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PAPER

The potential role of Th17 cells and Th17-related cytokines in the pathogenesis of lupus nephritis

D-Y Chen[1,2,3], Y-M Chen[1], M-C Wen[4], T-Y Hsieh[1,2], W-T Hung[1] and J-L Lan[1,2,3]
[1]Division of Allergy, Immunology and Rheumatology, Taichung Veterans General Hospital and Faculty of Medicine, National Yang Ming University, Taiwan; [2]Institute of Microbiology and Immunology, Chung Shan Medical University, Taiwan; [3]Institute of Biomedical Science, National Chung Hsing University, Taiwan; and [4]Department of Pathology, Taichung Veterans General Hospital, Taiwan

T helper type 17 (Th17) cells, a novel distinct subset of Th cell, can secrete interleukin (IL)-17 in humans. Although recent data suggest that Th17 cells and IL-17 play an important role in the pathogenesis of lupus nephritis (LN), the expression of Th17-related cytokines in the kidneys of SLE patients has not been studied in detail. In the present study, we investigated circulating Th17-cell frequencies using flow cytometry and serum Th17-related cytokine levels by enzyme-linked immunosorbent assay (ELISA) in 24 LN patients (17 patients with class IV and seven patients with class V) and 12 healthy controls. We also investigated glomerular Th17-related cytokine expression in LN patients and minimal change nephropathy (MCN) patients using immunohistochemistry. Our results showed significantly higher median frequencies of circulating Th17 cells in LN patients (0.68%) than in healthy controls (0.12%, $p < 0.001$). Serum levels of IL-17, IL-6 and IL-23 were significantly higher in LN patients (median 7.26, 232.60 and 37.01 pg/ml, respectively) than in healthy controls (median 0.82, 34.60 and 7.42 pg/ml, respectively; all $p < 0.001$). Circulating Th17-cell frequencies were positively correlated with SLEDAI, renal SLEDAI and histological activity index, the degree of cellular crescent and endocapillary proliferation. Significantly higher levels of glomerular IL-17 and IL-23 expression were observed in renal biopsies from class IV LN patients as compared to those from MCN patients and normal controls. Glomerular IL-17 and IL-23 expression levels were positively correlated with renal SLEDAI and histological activity index for LN patients. Our results suggest the potential role of the IL-23/Th17 axis in the intra-renal inflammation of SLE. *Lupus* (2012) **21,** 1385–1396.

Key words: Th17 cell; Th17-related cytokines; pathogenesis; lupus nephritis; systemic lupus erythematosus

Introduction

Lupus nephritis (LN), one of the most severe forms of organ involvement in systemic lupus erythematosus (SLE), may progress to end-stage renal failure unless properly treated.[1,2] Early detection and adequate therapy are associated with better renal outcomes in LN.[3,4] Examination of morphology and inflammation in renal biopsy specimens provides important information about classification of LN, the severity of renal injury, and pathological activity and chronicity.[5,6] It is well known that the pathological classification of LN is closely associated with therapeutic response and long-term renal outcome.[7,8]

Although the etiopathogenesis remains unclear, previous studies have focused on the cytokine profile in the peripheral blood of SLE patients.[9–11] T helper type 17 (Th17) cells, a novel distinct subset of Th cell, can secrete interleukin (IL)-17 in humans.[12–14] IL-17 is a pleiotropic cytokine that participates in tissue inflammation by inducing the expression of proinflammatory cytokines, chemokines and matrix metalloproteases.[15–17] Accumulating evidence indicates that Th17 cells and IL-17 play an important role in the pathogenesis of SLE.[18–20] Elevated IL-17 levels have been detected in sera, and enhanced expression of IL-17 has been observed in target tissues of SLE patients.[18,20–22] In addition, levels of IL-23 were elevated in the plasma and

Correspondence to: JL Lan, Division of Allergy, Immunology and Rheumatology, Taichung Veterans General Hospital, No. 160, Section 3, Taichung-Kang Road, Taichung City, 40705, Taiwan.
Email: jllan@vghtc.gov.tw
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peripheral blood mononuclear cells (PBMCs) of SLE patients,[18,23] and ex vivo induction of IL-17A by IL-23-costimulated leukocytes from LN patients was significantly higher than those from controls.[18] Recent studies show that IL-6 induces the differentiation of Th17 cells from naïve T cells,[24] and isolated PBMCs from LN patients were shown to produce higher IL-6 levels than in controls.[25] Th17 cells in SLE patients may be also closely influenced by IL-18 activation,[18] and IL-18 was shown to play a role in the induction of Th17 cells.[26] These observations suggested the involvement of Th17 cells and Th17-related cytokines in SLE.

In LN patients, renal biopsy specimens are the ideal materials for investigating intra-renal immunological disturbance. A study of SNF1 mice showed an increase of IL-17A production from splenocytes ex vivo and infiltration of IL-17A-associated T cells in the kidneys of these mice.[27] Crispin et al. found elevated numbers of IL-17-producing double negative (CD4-/CD8-) T cells infiltrating the kidneys in LN patients.[28] Laser microdissection-based cytokine analyses showed elevated expression levels of renal IL-17, which were closely correlated with clinical parameters in LN patients.[29] These data suggested that IL-17 may play an important role in LN. However, the expression of Th17-related cytokines in the kidneys of SLE patients has not been studied in detail.

In the present study, we investigated the frequencies of circulating Th17 cells using flow cytometry and levels of serum Th17-related cytokines by ELISA in 24 patients with LN and 12 healthy controls. We also examined the expression levels of Th17-related cytokines in renal biopsy tissues from LN patients using the immunohistochemistry method, and explored its relationship with histological morphology, including class of LN defined by the International Society of Nephrology/Renal Pathology Society (ISN/RPS), pathological activity indices and chronicity of this disease.

Patients and methods

Patients

Twenty-four consecutive patients (19 females and five males; mean age at study entry ± SD, 36.0 ± 11.3 years) who fulfilled the 1997 revised criteria of the American College of Rheumatology for SLE[30] were enrolled. Renal biopsy-proven LN specimens were evaluated according to the classification criteria defined by the ISN/RPS.[6] After diagnosis of LN, all patients were treated with corticosteroids (CSs), and hydroxychloroquine with or without other immunosuppressive agents including azathioprine, cyclophosphamide, mycophenolate mofetil or cyclosporine. On the day of renal biopsy, disease activity was assessed by an independent physician using the SLE Disease Activity Index (SLEDAI).[31] Renal activity of SLE was assessed by renal SLEDAI score, which consisted of proteinuria, urinary casts, hematuria and pyuria of the original SLEDAI score. Twelve healthy volunteers (nine females and three males; mean age ± SD, 35.5 ± 13.9 years) who had no rheumatic or renal disease served as controls. This study was approved by the Ethics Committee of Clinical Research, Taichung Veterans General Hospital, and informed consent was obtained from each participant.

