Original Article

Change of circulating lymphocyte subsets is related to disease activity and secondary infection in children with primary nephrotic syndrome—a retrospective study

Chuangkun Li[1,2#], Nisha Wu[1,3#^], Jingtong Huang[1,2#], Ying Gong[1,2], Hongxia Wang[1,2], Yunfei Liu[1,2], Chunfeng Wu[1,4], Lei Zheng[1,2], Shuai Chu[1,2]

[1]Department of Laboratory Medicine, Nanfang Hospital, Southern Medical University, Guangzhou, China; [2]Guangdong Engineering and Technology Research Center for Rapid Diagnostic Biosensors, Nanfang Hospital, Southern Medical University, Guangzhou, China; [3]Department of Clinical Laboratory, The Fifth Affiliated Hospital, Southern Medical University, Guangzhou, China; [4]Department of Laboratory Medicine, Lian Jiang People's Hospital, Zhanjiang, China

Contributions: (I) Conception and design: S Chu, L Zheng; (II) Administrative support: L Zheng; (III) Provision of study materials or patients: S Chu, J Huang; (IV) Collection and assembly data: C Li, N Wu, Y Liu; (V) Data analysis and interpretation: Y Gong, H Wang, C Wu; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Shuai Chu. Department of Laboratory Medicine, Nanfang Hospital, Southern Medical University, Guangzhou, China. Email: shine9533@smu.edu.cn; Lei Zheng. Department of Laboratory Medicine, Nanfang Hospital, Southern Medical University, Guangzhou, China. Email: nfyyzhenglei@smu.edu.cn.

Background: Primary nephrotic syndrome (PNS) is an immune-mediated glomerular disease that often reoccurs. However, the characteristics of circulating lymphocyte subsets in PNS children remain unclear. Immunosuppressive therapy can lead to temporary or persistent remissions, but also increases the risk of infection, and whether the circulating lymphocyte subsets can be used to predict the secondary infection also remains unclear. Here, we explored the distribution of lymphocyte subpopulations in the different stages of PNS, and its predictive value of secondary infection in pediatric patients.

Methods: We included 89 children who were first PNS episodes or diagnosed with PNS admitted to Nanfang Hospital from September 2019 to April 2021, and 19 healthy children were recruited as controls (C). PNS patients were divided into three groups according to their serum biochemical tests: active group (A), partial remission (PR) group, and complete remission (CR) group. PNS patients with infection symptoms were divided into a co-infection group, others were divided into the non-infection group. The peripheral lymphocyte subsets were analyzed by flow cytometry. The relationship between the peripheral lymphocyte subsets and PNS activity or infection was analyzed.

Results: Compared to the healthy controls, the PNS patients' $CD8^+CD28^+$ T cell (T_C) (C: 16.6%, 450.8/μL; A: 29.1%, P=0.000, 886.1/μL, P=0.012; PR: 25.7%, P=0.000, 817.3/μL, P=0.012; CR: 24.9%, P=0.001, 747.9/μL, P=0.020), and $CD4^+CD45RO^+$ ("memory" helper) T cells (C: 13.2%, 358.9/μL; A: 15.7%, P=0.036, 578.7/μL, P=0.001; PR: 17.6%, P=0.002, 610.0/μL, P=0.000; CR: 13.7%, P=0.676, 398.1/μL, P=0.525) were elevated. In addition, the regulatory T cells counts (non-infection: 117.9/μL; Co-infection: 73.3/μL, P=0.001) were significantly lower in patients with infection. We found that the predictive value measured by the area under the curve (AUC) showed that the AUC (t) T_{reg} cell counts (61.5–84.5%) were almost always higher than the AUC for the (t) $CD4^+$ T cell counts (55.1–77.1%).

Conclusions: In this study, we found that T cell subpopulations had different characteristics in PNS during different disease phases. The $CD8^+CD28^+$ T cells, and $CD4^+CD45RO^+$ T cells increased at the disease quiescence of PNS. Moreover, $CD4^+$ T cell subsets (regulatory T cell <82.5/μL) had higher predictive

^ ORCID: 0000-0002-9682-6418.

value than $CD4^+$ T cell counts for PNS infection.

Keywords: Primary nephrotic syndrome (PNS); lymphocyte subpopulations; infectious disease; risk factor

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Introduction

Primary nephrotic syndrome (PNS) is a common chronic illness in childhood (1). Clinically, PNS is a renal syndrome characterized by intense proteinuria, hypoalbuminemia, edema, and hyperlipidemia caused by damage to podocytes of the glomerular filtration barrier (2).

Immunosuppressive therapy can lead to temporary or persistent remissions. Currently, with the increased incidence of steroid-resistant patients, second-line immunosuppressive therapies for remission induction have been recommended, such as cyclophosphamide (CYC), mycophenolate mofetil (MMF), and rituximab (RTX) (3).

Effective treatment in different stages of the disease partly depends on dynamic monitoring of immune functioning. In 1974, Shalhoub *et al.* (4) reported that PNS was a disorder of T-cell homeostasis by inducing lymphocyte-derived permeability factors, which interfered with the expression and function of key podocyte proteins to causing proteinuria. Some studies have demonstrated that corticosteroid medication influences the distribution of lymphocyte subpopulations in childhood PNS (5,6). The T-cell subsets in PNS include Th1/Th2, the CD4/CD8 ratio, NK cells, T_{reg} populations, and cytokine disruption (7). Furthermore, T_{reg} cells, a group of inhibitory T cells, are correlated with the occurrence of proteinuria in patients with PNS (8). NK cells are considered indicators of prognosis in PNS. This evidence indicates that the lymphocyte subpopulations may play a crucial role in the pathogenic progression of PNS (9,10). However, the changes in peripheral lymphocyte subpopulations in children with PNS were also seldom seen.

More than 60% of steroid-sensitive patients experience relapses after being treated with glucocorticoids. Studies have found that glucocorticoids medication influence the distribution of lymphocyte subpopulations in PNS children (5,6). These changes in the distribution of lymphocyte subpopulations are associated with the occurrence of infection in children with PNS, which has become the leading cause of relapse, and some pediatric patients may even develop the end-stage renal disease. Therefore, it is critically important to seek some biomarkers for monitoring immune functions and preventing further infections in pediatric PNS patients.

With the development of precise detection techniques, an increasing number of lymphocyte subpopulations have been discovered. However, the present clinical application of molecular markers, such as $CD4^+$ T cells, has not met the needs of patients. Therefore, a detailed analysis of T cell subpopulations and their vital functions in childhood patients with PNS is necessary.

