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Original Article

Laser microdissection-based analysis of cytokine balance in the kidneys of patients with ANCA-associated glomerulonephritis

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Abstract: Antineutrophil cytoplasmic autoantibody (ANCA)-associated systemic vasculitis (AASV) includes a group of disorders characterized by autoimmune inflammation affecting small- to medium-sized vessels, which leads to vasculitides and systemic organ damage, especially in the kidneys. Cytokine imbalance among CD4+T-cell subsets has been known to have a close causal association with the pathophysiology of AASV. To determine the cytokine balance in patients with ANCA-associated glomerulonephritis (AAGN), we analyzed the expression of cytokines in the kidney-infiltrating T cells. The single-cell samples of both the glomelular and interstitial infiltrating cells were captured from 7 AAGN patients' renal biopsy by laser-microdissection. Nested reverse transcription polymerase chain reaction was performed with the samples for interleukin (IL)-2, IL-4, IL-13, IL-17, IL-23, and interferon (IFN)-γ. Then, the correlation between the mRNA expression levels of cytokines and clinical parameterswas analyzed. The results showed that the glomerular and interstitial infiltrating T cellsproduced IFN-γ, IL-2, IL-4, IL-13, IL-17 and IL-23 cytokines inthe kidneys of AAGN patients. The expression levels of IL-17 and IL-23 were closely correlated with clinical parameters, such as serum creatinine level, myeloperoxidase-ANCA level, and Birmingham Vasculitis Activity Score both in the glomeruli and the interstitium. The IL-17 levels also had a tendency to be positively correlated with percentage of glomerular crescent formation both in the glomeruli and the interstitiums. This suggests that the pathogenesis of AAGN in the patients is complex and may be associated not only with the imbalance between Th1 and Th2 but also with the predominance of Th17. IL-17/IL-23 axis might also play a critical role in the AAGN patients.

Keywords: ANCA, laser-microdissection, IL-17, IL-23, BVAS

Introduction

Antineutrophil cytoplasmic autoantibody (ANCA)-associated systemic vasculitis (AASV) is a generic term given to a group of disorders characterized by autoimmune inflammation affecting small- to medium-sized vessels, which leads to vasculitides and systemic organ damage [1, 2]. ANCAs are specific to the proteins in the cytoplasm of neutrophils and monocytes. The major target antigens in patients with vasculitis and glomerulonephritis are myeloperoxidase (MPO) and proteinase 3 (PR3). ANCAs are produced in more than 80% of patients with untreated active Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), and pauci-immune crescentic glomerulonephritis [3, 4]. There is compelling clinical and experimental evidence that ANCA IgG causes ANCA-associated vasculitis and glomerulonephritis. In general, ANCA with specificity to PR3 (PR3-ANCA) is predominant in WG patients, where as

Table 1. Clinical characteristics of patients

No.	Age	Sex	UP (g/day)	Cr (mg/dl)	BUN (mg/dl)	CRP (mg/dl)	MPO-ANCAEU	BVAS	R-BVAS	G-Sclerosis (%)	G-Crescent (%)	TTD (%)	NG (%)
1	77	F	0.64	0.58	14.0	2.52	62	13	10	12.5	42.9	5	50
2	82	M	0.2	2.40	35.2	9.00	386	16	12	36.4	35.7	30	40.9
3	75	F	0.2	1.36	22.7	15.13	33	29	12	9.1	10	80	81.8
4	81	F	2.53	2.59	46.7	0.43	95	18	10	10	11.1	25	80
5	76	F	0.73	2.48	37.9	5.00	105.0	21	21	41.7	85.7	70	8.3
6	70	M	0.6	2.77	33.3	2.5	232	35	20	23.3	21.7	30	60
7	53	M	0.06	1.08	18.6	6.81	119	14	0	0	15.4	10	84.6

UP = urinary protein; BUN = blood urea nitrogen; Cr = Serum creatinine; CRP = C-reactive protein; MPO-ANCA = Myeloperoxidase-anti-neutrophil cytoplasmic antibody; BVAS = Birmingham Vasculitis Activity Score; R-BVAS = Glomerulonephritis Activity was assessed at Renal function in BVAS; G-Sclerosis = the percentage of glomerular sclerosis; G-Crescent = the percentage of glomerular crescent formation; TTD = tubulointerstitial tissue damage; NG = Normal glomerular.

Table 2A. Oligonucleotide primer sequences

Gene name	PCR products	Oligonucleotide sequence(5'-3')		Product size (bp)	RT-PCR cycles
β-actin	First PCR	5'sense	GGCATCCTCACCCTGAAGTA	496	25
		3'antisense	CCATCTCTTGCTCGAAGTCC		
	Nested PCR	5'sense	AAATCTGGCACCACACCTTC	262	25
		3'antisense	AGGGCATACCCCTCGTAGAT		
TCR-Cβ	First PCR	5'sense	ACATAAGGAAGGCTGCATGG	249	30
		3'antisense	CGTTTTGATCATGGTGTGTGG		
	Nested PCR	5'sense	ATCAGGTGTGTGGGACTTTG	217	30
		3'antisense	GACTCAGGACAGTGACATCA		
IFN-γ	First PCR	5'sense	TCTGCATCGTTTTGGGTTCTC	346	25
		3'antisense	TCAGCTTTTCGAAGTCATCTC		
	Nested PCR	5'sense	TGTTACTGCCAGGACCCATAT	242	30
		3'antisense	ACTCTTTTGGATGCTCTGGTC		
IL-2	First PCR	5'sense	ACTACCAGGATGCTCACATT	267	25
		3'antisense	AAGGTAATCCATCTGTTCAGA		
	Nested PCR	5'sense	GCCACAGAACTGAAACATCTT	201	30
		3'antisense	TTCTACAATGGTTGCTGTCTC		
IL-4	First PCR	5'sense	CTTCCCCCTCTGTTCTTCCT	318	25
		3'antisense	TTCCTGTCGAGCCGTTTCAG		
	Nested PCR	5'sense	CTAGCATGTGCCGGCAACTTT	273	25
		3'antisense	TCGGATCAGCTGCTTGTGCCT		
IL-13	First PCR	5'sense	CTATGCATCCGCTCCTCAAT	391	30
		3'antisense	TTTACAAACTGGGCCACCTC		
	Nested PCR	5'sense	ATTGCTCTCACTTGCCTTGG	229	25
		3'antisense	TCCTGTGGGTCTTCTCGATC		
IL-17	First PCR	5'sense	CTTCACCCTGTGGAACGAAT	262	30
		3'antisense	CGGAATTGGTTCTGGAGTGT		
	Nested PCR	5'sense	GAGCACATGCACCACATACC	170	25
		3'antisense	AGGAAACAGTCGCGGAGTGT		
IL-23	First PCR	5'sense	GTTCCCCATATCCAGTGTGG	340	30
		3'antisense	CCTTGAGCTGCTGCCTTTAG		
	Nested PCR	5'sense	GTTCCCCATATCCAGTGTGG	220	25
		3'antisense	GAGGCTTGGAATCGTCTGAG		

RT-PCR = reverse transcription polymerase chain reaction; TCR-Cβ = T-cell receptor β chain; IL = interleukin; IFN-γ = interferon-gamma.

