

Research Article

## CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg Cells in Henoch-Schönlein Purpura Nephritis Patients

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### Abstract

**Objectives** CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells are regulators in almost all of the animal models of human organ specific diseases, transplant rejection and allergic diseases. The purpose of the present study was to observe the number of CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg in the patients with Henoch-Schönlein purpura nephritis(HSPN), and to study pathogenesis of HSPN.

**Methods** Thirty one patients with HSPN and thirty one healthy volunteers were studied. The CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells were examined by flow cytometry. Clinical and laboratory data, such as urinary protein, serum creatinine, were obtained from each patient and healthy volunteer. Glomerular injury was assessed by histopathology. Serum IgA, C3, IL-2, IL-4 and IL-6 were analysed by ELISA.

**Results** CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells significantly decreased in HSPN patients compared with the controls (P<0.05). CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells are negatively correlated with blood urea nitrogen, supernatant IL-4, and proteinuria in HSPN patients, and positively with estimated glomerular filtration rate (eGFR). CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells gradually decreased as the severity of renal histology increased. In addition, serum IgA, IL-2, IL-6 and supernatant IL-4 elevated while CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells decreased in HSPN patients(P<0.05).

**Conclusions** CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells are not only lower in HSPN patients, but also are correlated with disease severity in HSPN patients. CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells may play an important role in the pathogenesis of HSPN.

**Keywords:** CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells; Henoch-Schönlein Purpura Nephritis; Pathogenesis

## Introduction

Henoch-Schönlein purpura (HSP) is a form of systemic vasculitis characterised by vascular wall deposits of predominantly IgA typically involving small vessels in skin, gut and glomeruli and associated with purpura, colic, haematuria and arthralgia or arthritis. HSP nephritis (HSPN) is common glomerular disorders that can potentially progress to end-stage renal disease in some patients [1]. The postulation that HSPN is a systemic immune-complex mediated disease is supported by the clinical or histological recurrences of HSPN in some patients after transplantation [2]. Although detailed pathogenic mechanisms of HSPN have not been fully elucidated, perturbations in the immune system, including elevations in serum levels of IgA1, IgA1-containing circulating immune complexes and IgA-rheumatoid factors have been documented for patients with HSP [3,4]. Elevated serum levels of IgA and IgA-containing immune complexes were observed in patients with HSPN [5]. Furthermore, it was noted that all HSP patients have IgA1-circulating immune complexes of small molecular mass, but only those with nephritis have additional large-molecular-mass IgA1-IgG-containing circulating immune complexes [6]. However, it is still unclear why have the presence of various immune complexes until now.

Recent studies have shown that  $CD_4^+CD_{25}^+$  Treg cells are of critical importance to the maintenance of tolerance by inhibiting the activation and proliferation of autoreactive T cells [7]. Depletion of the minor  $CD_4^+CD_{25}^+$  Treg cells results in the development of organ-specific autoimmunity [8]. Autoimmune diseases can be prevented by reconstitution of the animals with  $CD_4^+CD_{25}^+$  Treg cells [8]. Powrie and colleagues [9] demonstrated that transfer of  $CD_4^+CD_{25}^+$  Treg cells protected mice from the development of inflammatory bowel disease and even reversed established gastrointestinal inflammation.  $CD_4^+CD_{25}^+$  Treg cells are regulators in almost all of the animal models of human organ specific diseases, transplant rejection and allergic diseases [10].

To date, there are only few studies of  $CD_4^+CD_{25}^+$  Treg cells in human HSPN. Some patients do not suffer from HSPN, implying that it is possible to find a balance between immunity and tolerance. However, other patients do suffer from HSPN. We therefore hypothesize that a numerical and/or functional deficit of  $CD_4^+CD_{25}^+$  Treg cells in the HSPN patients might trigger the development of disease. The purpose of the present study was to observe the number of  $CD_4^+CD_{25}^+$  Treg in the patients with HSPN.

## Methods

### Subjects

HSPN patient groups:

Thirty one patients with HSPN, hospitalized in Hunan Provin-

cial People's Hospital and Second Xiangya Hospital of Central South University Hospital between January 2009 and February 2013 and who had typical features of HSPN (13 men and 18 women; age range 23-45 years; mean age  $35.8 \pm 9.74$  years), were enrolled. Henoch-Schonlein purpura was defined based on non thrombocytopenic palpable purpura, arthralgia or arthritis, and gastrointestinal manifestations with abdominal pain or bleeding and renal involvement [11]. Patients with systemic disorders, vasculitis, drug hypersensitivity, and incomplete data were excluded from the study. Henoch-Schonlein nephritis was defined as the presence of gross or microscopic hematuria (more than 5 erythrocytes per high-power field in centrifuged urine) with or without proteinuria (urine protein greater than 150 mg/d), nephrotic syndrome (urine protein more than 3500 mg/d, serum albumin less than 30 g/L, edema and hyperlipidemia), and acute nephritis (hematuria plus one or more of the followings: increased serum creatinine, hypertension, and oliguria) [12].

