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Original Article

Pathogenic role of circulating $CD4^+CXCR5^+$ cell subpopulations in patients with chronic spontaneous urticarial

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Abstract: Accumulating evidence demonstrated that circulating $CD4^+CXCR5^+$, follicular helper T (Tfh) and follicular regulatory T (Tfr) cells maintain immune homeostasis and humoral immune response and were involved in the pathogenesis of certain autoimmune/inflammatory diseases. The current study was aimed to investigate the correlation between frequencies of $CD4^+CXCR5^+$, Tfh and Tfr cells, Tfh/Tfr and the disease activity in chronic spontaneous urticaria (CSU). Frequencies of $CD4^+CXCR5^+$, Tfh and Tfh/Tfr, but not Tfr, in peripheral blood mononuclear cells (PBMCs) were elevated in patients with CSU as compared with healthy controls. No difference was observed in the frequency of these cells between different subgroups of patients based on autologous serum skin testing (ASST), skin prick testing (SPT) and the level of total IgE. The expression of CXCR5 in PBMCs was higher in CSU than in controls, both at mRNA and protein levels. Higher levels of plasma IL-4, IL-6 and total IgE were observed in CSU, with positive correlation between IL-4/IL-6 and total IgE. The IL-21 level was lower and negatively correlated with total IgE. Using receiver operating characteristic (ROC) curve, we found positive correlation between the urticaria activity score (UAS) and $CD4^+CXCR5^+$, Tfh, Tfh/Tfr and total IgE, respectively, with area under the curves (AUCs) all greater than 0.7 ($P < 0.05$). These results indicated that frequencies of circulating $CD4^+CXCR5^+$ cells, Tfh cells and Tfh/Tfr were abnormal and correlated positively with disease severity, suggesting the possible involvement of these cells in the immunopathogenesis of CSU.

Keywords: Chronic spontaneous urticaria, $CD4^+CXCR5^+$ cell, follicular helper T cell, follicular regulatory T cell, urticaria activity score

Introduction

Chronic spontaneous urticaria (CSU) is a common recurrent skin disease, with a great impact on the life quality and health [1]. According to autologous serum skin test (ASST) and skin prick test (SPT), CSU can be divided into several subtypes [2-4]. A variety of factors, including autoreactivity, infections, genetic factors, coagulation cascade and autoimmunity, can contribute to the pathogenesis of CSU [1], but the complexity of the disease remains elusive. Recent studies suggested the association with IgE to autoallergens and IgG autoantibodies to IgE or its receptor [5].

Follicular helper T (Tfh) cell is a subgroup of $CD4^+CXCR5^+$ (C-X-C chemokine receptor 5) cells that activates germinal center formation and provides help for B cells in producing autoantibodies and their differentiation into memory cells and long-lived plasma cells [6, 7]. Follicular regulatory T (Tfr) cells are differentiated from T regulatory (Treg) cells that inhibit germinal center formation and B cell development [7]. Both Tfh and Tfr cells express CXCR5, but differ in that Tfr cells expresse CD25 and FoxP3 [8]. Increase or decrease of circulating $CD4^+CXCR5^+$, Tfh or Tfr cells was found to be associated with immune imbalance, contributing to the pathogenesis of certain diseases, such as atopic dermatitis [9], allergic asthma [10], psoriasis [11], bullous pemphigoid [12], systemic lupus erythematosus [13-15], primary sjogren·s syndrome [16], primary biliary cholangitis [17] and rthumatoid arthritis [18]. In addition, the complex cytokine signals network is essential for differentiation of Tfh cells and

production of autoantibodies in B cells. Tfh cells produce cytokines, such as IL-4, IL-6 and IL-21, which help the class switching of B cells. For example, IL-6 could regulate the number of Tfh cells and the production of total IgE in sensitized mice [19], while IL-4 produced from Tfh cells could regulate the production of IgG1 and IgE in allergic immune mice [20]. Since IgE and IgE autoantibodies have been shown their roles in pathognesis of CSU, it is important to explore the changes of $CD4^+CXCR5^+$ subpopulations which maybe helpful to understand the class switching of B cells in CSU.

The current study was aimed to detect frequencies of $CD4^+CXCR5^+$, Tfh and Tfr cells and the Tfh/Tfr ratio in peripheral blood mononuclear cells (PBMCs) of patients with CSU as compared to normal healthy controls (HC), regarding their correlations to the disease activity. The expression levels of CXCR5 in PBMCs and the concentrations of inflammatory cytokines (IL-4, IL-6 and IL-21) and total IgE in the plasma of studied samples were also evaluated.

