The gene expression of type 17 T-helper cell-related cytokines in the urinary sediment of patients with systemic lupus erythematosus

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Objective. We studied the role of type 17 Th cells (TH17) in the pathogenesis of SLE.

Methods. We quantified the mRNA expression of IL-17, -23, -27 and retinoic-acid-related orphan receptor (ROR)-γ, the regulator for the development and function of TH17, in the urinary sediment of 23 subjects with active lupus nephritis, 25 subjects with a history of lupus nephritis in remission, 30 SLE patients with no history of renal involvement and 8 healthy subjects.

Results. All three groups of lupus patients had a higher urinary expression of TH17-related cytokines than the controls. However, urinary expression of IL-17 and -27 was found to be inversely correlated with the SLEDAI score (r = -0.252 and -0.258, respectively; P < 0.05 for both). For patients with active lupus nephritis, the histological activity index of kidney biopsy was also found to be inversely correlated with the urinary expression of ROR-γ (r = -0.447; P = 0.032), IL-17 (r = -0.454; P = 0.029) and IL-23 (r = -0.455; P = 0.029). Urinary expression of IL-17, -23, -27 and ROR was also found to be inversely correlated with the urinary expression of IFN-γ and T-bet, the key transcription factor of type 1 Th cells. After 6 months of treatment, urinary IL-27 expression rose significantly in patients with complete response (from 2.07 ± 1.62 to 3.70 ± 1.69; P = 0.028) but remained unchanged in those with partial or no response (from 2.60 ± 1.87 to 2.52 ± 1.94; P = 0.9).

Conclusions. The urinary expression of TH17-related genes is increased in SLE patients. The degree of up-regulation, however, is inversely related to systemic and renal lupus activity, as well as urinary expression of TH1-related genes. Urinary expression of TH17-related genes increased again after successful immunosuppressive treatment of active disease. Our findings suggest a regulatory role of TH17-related cytokines in pathogenesis of lupus nephritis.

Key words: SLE, Lupus nephritis, Cytokine.

Introduction

SLE is a severe multi-system autoimmune disease with life-threatening complications. The intricate involvement of inflammatory mediators including cytokines seems fundamental in the aberrant immune system of SLE [1–2]. Traditionally, imbalance in the cytokines produced by the two subsets of Th cells, TH1 and -2, is believed to play an important role in the pathogenesis of SLE [3]. We have previously shown that patients with active lupus nephritis have increased expression of T-bet, the TH1 transcription factor, and depressed GATA-3, the TH2 transcription factor, in the urinary sediment and kidney tissue [4], and a high urinary T-bet expression was an independent predictor of lupus flare [5], suggesting an important role of TH1 type of T-lymphocyte activation.

However, reports on the TH1/TH2 imbalance in SLE have been inconsistent [6–9]. Many immunological phenomena, particularly in the area of T-cell-mediated tissue damage, could not be explained by TH1 and -2. For example, SLE is often considered to be a TH2-mediated disease at the early stage [7], but the TH1 commitment only replaces the TH2 pathway and takeover the progression of SLE to active nephritis [10]. A major paradigm shift in understanding how CD4+ T cells contribute to autoimmunity recently occurred with the discovery of a new T-cell population that produces the cytokine IL-17, termed as ‘TH17’. A major role for the TH17-related cytokines, such as IL-17, has now been described in various models of immune-mediated tissue injury, including organ-specific autoimmunity in the brain, heart, synovium and intestines, allergic disorders of the lung and skin and microbial infections of the intestines and the nervous system [11]. Although TH17 cells contribute to autoimmune disease in RA and Crohn’s disease, their role in SLE is far less clear. It is, however, known that the TH1 pathway antagonizes the TH17 pathway in an intricate fashion [11–13]. However, the role of TH17-related cytokines in SLE remains elusive. In this study, we examined the gene expression of TH17-related cytokines in the urinary sediment of SLE patients, and to study the relation between TH17-related cytokine expression and lupus disease activity.

Patients and methods

Patient selection

We recruited three groups of SLE patients, 23 consecutive patients with active lupus and renal involvement from April 2006 to March 2007 (the active group), 25 patients with history of biopsy-proved Class III or IV lupus nephritis whose disease became quiescent for at least 6 months after treatment (the Quiescent Group) and another 30 randomly selected patients who attended the rheumatology clinic without systemic disease activity and with no history of renal disease (the Non-renal Group). All patients fulfilled the ACR diagnostic criteria of SLE [14]. Active lupus was defined as an SLEDAI score of ≥6 [15, 16]. Active renal involvement was defined as any two of the following [17]: increase in proteinuria (≥1 g/day), an increase in serum creatinine (>20%) and new haematuria (≥10 red blood cell/high power field). We also recruited eight healthy volunteers as control (the control group).

