



RESEARCH ARTICLE

EVALUATION OF URINARY CD4 T-CELL LEVELS AS A BIO-MARKER FOR LUPUS NEPHRITIS AND ITS CORRELATION WITH DISEASE ACTIVITY.

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Abstract

Background:- Although renal biopsy is the most accurate method for assessing renal inflammation in patients with lupus nephritis (LN), the technique is invasive and cannot be performed frequently. There is a critical need to identify biomarkers for systemic lupus erythematosus (SLE) which has a high prevalence of renal failure.

Objective:- Our objective was to evaluate the level of urinary CD4 T-cells as a biomarker of active LN and indicator of treatment response in patients with SLE compared with normal control and its correlation with disease activity.

Subjects and methods:- 64 patients with SLE and 20 age & sex matched control group were included. Patients were classified into two groups A & B. Group A with LN and moderate to severe SLE disease activity, Group B without LN and mild to inactive SLE. Levels of urinary CD4 T-cell were quantified using flow cytometry in both patients' groups and controls at baseline and repeated for patients' groups only after 6 months.

Results:- We found that CD4 T-cell level in Group (A) was significantly higher at baseline than in Group (B). There was significant decrease in the CD4 T-cell number in group A, when repeated after 6 months. Number of CD4 T-cells was correlated significantly with the disease activity both at baseline and after 6 months. In healthy controls, the urine was almost devoid of CD4T-cells.

Conclusion:- Urinary CD4 T-cells is a good non-invasive marker for detecting active LN in SLE patients and also is a good monitor for treatment efficacy.

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Introduction:-

Systemic lupus erythematosus (SLE) is a typical autoimmune disease characterized by the production of a wide array of autoantibodies which potentially drive immune-complex related inflammation into various tissues and organs. Thus, the condition was traditionally classified as a "B-cell disease"[1]. Compelling evidence has however

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shown that without the assistance of the helper T lymphocytes, it is indeed difficult for the “helpless” B cells to become functional enough to trigger SLE-related inflammation [2]. T-cells have been recognized to be crucial in the pathogenicity of SLE through their capabilities to communicate with and offer enormous help to B cells for driving autoantibody production through stimulating the latter to differentiate, proliferate and mature. Abnormalities in adaptive immunity triggered by genetic and environmental factors are thought to play an important role in the pathogenesis of SLE[3]. Renal involvement becomes clinically apparent in approximately 75% of SLE patients; however, most of the remaining patients have subclinical disease that can be demonstrated if renal biopsy were performed. Renal affection usually develops in the first few years of illness and should be detected early by periodic urine analysis and estimation of the glomerular filtration rate [4]. Despite advances in the treatment of LN, the occurrence of nephritis is still associated with a significant burden of morbidity and mortality [5]. The patient is threatened from two sides: under-treatment, implicating uncontrolled autoimmunity and overtreatment, resulting in toxicity and danger of serious infections [6]. A diagnosis of LN is usually suspected in patients with systemic signs of SLE activity and urinary abnormalities and is confirmed by kidney biopsy. The sooner the diagnosis of LN is established, the better the prognosis [7]. However, urinary findings may be misleading or unspecific, while kidney biopsy is an invasive procedure not free from risk [8]. An ideal biomarker would non-invasively identify patients with acute LN and quantitatively reflect the inflammation in the kidneys to allow monitoring of the disease and patient tailored treatment. Enghard and his colleagues hypothesized that an SLE marker specific for LN would most likely to be found in urine. They reported that CD4 T-cells can be found in abundance in the urine of the patient with active LN [9]. Infiltration of inflammatory cells into the kidneys is a well-established element of LN, particularly the proliferative forms [10]. This infiltration predominantly consists of CD4 T-cells and the extent of the infiltration is one of the best predictors of renal outcome [11]. The phenotype of the CD4 T-cells in the urine is qualitatively reminiscent of the renal infiltrating cells rather than the T-cells circulating in the peripheral blood [12]. Hence, it is reasonable to assume that the CD4 T-cells found in the urine originate from the inflamed kidney interstitium and this is strengthened by the observed close correlation with LN activity.

Objective:-

Our objective was to evaluate the level of urinary CD4 T-cells as a biomarker of active LN and indicator of treatment response in patients with SLE compared with normal healthy control and its correlation with disease activity.