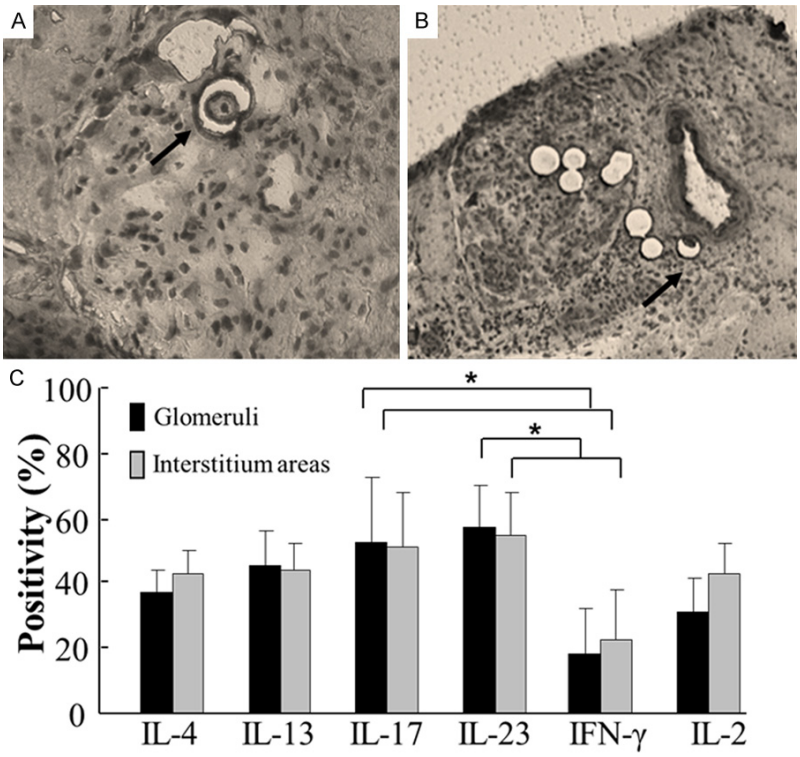


Figure 1. Targeted infiltrating cells and cytokine gene expression in lesions. A. Targeted infiltrating cells selected at the glomeruli areas. The glomeruli areas of a single infiltrating cell (black arrows) were selected and dissected with a laser microbeam one by one. B. Targeted infiltrating cells selected at the interstitium areas. The interstitium areas of a single infiltrating cell (black arrows) were selected and dissected with a laser microbeam one by one. C. Analysis of cytokine gene expression in lesions. Detection of cytokines in the lesions of the renal biopsy specimens from the patients by nested PCR. The mRNA of interferon-gamma (IFN-γ), interleukin (IL)-2, IL-4, IL-13, IL-17 and IL-23 expressed in both the glomeruli and interstitium areas fromthe renal biopsy specimens of the AAGN patients. Positive rates of both IL17 and IL23 cytokines were significantly higher than those of IFN-γ in the lesions of the glomeruli and interstitium areas. The number of positive samples is shown as a percentage. Error bars represent ± standard errors.

ANCA with specificity to MPO (MPO-ANCA) is predominant in MPA patients and Churg-Strauss syndrome (CSS) patients [5]. Although ANCAs are thought to be pathogenic [6, 7], previous studies have reported that cytokine imbalance among CD4+ T helper (Th) cells, which may act as the effectors of tissue injury, plays an important role and is associated with the pathogenesisbecause of its production of ANCA [8-10]. Masutani et al., [11] reported that glomerular lesions showed high interferon-γ (IFN-γ) and low IL-4 glomerular mRNA expression levels, and peripheral blood T cells showed a high IFN-γ: IL-4 cytokine ratio in patients with ANCA-associatedGN (AAGN) compared to those with non-proliferative forms of GN and Ig Adiseases, indicating a Th1-predominant effector response in the AAGN patients. Analysis of patients' sera with soluble markers associated with Th1 cells (IFN-γ) and Th2 cells (IL-4, IL-5, IL-10, IL-13) disclosed a shift towards a Th2-type response in patients with active generalized WG and CSS and the predominance of Th1 response in localized-WG patients and MPA patients [12, 13].

Recently, the Th1/Th2 paradigm has been changed by identification of the third IL-17-producing CD4+effector T cell subset termed Th17 [14]. IL-17 is a proinflammatory cytokine, as possibly known from the pathologic conditions of various inflammatory diseases in both humans and mice [15]. Recent studies have highlighted the potential importance of the Th17 immune response also in renal inflammatory diseases. This includes the identification and characterization of IL-17-producing T cells in nephritic kidneys of mice and humans, as well as evidence of the contribution of IL-17 and the IL-23/Th17 axis to renal tissue injury in glomerulonephritispatients [16].

We have previously reported that IL-17-producing cells were produced in murine and human lupus nephritis [17, 18]. The laser microdissection (LMD) technique has recently been adopted to obtain tissue samples exclusively from specific regions of interest. Thistechnique has been successfully used in various medical fields including oncology [19], endocrinology [20], gastroenterology [21], and nephrology [17, 18, 22]. In our study,we analyzed the single-cell expression levels of cytokines including IL-23 and IL-17 by infiltrating T cells in the kidneys of ANCA patients.

Table 2B. Positivity of cytokines in glomeruli and interstitium (%)

	Glomeruli	Interstitium
IFN-γ	17.6 ± 15.1	22.3± 15.2
IL-2	30.9± 10.5	42.3 ± 10.2
IL-4	37.4 ± 7.0	42.6± 7.1
IL-13	45.2± 10.7	42.2 ± 8.4
IL-17	52.2 ± 20.8	51.1 ± 16.8
IL-23	56.8 ± 13.9	54.8± 12.8

IFN-γ = interferon-gamma; IL = interleukin.

Materials and methods

Patients and samples

Renal biopsy samples were obtained from 7 patients with AAGN, for which clinical diagnosis was conducted in accordance with the Chapel Hill Consensus Criteria [23]. Disease activity was assessed at the time of sample collection by calculating the Birmingham Vasculitis Activity Score (BVAS) 2003 [24, 25]. The clinical characteristics and the calculated values for BVAS are shown in **Table 1**. This study was approved by the ethical committee of Yangzhou University Hospital. Prior written consent was given by the patients.

