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Original Article

Correlation analysis between urinary T lymphocyte subsets and lupus nephritis disease activity

Ze-Ming Zhang, Lin Zhao, Xiao-Li Zhang, Xiao-Fei Wang

Department of Rheumatology, Shengjing Hospital of China Medical University, Shenyang, Liaoning Province, China

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Abstract: Objective: In clinic, it's significantly important to monitor lupus nephritis (LN) disease activity for the long-term prognosis of patients. To explore the role of urinary T lymphocyte true count in the evaluation of LN disease activity, we enrolled 35 patients and 21 healthy controls. Methods: Fresh morning-midstream urine of all subjects was collected for preparing cell suspension by precipitation, filtration, centrifugation. Lymphocyte subsets were determined by flow cytometry using CD3-FITC, CD8-PE, CD4-APC, CD45-PerCP. The concentration of urinary MCP-1 and TWEAK were measured by ELISA. Contrastive analysis was made combining with the clinical data collected from patients. Rests: The urinary $CD3^+$, $CD4^+$ and $CD8^+$ T cells counts of LN patients are all significantly higher than that of the healthy controls, and positively correlated with the SLEDAI and rSLEDAI scores. Compared with other T cell subsets, experimental and clinical indicators, the counts of urinary $CD4^+$ T cells showed the most significant difference among different disease activity groups. Conclusions: Counts of the main urinary T lymphocyte subsets correlate with LN disease activity scores. And among them, $CD4^+$ T cells can be used to distinguish patients in different disease activity groups, revealing a new biomarker for LN disease activity evaluation.

Keywords: Lupus nephritis, systemic lupus erythematosus, T lymphocyte true count, urinary T lymphocyte subsets

Introduction

Systemic lupus erythematosus (SLE) is a kind of complex autoimmune disease with the characteristics of slow course, easily relapse and multiple organs involved, mainly damages young and middle-aged women. Delayed diagnosis due to diverse clinical manifestations of SLE makes it plague clinical doctors and patients for a long time. Renal involvement is common in lupus patients with high morbidity and mortality. There are up to 60% of the patients suffering the immune-complex lupus nephritis in the course of SLE [1]. The emergence of lupus nephritis makes 10-year-survival rate of SLE patients decrease from 92% to 88% [2]. About 10-30% of LN patients will develop into end-stage renal disease (ESRD) [3] which has higher mortality. Because of race, genetic, social-economic background and LN disease severity etc. [4], the percentage of ESRD did not reduce in the past decade [5]. In order to adjust the treatment plan in time and delay the appearance of ESRD, it has crucial importance to early diagnose and long-term disease activity monitor for LN patients.

In recent years, it focuses more and more on the noninvasive and sensitive urinary biomarkers for LN disease monitoring, such as some inflammatory cell-recruiting chemokines, adhesion molecules and cytokines reflecting kidney inflammation [6]. As well, studies in succession reported urine monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-like weak inducer of apoptosis (TWEAK), and other related indicators [7, 8]. However, it pays relatively less attention to the diagnose function of cells test for disease evaluation.

As we all know, inflammation caused by T lymphocyte homeostasis disorder is closely associated with LN pathogenesis, and T cells can infiltrate into LN patients kidney. These make scholars focus their attentions on urinary T cells determination to evaluate the state of LN. Sebastian Dolff *et al*. [9] firstly discussed the function of effector memory T in peripheral blood, kidney biopsy and urine of SLE patients. They also confirmed that the $CD8^+$ TEM lymphocytes of active LN patients could migrate to the kidney from peripheral blood and appear in the urine. Also, compared with lupus