In the present study, we aimed to compare the distribution of peripheral blood lymphocyte subpopulations in the active, partial remission, and complete remission stages of PNS patients to those of healthy controls. Furthermore, we aimed to explore the relationship between lymphocyte subsets and infectious complications to develop effective and promising biomarkers for diagnosing, monitoring progression, and predicting infection

Highlight box

Key findings

- We found that T cell subpopulations had different characteristics in PNS during different disease phases. Lower T_{reg} cell counts were an independent risk factor for PNS infection.

What is known and what is new?

- Primary nephrotic syndrome (PNS) is an immune-mediated disease while the changes in peripheral lymphocyte subpopulations in children with PNS remain unclear and which subpopulation can be used to predict the secondary infection also remains unclear.
- The $CD8^+$ T cells, T_C/T_S ratio, and $CD4^+CD45RA^+$ T cells increased, while the CD4/CD8 ratio and NK cells count decreased at the onset of PNS. The $CD4^+CD45RA^+/CD4^+CD45RO^+$ ratio was related to disease activity. Moreover, T_{reg} cell/μL <82.5 had a higher predictive value than $CD4^+$ T cell counts for PNS infection.

What is the implication, and what should change now?

- T_{reg} cell counts were independent risk factors of overall infection. Measuring peripheral blood lymphocyte subpopulations should be done to monitor disease progression and predicte infection in children with PNS.

throughout PNS. We present the following article in accordance with the STARD reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-22-581/rc).

Methods

Patients and study design

This study is a single-center observational, retrospective study conducted at the Nanfang Hospital, Southern Medical University (Guangzhou, China). Data of all patients aged 18 years or younger, who visited Nanfang Hospital and were diagnosed with PNS from September 2019 to April 2021 were extracted from the Hospital Information System (HIS) and retrospectively analyzed. Data on peripheral lymphocyte subpopulation from healthy controls who intended to do peripheral blood stem cell donation was collected. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Nanfang Hospital Ethics Committee board (No. NFEC-2022-076) and individual consent for this retrospective analysis was waived.

Enrollment criteria included: (I) age <18 years old, (II) first PNS episode or diagnosed PNS.

The exclusion criteria were as follows: (I) infantile or hereditary nephrotic syndrome; (II) secondary nephrotic syndrome; (III) hepatitis B virus associated glomerulone nephritis, Henoch-Schonlein Purpura and lupus nephritis (IV) function of heart, lung, and liver abnormal seriously.

According to the Improving Global Outcomes (KDIGO) guidelines for kidney disease in 2012 (11), PNS patients were divided into three groups according to their serum biochemical tests: an active group (n=40), a partial remission group (n=30), and a complete remission group (n=19). Nineteen children who intended to do peripheral blood stem cell donation were selected as the control group. The active PNS group (A) was defined by a low serum albumin concentration (<2.5 g/dL) and a high urinary protein excretion (>40 mg/m^2 per hour) with high cholesterol levels. The partial remission (PR) group was according to a serum albumin concentration (<3.5 g/dL) and a urinary protein excretion of <20 mg/m^2 per hour. While the PNS in the complete remission (CR) group was defined as no proteinuria using the colorimetric qualitative test and a urinary protein/creatinine ratio of <0.2 on a random urine sample.

PNS patients with infection symptoms were divided into a co-infection group, others were divided into a non-infection group.

Sample size: the sample size was estimated based on the prior reported study (6).

Blood samples and clinical data

Venous blood specimens were collected in an EDTA-Na_2 anticoagulant tube, and serum was obtained using a coagulation tube. All the tests were completed within 24 hours after the specimens were received. Clinical data such as age, gender, clinical diagnosis, infectious disease status, and biochemical tests that were related to PNS were recorded. For the PNS patients, 24-hour urine protein (UTP), serum albumin (ALB), total serum cholesterol (CHOL), and serum creatinine (CRE) were collected as the biochemical tests.

Reagents

Multitest^{TM} 6-color TBNK (Cat:662995), anti-CD4 (Cat:340133, 341115), anti-CD25 (Cat:340938), anti-CD3 (Cat:340663), anti-CD8 (Cat:335805), anti-CD28 (Cat:348047), anti-CD45RA (Cat:347723), and hemolysin for flow cytometry were purchased from BD Biosciences, USA. Flow tubes and FACS Lysing Solution were also purchased from BD Biosciences, USA. PE-anti-CD127 (Cat: P010034-B) and APC-anti-CD45RO (Cat: P010028-C) were bought from Jiangxi CELGENE Biotechnology Corporation, China.

Flow cytometry

The percentage and absolute counts of lymphocyte subpopulations, including CD3$^+$T cells, CD4$^+$ T cells, CD8$^+$ T cells, B cells, NK cells, Regulatory T cells, cytotoxic T cells, Inhibitory T cells, Naive CD4$^+$ T cells, and Effector memory CD4$^+$ T cells were analyzed on BD Canto II flow cytometry (FCM), according to standard operating procedure. The results were analyzed by BD FACS Diva software v8.02 (Becton, Dickinson and Company, USA).

Statistical analysis

The statistical analysis was conducted by GraphPad Prism 8.0 (GraphPad Software, USA) and SPSS 20.0 (IBM, USA). Results of the data in each group are displayed as means ± standard deviations (mean ± SD). Multiple comparisons

Table 1 Patient characteristics

Group	A	PR	CR	HC
Mean age (years)	9.5	9.9	8.1	9.3
Age range (years)	4–14	5–15	4–15	3–16
Male (case)	31	23	16	10
Female (case)	9	7	3	9
Total (case)	40	30	19	19

A, active group; PR, partial remission group; CR, complete remission group; HC, healthy control.

Table 2 Type and percentage of co-infection

Type of infection	Frequency (case)	Percentage (%)
Upper respiratory tract infection	23	69.70
Pneumonia	5	15.15
Acute bronchitis	1	3.03
EB virus infection	1	3.03
Viral rash	1	3.03
Urinary system infections	1	3.03
Skin fungal infection	1	3.03
Total	33	100.00

EB, Epstein-Barr virus.

were performed with an analysis of variance (ANOVA) and Kruskal–Wallis test followed by Tuckey or Dunn's post-test for sequential pairwise comparisons. The independent samples *t*-test was used for data with a normal distribution. For data with a non-normal distribution, the Mann-Whitney U test was used for the comparisons. A correlation analysis was conducted using Spearman's rank correlation test. Logistic regression was used to analyze the risk factors of PNS with infectious diseases. All statistical tests were two-sided and a P value <0.05 indicated a statistically significant difference.

