Imbalance of Th1/Th2 transcription factors in patients with lupus nephritis

R. W.-Y. Chan, F. M.-M. Lai¹, E. K.-M. Li, L.-S. Tam, K.-M. Chow, P. K.-T. Li and C.-C. Szeto

Objective. Systemic lupus erythematosus (SLE) is characterized by the aberrant activation of T lymphocytes. Since T-bet and GATA-3 are the principal transcription factors for the differentiation of type-1 and type-2 helper T lymphocytes, respectively, we studied their mRNA expression in the urinary sediment of SLE patients and compared this with their urinary and intra-renal protein expression.

Methods. We studied 100 SLE patients and 10 healthy subjects. Urinary mRNA expression of T-bet and GATA-3 were studied by the real-time quantitative polymerase chain reaction. Intra-renal and urinary expressions of T-bet and GATA-3 were studied by immunohistochemistry and western blotting, respectively.

Results. The urinary mRNA and protein expressions of T-bet were significantly higher in SLE patients with active nephritis than those with inactive disease (mRNA: \( P<0.001 \); protein: \( P=0.004 \)). The urinary mRNA expression of T-bet correlated with the SLE disease activity index (SLEDAI) score (\( r=0.55, P<0.001 \)) and the histological activity index (\( r=0.48, P=0.03 \)). On the other hand, the urinary mRNA and protein expressions of GATA-3 were significantly lower in SLE patients with active nephritis (mRNA: \( P<0.001 \); protein: \( P=0.006 \)), and GATA-3 mRNA expression inversely correlated with the SLEDAI score (\( r=0.38, P<0.001 \)). For the 22 SLE patients with kidney biopsy, tubular expressions of T-bet and GATA-3 significantly correlated with the histological activity index (T-bet: \( r=0.57, P=0.006 \); GATA-3: \( r=-0.79, P<0.001 \)).

Conclusions. Patients with active lupus nephritis have increased T-bet and depressed GATA-3 expression in the urinary sediment and kidney tissue, indicating a predominant Th1 type of T-lymphocyte activation.

Key words: SLE, Lupus nephritis, T-bet, GATA-3.
systemic disease activity and with no history of renal disease (the quiescent group). All patients fulfilled the American College of Rheumatology diagnostic criteria for SLE [24]. Active lupus was defined as a Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score of 6 or more [25, 26]. Active renal involvement was defined as any two of the followings [27]: increase in proteinuria ≥ 1 g/day, an increase in serum creatinine of >20% and new haematuria [≥10 red blood cells/high-power field (RBC/HPF)]. Previous lupus nephritis was defined as a history of biopsy-proven proliferative lupus nephritis. For the treatment of previous lupus nephritis, complete remission was defined as 24-h urine protein excretion less than 0.5 g/day and normal serum creatinine level, while a partial response was defined as urinary protein excretion less than 0.5 g/day and normal serum creatinine below 250 mmol/l. We also recruited 10 healthy volunteers as controls (the healthy group). The design of the study was approved by the Clinical Research Ethical Committee of the Chinese University of Hong Kong. Seventy three subjects were also participants in our previous cross-sectional study on gamma interferon gene expression in urinary sediment [23].

**Study of urinary mRNA expression**

The mRNA expression of T-bet and GATA-3 in the urinary sediment was studied by reverse transcription and then real-time quantitative polymerase chain reaction (RT-QPCR). A whole-stream early morning urine specimen was collected after informed consent according to the Declaration of Helsinki. Phase contrast microscopic examination was performed on the same specimen. The degree of erythrocyturia and leukocyturia were assessed semiquantitatively as number of cells per high-power field, as described previously [28]. For patients in the Active Group who required kidney biopsy, urine was collected on the morning of biopsy. The methods of mRNA extraction from urinary sediment have been described by Li et al. [29]. Briefly, the urine sample was centrifuged at 3000 g for 30 min at 4°C. Total RNA was extracted from the sediment by the RNasy Mini Kit (Qiagen Inc., Canada), following the manufacturer’s instructions. All specimens were pre-treated with deoxyribonuclease I (Invitrogen Life Technologies, USA) and then stored at −70°C. The integrity and purity of RNA was confirmed by the 18S to 28S rRNA ratio and the relative absorbance at the 260 to 280 nm ratio using the spectrometer.