Material and methods

Participants and blood sampling

A total of 24 healthy controls (HC) and 63 patients diagnosed with CSU, matched in sex and age distribution, were recruited in the current study. The study was approved by the Ethics Committee of Southwest Hospital, Army Medical University, Chongqing, with informed consent from all the participants. Diagnosis of CSU was made and the disease severity (urticaria activity score, UAS) was measured at the time of blood sampling according to the EAACI/GA^{2}LEN/EDF/WAO guideline [1]. PBMCs were prepared by density gradient centrifugation from peripheral blood and anticoagulated with EDTA-K2. Plasma samples were separated and stored at -80°C until used for ELISA. ASST and SPT were performed in patients with CSU as previously described [2, 3]. The allergens of SPT included dust mites, house dust mites, cockroaches, mugwort pollen, fish (squid), shrimp, duck feather, silk, crab, egg, cow milk and peanut. Patients with CSU and SPT+ displaying one or more positive allergens were indicated as allergic sensitization.

Flow cytometry

For detection of Tfh and Tfr cells, human PBMCs were stained with FITC mouse anti-human CD4 (BD Biosciences, San Diego, CA, USA), CD185 (CXCR5)-APC human (Miltenyi Biotec, CA, USA), PE-CY™7 mouse anti-human CD25 (BD Biosciences), and PE mouse anti-human CD127 (BD Biosciences). Cells were collected with FACSCanto™ II Flow Cytometer (BD Biosciences). Total $CD4^+CXCR5^+$ cells were gated by the forward/side scatter showing CD4/CXCR5 expression patterns. Circulating Tfh ($CD4^+CXCR5^+CD25^{low}CD127^{high}$) and Tfr ($CD4^+CXCR5^+CD25^{high}CD127^{low}$) cells were gated based on expression levels of CD25 and CD127. Data were analyzed using FlowJo software (Stanford University, San Francisco, CA, USA).

Quantitative real-time PCR (qRT-PCR)

PBMCs were resuspended in RNAiso Plus (TaKaRa, Dalian, China) and total RNA was extracted according to instructions of manufacturer. For *CXCR5* detection, cDNAs were synthesized using PrimeScript™ RT reagent Kit (TaKaRa). qRT-PCR was performed with TB Gerrn™ Premix Ex Taq™ II (TaKaRa) on a CFX Connect™ Real-Time System (BIO-RAD, Singapore, USA). The reaction parameters were as follows: 95°C for 30 s, then 40 cycles of 95°C for 5 s and 60°C for 45 s. The expression levels were calibrated to the *β-actin* control and determined by the $2^{-\Delta\Delta Ct}$ method. The primers used were as follows: *CXCR5* forward, 5'-CAACAACTCCCTGCCACGTT-3' and reverse, 5'-AGGAATCCCGCCACATGGTA-3'; *β-actin* forward, 5'-CTCTTCCAGCCTTCCTTCCT-3' and reverse, 5'-AGCACTGTGTTGGCGTACAG-3' (Sangon Biotech, Shanghai, China).

Western blotting

PBMCs were lysed in RIPA lysis buffer supplemented with phenylmethyl-sulfonyl fluoride protease inhibitor (Beyotime, Shanghai, China). Proteins were separated by electrophoresis in 4-20% SurePAGE™ gel (GenScript, Nanjing, China) and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodys against CXCR5 (Abcam, Cambridge, MA) or GAPDH (Bioss, Beijing, China), and then washed and incubated with the secondary antibody (Abclonal, Wuhan, China). Proteins were detected by scanning the membranes with ChemiDoc™ Touch Imaging System (BIO-RAD), and band intensities were quantified using CFX Manager (BIO-RAD).

Table 1. Demographics and clinical characteristics of the patients with chronic spontaneous urticaria (CSU) and healthy controls (HC)

	HC (n = 24)	CSU (n = 63)	*p* value
Gender (male/female)	11/13	23/40	0.305
Age (years), mean (range)	36.6 (16-59)	38.03 (13-70)	0.802
UAS, mean (range)	-	4.95 (2-6)	-
ASST positive (%)	-	15 (23.8%)	-
SPT positive (%)	-	42 (66.7%)	-

Note: CSU: chronic spontaneous urticaria; HC: healthy controls; UAS: urticaria activity score. ASST positive: positive reaction to autologous serum skin testing (ASST); SPT positive: positive reaction to skin prick testing (SPT). Significance between two groups was evaluated with a two-tailed Student's *t*-test and indicated by *p* value ($P < 0.05$ as significant).