Results

Patient characteristics

According to the curative effect, all PNS patients were divided into three groups: an active group (A), a partial remission (PR) group, and a complete remission (CR) group. This study included 89 PNS patients (A: mean age 9.5 years, 31 males, 9 females, range: 4–14 years; PR: mean age 9.9 years, 23 males, 7 females, range: 5–15 years; CR: mean age 8.1 years, 16 males, 3 females, range: 4–15 years) and 19 healthy controls (mean age: 9.3 years, 10 males, 9 females, range: 3–16 years) (*Table 1*). A total of 33 PNS patients had infections, of which 23 had upper respiratory tract infection (URTI), five had pneumonia, and there was one case each of acute bronchitis, EB virus infection, virus rash, urinary system infection, and skin and fungal infection (*Table 2*).

No significant difference in lymphocyte subpopulations between different genders

The following lymphocyte subpopulations were studied in all participants: total T lymphocytes ($CD3^+$), $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, total B lymphocytes ($CD3^-CD19^+$), natural killer (NK) lymphocytes ($CD3^-CD16^+CD56^+$), regulatory T lymphocytes (T_{reg}, $CD3^+CD4^+CD25^+CD127^{Low}$), cytotoxic T lymphocytes (T_C, $CD8^+CD28^+$), suppressive T lymphocytes (T_S, $CD8^+CD28^-$), naive $CD4^+$ T lymphocytes ($CD4^+CD45RA^+$), and effector memory T lymphocytes ($CD4^+CD45RO^+$). As shown in *Table 3*, the differences in lymphocyte subpopulations between genders were not statistically significant (P>0.05).

Alteration in lymphocyte subpopulations between PNS patients and healthy controls

A fluorescence-activated cell sorting (FACS) analysis revealed that all groups of patients with PNS had a higher percentage of total lymphocytes and $CD3^+CD8^+$ T cell counts (*Figure 1A*,*1B*). There was no significant difference in $CD4^+$ T cell percentage or counts in PNS patients and healthy controls (*Figure 1C*,*1D*). Meanwhile, the percentage and counts of $CD8^+$ T cell in PNS patients were significantly increased, which caused a decrease in CD4/CD8 ratio (*Figure 1E-1G*). Moreover, there was no significant difference in T_{reg} cell percentage or counts in PNS patients and healthy controls (*Figure 1H*,*1I*). The percentage and the counts of NK cells were significantly decreased in PNS patients compared to healthy controls (*Figure 2A*,*2B*). There was no significant difference in B lymphocyte percentage or counts in PNS patients compared to healthy controls (*Figure 2C*,*2D*). Interestingly, the percentage and counts of T_C cell were significantly increased in PNS patients compared to healthy controls (*Figure 3A*,*3B*), but there was no significant difference in

Table 3 Comparison of lymphocyte subpopulations between male and female PNS patients

Lymphocyte subpopulation	Male (n=71)	Female (n=18)	P value
LYM/μL	3,245.08±1,298.91	3,006.00±1,874.18	0.9064
$CD3^+$ (%)	73.40±8.11	71.96±6.99	0.5605
$CD3^+$/μL	2,398.23±1,030.64	2,152.22±1,338.10	0.7695
$CD3^+CD4^+$ (%)	34.86±7.55	34.12±4.90	0.9946
$CD3^+CD4^+$/μL	1,159.69±578.01	1,018.56±619.53	0.8422
$CD3^+CD8^+$ (%)	32.45±7.52	32.29±4.40	0.4975
$CD3^+CD8^+$/μL	1,042.06±465.37	988.67±719.45	0.8276
CD4/CD8 ratio	1.16±0.48	1.07±0.21	0.6756
B cells (%)	17.54±7.79	17.78±8.89	0.3397
B cells/μL	566.25±325.26	498.22±297.54	0.5715
NK cells (%)	7.96±4.88	9.31±3.78	0.4754
NK cells/μL	244.56±162.27	321.33±330.97	0.7365
T_{reg} (%)	9.42±2.24	8.68±2.89	0.5999
T_{reg}/μL	113.52±67.67	82.61±39.49	0.3488
$CD4^+CD45RA^+$ (%)	17.70±8.14	15.97±6.90	0.5835
$CD4^+CD45RA^+$/μL	618.20±417.79	510.69±413.12	0.6815
$CD4^+CD45RO^+$ (%)	16.00±4.96	16.62±5.54	0.7822
$CD4^+CD45RO^+$/μL	503.84±215.75	443.11±174.79	0.4505
$CD4^+CD45RA^+/CD4^+CD45RO^+$	1.23±0.70	1.17±0.85	0.8785
T_S (%)	26.88±5.66	24.80±5.23	0.3775
T_S/μL	878.02±413.74	726.18±428.49	0.5464
T_C (%)	6.14±3.54	8.28±4.35	0.5563
T_C/μL	188.16±118.20	286.53±334.04	0.2185
T_S/T_C	5.94±3.89	4.12±2.72	0.9857

Results of the data are displayed as means ± standard deviations (mean ± SD). Total T lymphocyte ($CD3^+$), helper T lymphocyte ($CD3^+CD4^+$), cytotoxic T lymphocyte ($CD3^+CD8^+$), total B lymphocyte ($CD19^+$), NK lymphocyte ($CD3^-CD16^+56^+$), regulatory T lymphocyte (T_{reg}, $CD3^+CD4^+CD25^+CD127^{Low}$), cytotoxic T lymphocyte (T_C, $CD8^+CD28^+$), suppressive T lymphocyte (T_S, $CD8^+CD28^-$), naive $CD4^+$ T lymphocyte ($CD4^+CD45RA^+$) and effector memory T lymphocyte ($CD4^+CD45RO^+$). LYM, lymphocyte; PNS, primary nephrotic syndrome; NK, natural killer.

T_S cell percentage or counts (*Figure 3C*,*3D*), indicating that the activation of PNS was related to an increase in T_C cells. In addition, T_C/T_S ratios were significantly increased in PNS patients compared to healthy controls because of the increase in T_C cell (*Figure 3E*).

There was no significant difference in naive T cell percentage and counts (*Figure 4A*,*4B*), but the effector memory $CD4^+$ T cell percentage and counts were significantly increased in PNS patients in the active and partial remission groups compared to healthy controls (*Figure 4C*,*4D*). However, the ratio of naive $CD4^+$ T cells/effector memory $CD4^+$ T cells was significantly increased in PNS patients in the complete remission group compared to the active and partial remission groups (*Figure 4E*). This result suggested that the counts and ratio of naive $CD4^+$ T cells and effector memory $CD4^+$ T cells may be important indicators for judging the progression and remission of PNS in children.