Quantitation of circulating Th17cells using flow cytometry analysis

For detection of circulating Th17 cells, phycoerythrin (PE)-conjugated anti-IL-17 (eBioscience, San Diego, USA) and phycoerythrin-cyanin 5 (PC5)-conjugated anti-CD4 (Beckman Coulter, Marseilles, France) were quantified using flow-cytometric analysis according to the manufacturer's protocol and the described technique.[32,33] Briefly, aliquots of 1000 µl of the sterile heparinized whole blood were stimulated with a combination of 25 ng/ml of phorbol myristate acetate (PMA) and 1 µg/ml of ionomycin (Sigma, Deisenhofen, Germany) and cultured for one hour at 37°C in a humidified 5% CO_2 incubator. Whole blood was then treated with 10 µg/ml of Brefeldin A (BFA) (Sigma, Germany) to inhibit intracellular protein transport. Activated cultures of blood samples were washed in wash buffer (phosphate buffered saline, 5% fetal bovine serum, 0.1% sodium azide; Merck, Darmstadt, Germany) and then stained with 20 µl of PC5-conjugated CD4-specific monoclonal antibody (mAb) (Beckman Coulter, Marseilles, France) for 15 minutes at room temperature (RT). Erythrocytes were lysed by adding 2 ml of fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson, Lincoln Park, NJ, USA). After 5 minutes' incubation, samples were centrifuged and washed with 0.1% bovine serum albumin-phosphate buffer saline (BSA-PBS) and subsequently fixed with 100 µl Reagent 1 (Beckman Coulter, USA) for 10 minutes. After washing, the pellet was incubated with 100 µl Reagent 2, saponin (Beckman Coulter, Marseilles, France) for 5 minutes at RT in the dark. The samples were washed twice with 0.1% BSA-PBS, and incubated with PE-conjugated

IL-17-specific mAb (eBiosciences, San Diego, CA, USA) for 30 minutes at RT in the dark. An isotype control IgG1-PE (eBiosciences, USA) was used for the IL-17 staining at RT in the dark. After staining, the cells were washed and immediately analyzed using flow cytometry (Beckman Coulter, USA). Lymphocytes were gated on the basis of forward- and side-scatter properties, and at least 10,000 $CD4^+$ cells were analyzed. Data were obtained using an Epics flow cytometer, and the results were analyzed using Expo32 software (Beckman Coulter, Miami, FL, USA).

Determination of serum levels of Th17-related cytokines

Serum levels of IL-6, IL-17, IL-18 and IL-23 were determined in LN patients and healthy controls using ELISA according to the manufacturer's instructions (eBiosciences, San Diego, CA, USA).

Renal histopathology of LN patients

A minimum of five glomeruli per biopsy specimen was required. The renal histopathological assessment included light microscopic examination (stained with hematoxylin and eosin, Masson's trichrome, periodic acid-Schiff, and silver methenamine), immunofluorescent evaluation and electron microscopic analysis. The pathological indices of activity and chronicity of each biopsy specimen were determined by standard methods.[34] The activity index (AI) is the sum of manual scores (0–3 each) of the following six parameters: endocapillary hypercellularity, leukocyte infiltration, hyaline deposits/wire loop, interstitial inflammation, necrosis/karyorrhexis and cellular crescents. The last two parameters are then weighted by a factor of two, making a total AI of 0–24. The chronicity index (CI) is the sum of manual scores (0–3 each) of the following four parameters: glomerular sclerosis, fibrous crescents, tubular atrophy and interstitial fibrosis. The total of CI is 0–12. To avoid inter-observer variability in reading and scoring, an independent pathologist (M-C Wen) evaluated biopsy specimens.

Glomerular expression levels of Th17-related cytokines in LN patients

Immunostaining for Th17-related cytokines (IL-6, IL-17, IL-18 and IL-23) of renal biopsies was performed according to the method described by Calvani et al.[35] with minor modifications in 24 patients with LN. Non-LN kidney tissues from four patients with minimal change nephropathy (MCN), a condition in which the immune reaction was minimal, were included as disease control. Normal kidney tissues from four patients who had received nephrectomy for tumor removal were included as normal controls. Paraffin-embedded samples were cut into 4-μm sections and then were deparaffinized for 2 hours at 56°C, followed by a series of descending alcohol concentrations. After washing with PBS, antigen retrieval was performed by boiling the slides in citrate buffer for 10 min at 121°C in a microwave oven. Peroxidase blocking with 0.3% H_2O_2 for 10 min and protein blocking with 2% horse serum for 20 min at room temperature were performed. After washing with PBS three times, sections were overlaid overnight with a goat mAb against Th17-related cytokines at 2 μg/ml including IL-6 (Abcam, Cambridge, UK), IL-17 (Santa Cruz, CA, USA), IL-18 (MBL, MA, USA) and IL-23 (Abcam, Cambridge, UK). After washing with PBS, binding of the secondary polymer-horseradish peroxidase (HRP) anti-goat IgG (Nichirei Biosciences, Tokyo, Japan) was detected by an immunodetection kit (Lab Vision Corporation, CA, USA) according to the manufacturer's instructions. Negative controls were performed without primary antibody. Finally, the specimens were counterstained with Mayer's hematoxylin, and all slides were dehydrated and mounted. Positive staining for Th17-related cytokines within the glomeruli in each biopsy specimen was expressed as mean value/glomerular cross section. The reader was unaware of the results of circulating Th17 frequencies and levels of serum Th17-related cytokines.

Statistical analysis

Results are presented as the mean ± SD or median (interquartile range). The nonparametric Mann–Whitney *U* test was used for between-group comparison of the levels of circulating Th17 cells and Th17-related cytokines in sera and glomeruli. The correlation coefficient was obtained by the nonparametric Spearman's rank correlation test. A probability of less than 0.05 was considered significant.