Enzyme-linked immunosorbent assays (ELISA)

Plasma levels of IL-4 (BD Biosciences), IL-6 (R&D Systems, Minneapolis, USA), IL-21 (Biolegend, USA) and total IgE (EUROIMMUN, Beijing, China) were measured using ELISAs according to instructions of the manufacturers.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Two-tailed Student's *t*-test was used to compare the two groups. Correlation analyses were conducted using Spearman's rank test. Data were recorded and analyzed with SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA), and $P < 0.05$ was considered as statistically significant.

Results

A total of 63 patients with CSU were recruited. Among them, there were 15 ASST+ *vs.* 48 ASST-, 42 SPT+ *vs.* 21 SPT-. The control group included 23 HC. Their demographic and clinical characteristics were listed in **Table 1**. Significant differences were not observed in gender and age between CSU patients and HC.

Increase in the frequencies of circulating CD4+CXCR5+ cells, Tfh cells and the Tfh/Tfr ratio in CSU

The specific gating strategy to identify Tfh and Tfr expressing CD4+CXCR5+ was shown in **Figure 1A**. The frequencies of circulating CD4+CXCR5+ cells, Tfh cells and the calculated Tfh/Tfr ratio were higher in CSU patients than in HC, without significant difference observed for the frequency of Tfr cells (**Figure 1B**).

Upregulation of CXCR5 expression in CSU

A significant upregulation of *CXCR5* at the mRNA level (**Figure 2A**) as well as in the protein expression (**Figure 2B** and **2C**) was observed in patients with CSU, as compared with HC.

Correlation between the plasma levels of IL-4, IL-6, IL-21 and the total IgE in CSU

Significantly higher plasma levels of IL-4 and IL-6 but lower IL-21 were found in patients with CSU than in HC (**Figure 3A**). The levels of total IgE were significantly higher in patients with CSU (**Figure 3A**), and positively correlated with IL-4 and IL-6, but negatively with IL-21 (**Figure 3B**).

No significant difference of circulating CD4+CXCR5+ cells, Tfh cells, Tfr cells or the Tfh/Tfr ratio among the different subgroups

We divided CSU patients into different subgroups based on ASST, SPT or the levels of total IgE as before [2] to see possible differences. There are no significant differences in the frequencies of circulating CD4+CXCR5+ cells, Tfh cells, Tfr cells and the Tfh/Tfr in different subgroups (**Figure 4**). After further division of the patients into four groups, ASST+/SPT+, ASST+/SPT-, ASST-/SPT+ and ASST-/SPT-, we still failed to identify any difference among them (data not shown).

There was no significant difference in patients with CSU between different subgroups, regarding gender, age and the UAS (**Table 2**).

CD4+CXCR5+ cells, Tfh cells, the Tfh/Tfr ratio and total IgE correlated with CSU disease activity

There was a positive correlation between CD4+CXCR5+ cells, Tfh cells, the Tfh/Tfr ratio, total IgE and the urticaria activity (UAS), respectively. While no such correlation was observed for Tfr cells (**Figure 5A**). The ROC curve

