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The prevalence of Th17 cells and FOXP3 regulate T cells (Treg) in children with primary nephrotic syndrome

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Abstract The aim of this study was to investigate the prevalence of interleukin (IL)-17-producing CD4+ T cells (Th17) and regulatory T (Treg) cells in children with primary nephrotic syndrome. The study cohort consisted of 62 children who were randomly divided into control, primary nephrotic syndrome, and isolated hematuria groups. Flow cytometric analysis revealed the presence of Th17 cells in the peripheral blood mononuclear cells (PBMCs) of 35 children and Tregs in the PBMCs of all children. In addition, mRNA expression of Th17-related factors [IL-17, -23p19 and retinoid orphan nuclear receptor (RORc)] and the concentration of plasma inflammatory mediators such as IL-6 and IL-1β were consistently detected in all children. Protein expression of IL-17 and transforming growth factor-β1 were also detected in renal biopsy tissue and compared between different groups. Patients with PNS were found to have an increased number of Th17 cells and decreased numbers of Tregs in their PBMCs, and there was significant difference in the prevalence of Th17 and Tregs between the patients with PNS and those with isolated hematuria. Our data show that among our study cohort, there was a dynamic equilibrium between Th17 and Treg cells in children with PNS following the development of PNS with apparent renal tubular epithelial cell and interstitium lesions. The dynamic interaction between Th17 and Treg cells may be important in the development of PNS.

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Introduction

Primary nephrotic syndrome (PNS) is a chronic inflammatory disease involving various immune cells, particularly T lymphocytes. It is one of the most commonly occurring inflammatory kidney diseases in children, accounting for a significant number of PNS-related chronic renal failures worldwide.

Primary nephrotic syndrome is characterized by massive proteinuria, hypoalbuminemia, hyperlipidemia, different degrees of edema and decreased renal function. A large body of evidence has demonstrated that the immune system may play a crucial role in PNS, but the precise mechanism is still unclear. The recent identification of a new member of the CD4+ effector T-cell family the IL-17-producing CD4+ T cells (Th17), has substantially deepened our understanding of T cell-mediated immunity [1–4]. Th17 cells have emerged as an important mediator in inflammatory and autoimmune diseases. These cells have been characterized as preferential producers of interleukin (IL-17A; also known as IL-17), IL-17F, IL-21, IL-22, and IL-26 in humans [5, 6]. Retinoid orphan nuclear receptor (RORc), which encodes the human ortholog of mouse RORrt, is a key regulator of Th17-cell lineage differentiation [7, 8]. Although human Th17-cell

development is less well understood, transforming growth factor-β1 (TGF-β1), IL-23, and pro-inflammatory cytokines (IL-1β and IL-6) have been shown to collectively mediate the differentiation of human Th17 cells in vitro [9–11]. In addition, Th17 cells and their effector cytokines are increasingly being recognized as key players in inflammation, autoimmune diseases and graft-versus-host disease (GVHD) [12–14].

Th17-related cytokines such as IL-17, IL-6 and IL-1β have been found in patients with PNS, including those with minimal change nephrotic syndrome (MCNS) [15–17]. These findings suggested Th17 cells may also play an active role in nephrotic syndrome pathogenesis.

The study reported here was designed to investigate the possible involvement of Th17 cells and regulatory T (Treg) cells in PNS. The population of Th17 cells and Treg cells as a percentage of total CD4+ cells was evaluated by flow cytometric analysis. We also determined the concentrations of IL-1β and IL-6 cytokines in serum, the mRNA expressions of Th17-related factors (IL-17, IL-23p19 and RORc) in peripheral blood mononuclear cells (PBMCs), and protein expression of the inflammatory mediators IL-17 and TGF-β1 in kidney biopsy samples from patients with PNS, and evaluated the potential association between Th17 cells and Treg cells in patients with PNS.

Materials and methods

Patients

Peripheral blood was collected from 36 pediatric patients with PNS, ten patients with isolated hematuria, and 16 healthy volunteers. Kidney biopsy samples were obtained from all 36 patients with PNS and the ten patients with isolated hematuria. The patients were divided into two groups: those with PNS ($n=36$) based on diagnostic criteria of: massive proteinuria (>100 mg/kg per day), hypoalbu-

minemia (albumin <25 g/l), hyperlipidemia, and edema (all indicating a disease limited to the kidney) and those with isolated hematuria (corresponding to standard classification criteria; $n=10$). Sixteen healthy children were selected as controls to provide normal blood samples. For the immunohistochemical analysis, there were also five samples of normal kidney tissues that had been obtained from normal kidney tissues in patients undergoing surgery. None of the patients received cortisone during this study. The characteristics of the study cohort are summarized in Table 1. This study was approved by the ethical committee of Chongqing Medical University, and written informed consent was obtained from all individuals.