The study was approved by the Clinical Research Ethical

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Committee of the Chinese University of Hong Kong, written consent was obtained according to the Declaration of Helsinki.

**Study of urinary mRNA expression**

A whole-stream early morning urine specimen was collected after informed consent. Phase contrast microscopic examination was done in the same specimen. The degree of erythrocyturia and leucocyturia was assessed semi-quantitatively as the number of cells per high power field, as described previously [18]. For patients in the Active Group who required kidney biopsy, urine was collected in the morning of biopsy. The methods of mRNA extraction from urinary sediment have been described by Li *et al.* [19]. Briefly, the urine sample was centrifuged at 3000 g for 30 min at 4°C. Total RNA was extracted from the sediment by the RNeasy Mini Kit (Qiagen, Ontario, Canada). All specimens were pre-treated with deoxyribonuclease I (Invitrogen and Life Technologies, Carlsbad, USA) and then stored at −80°C. The integrity and purity of RNA was confirmed by the 18S : 28S rRNA ratio and the relative absorbance at 260 : 280 nm ratio using the spectrometer.

The mRNA expression of cytokines related to the TH17 pathway, namely IL-17, -23 and -27, and retinoic-acid-related...
orphan receptor (ROR)-γ in the urinary sediment was studied by reverse transcription and real-time quantitative PCR (RT–QPCR). For each RT–QPCR reaction, 0.5 μg of RNA were reverse transcribed with the Superscript II RNase H− Reverse Transcriptase (Invitrogen™, Life Technologies). The RT–QPCR was done by the ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). The primer and probe sequences of all targets in this study were designed and synthesized by Applied Biosystems. RT–QPCR amplifications were done in a 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 were analysed by the Sequence Detection Software version 1.9 (Applied Biosystems), with the difference-in-threshold cycle (∆Ct) procedure according to the manufacturer’s instruction. Gene expression data are depicted as the log of the number of copies of mRNA per million copies of housekeeping gene (18S rRNA) mRNA.

Assessment of clinical and histological activity

On the day of urine collection, the disease activity of SLE was assessed clinically by an independent physician with the SLEDAI score [15]. For specific assessment of renal activity of the SLE, renal score of the SLEDAI, which consisted of proteinuria, urinary casts, haematuria and pyuria of the original SLEDAI score, was computed and analysed separately. In the 23 patients of the Active Group, the kidney biopsy specimens were evaluated according to the International Society of Nephrology pathologic classification of lupus nephritis [20]. For each specimen, the histological activity index and chronicity index were scored by standard methods [20, 21]. All biopsy specimens were evaluated by a single pathologist (F.M.-M.L.), who was blinded from the results of the SLEDAI score, blood and urinary tests.

Clinical follow-up

Patients of the Active Group were followed for 6 months. The clinical management was decided by individual nephrologist and not affected by the study. In general, the Active Group was treated with corticosteroid, together with cyclophosphamide or mycophenolate according to published protocols [22]. After 6 months of treatment, another whole-stream early morning urine specimen was collected for gene expression study. Therapeutic response was assessed at the same time and classified into complete remission, partial remission and no response [22]. Briefly, complete response was defined as a value for urinary protein excretion that was <0.3 g/g creatinine, with normal urinary sediment, a normal serum albumin concentration and values for both serum creatinine and estimated GFR that were ≤15%
above the baseline values. Partial response was defined as a value for urinary protein excretion that was between 0.3 and 2.9 g/g creatinine, with a serum albumin concentration of at least 30 g/dl and stable renal function. No response was defined as a value for urinary protein excretion that remained at a value of 5.3 g/g creatinine or a value of 0.3–2.9 g/g creatinine, but with a serum albumin value of <30 g/dl, an increase in the serum creatinine concentration ≥50 μmol/l, a value for estimated GFR that was >15% above the baseline value or the discontinuation of treatment due to side effects.

**Statistical analysis**

Statistical analysis was performed by Statistical Package for Social Sciences version 10.0 software (SPSS, Chicago, IL, USA). The mRNA expression levels were compared between groups by Kruskal–Wallis test or Mann–Whitney U-test as appropriate. Change in gene expression before and after treatment was compared by the Wilcoxon rank sum test. 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 <0.05 was considered as statistically significant. All probabilities were two tailed.