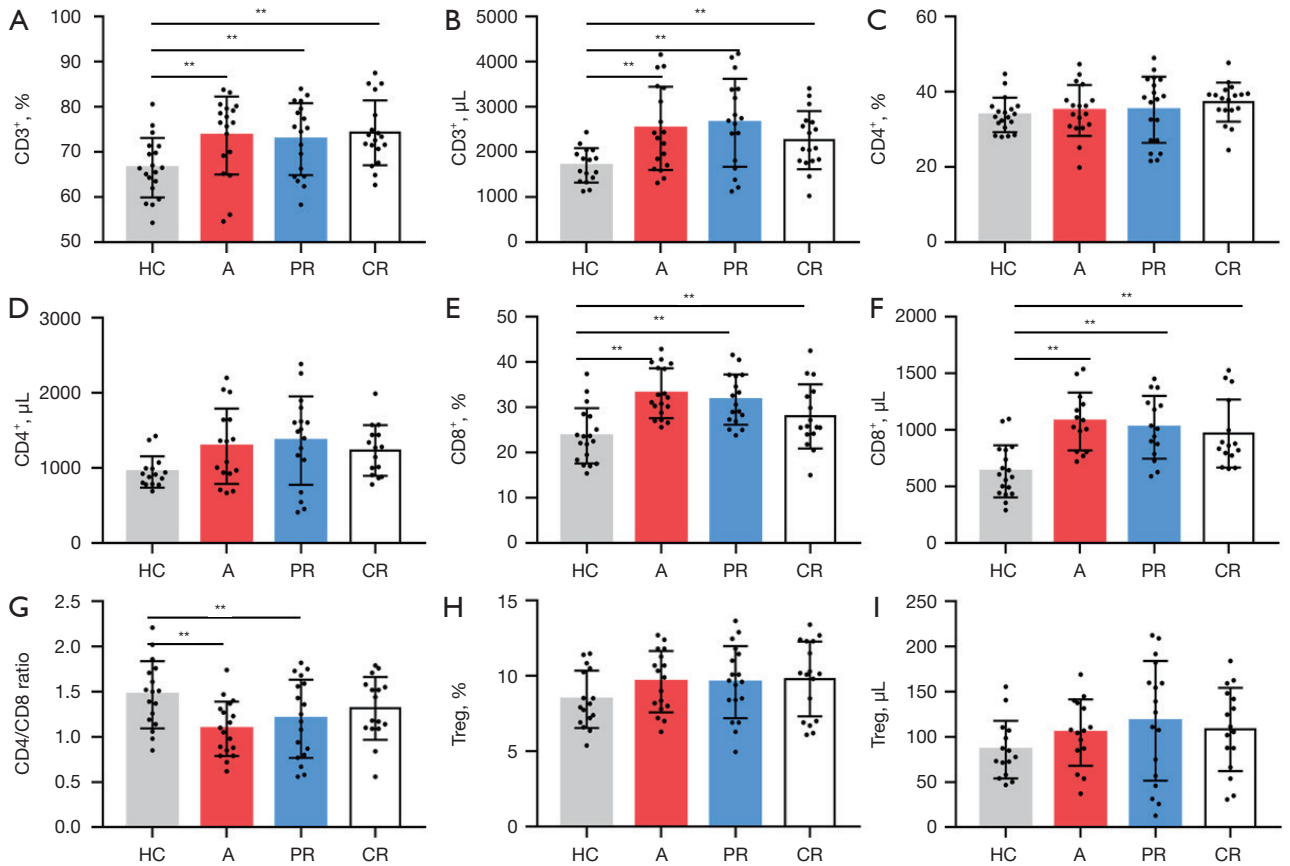

Figure 1 Comparative analysis of T cell subsets (total lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells, $T_{reg,}$ and CD4/CD8 ratio) between PNS patients and healthy control. (A) Percentage of total lymphocytes of PNS patients and healthy controls; (B) total lymphocyte counts of PNS patients and healthy controls; (C) percentage of $CD4^+$ T cells of PNS patients and healthy controls; (D) $CD4^+$ T cell counts of PNS patients and healthy controls; (E) percentage of $CD8^+$ T cells of PNS patients and healthy controls; (F) $CD8^+$ T cell counts of PNS patients and healthy controls; (G) CD4/CD8 ratio of PNS patients and healthy controls; (H) percentage of T_{reg} of PNS patients and healthy controls; (I) T_{reg} cell counts of PNS patients and healthy controls. **, P<0.01. A, active group; PR, partial remission group; CR, complete remission group; HC, healthy control. PNS, primary nephrotic syndrome.

Analysis of risk factors for infection in children with PNS

To evaluate the risk factors for infection in children with PNS, we compared the lymphocyte subpopulation percentage and counts in children without PNS infection and those with co-infections. As is shown in *Figure 5*, the $CD4^+$ T cell percentage and counts, CD4/CD8 ratios, percentage of naive $CD4^+$ T cell counts, and T_{reg} cell counts were significantly lower in patients with infection, which indicated that the reduction of $CD4^+$ T cells, naive $CD4^+$ T cells, and the CD4/CD8 ratios were related to PNS with infection. There was no significant difference between the other lymphocyte subpopulations of co-infection and non-infection (Figure S1). Using the receiver operating curve (ROC) method, curves were plotted to identify the cutoff values that best predicted PNS with infection (*Figure 6A-6D*, and Figure S2A,S2B). We found that lower $CD4^+$ T cell, naive $CD4^+$ T cell, and T_{reg} cell percentages and counts and the CD4/CD8 ratio reached statistical significance in predicting PNS with infection (*Table 4*). Accordingly, lower levels of these lymphocyte subpopulations in the peripheral blood were associated with significantly higher infection rates than subgroups with high levels (percentage of $CD4^+$ T cells: 47.27% *vs.* 18.28%, P<0.01; $CD4^+$ T cell counts: 52.38% *vs.* 20.00%, P<0.01; CD4/CD8 ratio: 52.38% *vs.* 22.22%, P<0.01; naive $CD4^+$ T cell counts: 61.90% *vs.* 20.93%, P<0.001; T_{reg} cell counts: 58.10% *vs.* 17.40%, P<0.001; (*Figure 6E-6H* and Figure S2C,S2D). Among them,