Cell and sample preparations

PBMCs were obtained by standard Ficoll–Hypaque density centrifugation of heparinized peripheral blood obtained from the subjects. The cells were centrifuged at 1500 rpm for 5 min. For the analysis of Th17 and Treg, the PBMCs were aliquoted into tubes for further staining.

For the analysis of Th17, PBMCs were suspended at a density of 2×10^6 cells/ml in complete culture medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10% heat-inactivated fetal calf serum; Gibco BRL, Gaithersburg, MD). The cell suspension was transferred to each well of 24-well plates. Cultures were stimulated with phorbol myristate acetate (PMA, 50 ng/ml) plus ionomycin (1 μM) for 4 h in the presence of monensin (500 ng/ml; all from Alexis Biochemicals, San Diego, CA). The incubator was set at 37°C under a 5% CO_2 environment. After 4 h of culture, the contents of the well were transferred to 5-ml sterile tubes and centrifuged at 1500 rpm for 5 min. For the analysis of Treg, PBMCs were aliquoted into tubes for further staining. For the analysis of IL-17, RORc, IL-23, IL-1β mRNA, and

Table 1 The characteristics of the patient cohort

Characteristics	PNS group ($n=36$)	Isolated hematuria group ($n=10$)	Normal control group ($n=16$)
Age (years)	6.8±1.70	5.9±2.19	5.6±1.20
Sex (male/female)	26/10	7/3	10/6
Histological classification[a]			
Minor change	0	5/10	
Focal glomerulonephritis	0	4/10	
MCNS	5/36	0	
MsPGN	21/36	1/10	
FSGS	5/36	0	
MN	5/36	0	

PNS, Primary nephrotic syndrome; MCNS, minimal change nephritic syndrome; MN, membranous nephropathy; MsPGN, mesangial proliferative glomerulonephritis; FSGS, focal segmental glomerulosclerosis

[a] Histological classification was according to World Health Organization (WHO) standards

PBMCs, the cells were suspended at a density of 2×10^6 cells/ml in complete culture medium. The cell suspension was transferred to each well of 24-well plates. Cultures were stimulated with phorbol myristate acetate (PHA, 10 ng/ml) for 24 h. The incubator was set at 37°C under a 5% CO_2 environment. After 24 h of culture, the contents of the well were transferred to 1.5-ml tubes and centrifuged at 1500 rpm for 10 min. Total RNA was extracted from the PBMCs. Fresh kidney biopsy tissues obtained from patients were fixed in 4% paraformaldehyde for routine histological examination and immunohistochemical study.

Flow cytometric analysis of Th17 and Treg

Cells were equated into tubes and washed once in phosphate-buffered saline (PBS). For the Th17 analysis, the cells were incubated with phycoerythrin (PE) anti-human CD4 (eBioscience, San Diego, CA) at 4°C for 20 min. For the Treg analysis, the cells were incubated with fluorescein isothiocyanate (FITC) anti-human CD4 and PE CD25 at 4°C for 30 min. Following surface staining, the cells were fixed and permeabilized according to the manufacturer's instructions, and then stained with PE anti-human IL-17A for Th17 detection or PC5 anti-human Foxp3 for Treg detection. Isotype controls were treated to enable correct compensation and confirm antibody specificity. All of the antibodies (Abs) and reagents were from eBioscience. Stained cells were analyzed by flow cytometric analysis.