Tissue sampling by laser microdissection

Frozen sections (10 μm thick) from the renal biopsy specimens of the AAGN patients were stained with 0.05% toluidine blue solution (pH 7.0) (Wako Pure Chemical Industries, Osaka, Japan) and the individual single cells infiltrating into the glomeruli and the interstitiums were selected followed by dissection by a laser-microdissection system (AS-LMD; Leica Microsystems China, Shanghai, China).

RNA extraction and nested reverse transcription polymerase chain reaction

Total RNA was extracted from the LMD samples by the Isogen method (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was prepared from total RNA by ThermoScript RT-PCR System (Invitrogen Life Technologies, Carlsbad, CA), and amplified with primers specific to β-actin, T-cell receptor β chain (TCR-Cβ), IL-2, IL-4, IL-13, IL-17, IL-23 and IFN-γ for nested RT-PCR (**Table 2A**).

Statistical analysis

All data were expressed as mean ± standard error of the mean. Statistically significant differences between groups were determined using the Mann-Whitney's U-test. A simple linear regression analysis was used to evaluate the correlation between the twoparameters. The statistical significance was defined as $P<0.05$.

Results

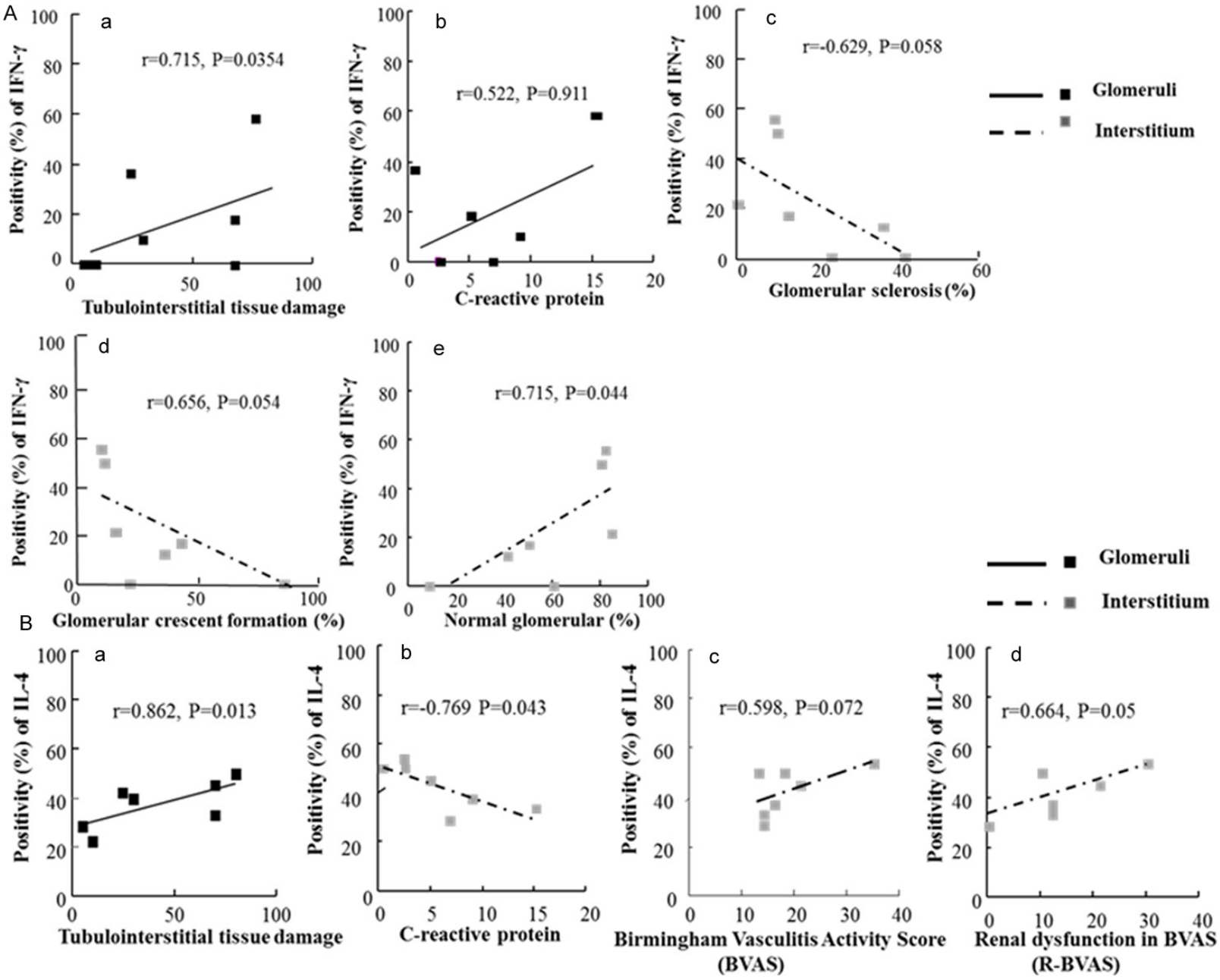
Analysis of gene expression bylaser microdissection and nestedreverse transcription polymerase chain reaction

Frozen sections from the renal biopsy specimens of the AAGN patients were stained with 0.05% toluidine blue solution and the individual single cells infiltrating into glomeruli and interstitiums were selected followed by dissection by a laser-microdissection system (**Figure 1A** and **1B**). Out of 672 glomerular and interstitial infiltrating cells, 503 (74.9%) were β-actin positive, among which 304 (60.4%) were TCR-Cβ positive; these 304 cells were deemed to be T cells and used for cytokines analysis. The number of positive samples for each cytokine/TCR-Cβ+ cellswas expressed in terms of percentage.

The glomerular and interstitial infiltrating T cells produced IFN-γ, IL-2, IL-4, IL-13, IL-17, and IL-23 cytokines in the kidneys of MPO-AAGN patients. The positivity levels of cytokines were shown in **Table 2B** and **Figure 1C**. The percentages of positive IL-2, IL-4, and IL-13 samples were over 30% (42%), 37% (42%), and 45% (42%), respectively in the glomerular and interstitial lesions. The expression level of IFN-γ was low both in the glomerular and interstitial lesions (17.6 ± 15.1% and 22.3 ± 15.2%) (**Figure 1C**). In the glomerular lesions, the percentages of positive IL-17 and IL-23 samples were 52.2 ± 20.8% and 56.8 ± 13.9%, respectively, while they were higher than those of IFN-γ ($P<0.05$, **Figure 1C**). In the interstitial lesions, the positivity levels of IL-17 (51.1 ± 16.8%) and IL-23 (54. ± 12.8%) were also higher than those of IFN-γ ($P<0.05$, **Figure 1C**).