**Results**

The demographic and baseline clinical data of the study subjects are summarized in Table 1. Renal biopsy of the Active Group showed pure membranous disease (Class V, 7 cases), pure proliferative glomerulonephritis (Class III or IV, 5 cases) and mixed proliferative and membranous disease (11 cases). Histological activity and chronicity indices of the Active Group were 6.7 ± 4.6 and 1.3 ± 0.9, respectively.

**TH1-related gene expression between groups**

The comparison of urinary mRNA expression of TH17-related genes between the Active, Quiescent, Non-renal and Control Groups is shown in Fig. 1. There was a significant difference in urinary mRNA expression of ROR-γ, IL-17, -23 and -27 between the groups (P < 0.001 by Kruskal–Wallis test for all comparisons). In general, all three groups of lupus patients had a higher urinary expression of TH17-related cytokines than the Control Group (Fig. 1). Post hoc analysis, however, showed that the Active Group had significantly lower urinary IL-17, -23 and -27 mRNA than the Quiescent and Non-renal Groups (P < 0.05 for all comparisons), whereas the urinary expression of these cytokines was similar between the Quiescent and Non-renal Groups. Urinary ROR-γ expression was not significantly different.
among Active, Quiescent and Non-renal Groups by post hoc analysis.

Relation with clinical and histological features

The correlation between urinary mRNA expression of TH17-related genes and baseline clinical and histological parameters are summarized in Figs 2–4. In short, urinary expression of IL-17 and -27 significantly correlated with the SLEDAI score ($r = -0.252$ and $-0.258$, respectively; $P < 0.05$ for both), whereas the correlation between SLEDAI score and urinary expression of ROR-$\gamma$ and IL-23 just fell short of statistical significance (Fig. 2). Urinary expression of IL-23, but not other TH17-related genes, significantly correlated with the degree of proteinuria ($r = -0.294$; $P = 0.004$) (Fig. 3). None of the urinary gene expressions correlated with the estimated GFR (details not shown).

For the Active Group, the histological Activity Index of the kidney biopsy correlated with the urinary mRNA expression of ROR-$\gamma$ ($r = -0.447$; $P = 0.032$), IL-17 ($r = -0.454$; $P = 0.029$), IL-23 ($r = -0.455$; $P = 0.029$) and IL-27 ($r = -0.389$; $P = 0.066$), although the last correlation did not reach statistical significance (Fig. 4). None of the urinary gene expression correlated with the histological Chronicity Index, degree of glomerulo-sclerosis or tubulo-interstitial fibrosis (details not shown).

Relation with TH1 and -2 pathways

We further explore the relation between the correlation between urinary mRNA expression of TH17-related genes and that of other targets related to the TH1 and -2 pathways. Urinary expression of T-bet, the key transcription factor of the TH1 pathway, inversely correlated with the urinary expression of ROR-$\gamma$ ($r = -0.402$; $P < 0.0001$), IL-17 ($r = -0.676$; $P < 0.0001$), IL-23 ($r = -0.426$; $P = 0.0001$) and IL-27 ($r = -0.704$; $P < 0.0001$). Similarly, urinary expression of IFN-$\gamma$ inversely correlated with the expression of ROR-$\gamma$ ($r = -0.453$; $P < 0.0001$), IL-17 ($r = -0.676$; $P < 0.0001$), IL-23 ($r = -0.484$; $P < 0.0001$) and IL-27 ($r = -0.716$; $P < 0.0001$). On the other hand, urinary expression of GATA-3, the key transcription factor of the TH2 pathway, did not correlate with the urinary expression of ROR-$\gamma$ ($r = 0.191$; $P = 0.1$), but urinary GATA-3 expression does significantly correlated with the expression of IL-17 ($r = 0.620$; $P < 0.0001$), IL-23 ($r = 0.464$; $P < 0.0001$) and IL-27 ($r = 0.435$; $P < 0.0001$).

Relation with treatment response

After 6 months of treatment, 12 patients of the Active Group had complete response, 9 had partial response and 2 had no response. In patients with complete response, urinary IL-27 expression rose significantly at the same time (from $2.07 \pm 1.62$ to $3.70 \pm 1.69$;
P = 0.028) (Fig. 5). By contrast, urinary IL-27 expression remained unchanged in patients with partial or no response (from 2.60 ± 1.87 to 2.52 ± 1.94; P = 0.9). There was a trend of increase in urinary IL-17 expression after 6 months of treatment in patients with complete response (from 1.31 ± 1.81 to 2.37 ± 1.51; P = 0.09) as well as those with partial or no response (from 0.88 ± 0.89 to 1.45 ± 1.61; P = 0.16), although the results did not reach statistical significance (Fig. 5). In the Active Group, there was no significant change in urinary expression of ROR-γ and IL-23 after 6 months, irrespective to the treatment response. Baseline urinary expression of the TH17-related genes did not predict treatment response (details not shown).