Table 1. Characteristics and laboratory data of patients and healthy controls

	Patients with LN (n = 35)	Severely active (n = 12)	Moderately active (n = 9)	Mildly active (n = 14)	Healthy controls (n = 21)
Female/Male	34/1	12/0	9/0	13/1	19/2
Age (years)	35 ± 13	33 ± 11	39 ± 16	35 ± 12	38 ± 16
$CD3^+$ T cells/μL	214 ± 284	381 ± 430	180 ± 105	93 ± 67	50 ± 58
$CD3^+CD8^+$ T cells/μL	157 ± 221	274 ± 341	132 ± 95	73 ± 55	36 ± 44
$CD3^+CD4^+$ T cells/μL	42 ± 58	87 ± 82	30 ± 12	10 ± 8	6 ± 5
$CD3^+CD8^+CD4^+$ T cells/μL	7 ± 10	12 ± 14	6 ± 7	4 ± 7	1 ± 3
$CD45^+$ cells/μL	346 ± 563	647 ± 883	309 ± 182	111 ± 70	64 ± 73
Ratio of $CD4^+/CD8^+$	0.29 ± 0.26	0.44 ± 0.36	0.29 ± 0.12	0.17 ± 0.14	0.27 ± 0.30
MCP-1 (10^{-2} pg/mgCr)	3.05 ± 2.41	3.42 ± 2.75	3.17 ± 1.27	2.65 ± 2.73	3.36 ± 3.78
TWEAK (pg/mgCr)	153.45 ± 134.67	173.28 ± 149.76	156.68 ± 102.18	134.39 ± 145.84	50.63 ± 50.81
SLEDAI	12 ± 6	18 ± 2	12 ± 2	6 ± 2	
rSLEDAI	7 ± 4	12 ± 3	7 ± 2	3 ± 2	
Proteinuria (g/24 h)	1.88 ± 2.19	2.40 ± 2.82	2.15 ± 2.11	1.27 ± 1.55	
Scr (umol/L)	71.94 ± 28.71	75.29 ± 32.97	70.50 ± 14.76	69.99 ± 32.96	
BUN (mmol/L)	5.62 ± 2.91	6.78 ± 4.02	5.66 ± 2.71	4.61 ± 1.28	
Cys C (mg/L)	1.43 ± 0.67	1.54 ± 0.75	1.76 ± 0.60	1.13 ± 0.55	
eGFR (ml/min • 1.73 m^2)	91.5 ± 43.34	87.22 ± 47.45	72.89 ± 30.05	107.16 ± 43.94	

Values are showed by mean ± SD. The data are from 21 healthy controls and 35 LN patients, who are divided into 3 groups with SLEDAI scores. LN: Lupus Nephritis, MCP-1: Monocyte chemoattractant protein-1, TWEAK: Tumor necrosis factor (TNF)-like weak inducer of apoptosis, SLEDAI: Systemic Lupus Erythematosus Disease Activity Index, Scr: Serum Creatinine, BUN: Blood Urea Nitrogen, Cys C: Serum Cystatin.

patients without renal involvement, LN patients show an increasing urinary $CD4^+$ T cell value and the number of $CD4^+$ T cells may be related to LN prognosis [10].

In this study, we wanted to identify the association of main urinary T cell subsets counts with the evaluation of LN disease activity by applying T lymphocyte true count, a common clinical detection method, to provide a new biomarker which is probably used in future clinic.

Patients and methods

Patients

Thirty-five LN patients hospitalized in our department were selected during Oct 2015 to Apr 2016. All the patients conformed to Systemic Lupus International Collaborating Clinics (SLICC) 2012 classification criterion for SLE [11]. Excluding other system severe diseases and autoimmune diseases, they also met the diagnostic criteria for LN [2] without urinary frequency, urgency, odynuria and any other clinical infection symptoms. And the basic characteristics of all subjects enrolled are showed in **Table 1**.

The patients were grouped by Systemic Lupus Erythematosus diseases Activity Index (SLEDAI): SLEDAI ≤ 9 was the mild or non-active group; 10 ≤ SLEDAI ≤ 14 was the moderately active group; SLEDAI ≥ 15 was the severely active group. Renal SLEDAI (rSLEDAI) score with a total score from 0 to 16 points, includes proteinuria, hematuria, pyuria and cylindruria four items and each of them is 4 points. LN disease was regarded as active when rSLEDAI scores ≥ 4. When urinary protein did not reach the diagnostic criteria of proteinuria (0.15 g/d < 24 h urinary protein < 0.5 g/d), it can not be regarded as active LN disease unless there is obviously abnormal renal function, namely, serum creatinine increased more than 30% in three months or estimated effective glomerular filtration rate (eGFR) was less than 60 ml/min.

Twenty-one gender and age matched healthy controls without urinary frequency, urgency, odynuria or any other clinical infection symptoms or system severe diseases or basic kidney disease were randomly selected.

Methods

Samples: 50 ml morning-midstream urine was collected from the patients and healthy controls. All samples needed to be tested in 4 to 6 hours after collecting to ensure they were fresh.

Flow cytometry: After precipitation, filtration, centrifugation (1500 rpm, 15 min), the cells

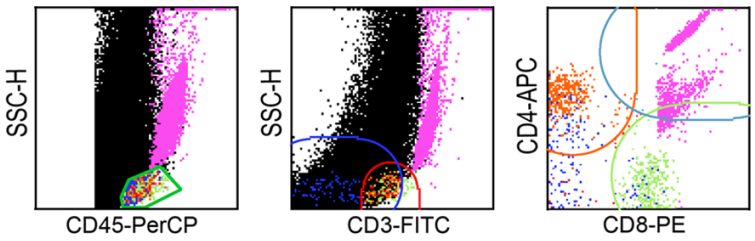

Figure 1. The results of flow cytometric analysis of urinary T subsets. Samples stained with mixed antibody (BD Company), containing peridin chlorophyll protein (PerCP)-conjugated anti-CD45, fluorescein isothiocyanate (FITC)-conjugated anti-CD3, phycoerythrin (PE)-conjugated anti-CD8 and allophycocyanin (APC)-conjugated anti-CD4. All the counting values were taken microglobulin in tubes as the standard.

