Urinary CD4 T cells identify SLE patients with proliferative lupus nephritis and can be used to monitor treatment response

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ABSTRACT

Objectives Proliferative lupus nephritis (LN) is one of the major concerns in the treatment of systemic lupus erythematosus (SLE). Here we evaluate urinary CD4 T cells as a biomarker of active LN and indicator of treatment response.

Methods Urinary CD4 T cells were quantified 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.

Results In SLE, urinary CD4 T cell counts ≥800/100 ml were observed exclusively in patients with active LN. Receiver operator characteristic analysis documented clear separation of SLE patients with active and non-active LN (area under the curve 0.9969). All patients with up-to-date kidney biopsy results showing proliferative LN had high urinary CD4 T cell numbers. In patients monitored under therapy, normalisation 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.

Conclusions Urinary CD4 T cells are a highly sensitive and specific marker for detecting proliferative LN in patients with SLE. Furthermore, monitoring urinary CD4 T cells may help to identify treatment responders and treatment failure and enable patient-tailored therapy in the future.

INTRODUCTION

Lupus nephritis (LN) is one of the most common manifestations of systemic lupus erythematosus (SLE) and implies a significant risk for the patient.1 Despite advances in the treatment of LN, the occurrence of nephritis is still associated with a significant burden of morbidity and mortality.2 The patient is threatened from two sides: undertreatment, implicating uncontrolled autoimmunity; and overtreatment, resulting in toxicity and danger of serious infections.3

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.4–6 However, urinary findings may be misleading or unspecifically elevated, while kidney biopsy is an invasive procedure not free from risk.7

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. We hypothesise that an SLE marker specific for LN would most likely be found in urine. In addition, we expect the sensitivity of such a marker to be a function of its involvement in the pathogenesis of LN, thus a marker that reflects an indispensable key event in the local pathogenesis is likely to be sensitive for the underlying disease.

The use of flow-cytometry-based analysis of cells that are considered crucial in the pathogenesis of SLE has yielded valuable biomarkers. Quantification of plasmablasts in the peripheral blood as well as assessment of the expression of interferon-induced markers on monocytes both correlate closely with disease activity in patients with lupus.8–9 In previous work, we reported that CD4 T cells can be found in abundance in the urine of patients with active LN. The CD4 T cells in the urine and in kidney biopsy specimens were enriched with CXCR3-expressing cells compared with the blood, suggesting CXCR3-mediated recruitment of CD4 T cells into the kidney.10 Importantly, the amount of urinary CD4 T cells in patients with SLE correlated with active renal involvement, suggesting urinary T cells as a potential biomarker for LN.10 Meanwhile, these results have been confirmed by another group.11 However, a larger trial on T cells as a biomarker for LN is still lacking.

Infiltration of inflammatory cells into the kidneys is a well-established element of LN, particularly the proliferative forms.12–14 This infiltration predominantly consists of CD4 T cells,15 16 and the extent of the infiltration is one of the best predictors of renal outcome.12–14 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.10 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.

In the present study we analysed a large cross-sectional cohort of patients with SLE, with and without renal involvement, for the presence of urinary CD4 T cells. Patients with other nephropathies and healthy volunteers were analysed as a
control group. Furthermore, a cohort of patients with LN under treatment was monitored during follow-up.

**PATIENTS AND METHODS**

**Patients**

We collected and analysed 186 samples from 147 patients with SLE; 36 patients were measured more than once (19 were measured twice, three were analysed three times; the respective measurements were all separated by at least 12 month). These datasets were included in all analyses (figures 1–5). Fourteen patients with SLE were monitored at shorter intervals, and these follow-up measurements were only included in the dataset presented in figure 6. Four further patients were analysed in whom the kidney biopsy revealed a non-SLE-related nephropathy. These four patients were only included in the correlation with histology and the receiver operator characteristic (ROC) analysis (figures 3 and 4). In summary, 168 samples form 143 SLE patients were analyzed and included in figures 1–5, 14 short term follow up measurements are included in figure 6 and four further SLE patients with a non-SLE related kidney disease are additionally included in the ROC curve. For all patients, the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was calculated, a score system containing 24 items reflecting disease activity. Patients with a SLEDAI ≥10 were considered to have severely active SLE disease; patients with a SLEDAI <10 were considered to have inactive to moderately active disease. A total of 104 of these samples were taken from patients with SLE with past or present involvement of the kidneys, and 64 samples were obtained from patients with SLE without any signs or history of renal affection.