Results

Clinical characteristics of SLE patients

As illustrated in Table 1, all LN patients had active disease (mean SLEDAI ± SD, 16.4 ± 3.9, range 10–24; mean renal score of SLEDAI ± SD,

Table 1 Demographic data, clinical characteristics and laboratory findings of patients with lupus nephritis (LN)#

	LN				*HC*
	Class IV-G (n = 8)	*Class IV-S (n = 6)*	*Class IV + V (n = 3)*	*Class V (n = 7)*	*n = 12*
Age at study entry, years	39.0 ± 12.6	41.5 ± 10.4	31.0 ± 3.0	30.1 ± 10.7	35.5 ± 13.9
Proportion of females	6 (75.0%)	6 (100%)	2 (66.7%)	5 (71.4%)	9 (75.0%)
Malar rash	8 (100%)	5(83.3%)	1 (33.3%)	5 (71.4%)	NA
Arthritis	5 (62.5%)	4 (66.7%)	1 (33.3%)	4 (57.1%)	NA
Serositis	3 (37.5%)	3 (50.0%)	0 (0.0%)	2 (28.6%)	NA
CNS involvement	2 (25.0%)	1 (16.7%)	0 (0.0%)	1 (14.3%)	NA
Histological activity index	5.63 ± 4.31*	3.67 ± 2.42	1.67 ± 0.58	0.29 ± 0.49	NA
Histological chronicity index	3.00 ± 2.51	3.00 ± 4.38	6.33 ± 2.31	0.57 ± 1.13	NA
Serum C3 levels (mg/dl)	53.0 ± 30.4	70.1 ± 25.7	56.0 ± 29.3	94.2 ± 30.9	NA
Serum C4 levels (mg/dl)	11.7 ± 8.0	15.0 ± 7.9	13.6 ± 10.9	19.6 ± 6.9	NA
Anti-dsDNA levels (U/ml)	232.7 ± 158.2	161.2 ± 178.7	228.4 ± 31.7	161.2 ± 178.7	NA
DUP (gm/24 hr)	3.22 ± 1.93	2.32 ± 0.81	2.25 ± 1.70	3.54 ± 3.23	NA
Ccr (ml/24 hr)	57.0 ± 33.0	63.7 ± 34.1	22.7 ± 30.1*	97.0 ± 29.6	NA
SLEDAI score	19.3 ± 2.5	15.5 ± 5.1	15.3 ± 5.0	14.4 ± 2.2	NA
Renal SLEDAI score	13.5 ± 2.1*	10.7 ± 4.8	9.3 ± 4.6	6.9 ± 3.0	NA
Daily dose of oral corticosteroids (mg/day)	25.6 ± 7.8	18.3 ± 10.3	18.3 ± 10.4	13.6 ± 5.4	NA
Hydroxychloroquine	8 (100%)	6 (100%)	2 (66.7%)	7 (100%)	NA
Azathioprine	6 (75.0%)	4 (66.7%)	2 (66.7%)	5 (71.4%)	NA
Mycophenolate mofetil	6 (75.0%)	4 (66.7%)	1 (33.3%)	3 (42.9%)	NA
Cyclophosphamide	5 (62.5%)	3 (50.0%)	0 (0.0%)	1 (14.3%)	NA
Cyclosporine	2 (25.0%)	1 (16.7%)	0 (0.0%)	3 (42.9%)	NA

#Values are mean ± standard deviation or the number (%) of patients. HC: healthy control; Class IV-G: diffuse global LN; Class IV-S: diffuse segmental LN; CNS: central nervous system; C3: complement 3; C4: complement 4; Anti-dsDNA: anti-double stranded DNA antibody; DUP: daily urinary protein; Ccr: creatinine clearance rate; SLEDAI: systemic lupus erythematosus (SLE) disease activity index; NA: not applicable. *$p < 0.05$, versus class V LN.

10.3 ± 4.2, range 4–16) at the time of study. According to ISN/RPS classification,[6] class IV LN could be divided into two groups: diffuse global (IV-G) LN when >50% of the involved glomeruli have global lesions, and diffuse segmental (IV-S) LN when >50% of the involved glomeruli have segmental lesions. Our results show significantly higher renal scores of SLEDAI and renal histological AI in class IV-G LN patients than those in class V LN patients ($p < 0.05$). Significantly lower glomerular filtration rates were observed in patients with overlapping class IV and class V LN than in those with class V LN. However, there were no significant differences in age at study entry, female proportion, disease duration, or frequencies of extra-renal manifestations between subsets of class IV LN patients and class V LN patients.

Frequencies of circulating Th17 cells in LN patients

Representative examples of flow cytometric dot-plots of intracellular IL-17 production in Th cells obtained from PB of one patient with class IV LN, from a class V LN patient, and from a healthy control (HC) are shown in Figure 1A. Significantly higher median frequencies of circulating Th17 cells were observed in LN patients (median 0.68%, interquartile range (IQR) 0.39%–1.10%) than in healthy controls (median 0.12%, IQR 0.05%–0.18%; $p < 0.001$, Figure 1B). Among LN patients, significantly higher median frequencies of circulating Th17 cells were observed in class IV-G LN patients (median 1.38%, IQR 0.99%–1.99%) compared with class IV-S LN patients (median 0.58%, IQR 0.43%–0.79%, $p < 0.05$), patients with overlapping class IV and class V LN (median 0.37%, IQR 0.20%–0.68%, $p < 0.05$), or class V LN patients (median 0.49%, IQR 0.34%–0.80%, $p < 0.05$, Figure 1C). However, there was no significant difference in circulating Th17 cell frequencies among class IV-S LN patients, patients with overlapping class IV and class V LN, and class V LN patients.

Serum levels of Th17-related cytokines in LN patients

As shown in Figure 2, serum levels of IL-17, IL-6 and IL-23 were significantly higher in LN patients (median 7.26, 232.60 and 37.01 pg/ml, respectively) than in healthy controls (median 0.82, 34.60 and 7.42 pg/ml, respectively; all $p < 0.001$). In addition,

