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Urinary T-Cells in Pediatric Lupus Nephritis

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Abstract: ***Lupus nephritis (LN) is a severe and frequent manifestation of systemic lupus erythematosus (SLE). Detection of initial renal manifestations and relapses during follow up is pivotal to prevent loss of renal function. Apart from renal biopsies, current urinary and serological diagnostic tests failed to accurately demonstrate the presence of active LN. Our objective is to assess the diagnostic value of urinary T-cells in comparison with traditional markers of active LN. T-cells in the urine in pediatric LN active and inactive were investigated. Twenty-five, in most cases biopsy proven, active LN patients, 25 inactive LN patients compared with 25 healthy subjects. Analysis of the urinary sediment in active and inactive renal disease showed a decrease of CD8+ T-cells as compared with healthy subjects. On the other hand, increasing of CD4+ T cells in active and inactive LN was observed when compared with healthy subjects. The expression of CD122+ on CD8+ showed an increase in active and inactive LN compared to healthy subjects. Data presented are compatible with the hypothesis that T-cells migrate from the PB to the kidney and appear in the urine of LN patients. These cells could serve as an additional marker of renal disease in patients with SLE.***

Keywords: Lupus nephritis (LN), systemic lupus erythematosus (SLE), Urinary T-cells, flow cytometry

1. Introduction

Lupus nephritis is one of the hallmarks of systemic lupus erythematosus (SLE) and one of the main determinants of prognosis. This nephritis is thought to be caused by local deposition of autoantibodies and immune complexes, but there is an increasing agreement that infiltrating leukocytes also contributes to kidney damage. SLE is a disease that is infrequent in childhood. It is a disease of immunological origin with autoantibodies, polyclonal B-cell activation and T-cell dysfunction. SLE has been reported in children in the first 1-2 years of life [**1**].

It has been frequently reported that pediatric patients have more severe and aggressive disease compared with adults [**2**].Maculopapular rash, diffuse or patchy non-scarring alopecia, and cutaneous vasculitis are also common in pediatric SLE **[3]**

Renal involvement is present in 40 to 80% of pediatric lupus patients and is second only to infection as the most common cause of mortality **[4]**.According to World Health Organization lupus nephritis is divided into six class **[5].**

Although the precise pathogenesis of lupus nephritis (LN) has not been fully elucidated, kidney infiltrating T-cells seem to contribute to the inflammatory pathology of LN **[6]** Podocyte effacement was obvious in most LN patients **[7].** Evaluation for LN includes dipstick and urine sediment analysis, urinary protein and creatinine excretion, determination of serum creatinine and assessment of serological markers such as anti-double stranded DNA (anti-ds) antibody titers, CRP, C3 and C4 levels **[8].** The combination of these markers is a powerful measure for the detection of active renal manifestations of SLE.

Multitarget therapy provides superior efficacy compared with intravenous cyclophosphamide as induction therapy for LN **[9].**

However, in clinical practice, traditional clinical markers for renal involvement such as proteinuria do not always discriminate between active and inactive disease, in particular for patients with a recent history of LN **[10].** For these patients, persistent proteinuria often limits the information of this test to detect renal flares or remission. This is due to the fact that proteiunuria might reflect both glomerular damage and renal activity. For these patients, strict guidelines defining renal flares based on laboratory information are lacking **[11].** Therefore, renal biopsies are crucial and still the gold standard to assess renal disease and to define the histo-pathologic class of LN **[12].** This invasive approach is associated with a risk of bleeding and repeated renal biopsies are not always applicable in daily clinical practice for patients with SLE. Thus, novel non-invasive urinary markers seem to be an attractive goal to detect renal flares in LN. Several studies demonstrated the presence of mononuclear cells in urine of patients with active IgA nephropathy, LN and Wegener's granulomatosis **[13].** . Urinary sCD25 may have the potential to predict poor response and relapse of LN **[14].** . In LN, the infiltrates in the nephritic kidneys consist mainly of CD4+T cells and, to a lesser extent, CD8+ T cells, macrophages, B cells, and plasma cells **[15].** CD4+ CD25+ T- cells were significantly decreased in pediatric patients with active **[16].**

This study aimed to present analysis of the expression of CD4+ and CD8+ urine of patients with lupus nephritis, as well as the expression of CD122+ on CD8+ T cells in the urine of LN patients. We demonstrate that infiltrating T cells express CD8+CD122+, their levels can be monitored in the urine, and they represent a promising biomarker of acute LN.