For each RT-PCR reaction, approximately 0.5 μg of RNA was reverse transcribed to complementary DNA (cDNA) with the Superscript II RNase H Reverse Transcriptase (Invitrogen). The RT-PCR was performed by the ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). The primer and probe sequences of human T-bet and GATA-3 were designed and synthesized by Applied Biosystems and are summarized in Table 1. As described in our previous study, the level of mRNA expression of each target was normalized to a housekeeping gene (18S rRNA). RT-QPCR amplifications were performed in 20 μl volume at 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was run in triplicate.

The results of RT-QPCR were analysed by Sequence Detection Software version 1.9 (Applied Biosystems), with the difference-in-threshold-cycle (ΔCt) procedure according to the manufacturer’s instructions. Briefly, the relative standard curve method was applied in the quantification of the mRNA expression of T-bet and GATA-3. A relative standard curve of each transcription factor to 18S RNA was prepared in each PCR run for comparing the relative expression across batches of reactions. Complementary DNA derived from phorbl 12-myristate 13-acetate (PMA)-stimulated human leucocyte was used for the generation of relative standard curves. For each sample, the relative T-bet and GATA-3 expression was calculated using linear regression analysis of the respective standard curves, and their values divided by the corresponding 18S rRNA value. The expression levels of T-bet and GATA-3 were expressed as dimensionless numbers relative to the corresponding average expression level of the healthy group.

**Western blotting of transcription factors**

The protein expression of T-bet and GATA-3 from 40 urinary sediment specimens, which included the last 10 consecutive cases recruited from each group, was studied by western blotting analysis. Briefly, 25 μg of total protein lysate from urinary cells was separated by SDS-PAGE and transferred to nitrocellulose membrane. T-bet and GATA-3 protein were detected after incubation with polyclonal goat anti-human T-bet (1:500) and monoclonal mouse anti-human GATA-3 (1:500) antibodies (Santa Cruz Biotechnology, CA, USA), respectively, followed by horseradish peroxidase (HRP)-conjugated donkey anti-goat-anti-γ (1:3000) (DakoCytomation, Denmark) or rabbit anti-mouse (1:3500) (Amersham Biosciences, NJ, USA) immunoglobulins as appropriate. Labelling signal on the membrane was detected by ECL Plus Western Blotting Detection Reagents (Amersham). For image analysis, the film was scanned with Imaging Densitometer GS-700 (BioRad, USA) and analysed. The level of protein expression of each target was normalized to β-actin.

**Immunohistochemistry of renal biopsy**

The intralobular renal level of T-bet and GATA-3 was determined by immunohistochemistry on the paraffin-embedded tissues (2 μm thick) from kidney biopsy of 22 patients in the active group with the conventional method. Briefly, the tissues were blocked with 0.3% H2O2 and then 3% normal serum after deparaffinization and antigen retrieval. Sections were then incubated with the anti-T-bet (1:400) and anti-GATA-3 (1:500) antibodies, respectively, and followed by the subsequent incubation in biotinylated rabbit anti-goat (1:100) and rabbit anti-mouse (1:400) immunoglobulins (DakoCytomation) as appropriate. After incubation with streptavidin (1:100) (DakoCytomation), sections were developed in diaminobenzidine and counterstained with haematoxylin. T-bet and GATA-3 staining was graded semiquantitatively from 0 to 3+.

**Assessment of clinical activity**

On the day of urine collection, the SLE disease activity was assessed clinically by an independent physician with the SLEDAI score [25]. For specific assessment of the renal activity of the SLE, the renal score of the SLEDAI, which consisted of proteinuria, urinary casts, haematuria and pyuria from the original SLEDAI

<table>
<thead>
<tr>
<th>T-bet</th>
<th>GATA-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>5'-GAT GTT TGT GGA</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>CTG GTT CTT G-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-CTC TCC ACA CTG</td>
</tr>
</tbody>
</table>

| 6FAM, 6-carboxyfluorescein; TAMRA, N,N,N',N'-tetramethyl-6-carboxyrhodamine. |

[Table 1. Primer and probe sequences for the real-time quantitative polymerase chain reaction (RT-QPCR)]
score, was computed and analysed separately. The overall SLEDAI score ranged from 0 to 32, the renal score ranged from 0 to 16.

**Assessment of histological activity**

Kidney biopsy was performed in 22 patients of the active group. The specimens were evaluated according to the WHO pathological classification of lupus nephritis [30]. For each specimen, the histological activity index and chronicity index were scored by standard methods [30, 31]. Briefly, the activity index was the sum of the semiquantitative scores (0 to 3 each) of the following parameters: endocapillary hypercellularity, leucocyte infiltration, subendothelial hyaline deposits, interstitial inflammation, necrosis, and cellular crescents, with the scores of the last two parameters counted double, making a total activity index of 0 to 24 [30, 31]. All biopsy specimens were evaluated by a single pathologist (FML), who was blinded to the results of the SLEDAI score, blood and urinary tests.