Correlation between Th1 and Th2 cytokines and clinical parameters

As known from the tendency of the point distributions in the charts, in the glomeruli, there


A
a
r=0.715, P=0.0354
Positivity (%) of IFN-γ
Tubulointerstitial tissue damage
b
r=0.522, P=0.911
C-reactive protein
c
r=-0.629, P=0.058
Glomerular sclerosis (%)
Glomeruli
Interstitium
d
r=0.656, P=0.054
Glomerular crescent formation (%)
e
r=0.715, P=0.044
Normal glomerular (%)
B
a
r=0.862, P=0.013
Positivity (%) of IL-4
Tubulointerstitial tissue damage
b
r=-0.769 P=0.043
C-reactive protein
c
r=0.598, P=0.072
Birmingham Vasculitis Activity Score (BVAS)
d
r=0.664, P=0.05
Renal dysfunction in BVAS (R-BVAS)

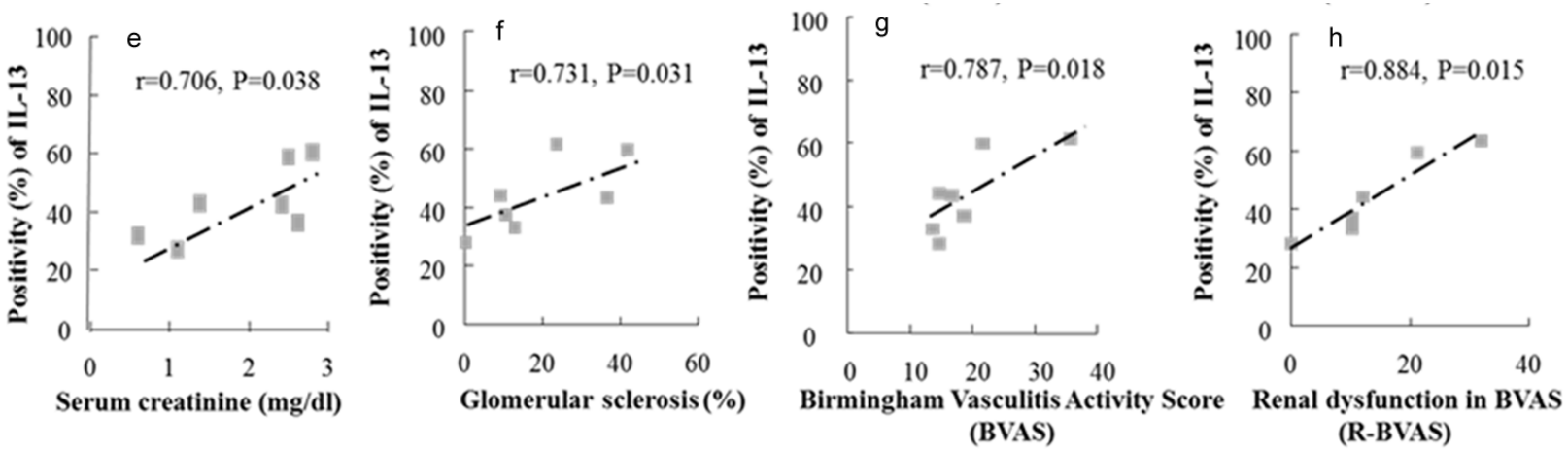


Figure 2. Correlation between Th1 and Th2 cytokines, and clinical and laboratory parameters in AAGN. (A) A positive correlation between the levels of IFN-γ andtubulointerstitial (TTD) (a) and tendency to be positively correlated with C-reactive protein (CRP) (b) were found in the glomeruli (black full line and points). The levels of IFN-γ showed a tendency to be negatively correlated with to Glomerular sclerosis (G-sclerosis) (c), Glomerular crescent formation (d), and a significantly positive correlation with Normal glomerular (d) in the interstitium (black dashed lineand gray points). R = 0.4-0.7 means good correlation, r = 0.7-0.9 means significantly correlation. (B) A significantly positive correlation between the levels of IL-4 andtubulointerstitial (TTD) (a) were found in the glomeruli (black full line and points). The IL-4 showed asignificantlynegative correlation with CRP (b), a tendency to be positivelycorrelated with Birmingham Vasculitis Activity Score (BVAS) (c), and a good positive correlation with the score of renal dysfunction in BVAS (R-BVAS) (d) in the glomeruli (black full line and points). The IL-13 also showed a significantly positive correlation with serum creatinine (Cr) (e), G-sclerosis (f), BVS (g), and R-BVAS (h) in interstitium (black dashed line andgray points). R = 0.4-0.7 means good correlation, r = 0.7-0.9 means significantly correlation.