Table 2. Increasing counts of urinary T lymphocyte in LN patients

	Patients with LN (n = 35)	Healthy controls (n = 21)	P
$CD3^+$ T cells/μL	214 ± 284	50 ± 58	0.002
$CD3^+CD8^+$ T cells/μL	157 ± 221	36 ± 44	0.003
$CD3^+CD4^+$ T cells/μL	42 ± 58	6 ± 5	0.001
$CD3^+CD8^+CD4^+$ T cells/μL	7 ± 10	1 ± 3	0.003

Compared with healthy controls, the urinary $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD4^+CD8^+$ T cells counts of LN patients all increased significantly.

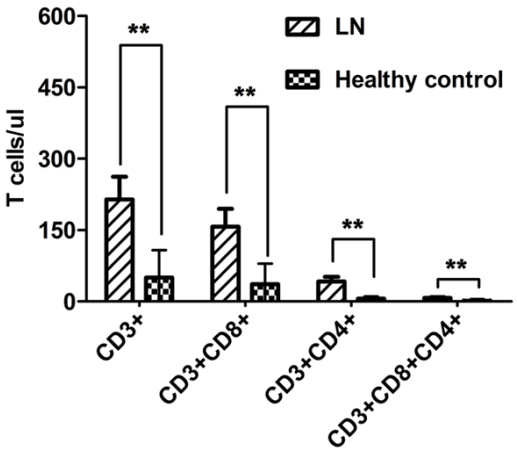

Figure 2. Increasing counts of urinary T lymphocyte in LN patients. Compared with healthy controls, the urinary $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD4^+CD8^+$ T cells counts of LN patients all increased significantly; $0.01 \leq P \leq 0.05$: *, $0.001 \leq P < 0.01$: **.

precipitated from 50 ml urine sample was repeatedly washed with PBS three times and made into 100 μL cell suspension which were then put into the true count tubes containing microglobulin producted by BD Company. To detecte the T cell subsets, mixed antibody containing CD45-PerCP, CD3-FITC, CD8-PE, CD4-APC (BD Company) was used at room temperature and reacted away from light. After erythrocyte lysing the samples were tested and analyzed by FACSCalibur (# E97500357) and software MultiSET V2.2 (**Figure 1**).

ELISA: According to the ELISA kit instructions, the concentration of MCP-1 (Abgent Company, United States) and TWEAK (NeoBioscience, Shenzhen) in the supernatant of the urine samples were determined and normalized to urinary creatinine concentration, expressed as pg/mg creatinine (pg/mg Cr).

Clinical data: Clinical data, including serum creatinine (Scr), blood urea nitrogen (BUN), cystatin C (Cys C), 24 hour urinary protein, were collected from the medical records and the effective glomerular filtration rate (eGFR) was calculated by CKD-EPI Cr-Cys C combinatorial formula [12].

Statistical analysis

One-Way ANOVA was used to compare the differences among three groups with different LN disease activity (equal variances assumed by F test of Levene method, multiple comparisons made by Bonferroni/Tamhane method). Independent sample t-test was used to identify whether the LN patients had difference from healthy controls or not. ROC curve analyzed the diagnosis meaning of indexes. The correlation analysis was expressed by Spearman correlation rate. All statistics data was calculated by SPSS19.0 and $P < 0.05$ was considered statistically different.

Results

Elevated counts of urinary T lymphocyte subsets in LN patients

The urinary $CD3^+$ cells count of LN patients (n = 35) was 214 ± 284 cells/μL, which was signifi-