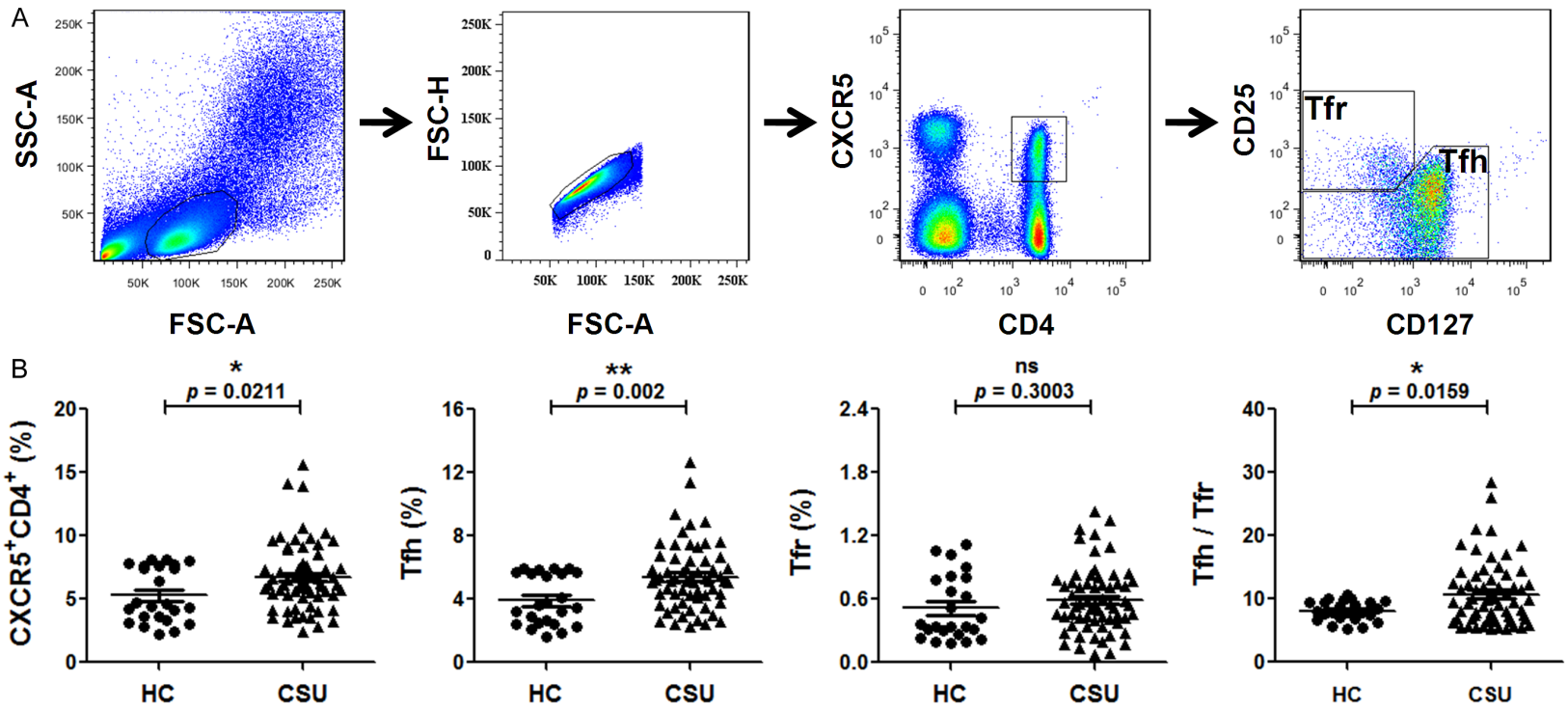

Figure 1. The status of circulating CD4⁺CXCR5⁺, Tfh, Tfr and Tfh/Tfr ratio in peripheral blood mononuclear cells (PBMCs) of patients with chronic spontaneous urticaria (CSU). A. Gating strategy to identify circulating Tfh ($CD4^+CXCR5^+CD25^{low}CD127^{high}$) and Tfr ($CD4^+CXCR5^+CD25^{high}CD127^{low}$) cells among the $CD4^+CXCR5^+$ T cells in human peripheral blood. B. Percentage of circulating $CD4^+CXCR5^+$, Tfh and Tfr cells among PBMCs and the Tfh/Tfr in patients with CSU and healthy controls (HC). Each plot represented individual subject. Horizontal bars indicated the mean and error bars the SEM. Significance between two groups was evaluated with a two-tailed Student's *t*-test and indicated by *p* value ($^*P < 0.05$, $^{**}P < 0.01$, $^{ns}P > 0.05$).