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2. Materials and Methods

Fifty children with LN disease and 25 healthy subjects were enrolled in this study; 25 patients were active and the other 25 patients were inactive LN. Median (range) anti-ds titers were 230 (3-1000 E/ml), median C3 and C4 were 0.55 g/l (0.05-1.03) and 0.14 g/l (0.04-0.30). Active LN was defined by at least two of the following items (a) new onset proteinuria > 0.5 g/24h, (b) an active urinary sediment representing glomerular injury (c) a renal biopsy providing evidence of active lupus nephritis.

2.1 Materials

Monoclonal antibodies (mAb) and flow cytometry.
The following mAb and reagents were used in this study: fluorescein isothiocyanate (FITC)–conjugated or peridinin chlorophyll A protein–conjugated anti-CD3 mAb, FITC-conjugated (PE)–conjugated anti-CD8 mAb, FITC - conjugated anti-CD4 mAb, PE-conjugated and anti-CD122 mAb, EDTA-blood and fresh urine samples were collected from patients. Urine samples from patients with signs of urinary tract infection were excluded.

2.2.1 Sample preparation and flow cytometry

The fresh urine sample was collected from each patient and control. For 3 ml urine was centrifuged at 2000 rpm for 20 min. The supernatant was removed and the pellet which contains lymphocyte was shaken. The lymphocytes from urine sample was Fixed with ice cold absolute alcohol 1ml for each tube and was preserved in +4 ^{o}C forever until analysis.

2.2.2 Isolation of peripheral blood

Peripheral venous blood samples were collected into tubes containing EDTA, and peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation for 20 min at 1500 rpm without break using Ficoll-Paque Plus solution. The bands of cell were pipette carefully into another centrifuge tube filled up with phosphate buffer saline (PBS) or hanks solution and mixed. After centrifugation for 10min at 1800 rpm, lymphocyte sediment were fixed with ice cold absolute alcohol 1ml and preserved at +4C^{o} until analysis.

This technique was applicable where the fluorochrome was directly linked to the primary antibody e.g.: phycoerythrin (PE) or fluorescein isothicyanate (FITC)-conjugate. 200 µl of lymphocytes were added in test tubes. The lymphocytes were washed with 1ml PBS, and then were centrifuged at 200 rpm for 5 minutes, the supernatant was discarded. The lymphocytes pellet was washed with PBS and mixed well. 3µl of required marker was added and was mixed well. The tubes were incubated at room temperature for 20 minutes. The cells were washed with 1ml PBS; then the tubes were centrifuged at 2000 rpm for 5 minutes. The supernatant was removed then 200ml of 4% paraformaldehyde were added for analysis by flow cytometer. Flow cytometric analysis was performed with FACClibur flow cytometer using Cell Quest software (Becton Dickinson, San Jose, CA). A total o10,000 to 20,000events were collected for each analysis. Lymphocyte gates were set on live cells using forward (FSC) and side scatter (SSC).

Statistical analysis: Data were explored, processed and analyzed using the statistical package for the social science, windows version 16, USA (SPSS PC+ version 16 software). Variable with normal distribution were expressed as mean± SD. In these variables, the T test was applied for group differences.

3. Results

We studied a total of 75 subjects. Their demographic and clinical data are summarized in Table (1).

Table 1: Demographic and clinical characteristics of patients and control subjects

	Control	*Lupus Nephritis patients*	
		Inactive	*Active*
Case no.	25	25	25
Sex (male\Female)	9\16	4\21	5\20
Age /years Range	9.91±3.75 5.5-17	13.41±3.66 7-17	14.7±3.48 4-19
Anti-dsDNA	7.5±5.5	50.77±23.53	247.38±168.23
creatinine mg/dl	0.44±0.12	0.59±0.4	0.96±1.1

T-cell count discriminates between LN patients and control

Table (2) detects the T-cells in the urine in all pediatric LN patients. The mean count of urinary $CD4^{+}$ %T-cells was significantly increased in pediatric LN patients in comparison to healthy subjects (34.16±7.18 vs. 14.84±4.89 respectively; P <0.0001). Insignificant differences were obtained in the mean count of urinary CD4+ T-cells in active LN patients compared to inactive LN patients ; P >0.05). The mean count of urinary $CD8^{+}$ T-cells was significantly decreased in LN patients in comparison to healthy subject (18.62±3.84 vs. 31.40±6.34, respectively; p <0.0001). Insignificant differences for urinary $CD8^{+}$ T cells (%) levels obtained from active LN as compared to inactive LN(P >0.05).