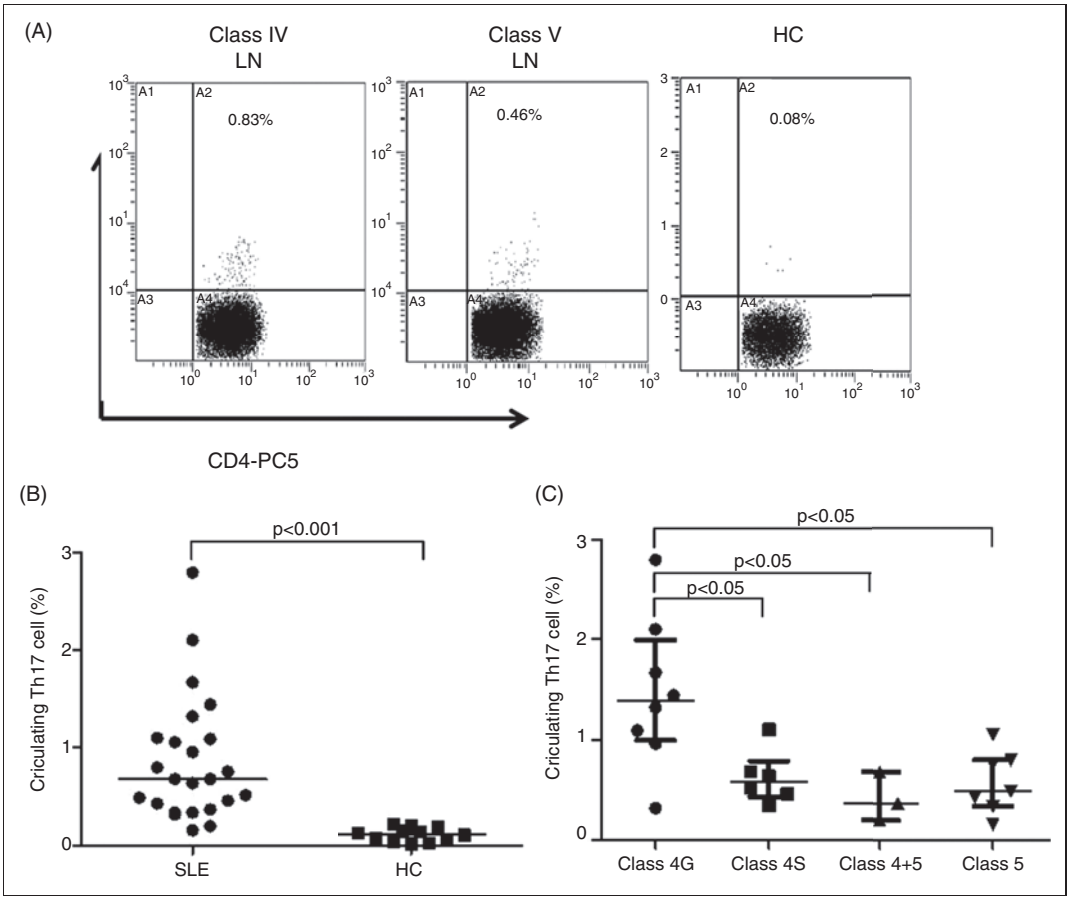

Figure 1 (A) Flow cytometric dot-plots of intracellular IL-17 production in Th cells obtained from peripheral blood of one represented patient with class IV lupus nephritis (LN), one patient with class V LN and one healthy control (HC). The comparisons of circulating Th17 cells frequencies are shown in (B) between two groups consisting of 24 patients with LN and 12 HC, and in (C) among four groups including eight patients with class IV-G LN, six patients with class IV-S LN, three patients with overlapping class IV and class V LN, and seven patients with class V LN. The middle horizontal line indicates median value, and the horizontal lines below and above the middle horizontal line represent the 25th percentile and the 75th percentile values, respectively, for each group. The *p*-value was assessed by Mann–Whitney test. IL: interleukin.

there was no significant difference in serum IL-18 levels between LN patients and healthy controls. However, there was no significant difference in serum levels of Th17-related cytokines between class IV LN patients and class V LN patients. Among LN patients, there was no significant difference in serum levels of Th17-related cytokines among class IV-G LN patients, class IV-S LN patients, patients with overlapping class IV and class V LN, and class V LN patients. Although previous studies revealed that IL-6 is eliminated by tubular excretion in kidney and its serum level may be affected by renal dysfunction,[36] we found no change in the results for between-group comparison of serum IL-6 levels (pg/mg creatinine) after correction by renal function (data not shown).

Expression levels of glomerular Th17-related cytokines in LN patients

The immunohistochemical study (Figure 3) showed representative examples of glomerular staining with IL-17, IL-6, IL-18 and IL-23 obtained from renal biopsy specimens of one patient with class IV LN (A1, B1, C1 and D1, respectively), a class V LN patient (A2, B2, C2 and D2, respectively), a patient with MCN (A3, B3, C3 and D3, respectively), and a normal control (A4, B4, C4 and D4, respectively). As shown in Figure 4, there were significantly higher levels of glomerular IL-17, IL-18 and IL-23 expression on renal biopsies from class IV LN patients (median levels, 9.16, 13.38 and 9.43, respectively) compared with those from MCN patients (median levels, 2.34, $p < 0.005$ for IL-17; 2.49, $p < 0.001$ for IL-18; and 2.58, $p < 0.001$