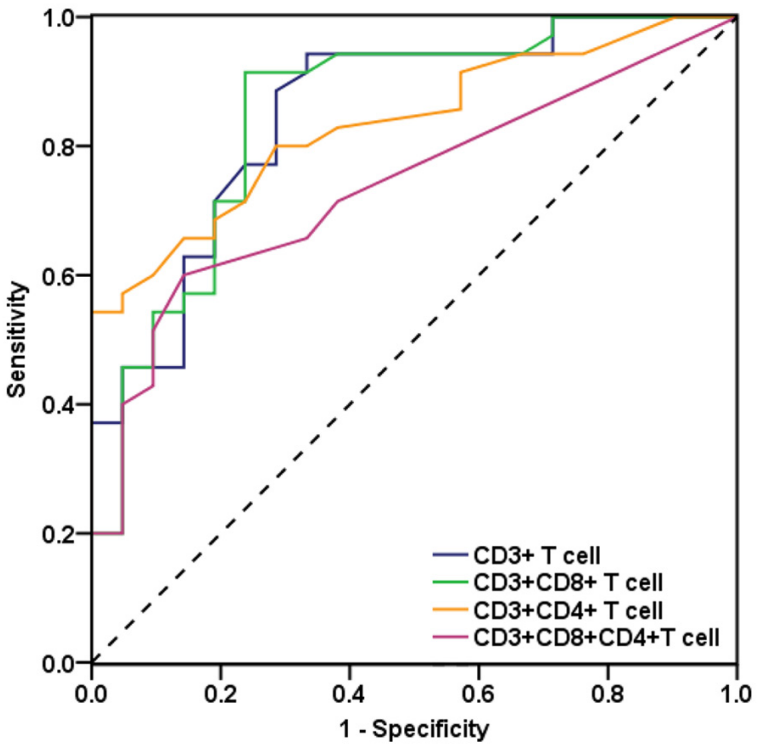

Figure 3. The receiver operator characteristic (ROC) curves of urinary T cell subsets. The curves showed the diagnostic performance of urinary $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD4^+CD8^+$ T cells counts in detecting LN patients and healthy controls.

cantly higher than that of the healthy controls (n = 21) was 50 ± 58 cells/μL. In addition, urinary $CD3^+CD8^+$ T cell and $CD3^+CD4^+$ T cells and $CD3^+CD4^+CD8^+$ T cells of LN patients were also obviously increased (**Table 2** and **Figure 2**). In addition, compared with that of the healthy controls, $CD45^+$ cell counts increased (346 ± 563 /μL *vs.* 64 ± 73 /μL, P = 0.006) in the urine of LN patients. These proved that urinary T lymphocyte count in LN patients was significantly higher than that in the healthy controls. However, the ratio of $CD4^+$ T cells to $CD8^+$ T cells had no statistical difference (0.29 ± 0.26 *vs.* 0.27 ± 0.30, P = 0.834).

Counts of urinary T lymphocyte subsets are highly sensitive and specific for diagnosing LN patients

To explore the sensitivity and specificity of urinary T cells to differentiate LN patients from healthy controls, Receiver Operator Characteristic curve (ROC curve) was made (**Figure 3**). The area under the curve (AUC) of $CD3^+$ T cells was 0.851 (P < 0.001), AUC of $CD3^+CD8^+$ T cells was 0.850 (P < 0.001), AUC of $CD3^+CD4^+$ T cells was 0.834 (P < 0.001), AUC of $CD3^+CD4^+CD8^+$ T cells was 0.739 (P = 0.003). The results showed that counts of urinary T lymphocyte subsets have relatively highly sensitivity and specificity to detect LN patients from the healthy controls.

Counts of urinary T lymphocyte subsets positively correlated with SLEDAI scores

Analyzing the relationship between T cell subsets true counts and the SLEDAI scores, we found that the count of $CD3^+$, $CD3^+CD8^+$, $CD3^+CD4^+$ and $CD3^+CD4^+CD8^+$ T cells in urine samples had positive correlation with SLEDAI scores (**Figure 4**). It means that, with the increase of LN patients disease activity score, all types of T cells in the urine would raise in different degrees.

Not only did it correlate with the SLEDAI scores, each T cells count was also found have the positive relationship with rSLEDAI (**Figure 5**). Moreover, $CD4^+$ T cell had the biggest r value which means it has strongest correlation with SLEDAI scores compared to the other three T cell subsets.

Urinary $CD4^+$ T cell count could distinguish LN patients in different disease activity groups better

We had multiple comparison of counts T cell subsets in different disease activity groups (the mean and standard deviation of the data showed in **Table 1**). There were statistical differences, for the count of $CD3^+CD4^+$ T cells, in the three groups with different disease activity of LN patients (**Figure 6B**). However, the $CD3^+$ T cells count between mildly active group and severely active (P = 0.041), between mildly active group and moderately active (P = 0.023) group had no statistical difference. Then we combined severely active group with moderately active, had the severe-moderately group