RNA isolation and reverse transcription–PCR) analyses

In total, 2×10^6 PBMCs were stimulated for 24 h with 10 ng/ml PHA (L8754, Sigma–Lectin) in 24-well plates. Upon harvest, the cells were equated into tubes and washed once in PBS. RNA was prepared with the TIANgen RNA extraction kit (Tiangen, China). The purity and integrity of the samples were confirmed by gel electrophoresis and ethidium bromide staining. Quantification of RNA was based on spectrophotometric analysis at 260/280 nm. Then, 2 μg of total RNA was reverse-transcribed with 0.5 μl reverse transcriptase (DRR037S; TaKaRa, China) using random 6-mers and oligo dT primer as primers in a 10-μl reaction mixture, following the manufacturer's instructions. The cDNA was used as a template in a multiplex PCR reaction with the housekeeping β-actin gene as an internal control. The PCR reaction mixture (25 μl) contained 0.25 mM deoxynucleoside triphosphate (dNTP), 1.5 mM $MgCl_2$, 0.05 U Taq polymerase, 10 mM Tris-HCl (pH 8.3), and 50 mM KCl (all from TIANGEN, China), 50 pmol of each rat-specific oligonucleotide primer (all from Invitrogen, China), and reverse transcriptase (RT) products (1/5 of RT reaction). The primers were: IL-17 (sense: 5'-ATGA CTCCTGGGAAGACCTCATTG-3', antisense: 5'-TTAG GCCACATGGTGGACAATCGG-3'); RORc (sense: 5'-GCTGGTTAGGATGTGCCG-3', antisense: 5'-GGA TGCTTTGGCGATGA-3'); IL-23P19 (sense: 5'-TGTGGA GATGGCTGTGAC-3', antisense: 5'-TTGAAGCGGAG AAGGAGA-3'); β-actin (sense: 5'-CATGTACGTTGCT ATCCAGGC-3', antisense: 5'-CTCCTTAATGTCACGC ACGAT-3').

The sizes of the reaction products were 467 bp for IL-17, 304 bp for RORc, 270 bp for IL-23P19, and 250 bp for β-actin. The PCR amplification conditions for IL-17 and β-actin included an intial heating of the samples to 94°C for 4 min, followed by 30 temperature cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min; this was followed by an extension for another 5 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gel containing 0.005% GoldView, and the gels were photographed under ultra-violet light. The bands were visualized with Quantity One ChemiDocXRS (Bio-Rad, Hercules, CA) and quantified by image analysis software (Quantity One). The relative intensity of the bands for IL-17 and the other products were normalized using the intensity of β-actin by calculating the ratio of the intensity values of each target product to that of β-actin. A 2-μg aliquot of total cellular RNA was reversely transcribed in a 10-μl volume using random hexamer as a primer. A cDNA equivalent of 1 ng RNA was amplified by PCR for Th17-related factors, as described previously.

Enzyme-linked immunosorbent assay

The serum concentrations of IL-1β and IL-6 were measured by an enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (Jingmei, China). All samples were measured twice.

Immunohistochemical analysis of kidney biopsy samples of IL-17 and TGF-β1

Immunohistochemistry (two-step method) was used to observe IL-17 and TGF-β1 expression. In brief, renal cortical paraffin sections (3 μm) were deparaffinized and rehydrated, treated with 0.3% H_2O_2 in methanol for 15 min to quench endogenous peroxide activity, and boiled at 100°C for 10 min in 10 mM citrate buffer (pH 6.0) to unmask antigens. Sections were incubated with rabbit anti-human IL-17 antibody (working dilution 1:100; sc-7927; Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-human TGF-β1 (working dilution 1:100; sc-146; Santa Cruz Technology) at 4°C overnight, followed by incubation with horseradish peroxidase-labeled polymer conjugated to secondary goat anti-rabbit antibody at 37°C for 30 min (PV-6001; Zhongshan, China). Sections were visualized with diaminobenzidene

(DAB) reagent and counterstained with hematoxylin. The PBS was substituted for primary antibodies as a negative control, whereas the positive control was from confirmed positive tissue specimens. Quantification of IL-17 and TGF-$\beta 1$ immunostaining was performed by calculating the proportion of area occupied by the brown staining in all glomeruli or tubular area per section using the Image-Pro Plus System associated with a video camera and computer as described previously by Qin [18].

Statistical analysis

The software used for statistical analysis was SPSS for Windows ver. 13.0 (SPSS, Chicago, IL). Data are presented as mean ± standard deviation (SD). Differences between the values were determined using Student's t test. Data of groups were analyzed using one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. Qualitative data were analyzed by the Kruskal–Wallis test. A value of $P<0.05$ was regarded as a significant difference.