Discussion

In the present study, we found an increase in the expression of TH17-related genes in urinary sediment in SLE patients. However, the degree of up-regulation is reduced with active disease. Our findings seem to contradict with the result of other published work. In a study of peripheral blood mononuclear cells (PBMC), Dong et al. [23] showed that IL-17 induces IgG, anti-dsDNA overproduction and IL-6 overexpression of PBMC in patients with lupus nephritis, and probably plays an important role in its pathogenesis. In another series of experiments on peripheral blood, Wong et al. [24] found that plasma IL-17 and -23 concentrations, as well as the number of Th17 cells, were significantly elevated in SLE patients than control subjects, and plasma IL-17 concentration correlated positively and significantly with SLEDAI score and plasma level of other cytokines related to the TH1 pathway. Neither of these studies, however, examines the immunological network within the kidney. More recently, Crispin et al. [25] reported that CD4/CD8 double-negative T cells from patients with SLE produce significant amounts of IL-17 and IFN-γ, and expand when stimulated in vitro with an anti-CD3 antibody in the presence of accessory cells. In their experiment, IL-17-positive double-negative T cells are found in kidney biopsies of patients with lupus nephritis [25], suggesting that IL-17 contributes to the pathogenesis of kidney damage in patients with SLE. It is, however, important to realize that in most of the biological systems [26], IL-17 largely comes from CD4-positive Th cells rather than CD4/CD8 double-negative ones. Our result should be viewed as complementary, rather than contradicting, to the findings of Crispin et al. [25]. To the best of our knowledge, CD4-positive TH17 cells in the kidney have not been studied in detail; further study on the pathobiology of TH17 cells in lupus nephritis is necessary.

We found that urinary mRNA expression of TH17-related genes is up-regulated in SLE patients, but the degree of up-regulation is inversely proportional to the disease activity. This pattern is distinctly different from our previous studies on the urinary mRNA expression of TH1- and TH2-related genes [4, 5], which showed an up-regulation of TH1-related genes and down-regulation of TH2-related genes in SLE patients, with a magnitude proportional to the disease activity. The cause of this paradoxical pattern of change in TH17-related gene expression remains elusive. It is possible that TH17 pathway may be a marker of the body's regulatory response to control intra-renal inflammation in SLE; with a loss of TH17 activity, the disease
flares. For example, recent studies showed that regulatory T cells favour IL-17 production and prevent the disease [27], and regulatory T cells may produce IL-17 and related cytokines [28, 29]. On the other hand, it is also possible that there is retention of TH17 cells within the kidney in patients with active lupus nephritis, resulting in reduction in urinary expression in the presence of high disease activity. Further studies are needed to clarify the role of IL-17 and related cytokines in lupus nephritis.

There are a number of inadequacies of our present study. First, we have not determined the cellular origin of the mRNA in the urine. Our previous study showed that CD3+ (i.e. T cells) and CD20+ cells (i.e. B cells) were the major components of urinary mononuclear cells in SLE patients [30], and IL-17 and other related cytokines are not produced by non-lymphoid cells. Therefore, it seems most likely that most of the mRNA tested in our present study comes from T cells. Nonetheless, since we have not performed flow cytometry or examination of a similar kind, we could not ascertain whether the IL-17 producing cells were traditional CD4-positive TH17 cells or CD4/CD8 double-negative T cells, as suggested by Crispin et al. [25].

Secondly, the sample size of our study was small. The result of some sub-group analyses (e.g. the change in cytokine gene expression in relation to treatment response) should be interpreted with caution. For logistic reasons, we did not include a group of SLE patients with active disease but no renal involvement, or patients with other types of glomerulonephritis. As a result, we could not conclude beyond doubt that the observed change in urinary gene expression is specific to lupus nephritis. Further, we have no data on the longitudinal change in urinary expression of TH17-related genes in SLE patients with quiescent disease, and it would be interesting to see if serial monitoring of urinary gene expression would predict disease flare.

In summary, we found an increase in expression of TH17-related genes in urinary sediment in SLE patients as compared with healthy controls. The degree of up-regulation, however, is inversely related to both systemic and renal lupus activity, as well as urinary expression of TH1-related genes. Urinary expression TH17-related genes increase again following successful immunosuppressive treatment of active disease. Our findings suggest a regulatory role of TH17-related cytokines in pathogenesis of lupus nephritis.

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### References

6. Wong CK, Ho CY, Li EK, Lam CW. Elevation of proinflammatory cytokine (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus. Lupus 2009;18:965–70.