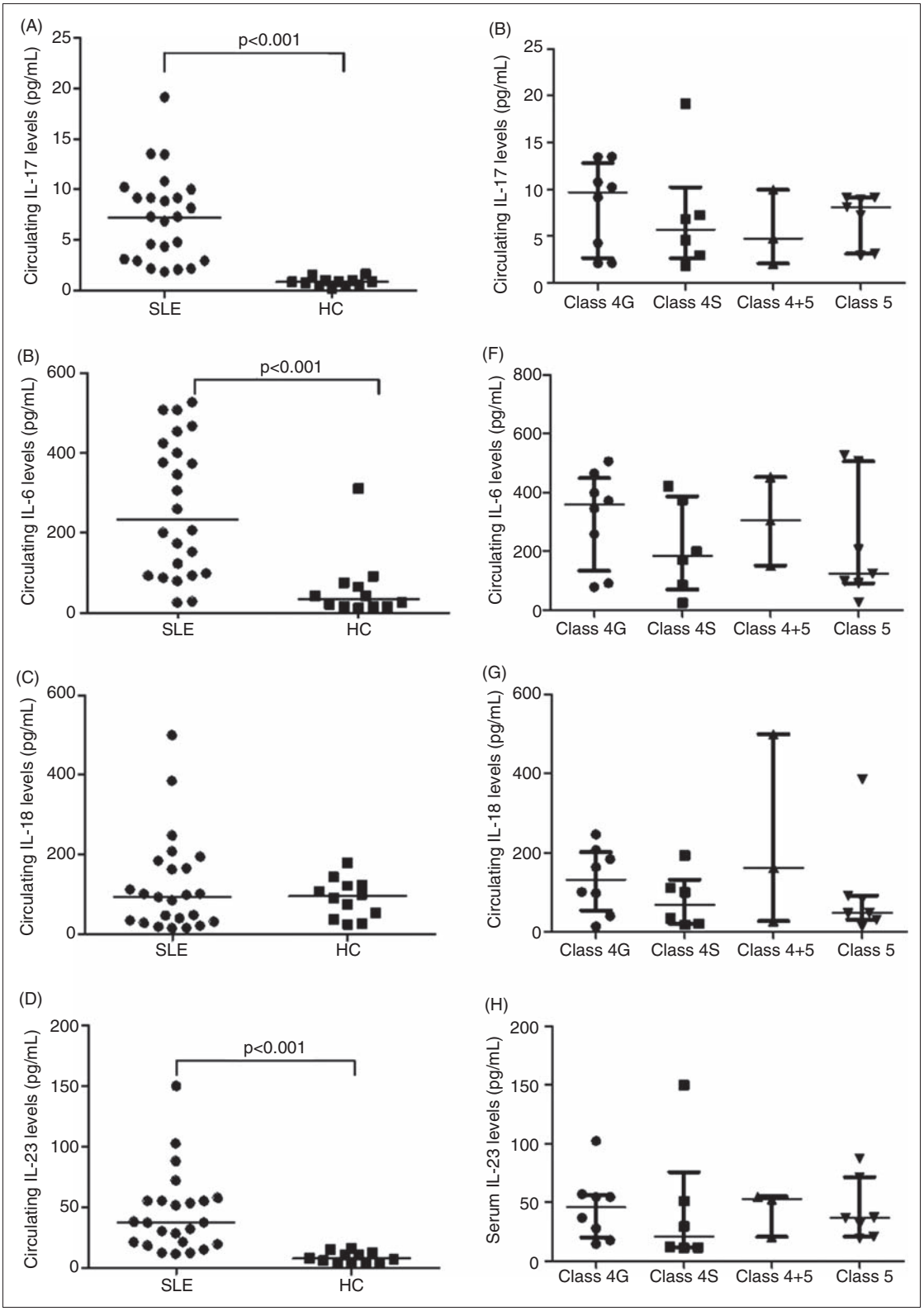

Figure 2 Levels of serum IL-17 (A), IL-6 (B), IL-18 (C) and IL-23 (D) were obtained from 24 patients with lupus nephritis (LN) and 12 healthy controls (HC). The comparison of serum levels of IL-17 (E), IL-6 (F), IL-18 (G) and IL-23 (H) are shown in eight patients with class IV-G LN, six patients with class IV-S LN, three patients with overlapping class IV and class V LN, and seven patients with class V LN. The middle horizontal line indicates median value, and the horizontal lines below and above the middle horizontal line represent the 25th percentile and the 75th percentile values respectively for each group. The *p*-value was determined by Mann–Whitney *U* test. IL: interleukin.

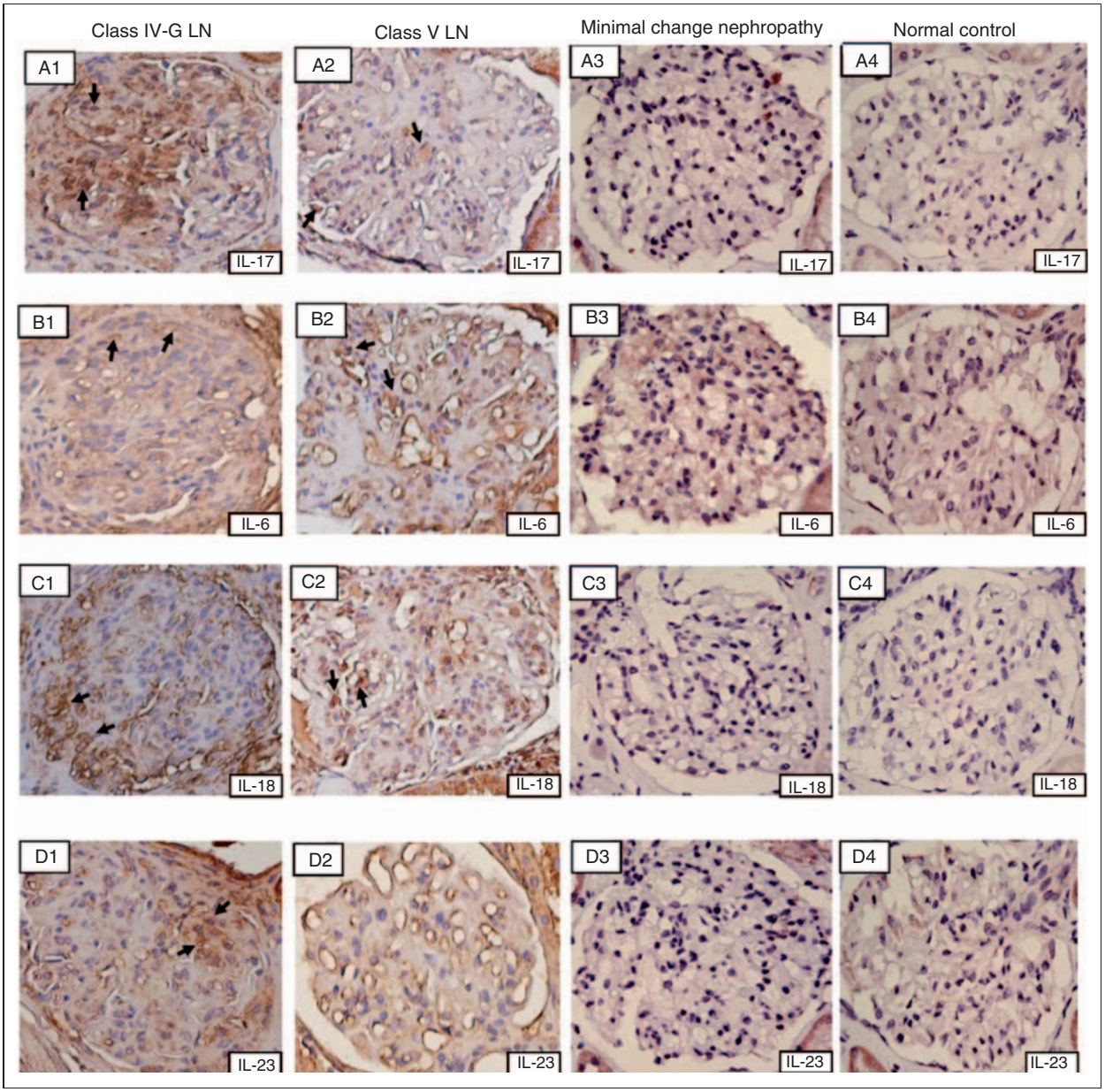

Figure 3 Representative examples of glomerular immunostaining with IL-17, IL-6, IL-18 and IL-23 (arrows, original magnification ×200) obtained from renal biopsy specimens of one patient with diffuse global (class IV-G) LN (A1, B1, C1 and D1, respectively), one patient with class V LN (A2, B2, C2 and D2, respectively), one patient with minimal change nephropathy (A3, B3, C3 and D3, respectively), and one control with normal renal tissue (A4, B4, C4 and D4, respectively). IL: interleukin.