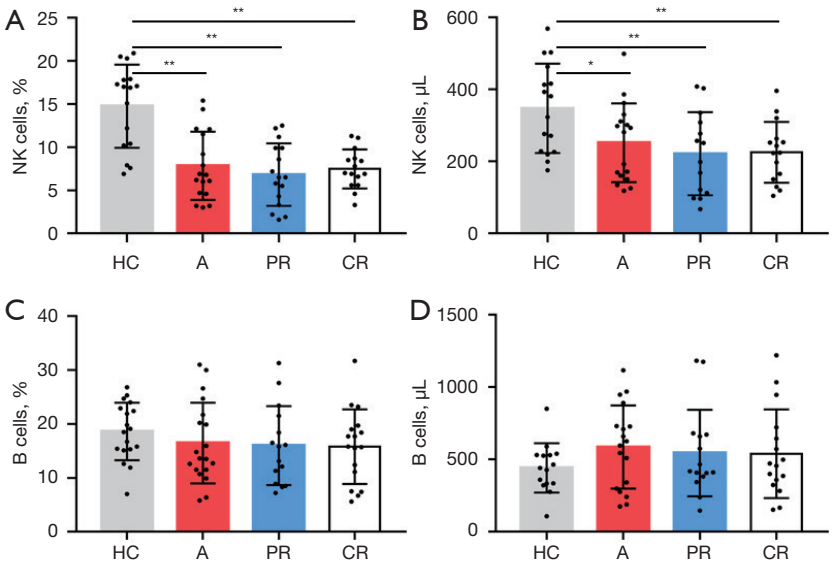

Figure 2 Differences in NK cells and B cells between PNS patients and healthy controls. (A) Percentage of NK cells in PNS patients and healthy controls; (B) NK cell counts of PNS patients and healthy controls; (C) percentage of B cells in PNS patients and healthy controls; (D) B cell count of PNS patients and healthy controls. *, P<0.05, **, P<0.01. A, active group; PR, partial remission group; CR, complete remission group; HC, healthy control. NK, natural killer; PNS, primary nephrotic syndrome.

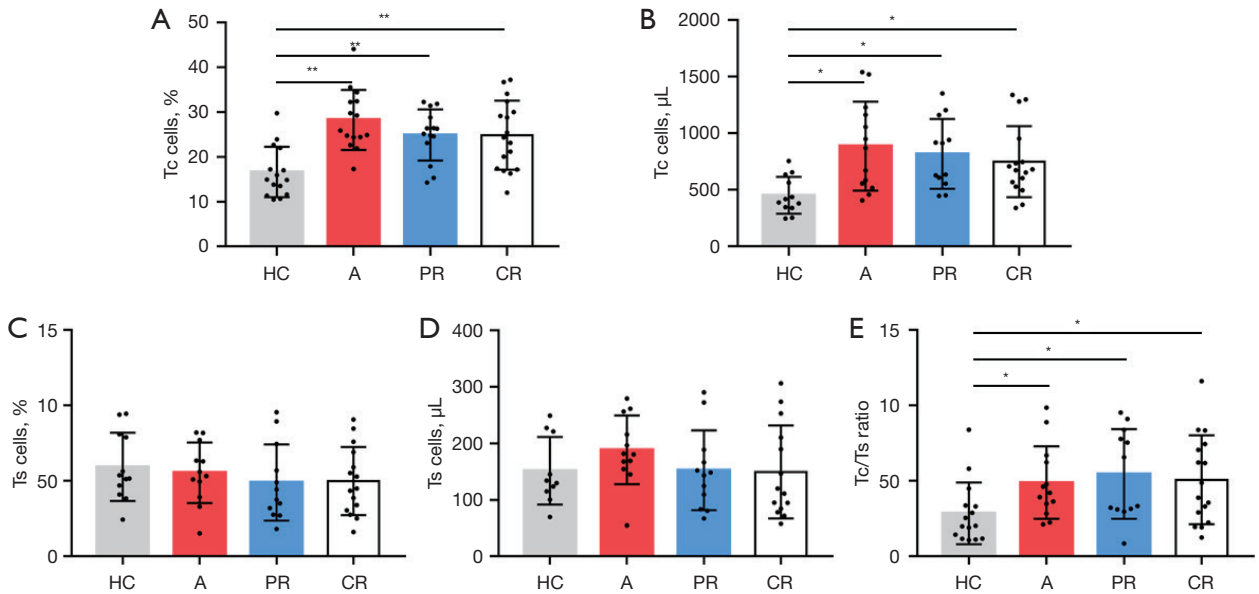

Figure 3 Differences in T_C cells, T_S cells, and the T_C/T_S ratio between PNS patients and healthy controls. (A) Percentage of T_C cells in PNS patients and healthy controls; (B) T_C cell counts of PNS patients and healthy controls; (C) percentage of T_S cells in PNS patients and healthy controls; (D) T_S cell counts of PNS patients and healthy control; (E) T_C/T_S ratio of PNS patients and healthy controls. *, P<0.05, **, P<0.01. A, active group; PR, partial remission group; CR, complete remission group; HC, healthy control. PNS, primary nephrotic syndrome.