The patient group with active SLE and past or present renal involvement contained patients with active nephritis, but not exclusively (n=104). Active nephritis was defined by a current renal biopsy demonstrating LN; in the absence of a current biopsy, active LN was defined as SLEDAI ≥10 and at least two renal elements of the SLEDAI (n=29). Proliferative/inflammatory nephritis was defined as patients with active nephritis, except cases of biopsy-proven pure class I or V nephritis (n=26).

Of the 29 patients defined as having active LN, 25 had undergone a current biopsy showing LN, and four were defined by SLEDAI and renal SLEDAI (rSLEDAI). Two of these four patients had undergone a previous kidney biopsy and now presented with worsening kidney function and increasing proteinuria. One further patient had active SLE disease with newly onset proteinuria, erythrocyturia and urinary casts, but was not biopsied. One patient had increasing creatinine levels, newly onset proteinuria and erythrocyturia combined with active SLE, but without undergoing kidney biopsy.

For all patients with active disease, the rSLEDAsi was determined by adding up the renal features of the regular SLEDAI (proteinuria, erythrocyturia, leucocyturia, casts). In cases without documented sediment, the feature ‘casts’ was omitted. Kidney biopsy was judged as being current if it had been undertaken ≤4 weeks distant from the urine analysis.

Thirty-one patients with other nephropathies and 20 healthy volunteers were included as a control group. The 31 patients with other nephropathies consisted of 13 patients with diabetic nephropathy, 10 patients with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis, and eight patients with
different proteinuric syndromes (one cast nephropathy, one renal amyloidosis, one minimal change, two focal segmental glomerulosclerosis, one global glomerulosclerosis, two membranous glomerulonephritis). All patients provided personal consent to participate in our study for research purposes. Samples were collected between 2006 and 2012 from patients of the Departments of Rheumatology and Clinical Immunology, Nephrology and Intensive Care Medicine, Charité University Hospital, Berlin, Germany. Patients from our initially reported cohort were reanalysed for clinical data not previously provided and included in the present study.

The study was approved by the ethics committee of the Charité University Hospital (Charité EA1/034/10) and was conducted according to the ethics guidelines at our institution and the Declaration of Helsinki.

Sample preparation and flow cytometry
Urine samples were collected, immediately centrifuged and washed with phosphate-buffered saline/bovine serum albumin. The median sample size was 100 ml urine. The usual standing time for the urine was 4–6 h; urine samples from the previous day were discarded. Cells were stained with anti-hCD3-PE (clone UCHT1 (DRFZ, Berlin, Germany) or clone HIT3a (Biolegend, San Diego, California, USA)) and anti-hCD4-FITC (clone TT1 (DRFZ) or clone OKT4 (Biolegend)). To block unspecific binding, cells were stained in phosphate-buffered saline/bovine serum albumin containing 10% human IgG (Flebogamma; Grifols, Langen, Germany); to exclude dead cells, propidium iodide (Sigma-Aldrich, Germany) was added immediately before flow cytometry. To calculate cell numbers, whole samples were acquired and the number of cells normalised as cells per 100 ml initial urine sample. The cells were analysed using a Calibur flow cytometer (BD Pharmingen, Heidelberg, Germany). Data were analysed using Flowjo Software (Tree Star, Ashland, Oregon, USA).