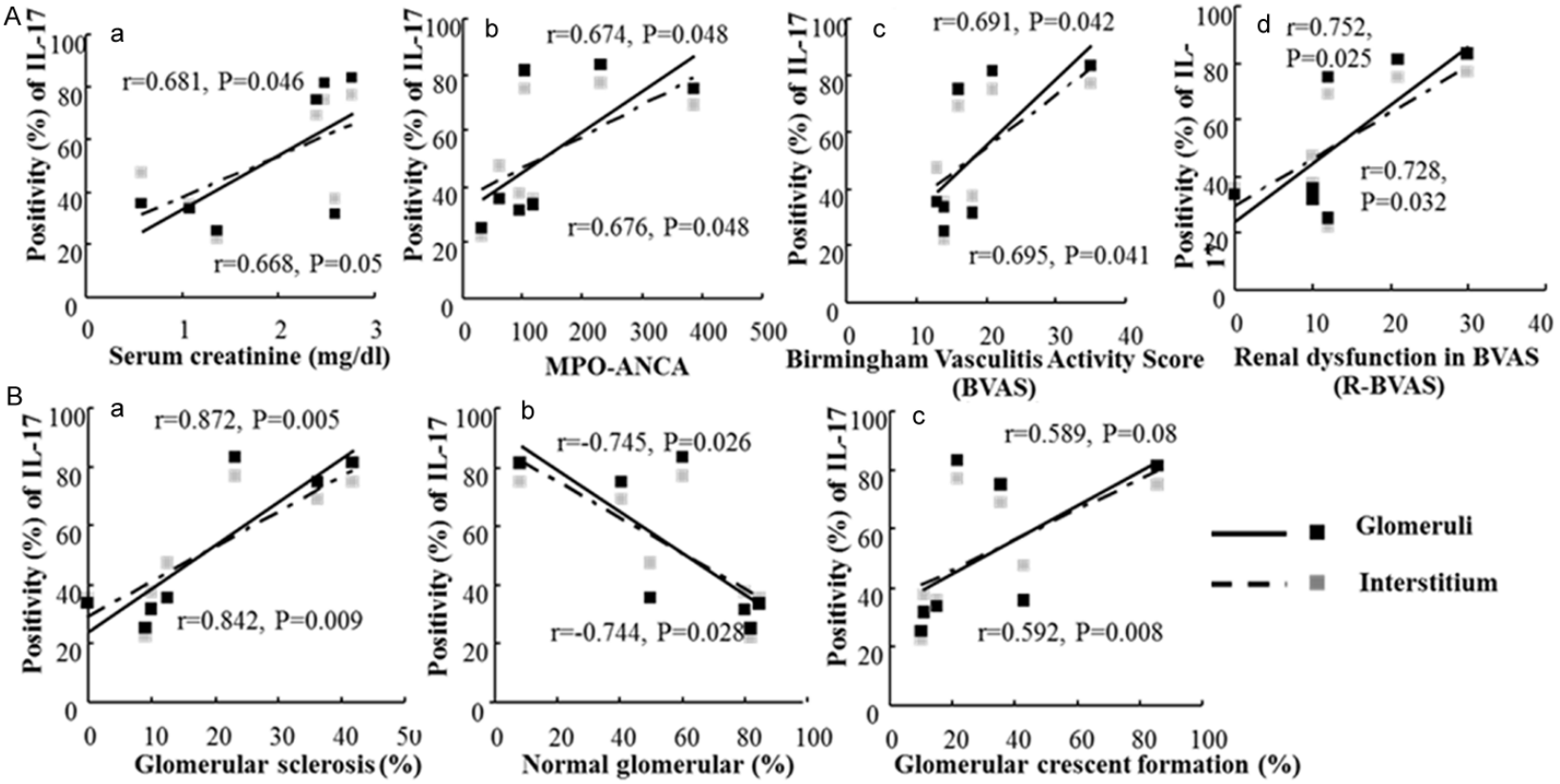


Figure 3. Correlation between IL-17 cytokines, and clinical and laboratory parameters in AAGN patients. (A) A positive correlation between the levels of IL-17 and Serum creatinine (Cr) (a), MPO-ANCA (b), Birmingham Vasculitis Activity Score (BVAS) (c), and the score of renal dysfunction in BVAS (R-BVAS) (d) was found in the glomeruli (black full line and points) and the interstitium (black dashed line andgray points). (B) Correlation between the levels of IL-17 and Glomerular sclerosis (a), Normal glomerular (b), and Glomerular crescent formation (c) in the glomeruli (black full line and points) and the interstitium (black dashed line andgray points). The level of IL-17 was positively correlated with Cr, MPO-ANCA, BVAS, R-BVAS and G-sclerosis, while being negatively correlated with NG both in the glomeruli and the interstitiums. R = 0.4-0.7 means good correlation, r = 0.7-0.9 means significantly correlation.

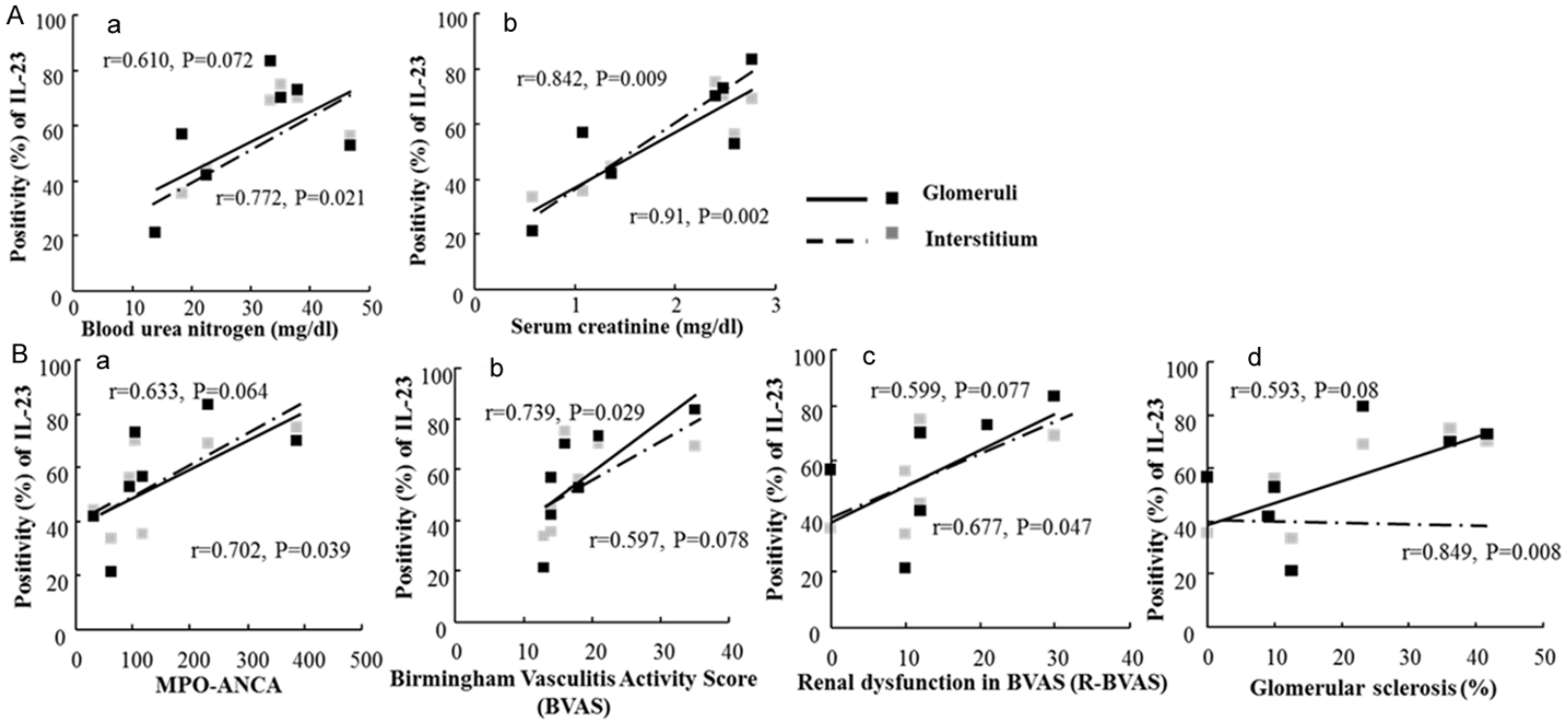


Figure 4. Correlation between IL-23 cytokines and clinical and laboratory parameters in SLE patients. (A) A positive correlation between the levels of IL-23 and blood urea nitrogen (BUN) (a), and serum creatinine (Cr) (b) was found in the glomeruli (black full line and points) and the interstitium (black dashed line andgray points). (B) A correlation between the levels of IL-23 and MPO-ANCA (a), Birmingham Vasculitis Activity Score (BVAS) (b), the score of renal dysfunction in BVAS (R-BVAS) (c), and Glomerular sclerosis (d) in the glomeruli (black full line and points) and the interstitium (black dashed line andgray points). The level of IL-23 hada significantly positive correlation with BVAS in the glomeruli, and positively correlated with MPO-ANCA, R-BVAS and a G-sclerosis in the interstitiums. A simple linear regression analysis was used to evaluate the correlation between the two parameters, $P<0.05$. r = 0.4-0.7 means good correlation, r = 0.7-0.9 means significantly correlation.