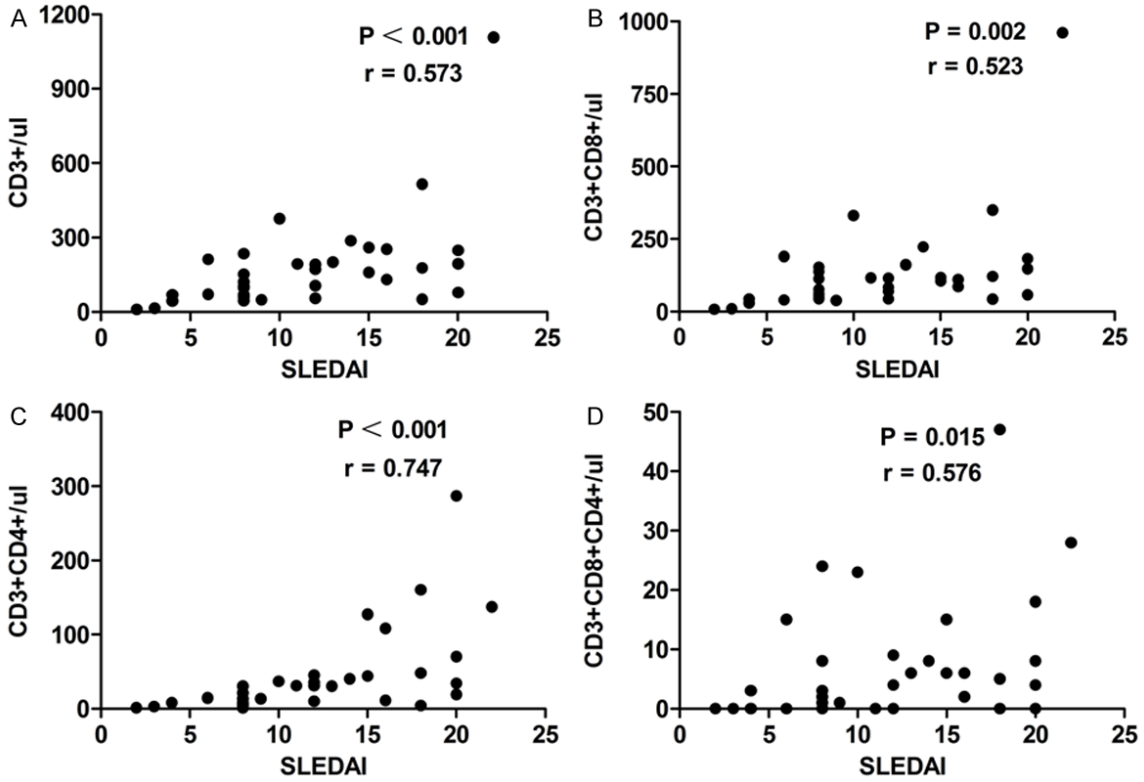

Figure 4. There was positive correlation of the numbers of urinary T cell subsets with SLEDAI scores. All the correlation had statistical significance and the "r" showed in the Figure means Spearman correlation coefficient.

(SLEDAI ≥ 10), and compared to mildly active group, finding an obvious difference (**Figure 6A**). Although compared with healthy control, the count of $CD3^+CD8^+$ T cells of moderately active group (P = 0.017) and mildly active group (P = 0.033) had statistical increase, but had no statistical differences in the 35 LN patients. By contrast, $CD3^+CD4^+CD8^+$ T cells count had no statistical in different groups.

The limitation of other laboratory indicators

Our results suggested the concentration of urinary TWEAK in LN group increased significantly (**Figure 7D**). However, the concentration of urinary MCP-1 of had no obvious difference between LN patients and healthy controls (P = 0.709). Moreover, neither urinary MCP-1 nor TWEAK did have statistical differences in diverse disease activity LN patients.

Analysis the clinical data of LN patients, we found that none of three indexes, including Scr, BUN, eGFR, had relationship with SLEDAI scores or differences in various LN disease activity patents yet. Although 24 h urinary protein of LN patients positively correlated with SLEDAI (**Figure 7A**) and rSLEDAI (**Figure 7B**), there was no statistical differences in patients with different disease activity. Serum Cys C had weak correlation (r = 0.338, P = 0.338) with SLEDAI scores, rather than the rSLEDAI. However, compared with mild-active groups, severe-moderate active group patients (SLEDAI ≥ 10) had higher serum Cys C concentration (**Figure 7C**).

Discussion

Lupus nephritis is one of the common and poor prognosis complications of systemic lupus erythematosus, seriously affecting the long-term survive of patients. There is a clear correlation between LN delayed diagnosis and renal insufficiency, even the appearance of end-stage renal disease [13]. As long-term medication, patient compliance and the disease severity, LN easily relapses. Therefore, it is extremely necessary to early diagnose and long-term disease activity monitor for LN patients.