Routine laboratory values
Values for creatinine, urinary sediment, proteinuria, erythrocytes/haemoglobinuria and leucocyturia were all retrieved from

Figure 2  Correlation of the number of urinary CD4 T cells with different markers of renal pathology. (A) Urinary CD4 T cells correlate with plasma creatinine (p=0.0007, Spearman r=0.2685). (B) Significant correlation between the number of CD4 T cells and proteins in the urine (p<0.0001, Spearman r=0.3883). (C) Urinary CD4 T cells and erythrocytes/haemoglobinuria correlate (p<0.0001, Spearman r=0.5397). (D) No correlation of urinary CD4 T cells with leucocyturia (p=0.4468, Spearman r=0.0629).

Figure 3  Results of concurrent kidney biopsies and number of urinary CD4 T cells in 29 patients. All patients with lupus nephritis (LN) IV and atypical inflammatory LN have greatly elevated urinary CD4 T cell counts. No elevated urinary CD4 counts are seen in pure class I or V LN.
the medical records. Creatinine was measured by the Jaffe reaction, and 24 h urinary protein excretion using a turbidimetric assay. Spot proteinuria, erythrocytes/haemoglobinuria and leucocyturia were determined using a dipstick assay (Bayer Diagnostics, Germany). Urinary sediments were analysed by trained nephrologists after centrifugation of fresh urine.

Statistical analysis
Medians, Mann–Whitney tests, Spearman correlations and the ROC curve were calculated using GraphPad Prism 3.0 (GraphPad Software, San Diego, California, USA).

RESULTS
Elevated amounts of urinary CD4 T cells are observed in patients with active LN
Urine samples from 143 patients with SLE were monitored for the presence of CD4 T cells; 25 repeated measurements separated by at least 12 month were included (n=168, patient characteristic are given in table 1). In patients with past or present renal involvement, the number of urinary CD4 T cells clearly correlated with disease activity (p<0.0001, Spearman r=0.5987, n=104), while in patients with no renal involvement, no significant correlation was observed (p=0.2114, Spearman r=0.2174, n=64; figure 1B).

In patients with known renal involvement and severely active disease (SLEDAI ≥10), a median of 2437 CD4 T cells/100 ml urine was detectable (n=32). This was significantly higher in patients with active disease and renal involvement compared with the other patients with SLE or healthy controls (p<0.0001). In patients with known previous renal involvement but only low to moderate disease activity (SLEDAI 0–15), we found only low numbers of CD4 T cells (median 19 CD4 T cells/100 ml, n=64). In healthy controls, the urine was almost devoid of CD4 T cells (median 3/100 ml, n=20), which was significantly fewer than observed in patients with SLE, regardless of disease activity or renal involvement (p=0.0003 and p=0.0074 comparing healthy controls with SLE patients with inactive LN and SLE patients without renal involvement, respectively) (figure 1C).

CD4 T cell counts of 800/100 ml or higher were exclusively found in patients with active disease and renal involvement; however, not all of these patients had elevated urinary T cell counts. To assess which patients with active disease and past or present renal infection had high urinary T cell counts, the number of urinary cells in the active patients was correlated with the rSLEDAI. The rSLEDAI correlated significantly with the number of urinary CD4 T cells (p=0.0171, Spearman r=0.4185, n=32). Patients with active disease and low urinary CD4 T cell counts (<800/100 ml) all had a relatively low rSLEDAI (0–8), while the patients with elevated T cell counts had a higher rSLEDAI (4–16) (figure 1D).

Urinary CD4 T cell counts correlate with plasma creatinine, proteinuria and erythrocytes/haemoglobinuria but not with leucocyturia
Urinary CD4 T cell numbers correlated with kidney function, assessed by plasma creatinine (p=0.0007, Spearman r=0.2685, n=157). The number of urinary CD4 T cells also correlated with proteinuria (p<0.0001, Spearman r=0.3883, n=141) and erythrocytes/haemoglobinuria (p<0.0001, Spearman r=0.5397, n=153). It is notable that high urinary CD4 T cell counts were also observed in patients without erythrocytes or haemoglobin in the urine, excluding bleeding as the main source of the T cells in the urine. No correlation was found between the presence of T cells and leucocyturia (p=0.4468, Spearman r=0.0629, n=153) (figure 2).

