Original Research Article

Regulatory T cells (CD4+CD25+FOXP3+) in lupus nephritis

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ABSTRACT

Background: Systemic lupus erythromatosus (SLE) is an autoimmune disease with 20–65% of patients developing lupus nephritis (LN). Studies have reported 10% of LN patients will end up with end stage renal disease and their mortality rate is higher compared to patients without LN. Abnormality of regulatory T cells (T_{regs}) level is thought to be a potential factor for this LN development. The aim of study was to evaluate the percentage of T_{regs} in LN patients.

Methods: This was a comparative cross sectional study involving LN patients and age and gender matched controls with a 2:1 ratio. The patients were grouped into active and inactive LN based on their lupus activity index; complement levels, ANA, dsDNA antibodies, ESR, SLE Disease Activity Index (SLEDAI2K) score and also urine PCI (uPCI>0.05 for active group). Disease history, demographic data, routine blood test, peripheral blood for differentials count were taken and recorded. Peripheral blood mononuclear cells were stained with CD4, CD25 and Foxp3 antibodies and percentage of T_{regs} was analysed using BD fluorescence-activated cell sorting (FACS) cytometer. We compared demographic and laboratory parameters between healthy controls and LN patients as well as active and inactive LN patients.

Results: A total of 34 LN patients (32 females, 2 males) were recruited. Their mean age and disease duration were 37.97±11.14 years and 110.95±65.07 months respectively. Thirteen matched controls with mean age 35.23±7.89 years were enrolled. There was no demographic difference between 2 groups of LN patients. T_{regs} were significantly lower in active LN compared to inactive LN and healthy control (0.44±0.37% vs. 1.89±0.46% vs. 3.12±0.56% of the $CD4^+$, *P<0.001*). C3 and C4 complement fragments were significantly reduced in patients with active disease (C3; 50.92±28.43 vs. 76.31±25.63, *P=0.011*) and (C4; 11.17±8.41 vs. 16.70±6.50 *P=0.044*). Proteinuria was significantly higher while serum albumin levels were significantly lower in active patients compared to inactive patients and healthy control (urine PCI; 0.25(0.15-0.3) vs. 0.03(0.01-0.05) vs. 0.01, *P<0.001*) and (albumin; 29.89±6.87 vs. 36.87±3.58 vs. 40.62±1.89mmol/L, *P<0.001*). We found positive inversely correlation between T_{regs} with SLEDAI2K (*r* = -0.572, P=0.011) and proteinuria (*r* = -0.451, *P=0.007*).

Conclusions: T_{regs}, C3 and C4 complements, and albumin were significantly lower while proteinuria was significantly higher in active LN. There was positive inversely correlation between the percentage of T_{regs} with SLEDAI2K score and proteinuria.

Keywords: CD4+CD25+Foxp3+, Lupus nephritis, Regulatory T cells, Systemic lupus erythematosus, Tregs

INTRODUCTION

Systemic lupus erythromatosus (SLE) is a chronic autoimmune disease with unclear aetiopathogenesis where the autoantibodies attack the host and cause deposition of these immune complexes in target organs such as the skin, kidney, lung, and central nervous system.[1] Kidney involvement or lupus nephritis (LN) is a major risk factor for morbidity and mortality in SLE with

10% of LN patients progressing to end stage renal disease (ESRD).[2] Patients with LN have a higher standardized mortality ratio (6-6.8 versus 2.4) and die earlier than those without LN.[3] Importantly, the 10-year survival improves significantly from 46% to 95% if disease remission can be achieved.[4]

Responsiveness of the patients to immunosuppressive therapy supports the hypothesis that there is an association between SLE and immune dysregulation. SLE was classically thought to be a B-cell driven disease, but compelling evidence has shown that T cells are crucial in its pathogenesis. T cells enhance the production of autoantibodies by offering substantial help to B cells to trigger SLE related inflammation.[5]

The majority of T cells are antigen-naive within the blood and lymphoid organs and small proportion of them are memory T cells. These naive T- cells will be activated and differentiated into T helper, T-cytotoxic and T-regulatory (T_{regs}) cells in the presence of any antigen presenting cells.[6] T helper cell initiate the immune response whereas T_{regs} are involved at the end of immune reaction by suppressing autoreactive T cells.[6,7]