Results

Circulating Th17 frequencies increased in patients with primary nephrotic syndrome

The prevalence of Th17 cells in the PBMCs of patients with PNS ($n=20$) and isolated hematuria ($n=7$) and in healthy donors ($n=8$) was investigated. As shown in Fig. 1, the frequencies of Th17 (CD4+IL17+/CD4+T cells) were markedly higher in patients with PNS ($0.9865\pm0.0886\%$) than in those with hematuria ($0.4433\pm0.0430\%$) and in the normal control ($0.4790\pm0.0560\%$) (all $P<0.01$), while there

was no obvious difference between the isolated hematuria patients and the controls ($P>0.05$).

Decreased populations of Treg cells in peripheral blood of patients with primary nephrotic syndrome

The prevalence of Treg cells in the PBMCs of patients with PNS ($n=36$) and isolated hematuria ($n=10$) and in healthy donors ($n=16$) was investigated. The proportion of Treg cells in total CD4+ cells was evaluated by flow cytometric analysis. Representative plots showed that the populations of Treg cells in gated CD4+ cells decreased in the patients with PNS in comparison with the healthy volunteers and patients with isolated hematuria (Table 2, Fig. 2).

Summarized data from all individuals indicated that the proportion of peripheral blood Treg cells in PNS patients was significantly lower than that in healthy donors (1.941 ± 0.613 vs. 4.029 ± 0.261, respectively; $P<0.001$; Table 2, Fig. 2). Moreover, patients with PNS had an distinct lower percentage of Treg cells than patients with isolated hematuria (1.941 ± 0.613 vs. 3.806 ± 0.3489, respectively; $P<0.01$), as shown in Table 2 and Fig. 2. These observations indicate that the patients with PNS had a predominantly decreased population of Treg cells in the PBMCs.

Expression of IL-17, IL-23p19, and RORc mRNA in PBMC of patients with PNS

mRNA expression levels of Th17-related factors in PBMCs from patients with PNS ($n=36$) were investigated by RT-PCR. The synchronous expression of IL-17 and RORC mRNA was detected in 20 of the 36 samples, and increased
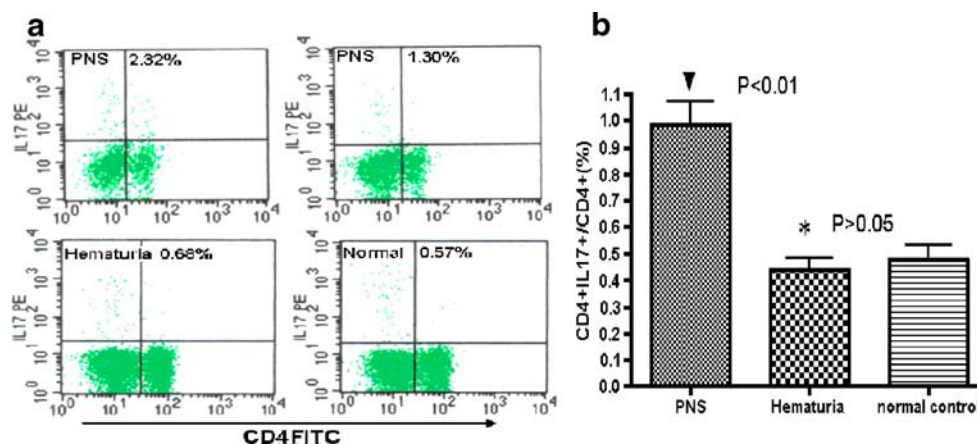
Fig. 1 Circulating Th17 (CD4+IL17+/CD4+T cells) frequencies increased in patients with primary nephrotic syndrome (*PNS*). **a** Representative fluorescence-activated cell sorting (FACS) pictures are from a single patient in each group, **b** collective analyses of results from all three groups. The percentage of positive cells is shown in each panel. *Arrowhead* indicates $P<0.01$ PNS vs. hematuria or normal control; *$P>0.05$ hematuria vs. normal control. *FITC* Fluorescein isothiocyanate, *PE* phycoerythrin, *IL* interleukin

Table 2 CD4+CD25+Foxp3+ Treg decreased in patients with PNS

	PNS ($n=36$)	Isolated hematuria ($n=10$)	Normal ($n=16$)
CD4+CD25+/CD4+ T cells (%)	6.8816±0.6969	7.78±1.55	8.118±1.906
CD4+CD25+Foxp3+/CD4+T cells (%)	1.941±0.613[#]	3.806±0.3489[##]	4.029±0.261*