for IL-23) or normal controls (median levels, 1.85, $p < 0.005$ for IL-17; 1.69, $p < 0.001$ for IL-18; and 1.89, $p < 0.001$ for IL-23). There were significantly higher levels of glomerular IL-6, IL-18 and IL-23 expression on renal biopsies from class V LN patients (median levels, 6.36, 12.82 and 6.33, respectively) compared with those from normal controls (median levels, 2.14, $p < 0.05$ for IL-6; 1.69, $p < 0.01$ for IL-18; and 1.89, $p < 0.01$ for IL-23). Among LN patients, class IV-G LN patients were shown to have significantly higher median levels of glomerular IL-17 expression (14.42) than patients with class IV-S LN (7.56, $p < 0.01$), patients with overlapping class IV and class V LN (4.16, $p < 0.005$), and class V LN patients (4.00, $p < 0.005$). Similarly, we observed significantly higher median levels of glomerular IL-23 expression in class IV-G LN patients (11.08) than in patients with overlapping class IV and class V LN (5.13, $p < 0.05$), and class V LN patients (6.33, $p < 0.05$).

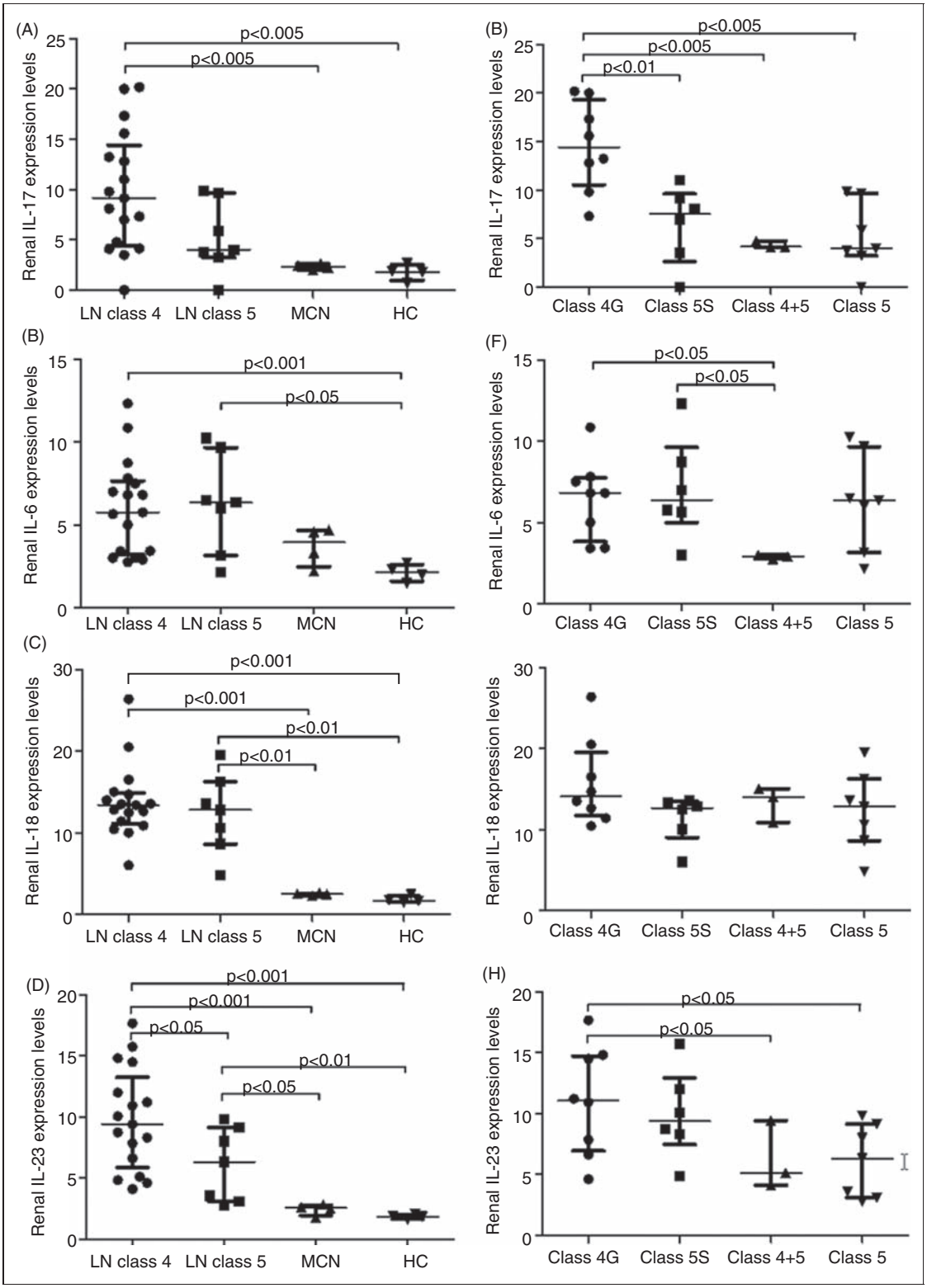

Figure 4 Median expression levels of glomerular IL-17 (A), IL-6 (B), IL-18 (C) and IL-23 (D) were shown in 17 patients with class IV LN, seven patients with class V LN, four patients with minimal change nephropathy (MCN) and four normal controls. The comparison of median expression levels of glomerular IL-17 (E), IL-6 (F), IL-18 (G) and IL-23 (H) is shown in eight patients with class IV-G LN, six patients with class IV-S LN, three patients with overlapping class IV and class V LN, and seven patients with class V LN. The middle horizontal line indicates median value, and the horizontal lines below and above the middle horizontal line represent the 25th percentile and the 75th percentile values, respectively, for each group. The *p*-value was determined by Mann–Whitney *U* test. IL: interleukin.