Table 2: Flow cytometery markers for urinary T-cells of studied subjects included in the study (n=75)

	Control(n=25)	Inactive LN (n=25)	Active LN (n=25)
$CD4^{+}$	14.84±4.89	33.20±4.16***	35.12±9.27***
$CD8^{+}$	31.40±6.34	17.28±4.37***	19.25±3.02***
$CD8^{+}CD122^{+}$	9.06±3.41	26.46±5.15***	28.18±6.64***

*** (P value <0.001)

The mean count of urinary $CD122^{+}$Tcells among $CD8^{+}$Tcells levels obtained from LN patients was significantly increased as compared to healthy control group (27.42±5.93 vs 9.06±3.41 respectively; P <0.001). Insignificant difference was found in the $CD8^{+}$ $CD122^{+}$Tcells levels in urine obtained from inactive LN group as compared to active LN group (P >0.05)**.(Fig.1)**

Table (3) represents the flow cytometry markers of T-cells % from blood samples. $CD4^{+}$ T cells (%) levels in blood samples obtained from LN patients were significantly elevated as compared to healthy control group (26.89% ± 4.42% and 12.83%±3.17%, respectively; p <0.001). Whereas,

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there were insignificant differences for $CD4^+$ T cells (%) levels in blood obtained from active LN group and inactive

Table 3: Flow cytometery markers for T-cells% from blood)

	Control (n=25)	Inactive LN (n=25)	Active LN (n=25)
$CD4^+$	12.75±3.02	26.95±6.24***	27.05±4.29***
$CD8^+$	29.04±7.11	15.16±1.68***	14.24±3.36***
$CD8^+CD122^+$	30.15±5.52	12.31±3.23***	9.5±3.57***

*** (P value <0.001)

The $CD8^+$ T cells (%) levels in blood samples obtained from active LN group were significantly decreased as compared to healthy control (15.25%±1.78% and 30.17%±7.63% respectively; P ≤0.01). There were no significant differences for $CD8^+$ T cells (%) levels in blood obtained from active LN group and inactive LN group (P >0.05). The mean count of $CD122^+$ cells among $CD8^+$ lymphocytes levels in blood samples obtained from LN patients was significantly decreased as compared to healthy control group (10.91%±3.65 versus 30.55%±5.5 respectively; P ≤0.001). On the other hand, insignificant difference was found in the $CD8^+$ $CD122^+$ T-cells levels in blood samples obtained from inactive LN group as compared to active LN group (P >0.05).(Fig.2)

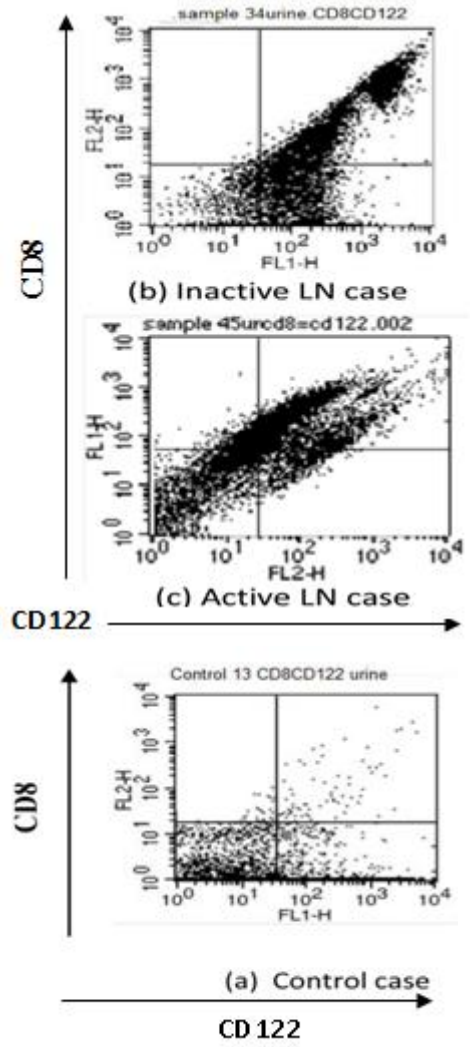


Figure 1: Comparison of flow cytometric analysis of double stain CD8 CD122T-cell populations between control and patient. Urine was stained with FITC-conjugated anti-CD8, PE-conjugated anti-CD122. Cells were gated on lymphocytes via their forward- and side-scatter properties.