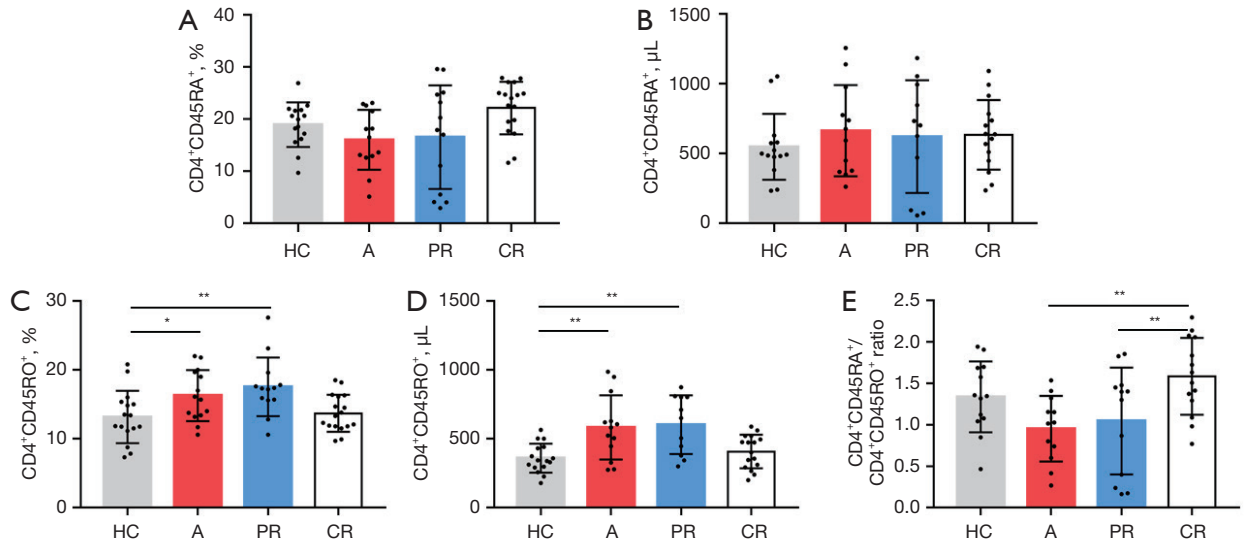

Figure 4 Differences in naive CD4$^+$ T cells, effector memory CD4$^+$ T cells, and the naive CD4$^+$ T cells/effector memory CD4$^+$ T cells ratio between PNS patients and healthy controls. (A) Percentage of naive CD4$^+$ T cells in PNS patients and healthy control; (B) naive CD4$^+$ T cell counts of PNS patients and healthy controls; (C) percentage of effector memory CD4$^+$ T cells in PNS patients and healthy controls; (D) effector memory CD4$^+$ T cell counts of PNS patients and healthy controls; (E) naive CD4$^+$ T cells/effector memory CD4$^+$ T cells ratio of PNS patients and healthy controls. *, P<0.05, **, P<0.01. A, active group; PR, partial remission group; CR, complete remission group; HC, healthy control. PNS, primary nephrotic syndrome.

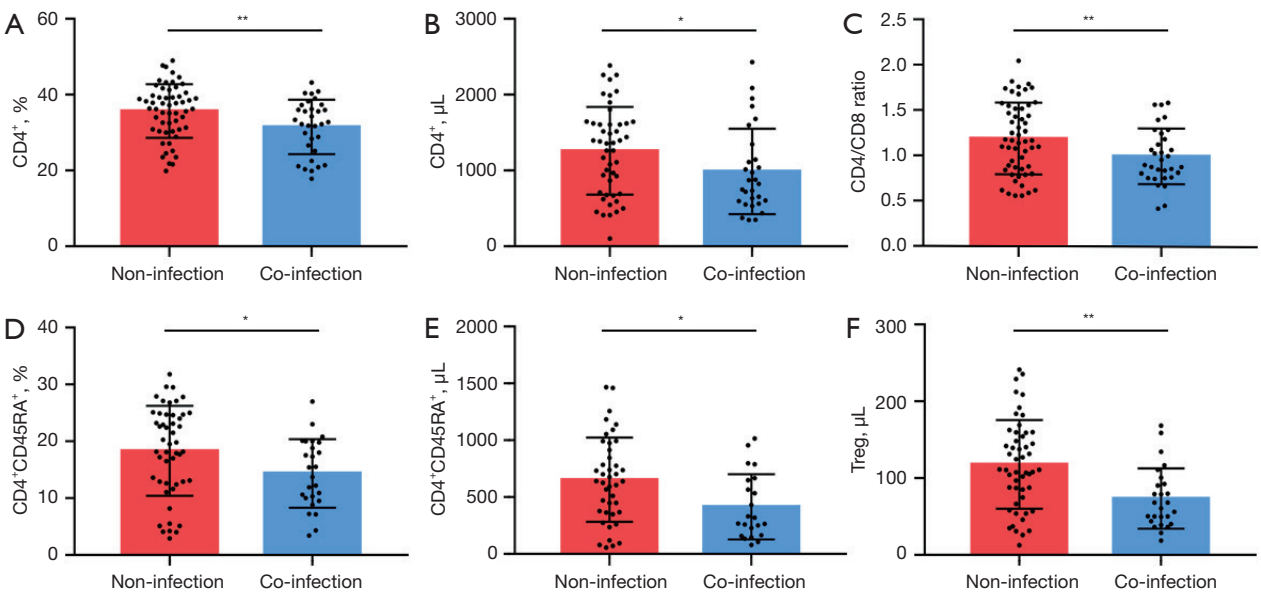

Figure 5 Risk factors for infection in children with primary nephrotic syndrome. (A) Percentage of CD4$^+$ T cells in infectious and non-infectious PNS patients; (B) CD4$^+$ T cell counts of infectious and non-infectious PNS patients; (C) CD4/CD8 ratio of infectious and non-infectious PNS patients; (D) percentage of CD4$^+$CD45RA$^+$ T cells in infectious and non-infectious PNS patients; (E) CD4$^+$CD45RA$^+$ T cell counts of infectious and non-infectious PNS patients; (F) T_{reg} cell counts of infectious and non-infectious PNS patients. *, P<0.05, **, P<0.01. PNS, primary nephrotic syndrome.