T_{regs} play a critical role in maintaining immune modulation and are present in low numbers in normal peripheral blood (5% to 10% of $CD4^+$ T cells or 1% to 2% of total lymphocytes).[8] T_{regs} prevent inappropriate immune responses by suppressing effector T cells through a transcription factor now recognized as Forkhead box P3 (Foxp3).[9] Both quantitative and/or qualitative deficiencies of the T_{regs} are known to be potential triggers for the development of autoimmune diseases.[10] A study in animal models of SLE demonstrated a significant reduction in the numbers of regulatory T cells.[11] On the other hand, another study has shown a decreased number of T_{regs} in peripheral blood in SLE patients.[12] In view of the limited and contradictory data on T_{regs}, we embarked on this study to see the association of T_{regs} in SLE patients with lupus nephritis. We also evaluated the correlations of this $CD4^+CD25^+Foxp3^+$ with disease activity and blood parameters in LN patients as well as healthy controls.

METHODS

Patients and healthy controls

This was a comparative cross sectional study involving SLE patients with LN. We screened all LN patients attending their routine nephrology clinic follow up at Universiti Kebangsaaan Malaysia Medical Centre between May to August 2017. Patients who fulfilled the SLE criteria based on the American College of Rheumatology (ACR) aged between 18 to 65 years old and consented were included.[13] All patients needed to have a renal biopsy within 2 years that graded according to the World Health Organisation (ISN/RPS) classification to be included.[14] We excluded patients with

ESRD on dialysis, malignancies, other autoimmune and mixed connective tissue diseases, ongoing infection and inflammatory diseases as these may interfere with the T_{regs} level. Their demographic data and laboratory parameters were recorded included history, clinical presentation, routine blood tests, peripheral lymphocyte count, ESR, complement levels, ANA, dsDNA antibodies, SLE Disease Activity Index (SLEDAI2K), and urine investigations. We grouped the LN patients into active and inactive LN based on recommendation from European League Against Rheumatism (EULAR) criteria.[15]

We also recruited consented healthy controls who matched in terms of age and gender in the ratio of 1:2 to our patients. We excluded anyone who had recent infection <4 weeks or was pregnant. The healthy controls were mainly staff who worked in the hospital. This study was funded and received ethics approval from our UKMMC Ethics and research Committee (FF-2017-006). Peripheral venous blood was drawn from each subject in heparin-containing vacutainer tubes on the day of the clinic visit after obtaining their consent. Each blood samples underwent few steps for analysis of the percentage of T_{regs} in these 3 populations (active LN, inactive LN and healthy control).

Phenotypic analyses and peripheral blood mononuclear cells separation

A total of 10ml blood was taken from the patients and controls and peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by layering over lymphocyte separation medium and density gradient centrifugation at 400g for 30minutes. Isolated lymphocytes were resuspended in BD Pharmigen TM Stain Buffer (FBS) and viable cells were counted manually under light microscope.

Cell surface staining

To determine the percentage of peripheral blood T_{regs} cells, PBMCs were incubated with fluorescently labelled anti-CD4 antibody (FITC Mouse Anti-Human CD4, BD PharmigenTM, San Diego, CA) and anti CD25 (PE Mouse Anti-Human CD25, BD PharmigenTM, San Diego, CA) for 20 minutes at room temperature in FBS for 20 minutes. The cells were then washed, and supernatant fluids were removed.

Intracellular staining for Foxp3

After the cell surface staining, the cells were fixed and permeabilized with the Foxp3 Staining Buffer Set (BD Pharmingen TM, San Diego, CA) for 30 minutes at room temperature, and stained with fluorescent Foxp3 antibody (Alexa Fluor® 647 Mouse Anti-Human Foxp3, BD PharmigenTM, San Diego, CA) for 30minutes at room temperature. The cells were then washed and supernatant fluids were removed.

