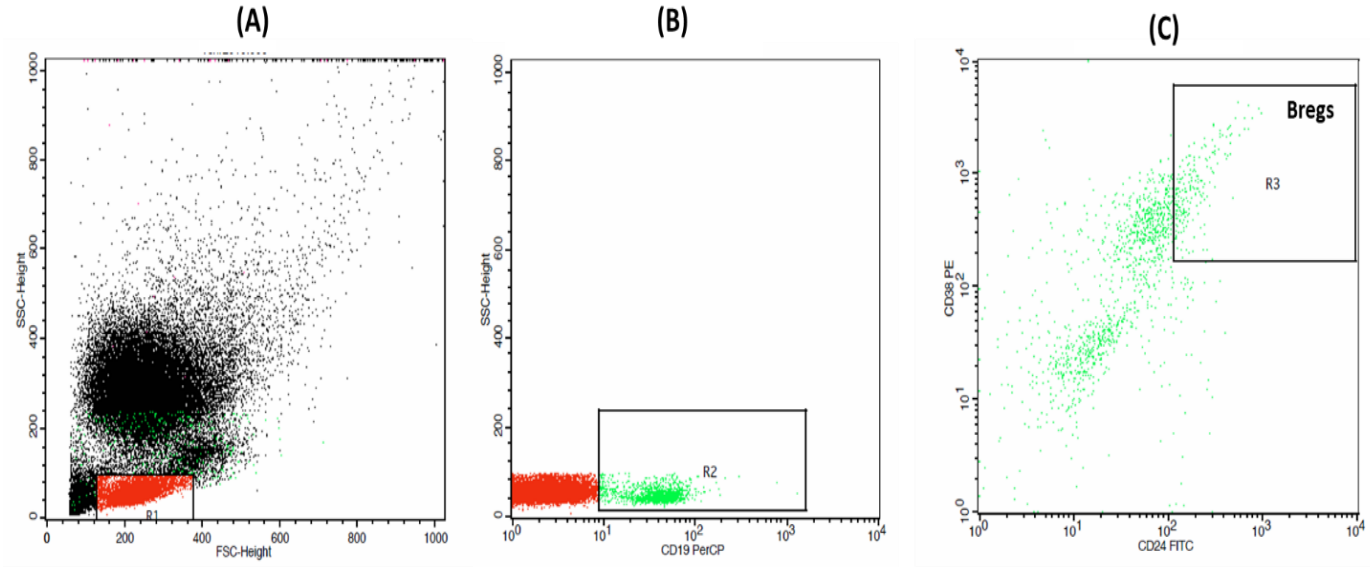

Figure (1): Flow cytometric identification of regulatory B cells. A. To define the lymphocyte population, a forward and side scatter plot was used (R1). B. The proportion of CD19+ cells (R2) in the lymphocyte population was evaluated. C. CD19+ CD24+hi CD38+hi cells were identified by measuring the expression of CD24 and CD38 in CD19+ cells

Statistical Analysis

SPSS (Statistical Package for the Social Sciences), version 22 was used to analyze the data. Qualitative data were statistically described in the form of mean ± standard deviation (SD), and median (range), and qualitative data were statistically described in form of number (percentage).

Student t-test and ANOVA test with Bonferroni corrections or Mann Whitney test were carried out to compare parametric/non-parametric data. Categorical data were compared by Exact test.

Pearson product-moment correlation coefficient was calculated for correlations between various variables. P-value was set significant at 0.05.

RESULTS

The study included 30 SLE patients; 27 (90.0%) were females and 3 (10.0%) were males with female: male of 9:1. B-lymphocytes (%) in SLE group was significantly higher in comparison to matched controls.

Meanwhile, Breg cell (%) was significantly lower in SLE cases in comparison to matched controls as shown in **Table 1**.

Table (1): B-lymphocyte and Breg cells (%) in SLE diseased patients and healthy control group (n=50)

Variable name	SLE (n=30)	Controls (n=20)	P value
B-lymphocytes (%)			**<0.002***
• Mean ± SD	17.28 ± 4.19	13.53 ± 3.29	
Breg cell (%)			**<0.001***
• Mean ± SD	2.37 ± 0.49	3.46 ±0.75	

LN: Lupus nephritis; Breg cells: Regulatory B cells. *: Significant

The characteristics of the patients with and without LN versus matched controls are presented in **Table 2**. We found that patients with LN were significantly younger in age, and younger at the age of onset of SLE development compared to non-LN patients, with no significant difference for disease duration. The three studied groups were matched regarding to sex. B-lymphocytes (%) was significantly higher in LN group compared to control group. Meanwhile, Breg cell (%) was significantly lower in studied SLE cases (with or without nephritis) compared to healthy matched controls.