PNS vs. normal: [#] $P<0.001$; PNS vs. isolated hematuria: [##] $P<0.01$; isolated hematuria vs. normal control: * $P>0.05$

Values are expressed as mean ± standard deviation (SD)

expression of IL-23p19 mRNA was observed in 33 of the 36 specimens. Only one tissue sample from isolated hematuria ($n=310$) and normal ($n=316$) patients showed this expression. These observations indicate that increased levels of Th17 cell-related factors were present in the PBMCs from patients with PNS. As shown in Table 3 and Fig. 3, patients with PNS had an increased expression of IL-17, IL-23P19, and RORc mRNA in comparison to healthy donors ($P<0.001$). In addition, there was a significant difference in the expression of IL-17, IL-23P19, and RORc mRNA between the PNS and isolated hematuria patients ($P<0.001$), but there was no significant difference in the expression of IL-17, IL-23P19, and RORc mRNA between the isolated hematuria patients and the normal controls ($P>0.05$).
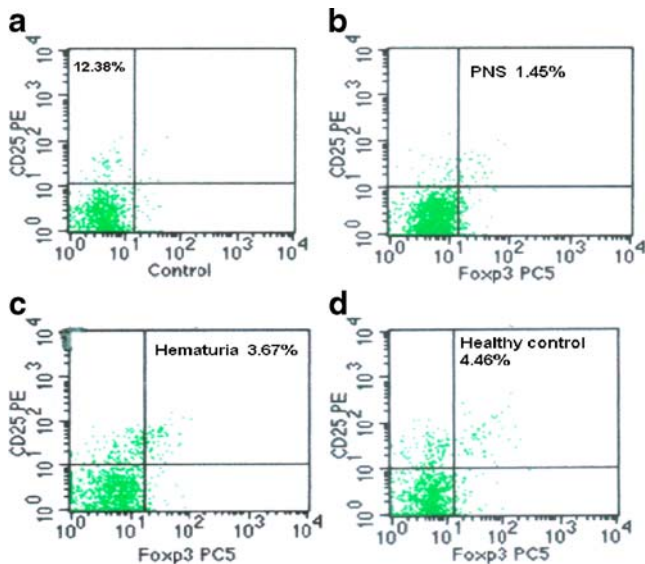
Increased IL-6 and IL-1β concentrations in serum with PNS

Serum IL-6 and IL-1β were determined in patients with PNS ($n=36$), patients with isolated hematuria ($n =10$) and

Fig. 2 Circulating regulatory T cell (Treg) frequencies decreased in patients with primary nephrotic syndrome (*PNS*). **a** CD4+CD25+T subsets were gated by flow cytometry. Plots in the *intern box* represent CD4+CD25+T cells. **b**, **c**, **d** Representative Foxp3 expression in CD4+CD25+T subsets from each group: **b** PNS, **c** isolated hematuria, **d** normal control. The percentage of positive cells is shown in each panel

normal controls ($n=16$). As shown in Fig. 4, cytokine concentrations were significantly higher in the patients than that in the control subjects (IL-6: 3.943±1.187 vs. 0.056± 0.024 pg/ml; $P<0.001$; IL-1β: 9.011±0.5078 vs. 2.079± 0.3526 pg/ml; $P<0.001$). There was no significant difference in IL-6 or IL-1β concentrations between patients with isolated hematuria and the normal controls (IL-6: 0.3330± 0.121 vs. 0.056±0.024 pg/ml; $P>0.05$; IL-1β: 3.606± 0.670 pg/ml vs. 2.079±0.3526 pg/ml; $P>0.05$). These results were consistent with the increased prevalence of IL-17, IL-23, and RORc mRNA in the PBMCs of patients with PNS following stimulation with PHA for 24 h.