Control groups:

Thirty one healthy volunteers (15 men and 16 women; age range 31-47 years; mean age  $38.2 \pm 9.74$  years) were selected as the control group in this study.

The study was approved by the Ethical Committee of Hunan Provincial People's Hospital and Second Xiangya Hospital of Central South University Hospital. Written informed consent was provided by all patients and healthy volunteers.

### Clinical Data and Clinical samples Collected

After acquiring informed consent, clinical data, peripheral blood and urine samples were collected. All patients did not undergo treatment with steroid, immunosuppressive agents, angiotensin-converting enzyme(ACE) inhibitor, or AT1 receptor blockers before clinical samples collected. The laboratory examinations before treatments included urinalysis, complete blood count, serum chemistries, IgA antibody, and complement components C3).

### Flow cytometry analysis of $CD_4^+CD_{25}^+$ Treg cells

Peripheral blood were mixed and incubated for 30 min at room temperature with 10 $\mu$ l monoclonal Cy5-labeled anti-human CD3 (Jingmei Biotech), FITC-anti-CD4, and PE-anti-CD25. After a short incubation period, the samples were fixed with 1% paraformaldehyde and analyzed by flow cytometry (Coulter EpicsXL, System 2 software; Beckman-Coulter). The analysis and gates were restricted to lymphocytes (The number of replicates of each sample was three).

Isolation of peripheral blood mononuclear cells and culture PBMC were isolated from heparinized peripheral blood by den-

sity gradient centrifugation, using Lymphocyte Separation Medium (Flow Labs, McLean, VA). Cells recovered at the interface were resuspended in RPMI1640 supplemented with penicillin (100 U/ml), streptomycin (100 pg/ml), glutamine (2 mM) and 10% heat-inactivated fetal calf serum (FCS), at a concentration of  $3 \times 10^6$  cells/ml. Duplicate cultures, with phytohemagglutinin (PHA; Sigma, St Louis, MO) 20ug/ml, were maintained for 24h at 37°C in a 5% CO<sub>2</sub> atmosphere. At the end of this period, cell-free supernatant was obtained by centrifugation at 800g for 10 min and frozen at -70°C until assayed.

### Enzyme-linked immunosorbent assay

The serum concentrations of IL-2, IL-6, and the cell-free supernatant concentration of IL-4 were measured respectively by an enzyme-linked immunosorbent assay (ELISA) with ELISA kits (R&D Systems, USA) according to the manufacturer's instruction (The number of replicates of each sample was three).

### Renal histopathology examination

Renal biopsy was performed in eighteen HSPN patients. All kidney biopsy samples were subjected to fluorescence, and light microscopic examination. For light microscopy, kidney biopsy samples were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin by conventional techniques. Sections were stained with hematoxylin and eosin, and periodic acid-schiff. IgG, IgA, IgM, complement component C3, activity (AI) and chronicity (CI) indices, as well as semiquantitative renal histological evaluation of renal biopsy specimens obtained at time of diagnosis, were compared. AI includes glomerular hypercellularity, leucocyte exudation, fibrinoid necrosis, cellular crescents, hyaline deposits and interstitial inflammation, while CI includes glomerular sclerosis, fibrous crescents, renal tubular atrophy and interstitial fibrosis. These alterations were graded semiquantitatively on a 1+ to 3+ scale (mild, moderate, or marked) [13]. The renal pathological lesions were graded single-blindedly according to the Haas classification [14].

### Statistical analysis

Data were checked for normality of distribution using the Kolmogorov-Smirnov test and were expressed as mean standard deviation or median and interquartile range. Comparison between the two groups was done by independent-sample t-test or non-parametric Mann-Whitney U-test as appropriate. Relationships between different values were examined using Spearman's correlation tests. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed using Graph Pad Prism 5.0 (Graph Pad Inc., USA) and Statistical Package for Social Sciences version 16.0 (SPSS Inc., USA).

## Results

### Flow cytometry analysis of CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup> Treg cells

We analyzed the population of CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup> Treg cells in peripheral blood by flow cytometry (Figure 1). CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup> Treg cells significantly decreased in HSPN patients compared to those of the control groups (<sup>a</sup>P<0.05) (Table 1).