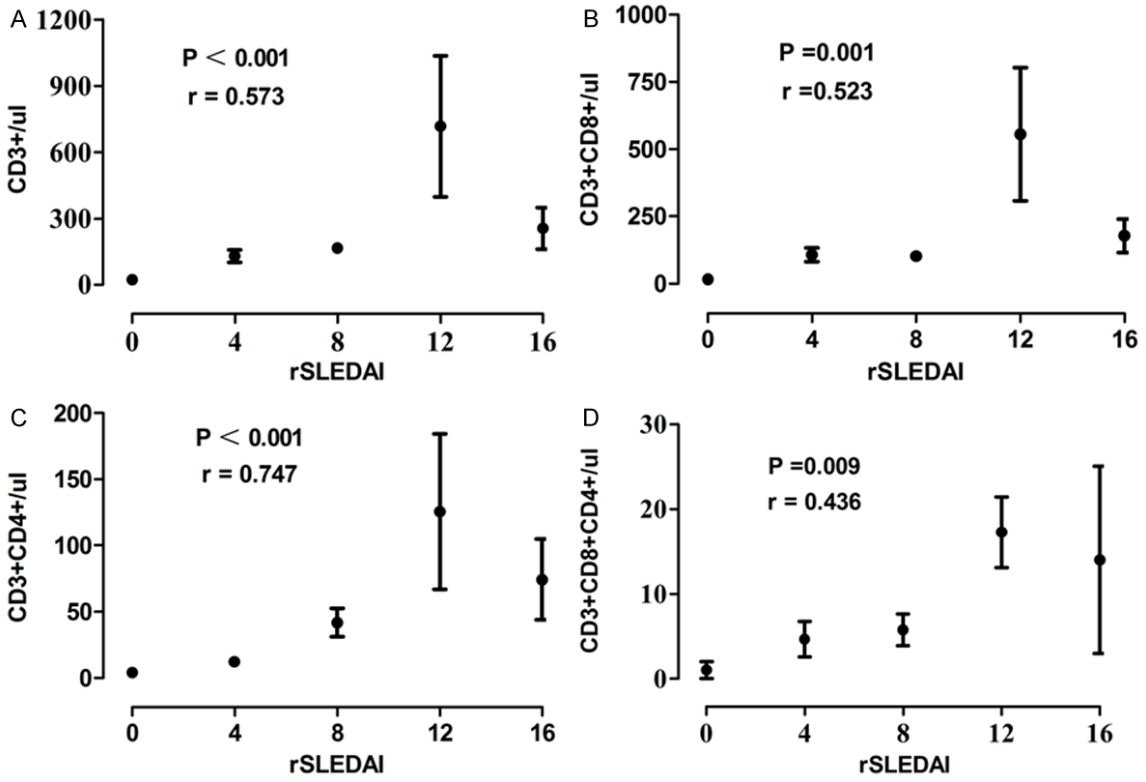

Figure 5. Correlation of urinary T cell subsets true counts with rSLEDAI. Urinary T cells count raised with the increasing of rSLEDAI scores. *P* values and Spearman correlation coefficient (r) were showed in the figure.

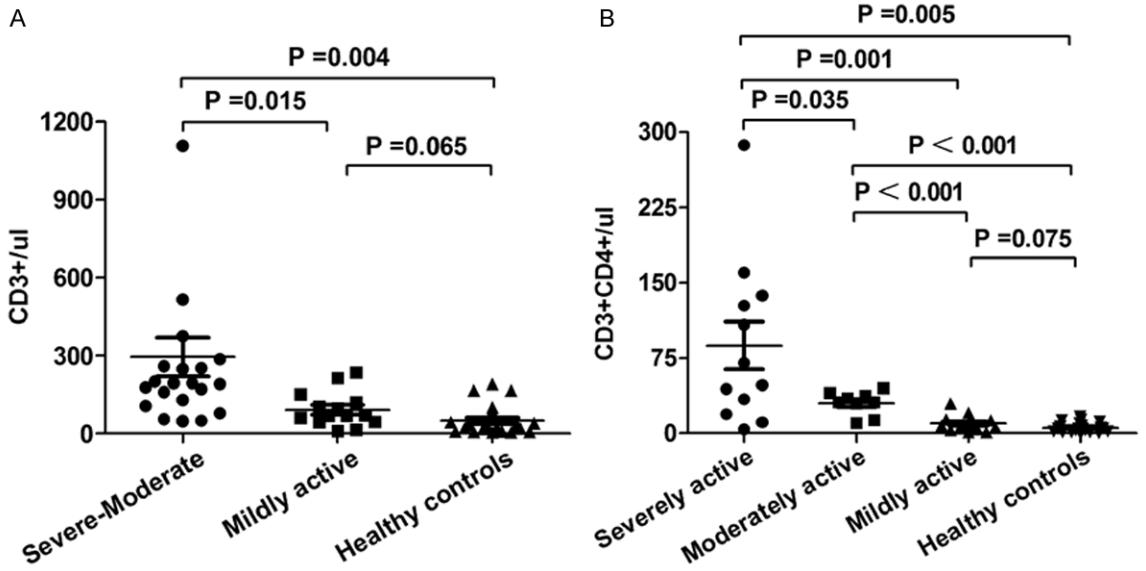

Figure 6. Counts of urinary T cells subsets had significant differences in different disease activity LN patients. A: Compared with mild-active LN patients, $CD3^+$ T lymphocytes in urine of severe-moderate active LN patients increased obviously in group. B: Urinary $CD3^+CD4^+$ T cells could detailedly distinguish severe, moderate, mild active LN patients.