Expression of IL-17 and TGF-β1 protein in kidneys of patients with PNS

The expression of IL-17 protein increased in patients with PNS when compared with that in the control subjects (12.54±0.7872 vs. 5.474±0.8144%; respectively; $P<0.001$; Table 4). This increase was remarkably in patients with FSGS with apparent tubular-interstitium injury, and IL-17 was found mainly in the glomerular cells and renal tubular epithelial cells. Patients with MCNS, MsPGN, MN, or FSGS showed an increasing trend in glomerulus and tubule immunostaining for IL-17 expression when compared with the controls (FSGS: 21.91±1.996 vs. 5.474±1.821%; MN: 18.94±2.366 vs. 5.474±1.821%; MsPGN: 10.50± 1.550 vs. 5.474±1.821%; MCNS: 7.084±1.122 vs. 5.474± 1.821%; all $P<0.01$; Table 5). The expression of IL-17 protein was significantly higher in FSGS, MN, and MsPGN patients with an apparent tubular-interstitium lesion than in MCNS and MsPGN patients without a tubular-interstitium alteration or in healthy subjects. No significant differences were found between normal controls and isolated hematuria patients (isolated hematuria 6.569±0.2774 vs. 5.474± 0.8144%, (control); $P>0.05$; Table 4). Moreover, the renal concentration of TGF-β1 was also found mainly in glomerular cells and renal tubular epithelial cells. Similar to the expression of IL-17 in the kidney, the expression of TGF-β1 protein increased in biopsy tissue from PNS kidneys when compared with that from the control subjects (14.640±0.3412 vs. 5.768±0.7812%, respectively; $P<0.01$; Table 4). Patients with MCNS, MsPGN, MN, or FSGS showed an increasing trend in glomerulus and tubulus

Table 3 Expression of IL-17, IL-23p19, and RORc mRNA in the PBMCs of patients with PNS and isolated hematuria

Th17-related factors	PNS mRNA (mean ± SD)	Isolated hematuria mRNA (mean ± SD)	Normal control mRNA (mean ± SD)
IL-17/β-actin	0.9742±0.0182*	0.4471±0.0175**	0.4379±0.0129***
IL-23p19/β-actin	0.9058±0.0173*	0.5105±0.0290**	0.4503±0.0259***
RORc/β-actin	0.5211±0.0583*	0.3071±0.0184**	0.2515±0.0126***

PBMCs, Peripheral blood mononuclear cells; IL, interleukin; RORc, retinoid orphan nuclear receptor; SD, standard deviation

Increased expressions of IL-17, IL23p19, and RORc mRNA in peripheral blood of patients with PNS. PNS vs. normal: *$P<0.001$; PNS vs. isolated hematuria: **$P<0.001$; isolated hematuria vs. normal control: ***$P>0.05$

immunostaining for TGF-β1 expression when compared with the controls (FSGS: 20.21±2.120 vs. 5.768±0.7812%; MN: 17.61±0.5561 vs. 5.768±0.7812%; MsPGN: 11.90± 2.730 vs. 5.768±0.7812%; MCNS: 6.849±0.7545 vs. 5.768±0.7812%; all $P<0.01$; Table 5). No significant differences were found between normal controls and isolated hematuria patients (isolated hematuria vs. control, $P>0.05$, Table 4).
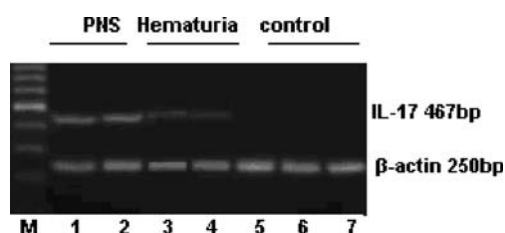
Discussion

Even though Shalhoub proposed in 1974 [19] that MCNS is a disorder of T-cell dysfunction, the nature and role of T-cells (including Th1, Th2, Treg, and Th17 cells) in PNS immunity remains unclear. The increased Th17 and decreased Treg levels may induce a number of autoimmune diseases, malignant tumors, and transplant rejection [20]. Indeed, the precise involvement of Th17 and Treg cells in PNS needs to be further elucidated.

The Th17/Treg balance maintains normal immune response. Any imbalance of Th17/Treg with increased Th17 cells and/or decreased Treg cells may induce local tissue inflammation, which in turn may be important in the pathogenesis of proteinuria emergence and the onset and progress of PNS. To assess whether this balance was disrupted in patients with PNS, we investigated Th17/Treg