Flow cytometry

The samples were analysed immediately on a BD fluorescence-activated cell sorting (FACS) cytometer. Lymphocytes were gated according to forward scatter (FSC) and side scatter (SSC). In addition gated CD4$^+$ cells were analysed for the expression of CD25 and Foxp3. Proportion of CD25$^+$Foxp3$^+$ cells within the gated CD4$^+$ cells were shown and recorded (Figure 1).
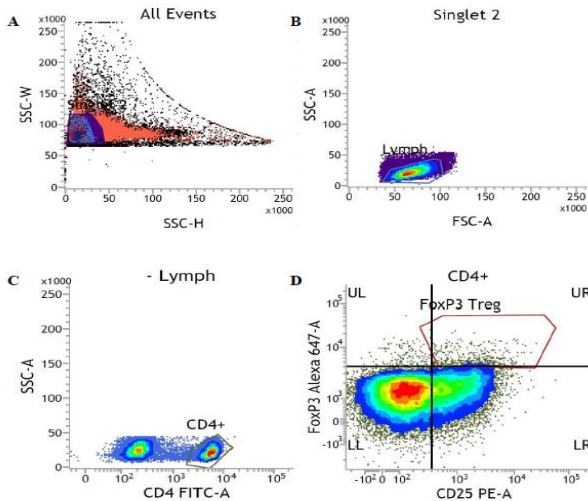

Figure 1: Plots from a PMBC labelled with anti-CD4, CD25 and Foxp3. (A) Cell population was identified by SSC-H and SSC-W. (B) FSC and SSC to identify lymphocytes. (C) CD4+ cells within the lymphocyte gate. (D) Cells were gated on the CD4+ cells.

Statistical analysis

All variables were tested for normality using the Statistical Package for Social Science version 22.0 (SPSS Inc. Chicago, IL). Normally distributed numerical data are expressed as mean±SD (standard deviation) whereas non normally distributed data are expressed as the median with the 25th percentile and 75th percentile value.

Non-parametric data was analysed using Mann U Whitney test and Kruskal-Wallis test whereas t-test or ANOVA was used for normally distributed variable. Correlation (*r*) between any two parameters was determined by Pearson coefficient/Spearman. A P<0.05 was considered to be statistically significance.

RESULTS

We recruited a total of 47 subjects; 34 LN patients (32 females, 2 males) with a mean age and disease duration of 37.97±11.14 years and 110.94±76.83 months respectively. Of these, 13 were healthy volunteers who served as controls. There were no significant differences in demographics and laboratory parameters such as serum creatinine, ESR and complement levels between LN patients and controls (Table 1).

However, we did find that LN patients had significantly lower peripheral lymphocytes; CD4 counts and T$_{regs}$ percentages. Serum albumin was significantly lower while urine protein/creatinine index (uPCI) was significantly higher in LN patients compared to controls.

Table 1: Demographic data and laboratory parameters in healthy controls and LN patients.

Parameter	Control (n=13)	LN patients (n=34)	P value
Age (years)	35.23±7.89	37.97$\pm$11.14	0.422
Gender, n (%)			
Female	13 (100%)	32 (94%)	-
Male	0 (0%)	2 (6%)	
Race, n (%)			
Malay	12 (92.3%)	24 (70.6%)	
Chinese	0 (0%)	9 (26.5%)	-
Indian	1 (7.7%)	1 (2.9%)	
Creatinine, µmol/l	67(64.8-71.35)	72.45(61.65-111.50)	0.419
eGFR (MDRD), ml/min/1.73m^2	91.97±8.42	79.19±32.40	0.170
Albumin, g/l	40.62±1.89	32.97±6.60	<0.001
C3, mg/dL	77.82±31.41	62.12±29.72	0.118
C4, mg/dL	17.94±9.85	13.61±8.02	0.128
ESR, mm/h	30.23±11.09	41.44±24.62	0.123
Urine PCI	0.01	0.09(0.03-0.28)	<0.001

eGFR: Estimated glomerular filtration rate; C3: Complement component 3; C4: Complement component 4; ESR: erythrocyte sendimentation rate; Urine PCI: urine protein creatinine index

We then compared demographic and laboratory parameter of those with active and inactive LN (Table 2).