Subjects and methods:-

Sixty-four patients with SLE were selected from the Outpatients Clinics and Inpatients Units of Rheumatology and Rehabilitation Department, Zagazig University Hospitals, were included in this study, their age ranged between 20-48 years. All SLE patients satisfied the Systemic Lupus International Collaborating Clinics (SLICC) group revised SLE Classification Criteria[13]. Twenty age and sex matched healthy volunteers were selected as a control group. For all patients, the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was calculated [14]. Patients with a SLEDAI ≥ 10 were considered to have moderate to severely active SLE disease, patients with a SLEDAI < 10 were considered to have inactive to mild active disease. The diagnosis of active lupus nephritis was made based on the fulfillment of any two of the following 3 criteria: (1) a level of glomerular erythrocytes of $> 50\%$ and/or hematuria and/or the presence of hyaline or cellular casts. (2) A quantitative 24-hour urinary protein level of $> 1\text{gm}$ or increase in the level of $> 0.5\text{gm}$ over a two to three-month period. (3) Evidence of one or more active lesions on a renal biopsy based on the Classification Criteria for LN established by the International Society of Nephrology/Renal Pathology Society [15]. Complete remission was defined as the absence of activity in the urinary sediment and a quantitative 24-hour urinary protein level of $< 0.5\text{ gm}$ within the previous three months [16].

Exclusion criteria:-

Patients with other nephropathy as diabetic nephropathy, ANCA-associated vasculitis were excluded. Patients with urinary tract infection, renal cancer or renal failure and patients with other autoimmune disease were also excluded.

The study was approved by the ethics committee of the Zagazig University hospitals, Faculty of Medicine, Zagazig University and the patients were included in the study after giving their informed consent after explanation of the purpose and procedures of the study.

Patients were classified into two groups:-

Group (A):- Included 40 patients with LN and a SLEDAI ≥ 10 (4 males, and 36 females with a mean age of 34.6 ± 6.4 years).

Group (B):- Included 24 patients with no LN and the SLEDAI < 10 (4 males, and 20 females with a mean age of 32.1 ± 5.7 years).

All patients were subjected to:-

1. Complete history taking including the duration of the disease, history of renal involvement (LN) or renal biopsy and drug therapy.
2. General and complete musculoskeletal examinations including assessment of disease activity by SLEDAI.
3. Complete neurological examination.
4. Routine laboratory investigations were done, as complete blood count (CBC) on SysmexKx 21 automatic cell counter (Japan). Liver, kidney function tests, 24h urine protein, CRP on CobasInegra 400 (Roche Diagnostic, Germany). Determination of complement C3 and C4 levels in serum was done on automated BN Prospec (Siemens Diagnostic, Germany). Antinuclear antibody (ANA) and Anti- double stranded DNA (anti-dsDNA) were measured by indirect immunofluorescence assay (IIF) on mouse kidney and stomach slides (Immuno-Diagnostic, USA).
5. **Urinary CD4 T-cell:** Urine samples were collected from 64 patients with SLE and 20 healthy controls were monitored for the presence of CD4T-cells at baseline and repeated measurements after 6 months were done for the patients only. The median sample size was 100 ml urine; the usual standing time for the urine was 4-6 hours.

Flow cytometry method:-

In this prescriptive study 4 colors was used to quantify the percent and events of CD4 in the monoclonal cells of urine sample , so for assessment we used CD4 PE , CD3 Prep , CD45 FITC and the urine sample were collected immediately, centrifuged and washed 3 times with phosphate buffer solution (PBS), then we incubate the pellet with monoclonal Abs for assessment with flow cytometry, also we added PromodiuIodid (Eugene ,Oregon , USA) immediately before assay on flow cytometry to exclude dead cells. For each sample up to 10,000 cells were acquired and according to the plot of Side Scatter Scale versus CD45 stain our data was visualized. Lymphocyte populations were identified based on low Side Scatter and a combination of low intensity CD45 expression with CD4 CD3. The data were collected using (FACS Caliber, Becton-Dickinson BD and San Diego, USA).

Statistical analysis:-

IBM SPSS statistics (V. 22.0, IBM Corp., USA, 2013) was used for data analysis. Data were expressed as Mean \pm SD for quantitative parametric measures in addition to both number and percentage for categorized data.

The following tests were done:

1. Comparison between two independent mean groups for parametric data using Student t test.
2. Comparison between 2 dependent groups for parametric data using Paired t test.
3. Pearson correlation test to study the possible association between each two variables among each group for parametric data.

The probability of error at 0.05 was considered significant, while at 0.01 and 0.001 was considered highly significant.