Table (2): Demographic and clinical data of the studied participants (n=50)

Variable name	LN Group (n=19)	Not LN Group (n=11)	Controls (n=20)	P value[1]	P value[2]	P value[3]	P value[4]
Age (years)				**0.045***	**0.046***	0.929	0.087
• Mean ± SD	30.37 ± 8.18	37.36 ± 7.06	31.25 ± 7.07				
• Median (range)	27 (20 – 46)	40 (24 – 45)	30.5 (23 – 45)				
Sex				0.393			
• Female	16 (84.2)	11 (100.0)	16 (80.0)				
• Male	3 (15.8)	0 (0.0)	4 (20.0)				
Age at onset (years)				**0.016***			
• Mean ± SD	25.76 ± 7.63	32.55 ± 5.61					
• Median (range)	24 (18 – 39)	35 (24 – 39)					
Disease duration (years)				0.264			
• Mean ± SD	4.61 ± 1.03	5.91 ± 1.04					
• Median (range)	5 (1 – 10)	6 (1 – 14)					
B-lymphocytes (%)				**0.005***	1	**0.037***	0.083
• Mean ± SD	17.27 ± 4.13	17.30 ± 4.01	13.53 ± 3.29				
Breg cell (%)				**<0.001**	0.951	**0.004***	**0.031***
• Mean ± SD	2.33 ± 0.46	2.45 ± 0.51	3.46 ± 0.62				

Breg cells: Regulatory B cells; LN: Lupus nephritis; BMI: body mass index. Data described as mean ± standard deviation, median (range) or frequency (%). *: Significant.

P value[1]: Comparison among all groups; P value[2]: Comparison between LN and non-LN groups; P value[3]:

Comparison between LN and control groups; P value[4]: Comparison between non-LN and control groups

According to rSLEDAI there were eight cases with active LN and 11 with inactive LN. Breg cell (%) showed no significant difference according to LN disease activity between the studied groups as shown in **Table 3**.

Table (3): Breg cells (%) according to LN disease activity "as defined by rSLEDAI score" (n=19)

Variable name	Active LN (n=8)	Inactive LN (n=11)	P value
Breg cell (%)			0.433
• Mean ± SD	2.44 ± 0.53	2.24 ± 0.54	

LN: Lupus nephritis; Breg cells: Regulatory B cells; *: Significant

No significant correlation was observed between r SLEDAI and % of Breg (r= - 0.064, p=0.796). Significant negative correlation was observed between % of Breg and Anti-ds DNA, **Figure 2**.

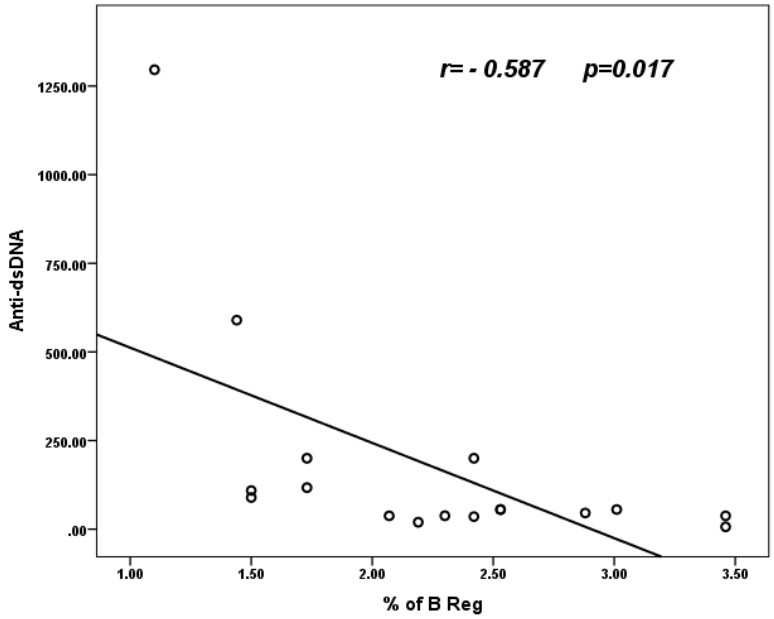

Figure 2: Scatter plot diagram shown the negative correlation between % of Breg and Anti-ds DNA in SLE patients