There were no significant differences between them in terms of age, disease duration, and comorbidities. As

expected, we found those with active LN had a lower serum albumin, complement levels, higher urine PCI and were on higher doses of corticosteroids. Patients with active LN had a significantly lower T_{regs} compared to inactive LN but lymphocyte count was not statistically significant different between these two groups. Further compared those with inactive LN and controls and found that those with inactive LN had a statistically significant lower peripheral lymphocyte, $CD4^+$ and T_{regs} (Table 3).

On correlation, we found the percentage of T_{regs} inversely correlated with SLEDAI2K disease ($r= -0.572$, $P=0.011$) (Figure 2). We also found an inverse correlation between T_{regs} and proteinuria ($r= -0.451$, $P=0.007$) (Figure 2).

All patients were on maintenance prednisolone either as monotherapy or combined with other immunosuppressants such as azathioprine (20.6%), mycophenolic acid (61.8%) ciclosporin A (23.5%) and tacrolimus (14.7%).
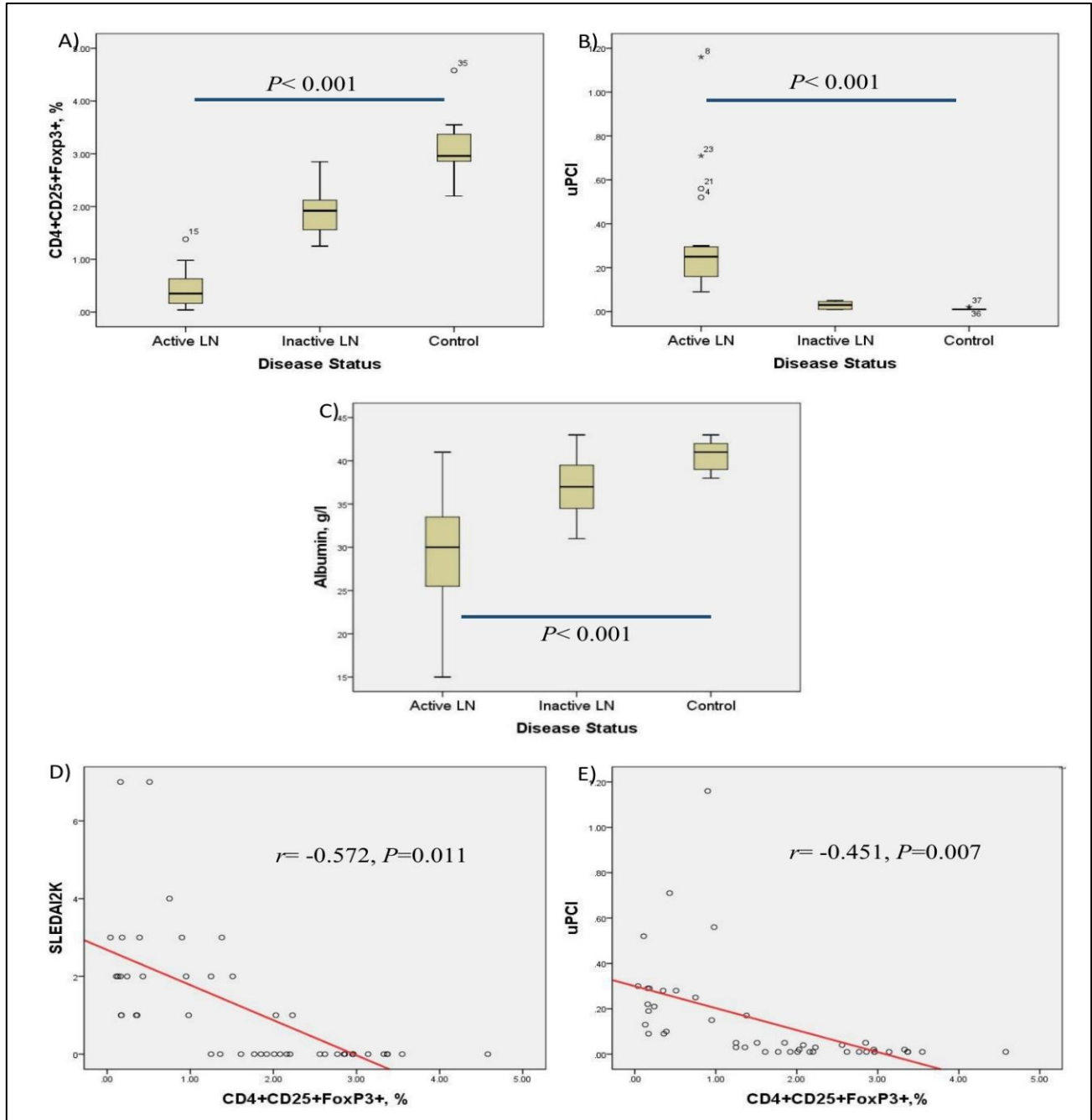