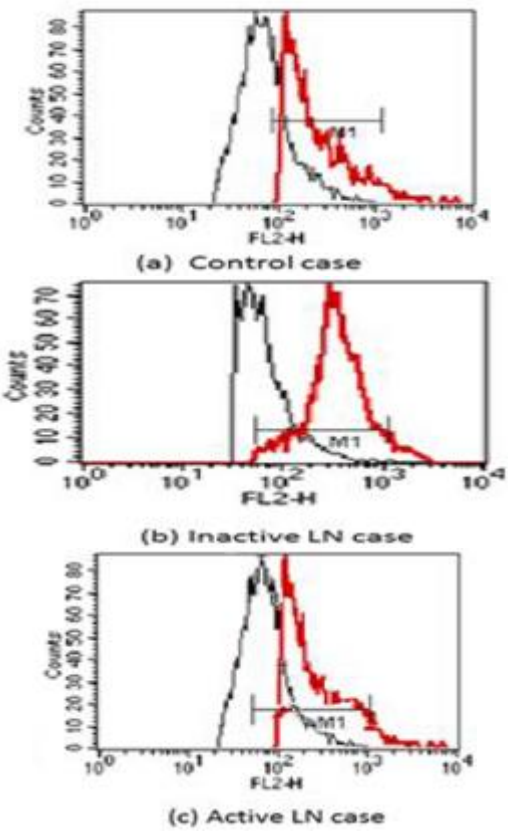


Figure 2: Flow cytometry histograms for $CD8^+CD122^+$ show positive expression for both two marker according to M1 (positive expression value) (black color CD8, red color CD122)

4. Discussion

In lupus nephritis, the severity of renal involvement depends on activated T cells and macrophages, which secrete a variety of inflammatory mediators into the kidney **[17]. Chan [18]** further supported the view that lupus nephritis involves intrarenal infiltration, activation, and alteration of the secretory phenotype of lymphocytes toward the Th1 pathway

Fritsch [19] hypothesis that effector memories T-cells migrate during active renal disease from the peripheral blood to the kidney and appear in the urinary sediment. The sediment was composed mainly of red blood cells, lymphocytes, and tubular epithelial cells.

Our data demonstrated the distribution frequency of $CD8^+$, $CD4^+$ and CD8/CD122 T-cells in the peripheral blood and urine of LN patients. In our findings the surface markers $CD4^+$ T-cells levels in urine samples obtained from LN patients were significantly elevated as compared to healthy control group. This result was confirmed by data obtained by **Enghard [20]** who demonstrated that urinary CD4 T-cells are a highly sensitive and specific marker for detecting proliferative LN in patients with SLE.

The surface markers $CD8^+$T-cells levels in urine samples obtained from LN patients were significantly decreased as compared to healthy control group. **Dolff [6]** showed that the

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percentage of circulating CD8+ TEM cells in SLE patients significantly decreased versus control (33.9 ±18.3 % vs. 42.9 ±11.0 %, p=0.008). On the other hand, **Dolff [8]** study demonstrated the analysis of the urinary sediment in active renal disease showed an increased number of CD8+ T-cells and absence of these cells during remission.

In the present study, the surface markers $CD4^+$T-cells levels in urine samples obtained from LN patients were significantly elevated as compared to healthy control group (15.14±5.73 and 34.53±8.17) respectively (p<0.001). This result was confirmed by data obtained by **Enghard [20]. Enghard [20]** demonstrated that urinary CD4 T-cells are a highly sensitive and specific marker for detecting proliferative LN in patients with SLE.

In the current study, The mean count of urinary $CD122^+$Tcells among $CD8^+$Tcells levels obtained from LN patients was significantly increased as compared to healthy control group (9.06±3.41 vs 27.42±5.93 respectively; P <0.001).
The present study is the first investigation $CD8^+CD122^+$ in the peripheral blood and urine of LN patients. The data provide strong evidence that $CD8^+$ $CD122^+$T-cells migrate from the peripheral blood to the kidney and appear in the urine during LN flares. Therefore, $CD8^+$ $CD122^+$Tcells may be a useful monitoring tool in LN patients with renal involvement.

Moreover, this analysis will be especially helpful in LN patients with a recent history of LN. This relates to the fact that persistent proteinuria is a frequent observation which limits the use of this marker for the judgment of renal activity. Measuring urinary CD8+ CD122+T-cells helps to discriminate between LN and healthy subjects. To introduce this test into routine diagnostics, further studies are needed to confirm these results in a larger cohort of biopsy proven LN patients.

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