**Statistical analysis**

Statistical analysis was performed by Statistical Package for Social Sciences version 10.0 software (SPSS Inc., Chicago, IL, USA). Because the data were highly skewed, the mRNA expression levels were compared between groups by Kruskal–Wallis test or Mann–Whitney U-test as appropriate. Correlations of mRNA expression with the SLEDAI scores, biochemical parameters and histological activity and chronicity indexes were determined by Spearman’s rank correlation coefficient. A P value of less than 0.05 was considered as significant. All probabilities were two-tailed. For the immunohistochemical grading, reproducibility between different observers was evaluated by the use of kappa statistics.

**Results**

We studied 110 subjects in total. Their baseline demographic and clinical data are summarized in Table 2. In the active group, 22 patients had kidney biopsy. Their subsequent renal diagnoses were focal proliferative nephritis (13 cases), diffuse proliferative nephritis (three cases), pure membranous nephropathy (five cases) and advanced glomerulosclerosis (one case). The mean histological activity and chronicity indexes of the 22 patients were 6.1 ± 4.9 and 3.6 ± 2.7, respectively. In the remission group, all 30 patients had a history of proliferative lupus nephritis: 14 were in complete remission and 16 in partial remission after treatment.

**Urinary T-bet and GATA-3 mRNA expression**

The urinary expression of T-bet was significantly elevated while the expression of GATA-3 was significantly reduced in the active group (P < 0.001 for both comparisons) (Fig. 1). There was a significant inverse correlation between the urinary expressions of T-bet and GATA-3 (r = −0.33, P < 0.001). Urinary expression of T-bet correlated with that of IFN-γ (r = 0.80, P < 0.001) and IL-2 (r = 0.28, P = 0.054), although the latter did

**Table 2. Baseline demographic and clinical data of the study patients**

<table>
<thead>
<tr>
<th>Group</th>
<th>Active</th>
<th>Remission</th>
<th>Quiescent</th>
<th>Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>40</td>
<td>30</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>2:38</td>
<td>2:28</td>
<td>1:29</td>
<td>3:7</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>38.0 ± 10.8</td>
<td>38.2 ± 10.1</td>
<td>41.6 ± 9.1</td>
<td>40.7 ± 4.0</td>
</tr>
<tr>
<td>Duration of disease (yr)</td>
<td>9.2 ± 6.3</td>
<td>11.0 ± 9.2</td>
<td>9.3 ± 5.4</td>
<td>–</td>
</tr>
<tr>
<td>Serum creatinine (μmol/l)</td>
<td>132.3 ± 119.7</td>
<td>166.3 ± 149.1</td>
<td>70.8 ± 29.8</td>
<td>–</td>
</tr>
<tr>
<td>Proteinuria (g/day)</td>
<td>4.8 ± 5.1</td>
<td>1.2 ± 1.8</td>
<td>0.6 ± 0.4</td>
<td>–</td>
</tr>
<tr>
<td>Previous immunosuppressives</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Prednisolone (g) (no. of cases)</td>
<td>11.0 ± 12.2</td>
<td>18.9 ± 12.2</td>
<td>0.9 ± 5.2</td>
<td>–</td>
</tr>
<tr>
<td>Cytoxic therapy</td>
<td>24</td>
<td>27</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>SLEDAI a</td>
<td>Total score</td>
<td>13.1 ± 4.7</td>
<td>1.7 ± 1.7</td>
<td>0.9 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>Renal score</td>
<td>8.3 ± 3.9</td>
<td>0.8 ± 1.7</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Current immunosuppressives</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Prednisolone (mg/day) (no. of cases)</td>
<td>5.8 ± 7.6</td>
<td>4.6 ± 2.3</td>
<td>0.3 ± 1.4</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>9</td>
<td>14</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± s.d.

aCumulative dose of prednisolone since diagnosis.

bSLEDAI, systemic lupus erythematosus disease activity index.
glomerulonephritis (either focal or diffused) had significantly
and leucocyturia (P = 0.29, P = 0.03) and inversely with IFN-γ (r = −0.45, P < 0.001).