Figure 2: Comparison of 3 groups of population (Active LN vs. Inactive LN vs. Healthy control), A) Tregs percentage, B) proteinuria and C) serum albumin (P<0.001), D) The percentage of Tregs was inversely correlated with SLEDAI2K disease score in LN patients (r= -0.572, P=0.011). E) The percentage of Tregs was inversely correlated with proteinuria (r= -0.451, p=0.007).

Table 2: Demographic data and laboratory parameters in inactive LN and active LN patients.

Parameter	Inactive LN (n=15)	Active LN (n=19)	*P* value
Age (years)	39.07±12.77	37.11±9.94	0.618
Disease duration (months)	103.66±79.10	116.67±76.64	0.631
Age of Diagnosis	29.00±9.23	25.00±11.05	0.198
Creatinine, µmol/l	66(60.6-84.9)	74(64-135)	0.259
eGFR (MDRD), ml/min/1.73m^2	86.44±22.02	73.46±38.32	0.252
Albumin, g/l	36.87±3.58	29.89±6.87	0.001
C3, mg/dL	76.31±25.63	50.92±28.43	0.011
C4, mg/dL	16.70±6.50	11.17±8.41	0.044
ESR, mm/h	32.87±20.72	48.21±25.84	0.071
SLEDAI2K	0	2(1-3)	<0.001
SLICC-ACR	0	1(0-2)	0.033
ANA +ve, n (%)	11 (73.3%)	12 (63.2%)	-
dsDNA +ve, n (%)	5 (33.3%)	9 (47.4%)	-
Urine PCI	0.03 (0.01-0.05)	0.25 (0.15-0.3)	<0.001
Peripheral lymphocyte, 10^9/l	1.4 (0.8-1.9)	1.8 (1.2-2.2)	0.424
CD4$^+$, %	26.3±10.3	27.2±10.9	0.792
CD4$^+$CD25$^+$Foxp3$^+$ (%CD4$^+$)	1.9±0.5	0.4±0.4	<0.001

SLEDAI2K: Systemic Lupus Erythematosus Disease Activity Index, SLICC/ACR: Systemic Lupus International Collaborating Clinics/American College of Rheumatology

Table 3: T cells analysis between control and inactive LN.

Parameter	Control (n=13)	Inactive LN (n=15)	*P* value
Peripheral lymphocyte, 10 9/l	2.4(2.0-2.6)	1.4(0.8-1.9)	0.004
CD4+, %	103.66±79.10	26.3±10.3	0.001
CD4+CD25+Foxp3+ (%CD4+)	39.2±6.5	1.9±0.5	<0.001

DISCUSSION

As reported in all published literature, our findings concur that SLE is a disease of predominantly childbearing aged woman. Malaysia is a multi-racial Southeast Asian country which comprises of three major ethnic groups. The largest ethnic composition is Malay, followed by Chinese and Indian and it is reported that Chinese have the highest prevalence of SLE in Malaysia (57/100,000), followed by Malays (33/100,000) and Indians (14/100,000).[16,17] Kidney function was preserved in our patients despite having proliferative LN for almost a decade. Most of our LN patients had extra renal involvement with haematological manifestation being the commonest followed by musculoskeletal, cutaneous neurological and serositis and consistent with others.[18]