was almost no finding that showed any correlation with the expression level of IL-2 except for CRP (r = -0.862, P = 0.0176) (data not shown). While, the expression level of IFN-γ showedatendency to be positively correlated with CRP (r = 0.522, P = 0.911). The expression levels of IFN-γ and IL-4 also had a significantly positive correlation only with TTD (r = 0.715, P = 0.035 and r =0.841, P = 0.013, respectively) (**Figure 2Aa**, **2Ab** and **2Ba**). In the interstitiums, it was found that the expression level of IFN-γ hadasignificantly positive correlation with NG (r = 0.715, P = 0.044), whileshowing a tendency to negatively correlated withG-sclerosis andG-crescent (r = -0.629, P = 0.058, and r = -0.656, P = 0.054) (**Figure 2Ac-e**). The expression level of IL-4, one of cytokine produced by Th2, had a significantly negative correlation with CRP (r = -0.769, P = 0.043), while showing a tendency to be positively correlated with BVAS (r = 0.598, P = 0.072). However, there was a good positive correlation with R-BVAS (r = 0.664, P = 0.05) (**Figure 2Bb-d**). The expression level of IL-13 also showed a tendency to have significantly positive correlations with Cr, G-sclerosis, and BVAS (r = 0.706, P = 0.038, r = 0.731, P = 0.031, and r = 0.787, P = 0.018, respectively) in especially with R-BVAS (r = 0.884, P = 0.015) (**Figure 2Be-h**).

Correlation between IL-17 and clinical parameters

As known from the tendency of the point distributions in the charts, inthe glomeruli, theparameters, Cr, MPO-ANCA, and BVAS showed a good correlationwith the expression level of IL-17. In the interstitiums, the positive IL-17 samples also showed a significantly positive correlation with Cr, MPO-ANCA, and BVAS (P<0.05, **Figure 3Aa-c**). In particular, they have a highly significant correlation with the R-BVAS values (r = 0.75, P = 0.025, and r = 0.728, P = 0.032, respectively). Furthermore, it was found that the positive IL-17 samples showed a highly significant positive correlation with G-sclerosis and a negative correlation with NG; they also had a tendency to be positively correlated withG-crescent both in the glomeruli and the interstitiums (**Figure 3Ba-c**). However, there was almost no finding that showed any correlation with the expression levels of IL-17 and UP, CRP, and TTD (data not shown).

Correlation between IL-23 and clinical parameters

In the glomeruli, Cr (r = 0.842) and BVAS (r = 0.739) showed a significant positive correlation with the expression level of IL-23 (P<0.05, **Figure 4Ab** and **4Bb**); and BUN, MPO-ANCA, R-BVAS, and G-sclerosis had a tendency to be positively correlated with IL-23 (**Figure 4**). In the interstitiums, there was a significant positive correlation between the expression level of IL-23, and BUN (r = 0.772), Cr (r = 0.91), MPO-ANCA (r = 0.702), and G-sclerosis (r = 849) (P<0.05, **Figure 4**). It was found that IL-23 had a tendency to be positively correlated with BVAS (r = 0.597, P = 0.78) and showed a good correlation with R-BVAS (r = 0.677, P = 0.047) (**Figure 4Bb**, **4Bc**). There was almost no finding that showed any correlation between the expression level of IL-23, and UP, CRP, TTD and NG (data not shown).

Discussion

AAGN is a generic term given to a group of disorders characterized by autoimmune inflammation affecting small- to medium-sized vessels, which leads to vasculitides and systemic organ damage, especially in the kidneys. Cytokine imbalance among CD4+T-cell subsets has been known to have a close causal association with the pathophysiology of AAGN. CD4+ T-helper cells, which play a central role in the regulation of immune response, are associated with the pathogenesis ofautoimmune diseases. In addition to the helper T cells classified into Th1 and Th2 types, recently, another helper T cell subset, Th17, has been discovered [15]. IL-17, which induces proinflammation, has been observed in many inflammatory conditions [15], which may contributeto the pathogenesis of autoimmune and inflammatory diseases including renal diseases such as systemic lupus erythematosis (SLE) and AAGN [26-28]. To clarify the cytokine balance in the kidneys of the AAGN patients, the single-cell cytokine profiles of the samples from the AAGN patients were analyzed by LMD. The result showed that the IFN-γ, IL-2, IL-4, and IL-13 were produced by glomerular and interstitial infiltrating T cells, while IL-17 and IL-23 cytokines were predominant inthe kidneys of the AAGN patients. This result also provided us with a finding that the

percentages of positive IL-17 and IL-23 samples were over 52% (51%) and 56% (54%), respectively in the glomerular and interstitial lesions, while the levels of IFN-γ and IL-4 were lower. This suggests that the pathogenesis of AAGN inpatients is complex and not only imbalance between Th1 and Th2 but also the predominance of Th-17 may contribute to the pathogenesis of AAGN. Abdulahad et al. [29] reported that the proportions of peripheral blood Th17 cells inpatients with ANCA-associated disease were high compared with the controls. The serum IL-17 and IL-23 levels and autoantigen-specific Th17 cell count are elevated in AAV patients [30]. Functional data on the relevance of the Th17 axis are limited in patients with renal disease. Recently, some studies reported that the Th17 immune response has close, causal relation to renal inflammatory diseases [31]. Gan et al. [27] showed that IL-17 play a crucial role in mediation of renal tissue damage in a murine model of MPO-AAGN. Importantly, IL-17 knockout mice were protected from kidney injury due to impairment of both the innate and adaptive arms of the immune response. In our study, it was successfully confirmed that IL-17 in the infiltrating T cells was produced in the kidneys (glomeruli and interstitiums) of the AAGN patients at a single cell level. This suggests that IL-17 may be closely associated with AAGN. It was reported that cyclosporine A might inhibit the production of IL-17 in the healthy control and RA patient groups [32]. Cyclosporine A also inhibits IL-15-induced IL-17 production in the $CD4^+$ T cells through down-regulation of PI3K/Akt and NF-Kb [33]. Inhibition of IL-15-induced IL-17 production by tacrolimuswas also observed in the $CD4^+$T cells [33]. It may be considered that the inhibition of IL-17 production is an essential mechanism of the efficacy of these two kinds of calcineurin inhibitors in steroid-resistant AAGN patients atthe early stage.