Urinary T-bet expression closely correlated with the overall
SLEDAI score (r = 0.55, P < 0.001) (Fig. 2) as well as the renal
score of SLEDAI (r = 0.62, P < 0.001). In contrast, the expression
of GATA-3 inversely correlated with the overall SLEDAI score
(r = −0.38, P < 0.001) and the renal score of SLEDAI (r = −0.37,
P < 0.001). Urinary T-bet expression directly correlated with
the degree of erythrocyturia (r = 0.55, P < 0.001) and leucocyturia
(r = 0.47, P < 0.001), while urinary GATA-3 expression inversely
 correlated with the degree of erythrocyturia (r = −0.42, P < 0.001)
and leucocyturia (r = −0.37, P < 0.001). In addition, the degree
of proteinuria strongly correlated with the urinary expressions
of T-bet (r = 0.51, P < 0.001) but not GATA-3 (r = −0.25,
P = 0.03). The urinary RNA expression of T-bet and GATA-3
did not correlate with patient age or serum creatinine level.

In the 22 cases with kidney biopsy, patients with proliferative
glomerulonephritis (either focal or diffused) had significantly
higher urinary expression of T-bet than those with pure
membranous nephritis (Mann–Whitney U-test, P < 0.001), while
there was no difference in their urinary expression of GATA-3
(Mann–Whitney U-test, P = 0.7) (Fig. 3). The histological activity
index strongly correlated with the urinary expression of T-bet
(r = 0.48, P = 0.03) but not GATA-3 (r = −0.09, P = 0.7) (Fig. 4).
Urinary T-bet expression significantly correlated with the degree
of endocapillary hypercellularity (r = 0.42, P = 0.017), necrotizing
lesions (r = 0.40, P = 0.025) and cellular crescent (r = 0.36,
P = 0.044). Neither the urinary mRNA expression of T-bet nor
GATA-3 correlated with the histological chronicity index (details
not shown).

Western blotting of urinary T-bet and GATA-3

The urinary protein level of T-bet was elevated (P = 0.004) while
GATA-3 was significantly reduced (P = 0.006) in the active group
(Fig. 5). Urinary T-bet level by western blotting did not correlate
with the overall SLEDAI score (r = 0.29, P = 0.088), but it
significantly correlate with the renal score in SLEDAI (r = 0.40,
P = 0.016). The urinary GATA-3 level did not correlate with the
overall and renal score in SLEDAI. Since only six patients in this
part of the experiment underwent kidney biopsy, the relationship
with histological findings was not analysed.

Intra-renal T-bet and GATA-3 expression

T-bet and GATA-3 were detected on the passenger lymphocytes
within glomeruli, and tubular epithelial cells per se (Fig. 6). The
immunohistochemical staining of T-bet in tubules significantly
correlated with the corresponding mRNA expression level in
urinary sediment (r = 0.57, P = 0.006). The immunohistochemical
score of tubular GATA-3 also significantly correlated with the
corresponding urinary mRNA expression level (r = −0.50,
P = 0.019).

Both the tubular T-bet and the GATA-3 level strongly
 correlated with the histological activity index (r = 0.57 and
r = −0.79, respectively, P < 0.001 for both), but not with the
histological chronicity index. The tubular T-bet level significantly
correlated with the degree of endocapillary hypercellularity
(r = 0.47, P = 0.022), necrotizing lesions (r = 0.62, P = 0.002) and
cellular crescent (r = 0.65, P = 0.001). Tubular GATA-3 level also
 inversely correlated with the degree of endocapillary hypercellu-
larity (r = −0.55, P = 0.006), necrotizing lesions (r = −0.60,
P = 0.003), cellular crescent (r = −0.56, P = 0.005) and leucocyte

![Fig. 2](https://academic.oup.com/rheumatology/article-abstract/45/8/951/1784684/fig2)

Fig. 2. Correlation between Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score and the T-bet mRNA expression in the urinary sediment of the SLE patients (Spearman’s r = 0.67, P < 0.001).

![Fig. 3](https://academic.oup.com/rheumatology/article-abstract/45/8/951/1784684/fig3)

Fig. 3 Comparison of urinary expressions of (A) T-bet and (B) GATA-3 between patients with proliferative lupus nephritis and pure membranous nephritis. The boxes indicate median, 25th and 75th percentiles; whisker caps indicate 5th and 95th percentile; closed circles indicate outliers. Data are compared by Mann–Whitney U-test.
infiltration ($r = -0.47$, $P = 0.024$). Neither the intra-renal T-bet nor the GATA-3 level correlated with the overall SLEDAI score.