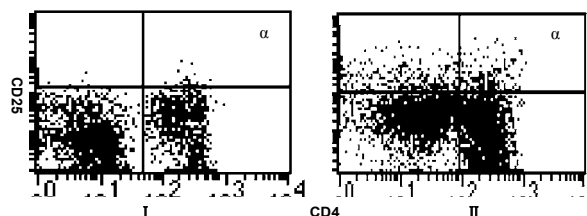


Fig.1 CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup> Treg cells in peripheral blood by flow cytometry.

CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup> Treg cells were counted using the indicated gates and are enumerated in Table 1. α : CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup> Treg cells, I: HSPN patients, II: Control groups.

CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup> Treg cells significantly decreased in HSPN patients compared to the control groups (<sup>a</sup>P<0.05).

Table 1. The number of CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup> Treg cells in peripheral blood (mean± SE) %

	HSPN groups	Controls
CD <sub>4</sub> <sup>+</sup> CD <sub>25</sub> <sup>+</sup> Treg cells	1.01±0.206 a	2.19±0.387

CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup> Treg cells significantly decreased in HSPN patients compared to the control group (<sup>a</sup>P<0.05).

### Clinical and histopathological findings in patients

Clinical and histopathological findings in patients were listed in Table 2. Serum IL-2, IL-6, and supernatant IL-4 significantly increased in HSPN patients compared to those of the control groups (all \*P<0.05). In contrast, serum levels of C3 did not differ between HSPN patients and control groups (P >0.05).

Table 2. Clinical and histopathological findings in HSPN patients

	HSPN groups	Control groups
Proteinuria(g/day)	2.14±0.31	
Hematuria (X10 <sup>6</sup> cells/mL)	364.75±4.21	
BUN(mg/dL)	17.3±3.98*	13.46 ±0.71
Serum Cr (mg/dL)	1.01±0.30*	0.75±0.16
Serum uric acid (μ mol/L)	322.64±54.09*	241.33±53.38
eGFR (mL/min/ 1.73 m <sup>2</sup> )	88.23±8.93*	110.78±8.36
Serum IgA(mg/dL)	276.31±51.55*	202.19±47.88
Serum C3 (mg/dL)	117.5±23.91	128.2±26.01
Serum IL-2(pg/ml)	101.2±11.85*	22.8±8.65
supernatant IL-4 (pg/ml)	337.35±74.01*	211.35±91.21
Serum IL-6 (pg/ml)	45.76±7.07*	28.83±6.71
AI (median)/ CI (median)	10.4±2.5/3.1±0.8	

AI, activity index; CI, chronicity index; \*P<0.05 compared with control.

## CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells and clinical data

The correlations between CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells and values for numerous clinical parameters were examined in HSPN patients (Table 3). CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells were negatively correlated with serum blood urea nitrogen (BUN), and uric acid in HSPN patients, and CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells were positively correlated with eGFR (all  $P < 0.05$ ). These results indicate that CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells were associated with renal function. CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells were negatively correlated with urine protein, supernatant IL-4 in HSPN patients (all  $P < 0.05$ ).

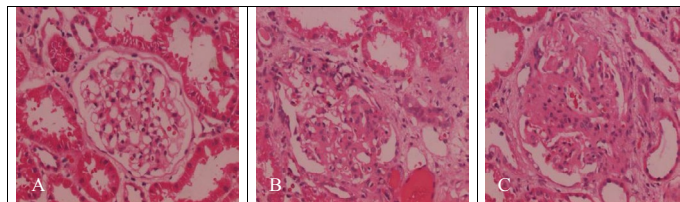
**Table 3. Correlation between the frequency of CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells and Clinical Parameters in HSPN patients**

	CD <sub>4</sub> <sup>+</sup> CD <sub>25</sub> <sup>+</sup> Treg cells (%)	
	r	p
SBP	-0.287	n.s.
DBP	-0.190	n.s.
BUN	-0.679	< 0.01
SCR	-0.502	< 0.01
SUA	-0.537	< 0.01
eGFR	0.547	< 0.01
24-h UP	-0.478	< 0.01
Serum IgA	-0.215	n.s.
Hematuria	-0.10	n.s.
Serum C3	-0.142	n.s.
Serum IL-2	-0.032	n.s.
Supernatant IL-4	-0.884	< 0.01
Serum IL-6	-0.313	n.s.

n.s., not significant; SBP, systolic blood pressure; DBP, diastolic blood pressure; Scr, serum creatinine; SUA, serum uric acid; eGFR, estimated glomerular filtration rate; 24-h UP, 24- hours urinary protein.