Table 4 shows studying the difference in B regulatory (%) with different disease characteristics, that revealed no significant differences in Bregs (%) in the presence or absence of neuropsychiatric, vasculitic lesions, musculoskeletal, muco-cutaneous, cardio-pulmonary, general manifestations, and/or urine analysis (casts, hematuria, proteinuria, or pyuria). Furthermore, Bregs (%) showed no significant differences with different lines of treatment received by the studied participants.

Table (4) : Differences in B regulatory (%) with different disease characteristics (n=30)

	% of Breg. in (-) Group	% of Breg. in (+) Group	
	Median (range)	**Median (range)**	**P value**
Neuropsychiatric	2.35 (1.10 - 3.84)	2.47 (2.07 - 2.90)	0.462
Vasculitic lesion	2.40 (1.10 - 3.84)	2.19 (1.84 - 2.53)	0.901
Musculoskeletal	2.41 (1.44 - 3.84)	2.13 (1.10 - 3.46)	0.447
Muco-cutaneous	2.30 (1.10 - 3.84)	2.42 (1.50 - 2.90)	0.773
Cardio-pulmonary	2.40 (1.10 - 3.84)		----
General			
- Fever	2.30 (1.10 - 3.84)	2.42 (2.07 - 2.88)	0.516
- Thrombocytopenia	2.40 (1.10 - 3.84)	2.13 (1.73 - 2.53)	0.777
- Leukopenia	2.30 (1.10 - 3.84)	2.42 (2.30 - 2.53)	0.746
Urine analysis			
- Castes	2.40 (1.10 - 3.84)	2.30 (1.73 - 2.88)	0.966
- Hematuria	2.40 (1.44 - 3.84)	2.13 (1.10 - 2.88)	0.536
- Proteinuria	2.35 (1.44 - 3.60)	2.47 (1.10 - 3.84)	0.836
- Pyuria	2.40 (1.44 - 3.84)	2.13 (1.10 - 2.88)	0.536
Medications			
- MTX	2.30 (1.10 - 3.84)	2.88 (2.30 – 3.46)	0.096
- HCQ	2.40 (1.10 - 3.84)	-----	----
- AZA	2.35 (1.50 - 3.46)	2.41 (1.10 – 3.84)	0.819
- Cyclophosphamide	2.40 (1.10 - 3.60)	2.47 (1.73 – 3.84)	0.617
- Steroid	2.25 (1.10 - 3.01)	2.53 (1.50 – 3.84)	0.134
- Statin	2.40 (1.10 - 3.84)	1.84 (1.84 – 1.84)	0.600

DISCUSSION

The SLE disease may cause multiple organs and systems damage including the cardiovascular, skin, kidneys, and central nervous systems [17]. Lupus nephritis is a serious and common presentation of SLE that is linked to substantial morbidity and mortality [18]. B-lymphocytes cells are pathogenic in the majority of autoimmune disorders as they generate autoantibodies [19]. Recent researches have focused on Breg as a crucial functioning subset of B cells that has a vital capacity in suppressing the autoimmune response and have been widely studied in many autoimmune disorders including vasculitis, rheumatoid arthritis, multiple sclerosis, and SLE [20-23]. **Wang *et al.*[24]** identified that Bregs augmented Treg cell expressions, which reduced inflammatory reactions in lupus-like mice. Additionally, Bregs are believed to contribute to immunological tolerance by harmonizing the immune system. However, the role of Bregs in SLE remains controversial [10,25]. Bregs have been found to include the CD molecules CD1d, CD19, CD20, CD21, CD23, CD24, CD25, CD27, and CD38 [11]. **Blair *et al.*** [7] demonstrated that CD19+CD24hiCD38hi cells could prevent T helper-1 cells from differentiating; they were chosen for research as Bregs phenotype. In order to ascertain the molecular phenotype of Bregs and their suppressive action, **Blair and colleagues** conducted a number of investigations. Furthermore, the authors confirmed that in SLE patients, the distributions of Bregs and Tregs displayed opposite patterns. However, there is little correlation between the numbers of CD19+CD24hiCD38hi cells and lymphocytes. This finding could explain the inconsistent results documented by the earlier literature. Thus, the role of $CD19^{+}CD24^{hi}CD38^{hi}$ cells remains poorly understood.