Discussion

Growing evidence suggests that the selection of Th1 and Th2 pathways by the immunological system is mastered by the upstream transcription factors rather than cytokine production downstream [3–6]. Inconsistent findings by various groups in studies with serological measurement [18–21] highlight the pathological relevance of measuring cytokines at the site of injury. Although Peng et al. [22] demonstrated that T-bet is not relevant to B-cell pathogenesis in the murine lupus model, our previous study found a substantial elevation of gamma interferon (a prototype Th1 cytokine) mRNA expression in the urinary sediment of patients with active lupus [23]. In the present study, both RNA and protein measurements demonstrate a marked up-regulation of T-bet and a corresponding depression of GATA-3 in the urinary sediment of patients with active lupus [23]. In the present study, both RNA and protein measurements demonstrate a marked up-regulation of T-bet and a corresponding depression of GATA-3 in the urinary sediment of patients with active lupus. Furthermore, the degree of T-bet expression in urinary sediment strongly correlated with the SLEDAI score, the degree of proteinuria and the histological activity index of kidney biopsy. In summary, our data demonstrate a shift towards the Th1 pathway of the immunological system in active lupus. The presence of a dose-responsive type of relation between the degree of Th1 pathway up-regulation and the severity of kidney involvement, which demonstrated in the urinary and intrarenal study, further supports the biological relevance of our observation.

In the present study, we did not recruit patients with clinically active SLE but no renal involvement. Since SLE is a complex multiorgan disease and the immunopathogenesis of individual organ involvement is likely to be different [32], we deliberately focused the present study on the renal involvement of SLE. We also did not recruit patients with inactive SLE with untreated nephritis, which is a rare occurrence in clinical practice.

It should be noted that renal biopsy is only performed in patients with clinically active renal involvement. Patients with no clinical renal involvement, nevertheless, could indeed have occult nephritis. Assessment of renal function by measuring the serum creatinine level and the degree of proteinuria alone cannot be used as a marker of renal involvement, as significant pathological changes in lupus can be seen in patients with minimal clinical changes. In the present study, we found that some SLE patients without clinical disease activity had a high urinary T-bet and low GATA-3 expression, indicating a skew towards the Th1 pathway of T-helper cell activation. It remains unknown whether these patients will be prone to lupus flare in the future. Since our local practice is to arrange renal biopsy early whenever there are features of renal involvement, a large proportion of our cases had class III rather than the florid class IV nephritis.

In the present study, we performed concomitant urine microscopic examination in all cases, and the major cellular components of the urinary sediments were red blood cells, epithelial cells and mononuclear cells (lymphocytes and macrophages). We did not perform immunohistochemistry to distinguish tubular epithelial cells from urothelial cells. Given that T lymphocytes are the common cell type that expresses T-bet and GATA-3 [33, 34], the infiltrating T lymphocytes rather than macrophages are probably, albeit not yet confirmed to be, the major source of the T-bet and GATA-3 mRNA being detected in urine. Theoretically, confocal microscopy with dual staining of urinary sediment for both lymphocyte markers (for example,
and T-bet would be needed to prove that T-bet expression is purely from lymphocytes. In addition, it should be noted that the immunohistochemistry of kidney biopsy tissue demonstrate that T-bet and GATA-3 are also expressed by renal tubular cells. Nevertheless, we found that the degree of intrarenal T-bet and GATA-3 expression correlated with the kidney severity by means of the histological activity index. This observation supports the pathophysiological relevance of T-bet and GATA-3 in lupus nephritis. Since immunohistochemistry is a semiquantitative method, further experiment with laser microdissection of kidney biopsy tissue [35, 36] may be necessary to investigate the relative contribution of glomerular and tubulointerstitial inflammation in detail.

In the present study, expression of the T-bet and GATA-3 genes was measured by their level relative to a housekeeping gene. As a result, we cannot define a cut-off level of expression for clinical use. Nevertheless, our results provide the proof-of-principle evidence that measurement of gene expression in urinary sediment by RT-QPCR has potential clinical and research applications in SLE and possibly other renal diseases. Further studies are needed to standardize the batch-to-batch comparison before the test is applicable for routine clinical practice.

In conclusion, the urinary sediment of SLE patients with active disease has increased mRNA and protein expressions of T-bet, and a reciprocal reduction in GATA-3, indicating a shift towards the Th1 pathway of T-lymphocyte activation. The measurement of T-helper cell transcription factor gene expression in the urinary sediment may provide valuable information for the assessment and risk stratification of SLE patients.

**Key messages**

- Patients with active lupus nephritis have increased T-bet and depressed GATA-3 expression.
- Urinary T-bet expression may be a surrogate marker of SLE renal activity.
Acknowledgements

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The authors have declared no conflicts of interest.

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