A urine sediment was documented in 14 patients with active SLE and renal involvement. All of these patients had urinary CD4 T cell counts >800/100 ml. Five had active sediment, five had inconclusive sediment with dysmorphic erythrocytes in insignificant numbers and no casts, and four had non-nephritic sediment. Twelve of these patients (five with active sediment, three inconclusive, four non-nephritic) had undergone a simultaneous kidney biopsy demonstrating proliferative LN.
Figure 6  Follow-up of patients under therapy. (A) Patients who normalised their urinary CD4 T cell counts <800/100 ml within 6 months showed a significant reduction in their Systemic Lupus Erythematosus Disease Activity Index (SLEDAI). In contrast, patients with persistent or increased urinary CD4 T cell numbers showed no treatment response in their SLEDAI. (B) Patients with normalised urinary CD4 cells had significantly decreased creatinine levels compared with patients with increased or persistent urinary CD4 cell count (6-month creatinine as % of initial creatinine). (C) Higher proteinuria levels in patients with increased or persistent urinary CD4 cell count, although not statistically significant (6-month proteinuria as % of initial proteinuria).

Elevated numbers of urinary T cells identify patients with proliferative LN
Twenty-nine patients with SLE who had undergone kidney biopsy were analysed in parallel. All patients with type IV LN or a combination of IV and V had high numbers of urinary T cells (n=20). Similarly, two patients with atypical inflammatory LN (one extracapillary, proliferative necrotising glomerulonephritis, one pauci-immune with interstitial lymphocytic nephritis) exhibited elevated CD4 numbers in the urine. In contrast, patients with class I or pure class V nephritis showed only low numbers of urinary T cells (n=3; figure 3).

In four patients with SLE biopsied for suspected LN, the histology revealed a non-SLE-related kidney pathology. Two patients had hypertensive nephropathy with CD4 T cell counts of 20 and 724/100 ml. One patient had focal segmental glomerulosclerosis with urinary CD4 T cell counts >1000/100 ml. The fourth patient had minimal-change nephropathy, which was thought by the treating clinicians to be potentially SLE associated, with very few urinary T cells (figure 3).

Urinary CD4 T cells are highly sensitive and specific for diagnosing inflammatory LN in patients with SLE
A ROC curve was used to calculate the sensitivity and specificity for identifying proliferative/inflammatory LN. The diagnostic performance of leucocytes in the urine and creatinine was even worse (AUC of 0.5890 and 0.76 for indicating active LN). The respective AUC for indicating active LN was 0.9209 for proteinuria and 0.8788 for erythrocytes/hemoglobinuria. When a cut-off of 500 mg/day was applied, proteinuria yielded a sensitivity of 88.9% and specificity of 83.5% for detecting active LN. The diagnostic performance of leucocytes in the urine and creatinine was even worse (AUC of 0.5890 and 0.6281 to detect active LN).

Increase in urinary CD4 T cell count is not a feature unique to LN
Given the high diagnostic precision for identifying inflammatory LN in patients with SLE in our cohort, we determined whether