Results:-

General and clinical characteristics of patients groups and the healthy controls were shown in Table (1). We found that in group (A) 22 patients (55 %) out of 40 with past history of renal biopsy revealed grade 2 and 3 proliferative nephritis. Laboratory results of patient groups at baseline shown in Table (2). The mean of CD4 T-cells in group (A) was high = 2400.9 ± 879.3 CD4 T-cells/100 ml urine at baseline, Table (3). In patients with LN group (A) the number of urinary CD4 T-cells in 100 ml urine clearly correlated significantly with the disease activity both at baseline and after 6 months (Table 5 and figures 1&2). And when repeated after 6 months the mean of CD4 T-cell number decreased significantly in group A indicating good response to treatment (Table 4). While, in group (B), SLE patients without LN and with mild or no active disease, a mean of 165 ± 22.4 CD4 T-cells/100 ml was found with no significant changes in number of CD4 T-cells were detected after 6 months and its number did not correlate with mean SLEDAI or therapy (Tables 4& 5). In healthy controls, the urine was almost devoid of CD4 T-cells and a mean of 2.3 ± 0.3 CD4 T-cells/100 ml urine were found, which was significantly fewer than that observed in patients with SLE, regardless of disease activity or renal involvement Table (3).

Table 1:- General and clinical characteristics of both patients and controls (Mean±SD).

The characteristic		Group (A) LN with SLEDAI ≥10	Group (B) No LN with SLEDAI <10	Healthy controls	P-Value
Number		40	24	20	---
Age (year)		22-45 34.6±6.4	20-42 32.1±5.7	20-40 31.7±6.9	>0.05
F/M		36/4	20/4	18/2	>0.05
Duration of disease (years)		2-13 6.1 ±2.4	1.5-10 5.7±1.2	-	>0.05
SLEDAI		(10-24) 15.1±3.7	(2-9) 6.2±2.2	-	<0.001
Treatment	Steroid	40 (100%)	24 (100%)	-	---
	CYX	26 (65%)	-	-	---
	AZA	6 (15%)	20 (83.3%)	-	---
	MMF	8 (20%)	-	-	---
	HCQ	38 (95%)	22 (91.6%)	-	---
Renal biopsy		22 (55 %)	-	-	---

SLEDAI=SLE disease activity index, CYX= Cyclophosphamide, AZA=Azathioprine
MMF=Mychophenolatemofetil, HCQ=Hydroxychloroquine.

Table 2:- Laboratory results of both groups at the baseline (Mean±SD):

Lab. Test	Group (A) (No.=40)	Group (B) (No.=24)	P-Value
ESR mm/h	46-76 61.7±11.2	23-37 30.1±6.9	<0.001
CRP	6-10 8.1±2.3	1-6 3.7±1.1	<0.001
S.Urea mg/L	25-55 34.9±10.3	12-25 18.4±6.4	<0.001
S.Creat mg/dl	0.9-2.8 1.9±0.7	0.5-1.1 0.8 ±0.3	<0.001
+ve ANA	36 (90%)	22 (91.6%)	>0.05
+ve Anti ds-DNA	28 (70%)	10 (41.6%)	<0.05
C3 mg/dl	52-72.5 64.5±10.9	75-135 99.3±20.4	<0.001
C4 mg/dl	9-13.2 10.1 ± 3.9	18.5 – 40 29.4 ± 11.7	<0.001
Protein in mg/ 24h urine	1300-5600 3070.3±500.4	150-350 290.4±80.9	<0.001

Table 3:- Range and mean levels of urinary CD4 T-cells at baseline in patients and controls:

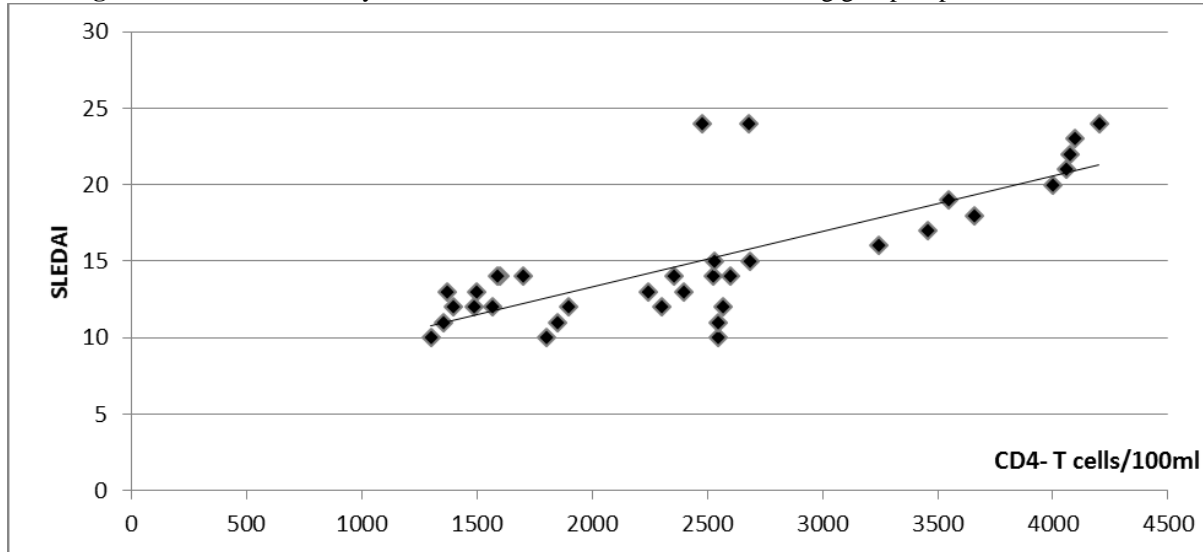
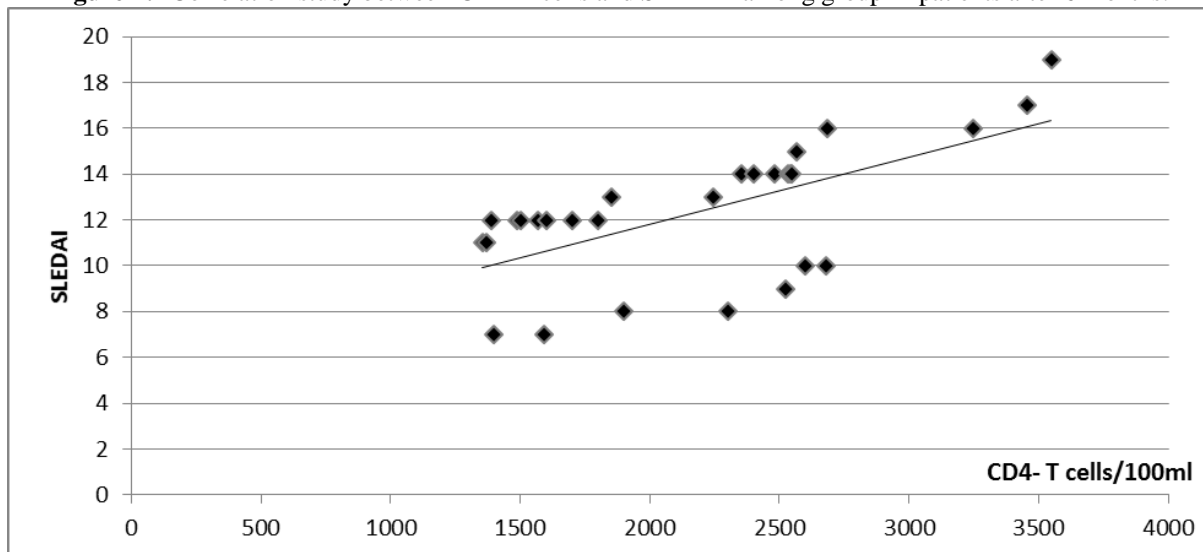
	Range (cells/100 ml)	Mean±SD	P-Value
Group (A)	1300-4200	2400.9±879.3	<0.001
Group (B)	150-240	165±22.4	
Control group	0-5	2.3±0.3	

Table 4:- Comparison between levels of urinary CD4 T-cells and SLEDAI in patients' groups at baseline and after 6 months:

	CD4 T-cells/100 ml (Mean±SD)		P-Value	SLEDAI (Mean±SD)		P-Value
	At baseline	After 6 months		At baseline	After 6 months	
Group (A)	2400.9±879.3	2054.8±411.4	<0.05	16.5±5.9	7.4±1.7	<0.001
Group (B)	165±22.4	154±18.4	>0.05	6.2±2.2	5.8±2.8	>0.05

Table 5:- Correlation between levels of urinary CD4 T- cells and SLEDAI in patients' groups at baseline.

Group (A)		CD4 T-cells/100 ml	SLEDAI	r	P-Value
	At baseline	2400.9±879.3	16.5±5.9	0.522	<0.001
	After 6 months	2054.8±411.4	7.4±1.7	0.436	<0.001

Figure 1:- Correlation study between CD4 T-cells and SLEDAI among group-A patients at baseline:**Figure 2:-** Correlation study between CD4 T-cells and SLEDAI among group-A patients after 6 months.