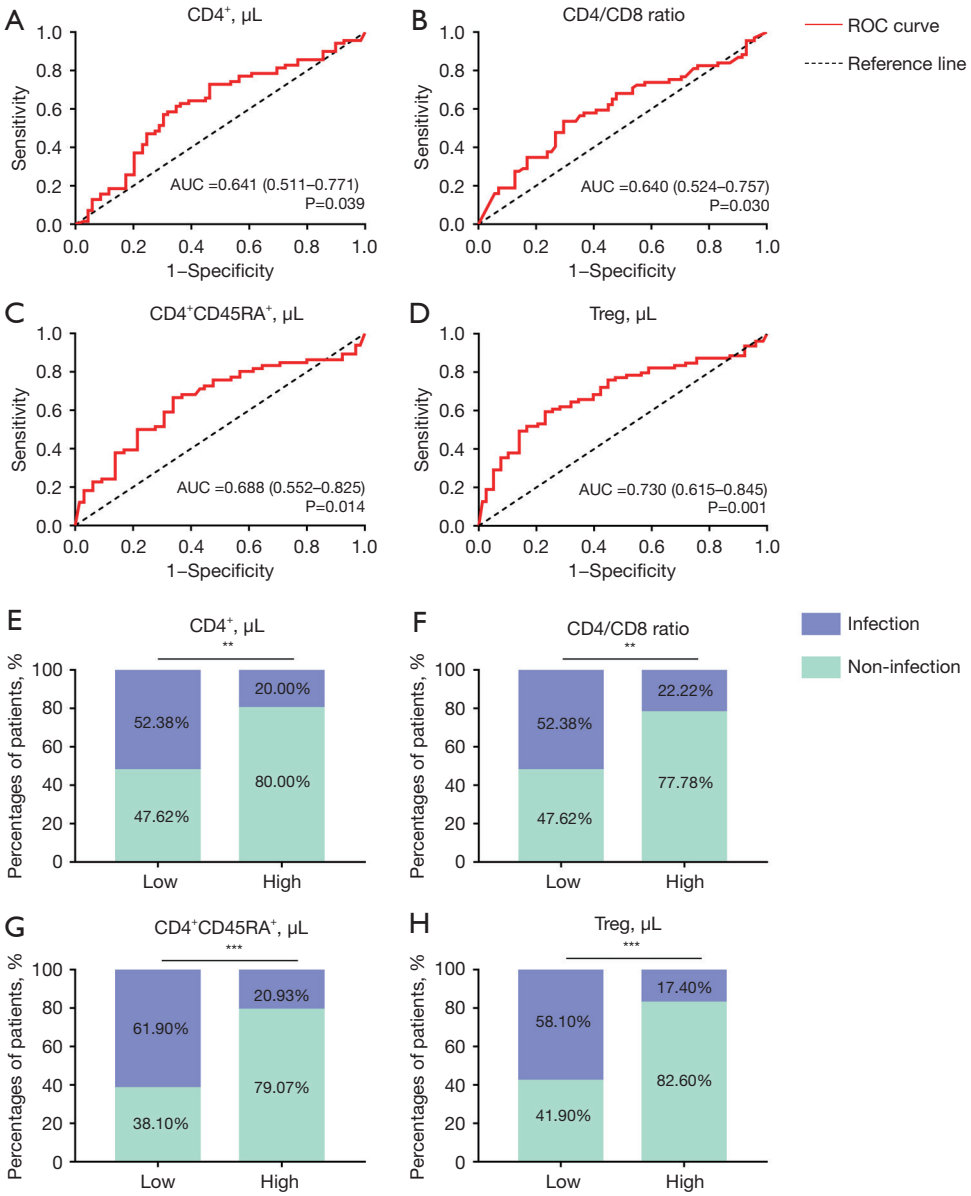

Figure 6 The ROC curve analysis of lymphocyte subpopulations predicts infection in children with primary nephrotic syndrome. (A) ROC curves for the correlation of $CD4^+$ T cells with infection in children with primary nephrotic syndrome. (B) ROC curves for the correlation of the CD4/CD8 ratio with infection in children with primary nephrotic syndrome. (C) ROC curves for the correlation of $CD4^+CD45RA^+$ T cells with infection in children with primary nephrotic syndrome. (D) ROC curves for the correlation of T_{reg} cells with infection in children with primary nephrotic syndrome. (E) Comparison of the frequency of infection in PNS patients with different $CD4^+$ T cell counts. (F) Comparison of the frequency of infection in PNS patients with different CD4/CD8 ratio levels. (G) Comparison of the frequency of infection in PNS patients with different $CD4^+CD45RA^+$ T cell levels. (H) Comparison of the frequency of infection in PNS patients with different T_{reg} cell levels. The cutoff value between high and low levels was determined using the Youden index. Fisher's test was used to compare the variables. **, P<0.01, ***, P<0.001. ROC, receiver operating characteristic; PNS, primary nephrotic syndrome.

Table 4 The AUC, P value, and cutoff value of lymphocyte subpopulations for predicting infection in children with PNS

Lymphocyte subpopulation	AUC	95% CI	P value	Cutoff value	Sensitivity	Specificity
$CD4^+$%	0.666	0.551–0.781	0.010*	37.5	0.188	0.982
$CD4^+$/μL	0.641	0.511–0.771	0.039*	1156	0.759	0.583
CD4/CD8 ratio	0.640	0.524–0.757	0.030*	1.075	0.688	0.636
$CD4^+CD45RA^+$%	0.670	0.548–0.791	0.016*	11.5	0.385	0.816
$CD4^+CD45RA^+$/μL	0.688	0.552–0.825	0.014*	340	0.591	0.810
T_{reg}/μL	0.730	0.615–0.845	0.001**	82.5	0.692	0.745

*, P<0.05, **, P<0.01. AUC, area under the curve; PNS, primary nephrotic syndrome; CI, confidence interval.

Table 5 Logistic regression analysis for risk factors associated with PNS

Variables	P value	OR (95% CI)
$CD4^+$%		
$CD4^+$%>37.5%		1.000
$CD4^+$%<37.5%	0.008**	4.034 (1.439–11.311)
$CD4^+$/mL		
$CD4^+$/mL >1,156		1.000
$CD4^+$/μL <1,156	0.005**	4.400 (1.577–12.275)
CD4/CD8 ratio		
CD4/CD8 ratio >1.075		1.000
CD4/CD8 ratio <1.075	0.004**	3.850 (1.523–9.735)
$CD4^+CD45RA^+$%		
$CD4^+CD45RA^+$%>11.5%		1.000
$CD4^+CD45RA^+$%<11.5%	0.062	2.778 (0.952–8.107)
$CD4^+CD45RA^+$/μL		
$CD4^+CD45RA^+$/μL >340		1.000
$CD4^+CD45RA^+$/μL <340	0.002**	6.139 (1.950–19.329)
T_{reg}/μL		
T_{reg}/μL >82.5		1.000
T_{reg}/μL <82.5	0.000***	6.577 (2.315–18.684)

**, P<0.01, ***, P<0.001. PNS, primary nephrotic syndrome.

T_{reg} cell counts were highly accurate in predicting PNS with infection (P=0.001). The cutoff value derived from the ROC analyses (the Youden index) for the T_{reg} cell counts and the AUC value was 82.5 and 0.730, respectively, and yielded a sensitivity and specificity of 69.2% and 74.5%, respectively (*Table 4*). Subsequently, we used logistic regression analysis to determine the independent clinical factors predicting PNS with infection. Patients were divided into two groups according to the cutoff value. The results showed that lower $CD4^+$ T cell, T_{reg} cell, and naive $CD4^+$ T cell percentages and counts and the CD4/CD8 ratio were independent risk factors for PNS with infection (*Table 5*). Patients with lower T_{reg} cell counts [<82.5 cells/μL, P<0.001, OR =6.577 (range, 2.315–18.684)] had a significantly higher risk of infection than those with higher counts (*Table 5*), which could be used as a predictor of infection in children with PNS.

Discussion

The role of the immune system in PNS has been investigated in relevant clinical and experimental studies (12). However, the evidence for potential biomarkers for patients with PNS is scarce. The present study comprehensively analyzed the relationship between lymphocyte subsets and disease activity, and the prediction of infection in childhood PNS. Moreover, our results showed that an upregulated expression of $CD8^+$ cells was significantly positively correlated with PNS-, dominated by T_C cells. Secondly, the immune response of $CD4^+CD45^+$ T cells may also vary in the different PNS disease phases, with an increased level of $CD4^+CD45RO^+$ T cells, a decreased $CD4^+CD45RA^+$/$CD4^+CD45RO^+$ ratio in disease activity, and downregulation of $CD4^+CD45RA^+$ T cells in patients with predicted infection, which has never been reported previously. Thirdly, a previous study demonstrated that the $CD4^+$ cell count was more predictive of overall infection than the total lymphocyte count (13). Our results indicated that $CD4^+$T cell subpopulations (T_{reg} cell counts and $CD4^+CD45RA^+$ T cell counts) have higher predictive values than the $CD4^+$T cell count for infection in PNS.