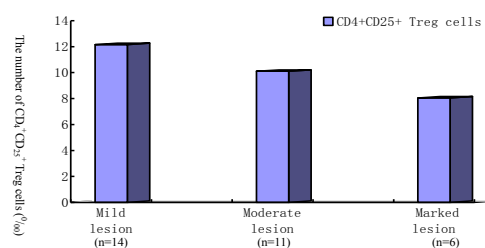
## Correlation between CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells and histological classification of HSPN

CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells in HSPN patients tended to decrease in parallel with the severity of the histological classification of nephropathy as determined by the Haas classification [14], although the difference was not significant (all  $P > 0.05$ ) (Figure 2, Figure 3).



**Fig.2** Histopathological findings in HSPN patients (40x)

A: Mild lesion B: Moderate lesion C: Marked lesion



**Fig. 3** The number of CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells in HSPN patients.

The number of CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells in HSPN patients tended to decrease in parallel with the severity of lesion, although the difference was not significant (all  $p > 0.05$ ).

## Discussion

HSP is a systemic disorder characterized by leukocytoclastic vasculitis involving the capillaries and the deposition of IgA immune complexes [15-18]. However, it is still unclear why have the presence of various immune complexes until now. CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells are regulators in almost all of the animal models of human organ specific diseases, transplant rejection and allergic diseases [10]. The most notable immunomodulatory property of CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells is their ability to limit the development of a proinflammatory CD<sub>4</sub><sup>+</sup>Th2 phenotype [19]; this inhibition is characterized by reduced cytokine production. Abnormality of peripheral T cell can result from an inappropriate balance between allergen activation of CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells and effector Th2 cells [20,21]. This imbalance could result from a deficiency in suppression by CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells or strong activation signals that overcome such regulation [22]. Recent work has shown that following antigen inhalation, CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg cells play a key immunomodulatory role [23]. It has been reported that Th1 responses are more prone to regulation by CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>T cells than Th2 responses [24]. Some patients do not suffer from HSPN, implying that it is possible to find a balance between immunity and tolerance. However, other patients do suffer from HSPN. We therefore hypothesize that a numerical and/or functional deficit of CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>Treg

cells in the HSPN patients might trigger the development of disease. The purpose of the present study was to observe the number of  $CD_4^+CD_{25}^+$  Treg in the HSPN patients.  $CD_4^+CD_{25}^+$  Treg cells significantly decreased in HSPN patients compared with controls. Our results are consistent with this suggestion. Serum IL-2, IL-6, and supernatant IL-4, IgA, urine protein, and urine erythrocytes significantly elevated in HSPN patients compared with controls.  $CD_4^+CD_{25}^+$  Treg cells were negatively correlated with serum BUN, uric acid, supernatant IL-4 and urinary protein, and were positively correlated with eGFR. The  $CD_4^+CD_{25}^+$  Treg cells tended to gradually decrease with increasing severity of histologically assessed nephropathy. A possible explanation for this is that when HSPN patients experiences a decrease in  $CD_4^+CD_{25}^+$  Treg cells, his/her lymphocytes react significantly more strongly to antigens, leading to higher levels of cytokine production (IL-2, IL-4, IL-6). Excessive cytokine production may enhance the switching of antibody production from one isotype to another (e.g. from IgM to IgA [25]). Therefore large amounts of IgA are present in the serum of HSPN patients. IgA are deposited in glomerular mesangium via the circulatory system where they activate complement or disturb the balance between blood coagulation and plasminogen; release inflammatory factors; and injure renal tissue.  $CD_4^+CD_{25}^+$  Treg cells may play an important role in the pathogenesis of HSPN. The findings mentioned above are both interesting and useful. Altering the  $CD_4^+CD_{25}^+$  Treg cells number might be useful in the prevention and treatment of HSPN. To date, there are only few studies of  $CD_4^+CD_{25}^+$  Treg cells in human HSPN.

Although some findings mentioned above are both interesting and useful, limitation is obvious.  $CD_{25}$  is also expressed on activated lymphocytes that limited the statistical power for comparisons. Foxp3 specially express on  $CD_4^+CD_{25}^+$  Treg cells. Examination of  $CD_4^+CD_{25}^+$  Foxp3 cells is necessary to identify Tregs and to demonstrate a functional deficit of  $CD_4^+CD_{25}^+$  Treg cells in HSPN patients in the future.

In conclusion, the present study indicated that  $CD_4^+CD_{25}^+$  Treg cells are not only lower in HSPN patients, but also are correlated with disease severity in HSPN patients.  $CD_4^+CD_{25}^+$  Treg cells may play an important role in the pathogenesis of HSPN. Altering the  $CD_4^+CD_{25}^+$  Treg cells number might be useful in the prevention and treatment of HSPN. Additional studies are necessary to demonstrate a functional deficit of  $CD_4^+CD_{25}^+$  Treg cells in HSPN patients and other biological properties of  $CD_4^+CD_{25}^+$  Treg cells.

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### Disclosure

All the authors have declared no competing interest.

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