Table 1  Characteristics and medication of patients

|                      | SLE, renal involvement, active disease | SLE, renal involvement, no active disease | SLE, no renal involvement | Other nephropathies | Healthy controls |
|----------------------|---------------------------------------|------------------------------------------|--------------------------|---------------------|-----------------|
| n                    | 32                                    | 64                                       | 31                       | 20                  |                 |
| Age (years)          | 32 (19–59)                            | 42 (21–74)                               | 44 (22–72)               | 57 (36–92)          | 26 (22–58)      |
| Female/male          | 29/3                                  | 59/13                                    | 59/5                     | 10/21               | 12/8            |
| Creatinine (mg/dl)   | 0.96 (0.51–2.76)                      | 0.81 (0.47–6.89)                         | 0.76 (0.56–2.1)          | 2.5 (0.49–12.48)   | Np              |
| Proteinuria (mg/24 h)| 1190 (60–20400)                       | 152 (40–4530)                            | 60 (40–320)              | 2110 (40–21000)     | Np              |
| SLEDAI               | 16 (10–25)                            | 4 (0–8)                                  | 4(0–15)                  | Na                  | Na              |
| Immunosuppressive    | 32×Pred, 19×Cyc                       | 59×Pred, 12×Cyc                          | 58×Pred, 2x Cyc          | 10×Pred, 2×Cyc      | –               |
| Treatment            | 6×MMF, 3×Aza, l×Borte, 15×HCQ         | 18×MMF, 15×Aza, 2×CyA, l×MTX, 33×HCQ    | 13×MMF, 32×Aza, 6×MTX, 35×HCQ | –                  |                 |

Values are mean (range). Data for SLE are from 143 patients, with the characteristics of the 25 patients measured on several occasions at least 12 months apart included (n=168). Aza, azathioprine; Borte, bortezomib; CyA, Ciclosporin A; Cyc, pulse cyclophosphamide; Dox, doxorubicin; HCQ, hydrochloroquine; MMF, mycophenolate mofetil; MTX, methotrexate; Na, not applicable; Np, not performed; Pred, prednisolone; Rtx, rituximab; SLE, systemic lupus erythematosus; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

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an increased urinary T cell count is specific for LN compared with other kidney pathologies. A fraction of patients with diabetic nephropathy and ANCA-associated vasculitis exhibited elevated numbers of urinary CD4 T cells. In contrast, patients with proteinuric syndromes with different aetiologies (minimal-change glomerulonephritis, cast nephropathy and renal amyloidosis) showed no increase in urinary T cell count (figure 5).

Monitoring urinary CD4 T cells indicates treatment response

Twelve patients with acute LN (nine with biopsy-proven class IV LN, one with biopsy-proven extracapillary, proliferative necrotising glomerulonephritis, two diagnosed without biopsy) were monitored under treatment. In nine of these, the urinary CD4 T cell count had normalised to below the cut-off value of 800 cells/100 ml urine within 6 months. These patients were considered ‘responders’. In three patients, the elevated urinary T cell count >800/100 ml persisted despite 6 months of treatment. Two further patients who initially presented with normal urinary T cell counts were monitored and showed an increase in urinary CD4 T cell count >800/100 ml 6 months later. The patients in whom the urinary CD4 T cell count had not normalised or had increased during the observation period were considered ‘non-responders’.

Six months after the initial analysis, the responders showed a significant reduction in disease activity (initial median SLEDAI, 16; SLEDAI 6 months later, 2; p=0.0006). The non-responders showed no reduction in disease activity (initial median SLEDAI, 12; SLEDAI 6 months later, 12). In addition, the responding group showed lower disease activity at 6 months than the non-responders (median SLEDAI 3 vs 12, p=0.0141) (figure 6A). Plasma creatinine levels were compared by calculating creatinine at the 6-month visit as a percentage of the initial creatinine concentration. The responders had lowered their creatinine levels (median 73.55% of initial, 9/9 values available), which was significantly lower than the non-responding group (median 103.1% of initial, p=0.0120, 5/5 values available) (figure 6B). Similarly, the responders had a lower median proteinuria (percentage of the initial proteinuria, median 64.48%, 6/9 values available) than the non-responders (median 104.4%, 5/5 values available); however, this did not reach statistical significance (p=0.1255; figure 6C).

DISCUSSION

In this paper, we report that urinary CD4 T cells (i) are increased in patients with SLE with active proliferative/inflammatory nephritis, (ii) show excellent sensitivity and specificity for identifying proliferative nephritis in patients with SLE, and (iii) can be used to monitor the response of LN to treatment. In our study, a urinary CD4 T cell count >800/100 ml identified patients with SLE with active inflammatory nephritis. The high sensitivity and specificity for detecting proliferative LN in patients with SLE is close to providing clinicians with the ‘black or white’ performance of an optimal biomarker.