A disease state biomarker depicts the intensity of the

disease process, while a disease stage biomarker depicts how far the process has progressed. Thus, we conducted lymphocyte subpopulation investigations between groups with three clinically distinct progressions (active, partial remission, and complete remission) and a healthy group. For example, $CD8^+$ T cells may injure the kidneys through the production of chemokines and the recruitment by antigen capture (10). Furthermore, $CD8^+$ T lymphocytes contain two functionally distinct subsets: T_C cells and T_S cells. Tc cells produce IFN-γ and conduct cytotoxic activities, while Ts cells restrain the Th1 responses (14).

NK cells may play a vital role in the pathogenesis and the progression of PNS disease when followed by immunogenic challenges (such as vaccine immunizations, allergens, or viral infections). In our study, we found that some laboratory indicators may play an important role in the PNS, such as an increase in T_C cell counts, an elevated ratio of T_c/T_s cells, and a decline in the percentage of NK cells. More recently, the role of B cell depletion in inducing disease remission has become evident (15). A previous report suggested that the effectiveness of anti-CD20 monoclonal antibodies, such as rituximab, for refractory PNS strongly implicates B cells in the pathogenesis of PNS (16). However, our findings were inconsistent with previous reports. We found no statistical significance in the level of B cells over the disease course. The reason may be due to the insufficient sample size in this study. Currently, the immune mechanisms underlying the effect of B cell depletion are unclear and are worthy of further investigation.

Lama *et al.* observed a significant decrease in $CD4^+$ cells in short and long remissions in PNS children compared with relapsed patients (17). In the last decade, one potential target of T cells on the podocyte has been found in pediatric patients during disease relapse (18). In metastatic colorectal cancer (mCRC) patients, $CD4^+$ T cell levels and the ratio of CD4/CD8 were shown to be potential independent biomarkers for the objective response rate and overall survival (19). Notably, no statistically significant difference was found in $CD4^+$ T cell counts between our groups in this study. Therefore, given the controversy about the function of $CD4^+$ T cells, our group conducted further exploration of the peculiar subgroup of $CD4^+$ T cells. $CD4^+$ T cells can be divided into mutually exclusive subsets based on the expression of the common leukocyte antigen $CD45^+$ isoforms, namely $CD45RA^+$ and $CD45RO^+$ (20). $CD45RA^+$ cells differentiate into $CD45^+RO^+$ "memory" cells, defined as "naïve" lymphocytes. Upon antigenic stimulation, the $CD45RA^+$ phenotype is switched to the $CD45RO^+$ phenotype. A previous article indicated that the proportion of $CD4^+CD45RO^+$ T cells and $CD4^+CD45RA^+$ T cells was significantly higher in patients with steroid-resistant nephrotic syndrome than in controls (21). Similarly, we found that the percentage and $CD4^+CD45RO^+$ T cell counts in the active and partial remission stages were significantly higher than in healthy controls. The active phase of the disease plays a crucial role in treatment. In recent retrospective studies (22,23), MMF and corticosteroids were shown to be effective in treating active disease. Moreover, a previous study suggested that TFH1, TFH2, and TFH17 are indicators of the active state (24). However, knowledge of the clinical usefulness of biomarkers is limited, and the relevant indicators of active disease deserve further investigation. Meanwhile, the $CD4^+CD45^+$ cells responded differently in different phases of PNS in this study. Furthermore, our finding that the $CD4^+CD45RA^+/CD4^+CD45RO^+$ ratio was reduced significantly in the active group compared with the partial and complete remission groups was unexpected. These findings demonstrated that the $CD4^+CD45RA^+/CD4^+CD45RO^+$ ratio might be associated with disease activity.

Nephrotic syndrome in children requires additional treatment to prevent complications, particularly serious infections (25). Based on different types of pathology in children and adults, different types of infections were documented. The most common adult infections are hepatitis B and C (26), whereas children more commonly experience URTIs (27). Consistent with the above conclusion, our study indicated that approximately 70% of children suffered from URTI. Several studies have reported that the detection of lymphocyte subsets is significantly related to infection prediction (4,28). As shown in the results, the percentage and the counts of $CD4^+$ T cells, CD4/CD8 ratio, the percentage and the counts of $CD4^+CD45RA^+$ T cells, and T_{reg} cell counts were significantly lower in patients with infections. Tregoning *et al.* reported that T cells play a critical role in defending neonates against respiratory syncytial virus infection (29). The results also showed that lower T_{reg} cell counts were an important risk factor for infection in children with PNS. Previous research has demonstrated that impaired T_{reg} cell function may elucidate the mechanism for patients with minimal in-change nephrotic syndrome and regulatory T cells induce remission of the disease (30). In addition, we found that T_{reg} cell counts and $CD4^+CD45RA^+$ T cell counts were both independent risk factors of overall infection.

The current study has some limitations. Firstly, selection

bias cannot be excluded due to the retrospective nature of the study. For instance, early glucocorticoid exposure for the pretreatment samples ensured that all pretreatment samples reflected only the disease state at presentation, whereas the post-treatment samples reflected the cumulative effects of glucocorticoid treatment on the disease. Secondly, regulatory T cell secretions may have a role to play in regulating atopic reactions and humoral immunity via their cytokines.

Additionally, the clinical sample size was relatively small and should be increased in future studies to confirm our current findings.

Conclusions

Measuring peripheral blood lymphocyte subpopulations by flow cytometric analysis aids in monitoring disease progression and predicting infection. This study indicated that $CD8^+$ T cells, dominated by T_C cells, were associated with the occurrence of disease. Moreover, the $CD4^+$ T cell counts, CD4/CD8 ratio, $CD4^+CD45RA^+$ T cells, and T_{reg} cell counts were dependent factors in the PNS. The $CD4^+CD45RA^+/CD4^+CD45RO^+$ ratio is an indicator of the active state in PNS. In addition, the T_{reg} cell counts and $CD4^+CD45RO^+$ T cell counts have higher predictive values than the $CD4^+$ T cell counts for overall infection. However, additional studies are required for more detailed typing of lymphocyte subgroups in childhood PNS.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Nanfang Hospital Ethics Committee board (No. NFEC-2022-076) and individual consent for this retrospective analysis was waived.

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