The correlation between the expression levels of IL-17 and IL-23 cytokines and clinical parameters was analyzed. The result showed that the levels of IL-17 and IL-23 have a correlationwith some clinical and laboratory parameters (**Figures 3**, **4**). The level of IL-17 was positively correlated with Cr, MPO-ANCA, BVAS, R-BVAS and G-sclerosis, while being negatively correlated with NG both in the glomeruli and the interstitiums (**Figure 3**). It is suggested that IL-17 may be closely associated with the inflammatory process of a renal disease in AAGN patients. IL-17 also had a tendency to be positively correlated with G-crescent (**Figure 3Bc**). This finding indicates that IL-17 may playan important role in the formation of crescents. It was also found that the level of IL-23 was a positively correlated with BUN, MPO-ANCA and G-crescent in the interstitiums; and significantlycorrelated with Cr bothin the glomeruli and the interstitiums (**Figure 4**). Nogueira et al. [30] showed that the serum IL-17A and IL-23 levels were significantly elevated in acute AAV patients compared to healthy controls; however, importantly, they remained elevated in a proportion of convalescent patients. Furthermore, patients with elevated levels of IL-23 had high BVAS and ANCA titer values compared to patients with IL-23 levels within the normal range.In our study, not only IL-23 but also IL-17 had significantly positive correlations with BVAS and MPO-ANCA (**Figures 3**, **4**). In particular, the level of IL-17 had a significantly positive correlation with R-BVAS (**Figure 3Ad**). This suggests that IL-17 may have close causal relation to the inflammatory process of a renal disease in AAGN patients, especially in renal damage. IL-17/IL-23 may be closely associated with the pathogenesis of AAGN in patients. Our results showed that the level of IFN-γ was low both in the glomeruli and the interstitiums; this corresponds to the results of the experiments on the serum in AAV patients reported by Nogueira et al. [30]. Since the successful characterization of Th17 lineage, it has been recognized that IFN-γ takes inhibitory action in the differentiation process of Th17 cells [34, 35]. Nakae et al. [36] also found that IL-17 can suppress Th1 cell differentiationin the presence of exogenous IL-12 *invitro*, and IFN-γ can down-regulate Th17 cell differentiation. Therefore, the finding that Th17, as the most definite pathogenesis of ANCA, may suppress Th1 cell differentiation is consistent with the finding that IFN-γ levels should below. In our recently study, theexpression level of IFN-γ was even lower; this finding was observed only in the glomeruli of the International Society of Nephrology/Renal Pathology Society (ISN/RPS) Class III-predominant and ClassV groups of lupus patients, and the IFN-γ expression was almost not detected in the interstitiums [17]. However, the IL-4 levels in lupus were significantly higher than those in the group of AAGN patients both

in the glomeruli and the interstitiums (data not shown). The high IL-4 level may suppressIFN-γ cell differentiation. This might be the reason why the expression levels of IFN-γ were higher in the interstitiums of AAGN than lupus nephritis patients. This suggests that Th1 cytokine may play a more important role in AAGN patients than lupus nephritis. The expression level of IFN-γ had a significantly positive correlation with TTD, while havinga good negative correlationwith G-crescent (**Figure 2**). This finding indicates that IFN-γ may be an important role in the inflammatory process of interstitiumlesion but not in the formation of crescents.

In conclusion, it has been shown that the glomerular and interstitial infiltrating T cellsproduced IFN-γ, IL-2, IL-4, IL-13, IL-17 and IL-23 cytokines inthe kidneys of the AAGN patients. IL-17 and IL-23 levels were correlated with clinical disease activity BVAS and MPO-ANCA values. This suggests that the pathogenesis of AAGN inpatients is complex and not only the imbalance between Th1 and Th2 levels but also the predominance of Th17 may contribute to the pathogenesis of AAGN. IL-17/IL-23 axis might play a critical role in AAGN development.

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Disclosure of conflict of interest

None.

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References

[1] Jennette JC and Falk RJ. Small-vessel vasculitis. N Engl J Med 1997; 337: 1512-1523.

[2] Kallenberg CG, Brouwer E, Weening JJ and Tervaert JW. Anti-neutrophil cytoplasmic antibodies: current diagnostic and pathophysiological potential. Kidney Int 1994; 46: 1-15.

[3] Jennette JC, Xiao H and Falk RJ. Pathogenesis of vascular inflammation by anti-neutrophil cytoplasmic antibodies. J Am Soc Nephrol 2006; 17: 1235-1242.

[4] Koyama A, Yamagata K, Makino H, Arimura Y, Wada T, Nitta K, Nihei H, Muso E, Taguma Y, Shigematsu H, Sakai H, Tomino Y and Matsuo S. A nationwide survey of rapidly progressive glomerulonephritis in Japan: etiology, prognosis and treatment diversity. Clin Exp Nephrol 2009; 13: 633-650.

[5] Sinico RA, Di Toma L, Maggiore U, Bottero P, Radice A, Tosoni C, Grasselli C, Pavone L, Gregorini G, Monti S, Frassi M, Vecchio F, Corace C, Venegoni E and Buzio C. Prevalence and clinical significance of antineutrophil cytoplasmic antibodies in Churg-Strauss syndrome. Arthritis Rheum 2005; 52: 2926-2935.

[6] Pfister H, Ollert M, Frohlich LF, Quintanilla-Martinez L, Colby TV, Specks U and Jenne DE. Antineutrophil cytoplasmic autoantibodies against the murine homolog of proteinase 3 (Wegener autoantigen) are pathogenic in vivo. Blood 2004; 104: 1411-1418.

[7] Xiao H, Heeringa P, Hu P, Liu Z, Zhao M, Aratani Y, Maeda N, Falk RJ and Jennette JC. Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. J Clin Invest 2002; 110: 955-963.

[8] Abdulahad WH, Stegeman CA and Kallenberg CG. Review article: The role of CD4(+) T cells in ANCA-associated systemic vasculitis. Nephrology (Carlton) 2009; 14: 26-32.

[9] Griffith ME, Coulthart A and Pusey CD. T cell responses to myeloperoxidase (MPO) and proteinase 3 (PR3) in patients with systemic vasculitis. Clin Exp Immunol 1996; 103: 253-258.