As clinical common indexes, 24 h urinary protein, Scr, BUN, urine routine examination and eGFR, are widely used in renal function evaluation. In our study, the average Scr of LN patients

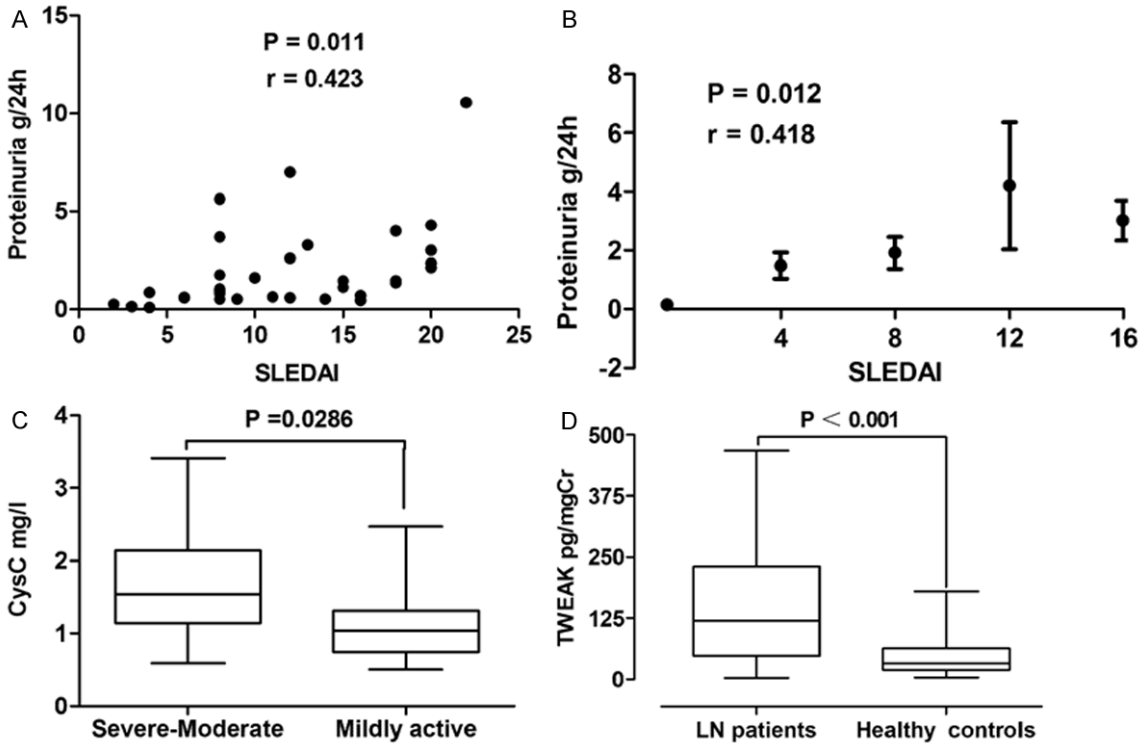

Figure 7. Analysis results of other indicators. A, B: 24 h urinary protein of LN patients positively correlated with SLEDAI and rSLEDAI scores, while has no statistical differences in patients with different disease activity. C: Compared with the mild-active group, the concentration of Cys C was higher in severe-moderate active group. D: Urinary TWEAK concentration of LN patients is obviously higher than the healthy controls.

was 71.94 ± 28.70 umol/l within our hospital normal range (59-104 μmol/L) and only 4 cases' Scr level beyond normal standard. The average level of BUN (5.62 ± 2.91 mmol/L) was also in our hospital normal range (3-9.2 mmol/L). These suggested that clinical Scr and BUN level is delayed to actually reflect the LN patients disease activity in some extent. For eGFR, the average level in our study was 91.5 ± 43.34 ml/min and the 20 patients with eGFR less than 90 ml/min scattered in different groups. As this, eGFR cannot reflect the disease condition changes flexiblely when there was basic kidney damage, despite it can be used to evaluate the renal function. 24 h urinary protein correlated with SLEDAI scores, but had no differences in different disease activity groups. Moreover, except for 1 case in severe-active group was with 10.56 g urinary protein, the rest patients had various urinary protein levels in different groups.

According to our results, there are still some limitations of existing clinical indexes. Serum creatinine and urinary protein, can be maintaining for a long time at a certain level once elevated. They are difficult to distinguish the active condition in the process of continuous renal damage [10]. Renal biopsy is intuitionistic gold standard for LN diagnosis. Nevertheless, it is not easy to be accepted, repeated and long-term follow-up due to it's an invasive operation. The representativity of biopsy samples cannot be ensured by blind needle puncture [6] and it will increases risk in centers with limited experience [14]. If encounter LN patients with high Scr, urinary protein and low eGFR in clinical, we need to keep vigilance disease active, but only depending on these indexes is still insufficient.

Serum anti-double-stranded DNA antibodies (anti-dsDNA) and complement levels are also normally used to evaluate LN disease, sensitive but less specificity [15]. Therefore, qualitative and quantitative laboratory indicators which can reflect the disease activity specially become the focus in recent years [16].