In current clinical routine, 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 sediment is potentially able to close this diagnostic gap; however, it is only semiquantitative and observer dependent. The mainstay for diagnosing LN is renal biopsy, which is not free from risk and is not suitable for monitoring follow-up. Furthermore, interobserver agreement between renal pathologists classifying LN biopsy results has been reported to be surprisingly low, including treatment-relevant features such as grading a sample as proliferative or not.18

Several candidate urinary biomarkers have been proposed in recent years, most detected by ELISA or reverse transcription PCR.19–21 Among the most promising novel biomarkers are molecules involved in the recruitment of cells into the kidney (chemokines and adhesion molecules) and molecules reflecting renal inflammation (cytokines). Prominent examples are urinary monocyte chemotactic protein (MCP)-1, vascular cell adhesion protein-1, Chemokine (C-X-C motif) ligand (CXCL)16 and tumor necrosis factor-like weak inducer of apoptosis (TWEAK), which have all been reported in several independent publications.22–26 However, the levels of these soluble urinary biomarkers show considerable overlap between SLE patients with active LN and those without active LN. Therefore, these molecules are unable to clearly distinguish between patients with and without nephritis, limiting their clinical application.

Not surprisingly, increased CD4 T cell count in the urine is not unique to LN. Patients with other nephropathies known to be associated with inflammatory infiltration also have high urinary CD4 T cell counts. This limitation is presumably shared by all urinary biomarkers reflecting interstitial inflammation. Consequently, quantification of urinary T cells will not be useful for discriminating between LN, ANCA-related vasculitis and other inflammatory nephropathy, but may be appropriate for deciding whether or not an SLE patient has active proliferative/inflammatory renal involvement.

A cohort of patients was monitored for urinary CD4 T cells during follow-up. Under treatment, some patients normalised their urinary CD4 T cell count, while others showed persistence or even an increase. Similar observations of responders and non-responders have been reported for urinary levels of MCP-1 and urinary mRNA levels of CXCL10, CXCR3, transforming growth factor (TGF)β and Vascular Endothelial Growth Factor.23–27 Here we were able to demonstrate that patients with normalised urinary CD4 T cell count within 6 months of a nephritis diagnosis had a better outcome than patients with persistence of, or increase in, these cells. Interestingly, three of the eight responders had normalised their urinary CD4 T cell counts as early as 2–3 months after initiation of treatment, suggesting that monitoring urinary T cells may be predictive of the outcome. Moreover, it is reasonable to hypothesise that patients with persistent urinary CD4 T cells would benefit from more immunosuppression, while treatment of patients with normalised cells may be reduced more rapidly.

Flow cytometry of blood cells is routinely performed in most hospitals, so the requirements for quantification of urinary CD4 T cells are widely available. Although CD4 T cells have a limited survival in urine (82% die within 24 h, data not shown), the survival over a time span of 4–6 h was sufficient to detect the reported differences between patients with active and inactive LN. Furthermore, in our dataset neither urinary tract infections nor menstruation resulted in urinary CD4 T cell counts ≥800/100 ml (data not shown). Hence quantification of urinary CD4 T cells is an accessible technique with a robust readout, which awaits exploitation by the rheumatology and nephrology community.

Taking the results together, urinary CD4 T cells represent a promising biomarker in the assessment of proliferative LN. They should be useful for monitoring treatment response and enabling patient-tailored treatment of LN in the near future. Current limitations include the sparsity of data on non-proliferative forms of LN and the lack of extensive data on follow-up measurements. We hope that this paper will lay the basis for a large, multicentre study on urinary CD4 T cells as biomarkers for LN.
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Competing interests None.

Data sharing statement Data not shown in the manuscript include a kinetic analysis of the survival of CD4 T cells in urine and urinary T cell quantification in patients with urinary tract infections and healthy volunteers during menstruation. These datasets are available via email to the corresponding author.

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