Discussion:-

Currently, patients with SLE are mainly screened for renal involvement using creatinine levels, proteinuria and urinary sediment[17]. Although useful, these variables have certain limitations. Creatinine levels and proteinuria can be persistently elevated and therefore unable to differentiate present kidney damage from acute nephritis. The mainstay for diagnosing LN is the renal biopsy, which is not free from risk and is not suitable for monitoring follow-up[9]. Several urinary biomarkers have been proposed in recent years, most of them detected by ELISA or reverse transcription PCR [18 & 19]. Massengill and his colleagues were the first to report oligoclonal expansions of T cells in lupus nephritis [20] and this was confirmed by Murata and his colleagues[21]. However, they found no evidence

of clonal sharing with the blood, and didn't determine whether these expansions were members of the CD4 or CD8 T-cell subset or how they related to lupus nephritis.

To better define the immunologic character of the T cell infiltrate in lupus nephritis, Winchester and his colleagues reported that SLE kidneys have a variably patterned and often extensive infiltrate of predominantly expanded T cells of CD4 and CD8 lineages. CD4 T-cells were prominent in nearly two thirds of SLE biopsies and distributed as broad aggregates or intermixed with CD8 T-cells forming periglomerular caps. The immunological characteristics of the infiltrating CD4 and CD8 T-cells in the lupus kidney indicate they have the potential to mediate injury, which may be relevant to development of progressive renal failure. The high frequency of CD4 T-cells in the interstitial infiltrates of patients with LN suggests a contribution of these cells to the local pathogenesis[22].

In a study done by Moon and his colleagues, they indicated that intra-renal infiltration and activation of T-cells in the interstitium is the main mechanism of kidney injury and showed a predominant CD4 T-cell renal infiltration [23]. Another study reported that in LN the interstitial infiltration consists mainly of T-cells and to lesser extent of macrophages, B-cells and plasma cells [24]. Enghard and his colleagues determined whether the infiltrating T cells could be monitored in the urine to provide a reliable biomarker for acute LN. The frequency of CD4 T-cells was determined by flow cytometry of peripheral blood and urine from 38 patients with SLE and the values were compared with disease activity as determined by the SLEDAI. The number of urinary CD4 T-cells reflected nephritis activity and elevation above 800 CD4 T-cells per 100 ml of urine sharply delineated active from inactive nephritis. Moreover, the frequency of urinary CD4 T-cells correlated with the disease activity [12].

In our study, we found that urinary CD4 T-cell counts were increased significantly in active SLE patients with severe nephritis and the SLEDAI ≥ 10 , with a mean urinary CD4 T-cell count of $(2400.9 \pm 879.3 \text{ cells}/100 \text{ ml urine})$ in patients of group (A). While in group (B) the mean of CD4 T-cells was low $(165 \pm 22.4 \text{ cells}/100 \text{ ml urine})$ and there was significant correlation between the level of CD4 T-cells and SLEDAI score. These results were in agreement with Enghard and his colleagues[9] who measured the level of urinary CD3CD4 T-cells using flow-cytometry in 186 urine samples from 147 patients with SLE. Fourteen patients were monitored as follow-up. Thirty-one patients with other nephropathies and 20 healthy volunteers were included as controls. In SLE, urinary CD4 T-cell counts $\geq 800/100 \text{ ml}$ were observed exclusively in patients with active LN. In patients monitored under therapy, normalization of urinary CD4 T-cell counts indicated lower disease activity and better renal function. In contrast, patients with persistence of, or increase in, urinary T-cells displayed worse outcomes. Our results agreed also with Kopetschke and his colleagues[25], who studied 98 patients with SLE, 19 with active renal involvement defined by (SLEDAI ≥ 10 & a current renal biopsy showed LN or at least two elements of the renal SLEDAI in the absence of a biopsy) and 79 patients with non-active renal involvement. Urine samples from patients with active renal involvement had higher numbers of CD4 (median of 1415 cells/100 ml urine), in SLE patients without active renal involvement only low number of T-cells was detected with a median of 29 CD4 T-cells/100ml urine).

Conclusion:-

Urinary CD4 T-cells marker has a valuable role in detecting LN in SLE patients and has significant correlation with disease activity index. Furthermore, monitoring urinary CD4 T-cells may help to identify treatment responders and treatment failure and enable patient-tailored therapy in the future.

Conflict of interest:-

The authors declare they have no conflicts of interest.

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