Fig. 3 Increased expressions of IL-17 mRNA in peripheral blood of patients with PNS. The graph represents the IL-17 mRNA levels from reverse transcription (RT)-PCR relative to the housekeeping gene, β-actin in PBMCs of patients with PNS, isolated hematuria and in the normal controls. *Lanes*: *M* Molecular weight marker, *1*, *2* PNS, *3*, *4* isolated hematuria, *5–7* normal controls
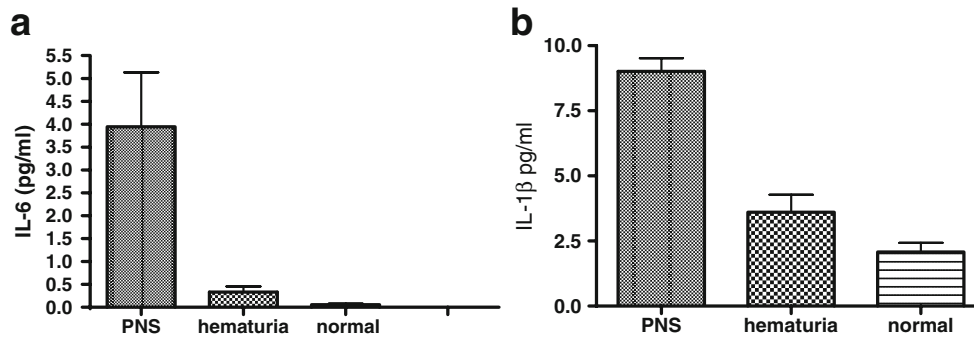
functions on different levels, including cell frequencies, key transcription factors, and related cytokine secretion in PBMCs and kidney biopsy tissue.

We found that the frequency of Treg cells was significantly lower in patients with PNS than in normal controls and that it was markedly decreased in patients with PNS compared to patients with isolated hematuria. These results suggest that Treg cells plays a potentially protective role in proteinuria emergence and chronic development of PNS. Treg cells exert their function partly through the secretion of anti-inflammatory cytokines (IL-10 and TGF-β1), while TGF-β1 may promote the expression of Foxp3 and induce the differentiation of Treg cells that which originate from CD4+CD25−T cells [21, 22]. Interleukin-1β, IL-6, and IL-23 are the inducers and TGF-β is the inhibitor of Th17 differentiation in humans [23]. In this study, we measured the expression of IL-17 and TGF-β1 protein in kidney biopsy tissue of PNS patients and found that the levels of both IL-17 and TGF-β1 increased in the PNS group. However, we did not observe a significantly positive correlation between TGF-β1 and the prevalence of Treg cells in PNS tissue; rather, there was a negative correlation between Treg and TGF-β1. The different objects of study may account for these discrepant results. Thus, the role of TGF-β1 in inducing the differentiation of Treg cells originating from CD4+CD25− T cells in children with PNS requires further investigation.

The Th17 cell has a lineage that is distinctly different from that of the Th1 and Th2 cells which regulate tissue inflammation. Its identification emerges from the discovery of a new type of cytokine, IL-17 [13]. Th17 cells expressing RORc play critical roles in the development of autoimmunity and allergic reactions by producing IL-17 and, to a lesser extent, TNF-α and IL-6 [7, 13]. Interleukin-17, the hallmark of Th17 cells, acts in vitro and in vivo as a proinflammatory cytokine [12]. Mouse Th17 cells are differentiated from naive T cells in response to IL-6 plus TGF-β and need the presence of IL-23 for their expansion and/or maintenance [2, 4]. However, TGF-β1 plus IL-6 can not induce the differentiation of human Th17 from naive CD4+ T cells, while IL-23 is an important inducer of the differentiation of human Th17 cells [23, 24]. Consequently,

a

b

Fig. 4 Increased concentration of serum IL-6 (**a**) and IL-1β (**b**) in patients with PNS. Patients with PNS had an increased concentration of IL-6 and IL-1β in comparison to the normal controls and isolated hematuria patients ($P<0.001$; $P<0.001$). There was a significant difference in the concentration of IL-6 and IL-1β between the PNS and isolated hematuria patients ($P<0.001$; $P<0.001$), but there was no significant difference in the expression of IL-6 and IL-1β between the isolated hematuria patients and normal controls ($P>0.05$)

IL-1β, IL-6 and IL-23 are the inducers and TGF-β is the inhibitor of Th17 differentiation in humans [23]. Moreover, IL-6 and IL-23 have been identified as inflammation-associated cytokines because they promote the incidence and growth of inflammation.