In the present prospective observational cross-sectional study of 30 SLE patients, we observed that, B-lymphocytes (%) was significantly higher, while; Breg cell (%) was significantly decreased in SLE cases (with or without LN) in comparison to healthy control. Also Breg cell (%) shows no significant association with LN disease activity. This finding could be clarified by the fact that activated B cells can exacerbate illness by generating localized autoantibodies, activating pro-inflammatory cytokines, and effector leukocytes [26]. Additionally, the reported Bregs impairment in the LN could be a result of or a cause of the activity in the LN. In either situation; this depletion may increase the extent of tissue damage [27].

This finding is supported by the previous study of **Wang *et al.*[28]**, who described decreased numbers of Bregs in new-onset SLE patients compared to healthy controls. Also, similar finding was reported by previous studies, which reported a functional defect in $CD19^{+}CD24^{hi}CD38^{hi}$Breg population in SLE [7, 12, 29, 30].

Conversely; **Sims *et al.*[31]** observed significant higher $CD19+CD38^{high}CD24^{high}$ B cells (%) in the peripheral blood mononuclear cells of SLE diseased patients compared to healthy control (HC), also **Wang *et al.*[12]** reported higher $CD19^{+}CD24^{high}CD38^{high}$ Bregs percentage among circulating lymphocyte from SLE patients compared with HC ($P<0.001$). In comparable with our finding **Wang *et al.*[12]** reported that $CD19^{+}CD24^{high}CD38^{high}$ Bregs show no significant correlation with the presence of LN.

Comparable percentage of $CD19+CD24^{hi}CD38^{hi}$ Breg population in SLE patients and matched healthy controls was observed in stimulated peripheral blood mononuclear cells of 34 patients with SLE patients and 21 HC examined by **Heinemann *et al.*** [1].

The variability in patient geographic origin, genetic background, and clinical features, particularly disease duration or received medications, may be reflected in the discrepancies in circulating Breg levels between these studies. However; the underlying mechanisms for Breg proliferation in SLE diseased patients, if present, still not fully understood.

In the current study we found no significant association between regulatory B lymphocytes (Breg) % in SLE patients and different lines of treatment received by the studied participants. Conversely; in SLE, **Wang *et al.*[28]** assessed the impact of immunosuppression on Breg and found that, therapy increased the percentage of CD19+CD5+CD1dhiIL-10+ regulatory B cells. The proportion of Bregs in LN patients and daily steroid dose were found to be negatively correlated by **Heinemann *et al.*[1]**. Also recently **Girimaji *et al.*[19]** reported that after six months of immunosuppression initiation, Bregs in LN patients become comparable to HC with no significant difference between them. This finding was observed in all cases irrespective of the type of immunosuppression received by the studied cases. The Bregs in responders and non-responders were subsequently examined by the same author. Following immunosuppression in responders, Breg populations increased. In contrast, no noticeable change was seen in non-responders [19].

These finding imply that the response to immune-suppression was associated with Breg population improvement. As these medications could influence B cell activation and proliferation or, alternatively, the bone marrow and, consequently, the circulating B cells [27]. We failed to find such association that may be contributed to difference in disease duration, type, dose, and duration of the received therapies, and also to the level of patient complaince for the given medications. This finding highlight the need for further larger studies to determine the effect of different lines of immunosuppressor on Breg (%) among SLE patients. Also in the current study we showed significant negative correlation between % of Breg and Anti-ds DNA. Anti-dsDNA antibodies can be identified in SLE patients before the onset of disease diagnosis for at least 2 years. Additionally, in individuals with lupus nephritis, serum anti-dsDNA antibody levels frequently indicate disease activity [32]. Based on this finding we could say that Breg depletion

could reflect the extent of disease progression not only in SLE diseased patients, but also in LN patients.

CONCLUSION

In conclusion, we found that B lymphocytes increased while Bregs were deficient in SLE patients with no correlation with immunosuppression. The present findings support the possible role of total B lymphocytes and their subtype-Breg (%) in aetio-pathogenesis of SLE and also contribute to the onset of LN. Larger prospective studies are needed to effectively study the impact of different immunosuppression therapies on B lymphocytes and Bregs population.

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Author contribution: Authors contributed equally in the study.

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