As reported in the previous literature, we also demonstrated that high urine PCI, hypoalbuminaemia, C3 and C4 hypocomplementaemia, increased SLEDAI2K and SLICC-ACR were associated with disease activity.[13] Surprisingly, we found that ESR was not significantly different between patients with active and inactive LN. We believe patients with active LN were already treated with high dose corticosteroids that resulted in reduction in the degree of systemic inflammation and hence lower ESR.[19]

We found that LN patients have significantly low peripheral lymphocyte compared to healthy controls regardless of disease activity and is a well-known association. However, we no found significant difference in peripheral lymphocyte count between those with active and inactive LN. We believe this may be because patients with active LN were on significantly higher dose of steroids compared to inactive LN (*P*=0.036). LN patients also had lower CD4$^+$ lymphocyte count compared to healthy controls. The reason for decreased these CD4$^+$ lymphocyte count in SLE is poorly explored but the depletion of lymphocytes may be linked with anti-lymphocyte antibodies, which are detected in almost 50% of SLE patients presenting with lymphopenia.[20] There is emerging evidence that T$_{regs}$ are useful marker for monitoring disease activity in SLE. To date, there is conflicting results on the percentages of CD4$^+$CD25$^+$Foxp3$^+$ T$_{regs}$ in SLE patients when compared to healthy individuals.[21-25] We demonstrated that the percentage of peripheral CD4$^+$ T$_{regs}$ in general was significantly lower in LN patients compared to healthy controls regardless of their disease activity and in keeping with previous literature.[21,26-29] We chose to include healthy controls as there is no published data on the true

"normal value" of the percentage of T_{regs}, but one of the study has reported it to be 5% to 10% of $CD4^+$ T cells or 1% to 2% of total lymphocytes in normal peripheral blood.[8] However, we found the % of $CD4^+$ T cells was lower in our healthy controls than the reported study and we believe this maybe because of technical analysis. There were a slightly reduced number of lymphocytes in the flow cytometry sample compared to the actual measured lymphocytes in the laboratory which could attribute to this result.

We found that T_{regs} percentages were significantly lower in inactive LN group compared to healthy controls as expected.[27] Furthermore, patients with active LN had lower T_{regs} compared to those with inactive LN. As there was no difference in $CD4^+$ T cells between active and inactive LN patients, one would assume there would be no difference in the $CD4^+CD25^+Foxp3^+$ T_{regs} between both groups. However, we found that there was a reduction in $CD4^+CD25^+Foxp3^+$ T_{regs} in active LN. Studies have shown in autoimmune disease $CD4^+$ T cells differentiate into both T_{regs} and Th17 and Ma et al, and Dolff et al, had reported a compensatory increment in Th17 when t_{regs} are reduced.[30,31] This supports present study finding even though the $CD4^+$ T cells levels are restored, the $CD4^+CD25^+Foxp3^+$ T_{regs} were reduced. It would have been beneficial if we had measured Th17 to support our hypothesis. There is conflicting data with some studies reporting lower $CD4^+CD25^+Foxp3^+$ cells in SLE and it inversely correlated with disease activity whereas Barath et al, found no correlation with disease activity.[22,26,23] Authors demonstrated that T_{regs} cells inversely correlated with SLEDAI2K and consistent with recent reports.[11,28,32] The number of functional T_{regs} in SLE were found to be restored in patients receiving glucocorticoids therapy supporting that T_{regs} percentage will improve with improvement of underlying disease activity.[33] Karagiannidis et al, reported that the patients with corticosteroid might have higher percentage of T_{regs} but Suárez et al reported of no change in the level of T_{regs} with steroids.[34,35] Authors found that the patients with inactive LN had a higher level of T_{regs} but still lower than controls suggesting that although glucocorticoids may have an effect in restoring the immunity, the level of T_{regs} do not go to baseline. Few literatures have reported a significant increment of T_{regs} levels following cyclophosphamide and rituximab therapy, but the increment was not significant with other agent such as methotrexate, azathioprine and mycophenolic acid.[10,36,37]

The main limitation of present study is small sample size and we only measured T_{regs} from peripheral blood. It would be helpful to assess Th17 at the same time and also T_{regs} in the urine and tissue as well.

CONCLUSION

This study demonstrated that T_{regs} were significantly lower in patients with active LN hence it can be a useful marker for disease activity in LN patients.

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