Our results show that patients with PNS exhibited a significant increase in the frequency of Th17 cells, Th17-related cytokines (IL-17, IL-1β, IL-6, and IL-23) and RORc levels compared with patients with isolated hematuria and normal subjects, thereby demonstrating that the presence and differentiation of Th17 cells play a prominent role in the pathogenesis of PNS. Moreover, the increased expression of IL-17 protein in kidney biopsy tissue may suggest that an increased number of cells that mainly produce IL-17 may play a role in the PNS microenvironment. Intriguingly, the increase in IL-17 expression was associated with kidney pathology, especially in patients with FSGS, MN, and MsPGN with apparently renal tubular-interstitium injury. Our data show that Th17 cells and Th17-related factors and cytokines are involved in the development or progression of kidney disease in children with PNS, suggesting that Th17 cells may potentially mediate proteinuria emergence and renal

tubular-interstitium lesion. Thus, Th17 may participate in the onset of the inflammatory process and chronic progression in PNS.

It is not clear whether Th17/Treg imbalance contributes to the development of PNS or whether tubule-interstitial alteration caused by severe proteinuria leads to the imbalance. We found that there was a correlation between proteinuria and the reduced number of Treg cells and the increment in IL-17 protein expression in the kidney tissue of PNS patients. It has been reported that overload proteinuria is followed by renal infiltration of the immune cells [25] and that lymphocytes actively participate in the progression of chronic kidney disease. Based on our results, we speculate that the reduction in Treg cells and increment of cells that mainly produce IL-17 may be involved in the pathogenesis of proteinuria production and progressive renal damage.

Table 4 Immunohistochemistry assessment of IL-17 and TGF-β1 in kidney tissue of patients with PNS

Th17-related factors	PNS ($n=36$)	Isolated hematuria ($n=10$)	Normal control ($n=5$)
IL-17 ($\overline{x} \pm s$)%	12.540± 0.7872*	6.569±0.2774**	5.474±0.8144***
TGF-β1 ($\overline{x} \pm s$)%	14.640± 0.3412*	6.461±0.3412**	5.768±0.7812***

TGF-β1, Transforming growth factor-β1

Increased expressions of IL-17 and TGF-β1 protein in kidney biopsy tissue of patients with PNS. PNS vs. normal control: *$P<0.001$; PNS vs. isolated hematuria: **$P<0.001$; isolated hematuria vs. normal control: ***$P>0.05$

Table 5 Immunohistochemistry assessment of IL-17 and TGF-β1 in kidney tissue of different histological classifications

Histological classification	n	IL-17 ($\overline{x} \pm s$) (%)	TGF-β1 ($\overline{x} \pm s$) (%)
Normal control	5	5.474±1.821	5.768±0.7812
Minor change	5	6.156±0.5134**	6.676±1.941
Focal glomerulonephritis	4	6.559±0.4435**	6.470±1.518
MCNS	5	7.084±1.122*	6.849±0.7545
MsPGN	22	10.50±1.550*	11.90±2.730
MN	5	18.94±2.366*	17.61±0.5561
FSGS	5	21.91±1.996*	20.21±2.120

Increased expressions of IL-17, TGF-β1 protein in kidney biopsy tissue of patients with PNS. Patients with PNS had an increased expression tend of IL-17 and TGF-β1 protein in comparison to controls (FSGS > MN > MsPGN > MCNS, vs. normal control; *P all <0.001). There were no significant differences between the isolated hematuria patients and normal controls (**$P>0.05$)

This is the first report on the relationship between Th17 cells and Treg cells from cells, related-cytokines, transcription factor levels, and kidney biopsy tissues in children with PNS.

In summary, our data demonstrate that a Th17/Treg functional imbalance exists in patients with PNS and that Th17 and Treg cells are in a dynamic equilibrium in children with nephrotic syndrome following PNS development with apparent renal tubular-interstitium lesion. Therefore, the dynamic interaction between Th17 and Treg cells may be important in the development of PNS, suggesting a potential role for Th17/Treg imbalance in the pathogenesis of proteinuria emergence and the onset and development of PNS. Because of the reciprocal developmental pathway for the generation of Th17 and Treg cells, and their opposing effects, Th17/Treg subsets may have been evolved to induce or regulate tissue inflammation, analogous to the dichotomy of Th1/Th2 T-cell subsets [2, 26]. We need to prove our conclusion in a larger scale of the population, and ongoing efforts should be made to identify the precise effect and mechanism of Th17/Treg imbalance in PNS. A better understanding of the nature, regulation, and function of Th17 cells and Treg cells in PNS immunity may provide a new target for the treatment on children with PNS and even facilitate the development of a more novel and effective immunotherapy for PNS.

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