Table 2 The correlation between the frequencies of circulating Th17 cells as well as levels of Th17-related cytokines and clinical parameters, levels of Th17-related cytokines in sera and glomerular areas, and renal histological parameters in patients with lupus nephritis

	Th17	*IL-17*	*IL-6*	*IL-18*	*IL-23*	*IL-17S*	*IL-6S*	*IL-18S*	*IL-23S*
SLEDAI	0.471*	0.383	0.231	0.341	0.251	0.607**	−0.073	0.280	0.371
RSLEDAI	0.479*	0.242	0.087	0.281	0.028	0.606**	0.011	0.210	0.442*
C3	−0.197	−0.108	0.155	−0.071	0.112	−0.114	−0.156	−0.441*	−0.376
Anti-DNA	−0.164	0.218	−0.082	0.464*	−0.084	0.237	0.137	0.427*	0.215
DUP	0.206	−0.134	−0.296	−0.253	−0.060	0.222	0.167	0.250	0.458*
Ccr	−0.178	0.029	−0.021	0.156	−0.037	0.090	0.159	−0.160	0.120
IL-17	0.351	–	0.164	0.353	0.585**	0.105	0.074	−0.230	0.081
IL-6	0.233	0.164	–	0.339	0.586**	−0.015	−0.64***	−0.268	−0.257
IL-18	−0.043	0.353	0.339	–	0.378	0.372	−0.047	−0.026	0.184
IL-23	0.201	0.585**	0.586**	0.378	–	−0.089	−0.358	−0.237	−0.056
IL-17S	0.537**	0.105	−0.015	0.372	−0.089	–	0.175	0.177	0.333
IL-6S	0.028	0.074	−0.638**	−0.047	−0.358	0.175	–	0.162	0.455*
IL-18S	−0.022	−0.028	−0.268	−0.026	−0.239	0.177	0.162	–	0.253
IL-23S	0.088	0.081	−0.257	0.184	−0.056	0.333	0.455*	0.253	–
AI	0.447*	0.355	−0.010	0.227	−0.097	0.514*	0.192	0.226	0.567**
CCres	0.426*	0.518**	0.376	0.219	0.439*	0.313	−0.195	−0.249	0.210
Necro	0.258	0.560**	0.278	0.438*	0.347	0.435*	−0.160	0.029	0.183
EndoP	0.427*	0.193	−0.134	0.016	−0.229	0.374	0.312	0.198	0.495*
WireL	0.182	0.266	−0.220	0.126	−0.288	0.299	0.301	0.189	0.423*
NeutI	0.228	0.137	−0.209	0.355	−0.118	0.300	0.328	0.391	0.300
InterI	0.209	0.162	−0.062	0.075	−0.107	0.137	0.167	0.256	0.538**
CI	−0.010	−0.265	0.131	−0.138	0.007	−0.173	−0.289	0.153	−0.130
GloS	−0.060	−0.127	0.129	−0.061	0.016	−0.156	−0.263	0.152	−0.102
TubuA	0.013	−0.240	0.133	−0.270	−0.041	−0.221	−0.227	0.137	−0.122
InterF	0.023	−0.399	0.072	−0.284	−0.069	−0.209	−0.244	0.101	−0.150
FCres	0.018	0.292	0.373	0.209	0.264	−0.055	−0.391	−0.046	−0.282

SLEDAI: SLE disease activity index; RSLEDAI: renal score for SLEDAI; IL: interleukin; IL-17S: glomerular stained IL-17; IL-6S: glomerular stained IL-6; IL-18S: glomerular stained IL-18; IL-23S: glomerular stained IL-23; C3: complement 3; anti-DNA: anti-double stranded DNA antibody; DUP: daily urinary protein; Ccr: clearance for creatinine; AI: histological activity index; CCres: cellular crescent; Necro: necrotizing change; EndoP: endocapillary proliferation; WireL: wire loop; NeutI: neutrophil infiltration; InterI: interstitial inflammation; CI: chronicity index; GloS: glomerulosclerosis; TubuA: tubular atrophy; InterF: interstitial fibrosis; FCres: fibrous crescent. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ was obtained by the nonparametric Spearman's rank correlation test.

However, there was no significant difference in the levels of glomerular Th17-related cytokines expression on renal biopsies between MCN patients and normal controls.

Correlations of levels of circulating Th17 cells and glomerular Th17-related cytokine expression with clinical parameters in LN patients

As illustrated in Table 2, the frequencies of circulating Th17 cells were positively correlated with SLEDAI score and renal SLEDAI score ($r = 0.471$ and $r = 0.479$, $p < 0.05$) in LN patients. The levels of glomerular IL-17 expression were also positively correlated with SLEDAI score and renal SLEDAI score ($r = 0.607$ and $r = 0.606$, both $p < 0.01$). The expression levels of glomerular IL-23 were positively correlated with renal SLEDAI score and urinary protein levels ($r = 0.442$ and $r = 0.458$, both $p < 0.05$). The expression levels of glomerular IL-18 were positively correlated with serum levels of anti-dsDNA and negatively correlated with serum levels of complement ($r = 0.427$ and $r = -0.441$, both $p < 0.05$). Levels of serum IL-18 were also positively correlated with serum levels of anti-dsDNA ($r = 0.464$, $p < 0.05$).

Correlations of levels of circulating Th17 cells and glomerular Th17-related cytokine expression with histopathological parameters in LN patients

As illustrated in Table 2, the frequencies of circulating Th17 cells were positively correlated with histological activity index ($r = 0.447$, $p < 0.05$), the degree of cellular crescent ($r = 0.426$, $p < 0.05$) and endocapillary proliferation ($r = 0.427$, $p < 0.05$). Serum IL-17 levels were positively correlated with the degree of cellular crescent ($r = 0.518$, $p < 0.01$) and the degree of necrotizing change ($r = 0.560$, $p < 0.01$). Expression levels of glomerular IL-17 were positively correlated with histological activity index ($r = 0.514$, $p < 0.05$) and the degree of

necrotizing change ($r = 0.435$, $p < 0.05$). Expression levels of glomerular IL-23 were also positively correlated with histological activity index ($r = 0.567$, $p < 0.01$), the degree of endocapillary proliferation ($r = 0.495$, $p < 0.05$), wire loop ($r = 0.423$, $p < 0.05$) and interstitial inflammation ($r = 0.538$, $p < 0.01$). Th17-related cytokine levels in sera and glomerular expression did not correlate with histological chronicity index or its parameters in LN patients.