[10] Ruth AJ, Kitching AR, Kwan RY, Odobasic D, Ooi JD, Timoshanko JR, Hickey MJ and Holdsworth SR. Anti-neutrophil cytoplasmic antibodies and effector CD4+ cells play nonredundant roles in anti-myeloperoxidase crescentic glomerulonephritis. J Am Soc Nephrol 2006; 17: 1940-1949.

[11] Masutani K, Tokumoto M, Nakashima H, Tsuruya K, Kashiwagi M, Kudoh Y, Fukuda K, Kanai H, Akahoshi M, Otsuka T, Hirakata H and Iida M. Strong polarization toward Th1 immune response in ANCA-associated glomerulonephritis. Clin Nephrol 2003; 59: 395-405.

[12] Lamprecht P, Bruhl H, Erdmann A, Holl-Ulrich K, Csernok E, Seitzer U, Mack M, Feller AC, Reinhold-Keller E, Gross WL and Muller A. Differences in CCR5 expression on peripheral blood CD4+CD28- T-cells and in granulomatous lesions between localized and generalized Wegener's granulomatosis. Clin Immunol 2003; 108: 1-7.

[13] Muller A, Trabandt A, Gloeckner-Hofmann K, Seitzer U, Csernok E, Schonermarck U, Feller AC and Gross WL. Localized Wegener's granulomatosis: predominance of CD26 and IFN-gamma expression. J Pathol 2000; 192: 113-120.
[14] Steinman L. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. Nat Med 2007; 13: 139-145.
[15] Afzali B, Lombardi G, Lechler RI and Lord GM. The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. Clin Exp Immunol 2007; 148: 32-46.
[16] Paust HJ, Turner JE, Steinmetz OM, Peters A, Heymann F, Holscher C, Wolf G, Kurts C, Mittrucker HW, Stahl RA and Panzer U. The IL-23/Th17 axis contributes to renal injury in experimental glomerulonephritis. J Am Soc Nephrol 2009; 20: 969-979.
[17] Wang Y, Ito S, Chino Y, Goto D, Matsumoto I, Murata H, Tsutsumi A, Hayashi T, Uchida K, Usui J, Yamagata K and Sumida T. Laser microdissection-based analysis of cytokine balance in the kidneys of patients with lupus nephritis. Clin Exp Immunol 2010; 159: 1-10.
[18] Wang Y, Ito S, Chino Y, Iwanami K, Yasukochi T, Goto D, Matsumoto I, Hayashi T, Uchida K and Sumida T. Use of laser microdissection in the analysis of renal-infiltrating T cells in MRL/lpr mice. Mod Rheumatol 2008; 18: 385-393.
[19] Lechner S, Muller-Ladner U, Renke B, Scholmerich J, Ruschoff J and Kullmann F. Gene expression pattern of laser microdissected colonic crypts of adenomas with low grade dysplasia. Gut 2003; 52: 1148-1153.
[20] Hong SH, Nah HY, Lee JY, Gye MC, Kim CH and Kim MK. Analysis of estrogen-regulated genes in mouse uterus using cDNA microarray and laser capture microdissection. J Endocrinol 2004; 181: 157-167.
[21] Shi X, Kleeff J, Zhu ZW, Schmied B, Tang WH, Zimmermann A, Buchler MW and Friess H. Gene-expression analysis of single cells-nested polymerase chain reaction after laser microdissection. World J Gastroenterol 2003; 9: 1337-1341.
[22] Chan RW, Lai FM, Li EK, Tam LS, Chow KM, Lai KB, Li PK and Szeto CC. Intrarenal cytokine gene expression in lupus nephritis. Ann Rheum Dis 2007; 66: 886-892.
[23] Jennette JC, Falk RJ, Andrassy K, Bacon PA, Churg J, Gross WL, Hagen EC, Hoffman GS, Hunder GG, Kallenberg CG and et al. Nomenclature of systemic vasculitides. Proposal of an international consensus conference. Arthritis Rheum 1994; 37: 187-192.
[24] Luqmani RA, Bacon PA, Moots RJ, Janssen BA, Pall A, Emery P, Savage C and Adu D. Birmingham Vasculitis Activity Score (BVAS) in systemic necrotizing vasculitis. QJM 1994; 87: 671-678.
[25] Mukhtyar C, Lee R, Brown D, Carruthers D, Dasgupta B, Dubey S, Flossmann O, Hall C, Hollywood J, Jayne D, Jones R, Lanyon P, Muir A, Scott D, Young L and Luqmani RA. Modification and validation of the Birmingham Vasculitis Activity Score (version 3). Ann Rheum Dis 2009; 68: 1827-1832.
[26] Free ME and Falk RJ. IL-17A in experimental glomerulonephritis: where does it come from? J Am Soc Nephrol 2010; 21: 885-886.
[27] Gan PY, Steinmetz OM, Tan DS, O'Sullivan KM, Ooi JD, Iwakura Y, Kitching AR and Holdsworth SR. Th17 cells promote autoimmune anti-myeloperoxidase glomerulonephritis. J Am Soc Nephrol 2010; 21: 925-931.
[28] Yap DY and Lai KN. Cytokines and their roles in the pathogenesis of systemic lupus erythematosus: from basics to recent advances. J Biomed Biotechnol 2010; 2010: 365083.
[29] Abdulahad WH, Stegeman CA, Limburg PC and Kallenberg CG. Skewed distribution of Th17 lymphocytes in patients with Wegener's granulomatosis in remission. Arthritis Rheum 2008; 58: 2196-2205.
[30] Nogueira E, Hamour S, Sawant D, Henderson S, Mansfield N, Chavele KM, Pusey CD and Salama AD. Serum IL-17 and IL-23 levels and autoantigen-specific Th17 cells are elevated in patients with ANCA-associated vasculitis. Nephrol Dial Transplant 2010; 25: 2209-2217.
[31] Turner JE, Paust HJ, Steinmetz OM and Panzer U. The Th17 immune response in renal inflammation. Kidney Int 2010; 77: 1070-1075.
[32] Crispin JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, Kyttaris VC, Juang YT and Tsokos GC. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. J Immunol 2008; 181: 8761-8766.
[33] Zhang C, Zhang J, Yang B and Wu C. Cyclosporin A inhibits the production of IL-17 by memory Th17 cells from healthy individuals and patients with rheumatoid arthritis. Cytokine 2008; 42: 345-352.
[34] Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM and Weaver CT. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005; 6: 1123-1132.
[35] Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q and Dong C. A distinct lineage of CD4 T cells regu-

lates tissue inflammation by producing interleukin 17. Nat Immunol 2005; 6: 1133-1141.
[36] Nakae S, Iwakura Y, Suto H and Galli SJ. Phenotypic differences between Th1 and Th17 cells and negative regulation of Th1 cell differentiation by IL-17. J Leukoc Biol 2007; 81: 1258-1268.