Analyzing the experimental data, we found that $CD45^+$ cells in urine of patients with LN incre-

ased significantly (P = 0.006). Also, compared to 50 ± 58/μL of healthy controls, the urinary $CD3^+$ T cell count of LN patients could be up to 214 ± 284/μL. These reminded us the number of urinary T lymphocyte would elevate in LN patients. Then we also found the counts of LN patients urinary T cell subsets all increased significantly (**Table 2**). And the AUCs of T cell subsets in ROC curves were all greater than 0.8 with P < 0.001, apart from the $CD3^+CD8^+CD4^+$ T cells. These results showed urinary T cell subsets true count not only do increase in LN patients, but also have high sensitivity and specificity to distinguish LN patients from the healthy people.

In spite of correlating with the SLEDAI and rSLEDAI scores, $CD3^+CD8^+$ and $CD3^+CD8^+CD4^+$ T cell counts had no differences in LN patients. To contrast, urinary $CD3^+$ T cell count of LN patients with severely moderate active disease was significantly higher than mildly active patients and healthy controls (**Figure 6A**). Furthermore, the true count of urinary $CD4^+$ T cells of the three groups with different disease activity had obviously statistical difference each other (**Figure 6B**). However, the urinary T cell subsets counts of mildly active LN patients are similar to healthy people. These could reveal that the raise of urinary T cell subsets could be used as an estimated index to severe, moderate activity of LN disease and provide some guides for clinical practice. And T cell counts in urine decrease or recover, especially $CD4^+$ T cells, was likely to be an indicator for LN remission, which was also mentioned in previous research [10]. In addition, we found that blood Cys C of patients with LN (1.43 ± 0.67 mg/l) is beyond the normal range (0.59-1.03 mg/l), and increased significantly in the severely moderate active group (**Figure 7C**). It reminded us also need to pay more attention to the importance of Cys C in future clinical work. But it still cannot directly reflect the detail disease activity, compared with urinary $CD4^+$ T cells.

T lymphocytes play a vital role in the pathogenic process of LN, which can directly infiltrate in kidney and play toxic effects on the renal interstitial cells [17]. Although we didn't have deep research to the mechanism of urinary T cell subsets increasing, lots of previous results could help us to clear the basic processes. Yang Sen et al. [18] confirmed that central memory T cells can migrate to inflammatory tissue from secondary lymphoid organs of SLE patients by expressing CCR7. Dendritic cells derived from normal monocytes and the raise of IFN genes expression can stimulate $CD8^+$ T cells increasing in periglomerular tissue of patients with severe LN [19]. $CD4^+$ T cells of MRL/lpr mice kidney can accelerate nephritis progress [20]. Also there was study showing $CD4^+$ T cells could exist in the urine of active LN patients by CXCR3 [21].

Monocyte chemoattractant protein (MCP-1), belongs to the cytokines CC family, is a kind of signal cytokine respond to inflammatory reaction, which is secreted by the renal tubular endothelial, interstitial and epithelial cells [22]. Tumor necrosis factor-like inducer of apoptosis (TWEAK), is a kind of multiple-effective cytokines belonging to the TNF ligand superfamily, involved in cells proliferation, differentiation, migration and other biological processes by bonding to Fn14 receptor [23]. Two above kinds of cytokines are the popular biomarkers for LN disease evaluation in present. However, in our study, only the difference of urinary TWEAK concentration between LN patients and healthy controls got the statistical significance (P < 0.001) but without exact differences among diverse disease activity groups. Many factors might contribute to the results, such as the experimental subjects, samples, ELISA kits and related operation in the experimental process, and it needs to be further discussed. In some extent, these may also reflect the instability of them.

Certainly, there were still some shortcomings in this study, such as lacking of kidney biopsy as the support and the relatively small size of experimental samples. It will be improved in the future study. And it is necessary to have large sample, multiple-group research to indentify the clinical significance of urinary T cell subsets true count in LN patients.

In conclusion, T cells are crucial for the pathogenesis of LN, which can directly invade into inflammatory kidney and have cytotoxic effect. Urinary $CD3^+$ and $CD3^+CD8^+$ and $CD3^+CD4^+$ and $CD3^+CD8^+CD4^+$ T cell subsets true counts of LN patients increased significantly and were correlated with lupus disease activity. Moreover urinary $CD3^+CD4^+$ T cells count can better distinguish LN patients with different disease activity. The data reveal that the count of urinary T

cell subsets could be a potential clinical biomarker to evaluate LN disease activity. In addition, T lymphocyte true count is a mature clinical laboratory testing technology, generally used in hospitals, will provide the practical basis for clinical detection of urinary T cells.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiaofei Wang, Department of Rheumatology, Shengjing Hospital of China Medical University, Sanhao Street 36, Heping District, Shenyang 110004, Liaoning Province, China. Tel: 0086-18940251629; E-mail: wangxf@sjhospital.org

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