Discussion

Very little is known about the relationship between the IL-23/Th17 axis and clinical as well as histolopathological parameters in LN patients. This study is the first attempt to investigate the frequencies of circulating Th17 cells and levels of Th17-related cytokines in sera as well as in glomeruli of LN patients. We showed that significantly higher frequencies of circulating Th17 cells were found in LN patients compared with those found in healthy controls. Our results are consistent with the findings of recent studies showing elevated frequencies of Th17 cells using enzyme-linked immunospot assay (ELISPOT)[18] and using flow cytometry assay in SLE patients.[19,37,38] Our findings also support previous studies showing augmented mRNA expression of retinoic acid receptor-related orphan nuclear receptor RORγt/RORC (mice/humans) that is the specific transcription factor for Th17 cells in lupus-prone mice[39] and in new-onset SLE patients.[21] Among LN patients, significantly higher frequencies of circulating Th17 cells were observed in patients with class IV-G LN than those with class IV-S or class V LN. In addition, we demonstrated that the frequencies of circulating Th17 cells were positively correlated with SLEDAI score in LN patients, which was in accordance with the findings of other recent studies.[19,21,37] Taken together, these observations strongly implicate a potential role for Th17 cells in the pathogenesis of LN, especially in patients with diffuse global proliferative LN.

Th17 cells have a specific role in immune function through the production of effector cytokines, including IL-17. In the present study, we did not show a positive correlation between the frequencies of circulating Th17 cells and levels of serum IL-17 in LN patients. This discordant result may be explained by the findings of recent studies showing that IL-17 levels are produced by several cell types,[40] including CD4+Th cells,[41] γδ T cells,[42] double negative T cells[28] and mast cells.[43] Nevertheless, we demonstrated that serum IL-17 levels were significantly higher in LN patients than in healthy controls, in accordance with the results of recent studies describing an increase in serum IL-17 levels in active SLE patients.[18,20–22] Accumulating evidence indicates that IL-6 can enhance Th17 cell differentiation by promoting the sequential engagement of IL-21/IL-23 pathways and that it plays a critical role in Th17-dependent autoimmune diseases.[24] Previous studies also suggested that IL-6 is an important inhibitor of T-cell regulatory functions.[44] Our results show significantly higher levels of serum IL-6 in LN patients when compared to healthy controls, which is consistent with the findings of Dong et al. showing that PBMCs isolated from LN patients secreted higher IL-6 levels.[45] These observations were in keeping with the results of a recent Phase I trial showing that blockade of IL-6 receptor had therapeutic benefits for SLE.[46]

In contrast to IL-12, IL-23 does not promote the development of Th1 cells, but is crucial for the expansion and maintenance of Th17 cells.[47] IL-23 has been shown to mediate renal manifestations in Ro52 knockout murine lupus model.[48] Our results showed that serum IL-23 levels were significantly higher in LN patients than in healthy controls, which is consistent with the findings of previous studies.[18,23,33,49] Taken together, these observations support the role of Th17-related cytokines in the pathogenesis of LN. However, serum levels of Th17-related cytokines were not related to SLEDAI score in our LN patients. We cannot exclude the possible effect of concurrent medications on cytokine levels. Therefore, a large prospective cohort study enrolling untreated active patients should be conducted to confirm our findings.

To confirm Th17-related cytokine production in the kidney, we investigated the expression levels of Th17-related cytokines in renal biopsy tissues from LN patients using the immunohistochemistry method, and explored its relationship with histological parameters and circulating Th17 cells. Our results showed significantly higher levels of glomerular IL-17, IL-6, IL-18 and IL-23 expression on renal biopsies from class IV LN patients compared with normal controls (Figure 4), supporting the findings of recent studies showing infiltration of Th17 cells into the glomeruli[27,29,38,50] and a higher urinary expression of Th17-related cytokines compared with those in healthy controls.[51] Moreover, we showed that the expression levels of glomerular IL-17 and IL-23 were positively correlated with SLEDAI score, renal score of SLEDAI and

histological activity index, in accordance with the findings of a recent study.[29] However, our findings seem to contradict the results of a recent study showing an inverse correlation between expression levels of Th17-related gene in urinary sediments and histological activity index as well as SLEDAI.[51] This discrepancy may be explained by dissimilar disease activities at baseline, different methods and the types of samples analyzed.

Interestingly, we demonstrated significantly higher levels of glomerular IL-17 and IL-23 expression in class IV-G LN patients compared with overlapping class IV plus class V LN and class V LN patients. Moreover, glomerular expression levels for IL-17 and IL-23 were significantly correlated with histopathological parameters of activity index. This may explain why the levels of glomerular IL-17 and IL-23 expression were higher in proliferative (class IV) LN than in membranous (class V) LN in our patients. Our data are consistent with the findings of a recent study showing that injection of ovalbumin-specific Th17 cells induced proliferative glomerulonephritis.[52] Based on these findings, it is likely that glomerular IL-17-producing cells may induce downstream proinflammatory cytokines perpetuating inflammation in the kidneys of LN patients.[53] Among Th17-related cytokines, we showed that the levels of IL-17 and IL-23 were significantly elevated and were positively correlated with renal SLEDAI score, suggesting both cytokines as plausible markers for disease activity of LN. The findings of previous research and our study point to the potential use of therapy targeting the IL-23/Th17 axis for LN patients.[54–56]

There were some limitations in the present study. First, we did not determine the cellular origin of glomerular expression of Th17-related cytokines. Although cytokines normally produced within the renal parenchyma are overexpressed in LN by both resident and infiltrating cells,[57] we could not ascertain whether IL-17-producing cells were CD4 + T cells[41] or double negative T cells.[28] Second, the sample size of our study was small. The analysis of cytokine profiles between class IV LN and class V LN should be interpreted with caution. Finally, we did not collect data on longitudinal changes in Th17-related cytokines. Therefore, a larger and longer study is required to clarify the role of Th17-related cytokines in the pathogenesis of LN.

In conclusion, our results show that the frequencies of circulating Th17 cells were elevated and positively correlated with lupus activity, and the expression levels of glomerular IL-17 and IL-23 were positively correlated with renal score of SLEDAI as well as the histological activity index in LN patients. Although the sample size of this study was too small to obtain a definitive conclusion, our findings may contribute to a better understanding of the potential roles of the IL-23/Th17 axis in the intra-renal inflammation of SLE. Determination of the levels of circulating Th17 cells and Th17-related cytokines may allow for the possibility of using agents designed to neutralize Th17 cells and IL-23p19 in LN patients.[55,56] However, further study on the pathobiology of Th17 cells and Th17-related cytokines in LN patients is needed.

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Conflict of interest statement

None declared.

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