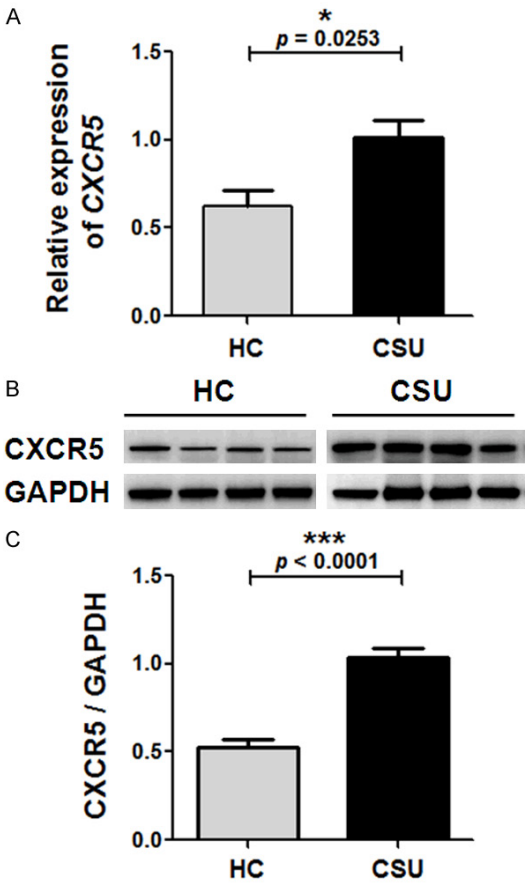

Figure 2. Relative expression of *CXCR5* mRNA and protein levels in peripheral blood mononuclear cells (PBMCs) of patients with chronic spontaneous urticaria (CSU) and healthy controls (HC). A. The *CXCR5* mRNA expression levels were determined by quantitative real-time (qRT)-PCR, with β-actin as an internal reference gene. B and C. The CXCR5 protein expression levels were determined by western blot, with GAPDH as the endogenous control. B. Representative data (n = 4) by the western blot analysis of total protein samples from PBMCs of CSU patients and HC. C. The CXCR5/GAPDH in PBMCs were calculated from the western blot results. Vertical bars represent SEM. Significance between two groups was evaluated with a two-tailed Student's *t*-test and indicated by *p* value ($^{*}P < 0.05$, $^{***}P < 0.001$).

analysis showed that area under the curve (AUC) of Tfr cells was 0.587, there are no statistical difference ($P > 0.05$). The AUCs of $CD4^+CXCR5^+$, Tfh, Tfh/Tfr and total IgE were 0.704, 0.788, 0.708 and 0.900, respectively, with statistical significance ($P < 0.05$) (**Figure 5B**).

Discussion

The pathogenic roles of $CD4^+CXCR5^+$, Tfh and Tfr cells in autoimmune and autoinflammatory diseases have received much attention in recent years. In children with atopic dermatitis, the percentages and absolute numbers of circulating Tfh-like cells ($CD4^+CXCR5^+ICOS^+PD\text{-}1^+$) were increased and their production of IL-21 cytokine positivily correlated with the disease severity index SCORAD [9]. In patients with systemic lupus erythematosus, the frequencies of Tfh and Tfr cells as well as the Tfh/Tfr ratio in peripheral blood were abnormal (increased or decresed) and correlated with the disease activity [13-15]. In bullous pemphigoid, the frequency of Tfh cells in peripheral blood of patients was increased and correlated with the production of anti-BP180-NC16A autoantibodies [12]. All these studies suggested the potential involvement of these cells in the pathogenesis of autoimmunity and disease.

There are at least two distinct pathways, type I (IgE to autoallergens) and type II (IgG autoantibodies to IgE or its receptor) autoimmunity, have been proposed to contribute to the pathologic mechanisms of CSU [5]. Antibodies, which are produced by the associated B cells and helper T cells, may play an important role in the pathogenesis of urticaria [4, 21]. Human studies have demonstrated a central role of CXCR5 in the migration of T and B cells into follicles, formation of germinal center, production of pathogenic autoantibodies, and governing the ability of Tfh cells to help B cells [22-24]. Our study provided the primary evidence of an upregulation of CXCR5 in PBMCs and the increased $CD4^+CXCR5^+$ T cells, Tfh cells and the Tfh/Tfr ratio in patients with CSU.

Studies reported that the production of IgE was mainly derived from two pathways, extrafollicular and germinal centers [25]. Early IgE antibody response arises from extrafollicular, whereas late IgE antibody response arises from germinal centers. IgE germinal center B cells mainly differentiate into short-lived IgE plasma cells, which produce IgE of high-mutation and mature affinity to bind antigens with higher affinity in the environment [25]. Most IgE cells are plasma cells and high affinity IgE is produced by the switching of IgG1 cells to IgE [26]. $CD4^+CXCR5^+$ cells and Tfh cells were found to regulate B cells to produce specific IgE and total IgE. In patients with asthma, the frequency of $CD4^+CXCR5^+$ cells in peripheral blood was increased and associated with the production of pathogenic total IgE antibody [10]. Tfh cells could regulate B cells to produce anti-OVA

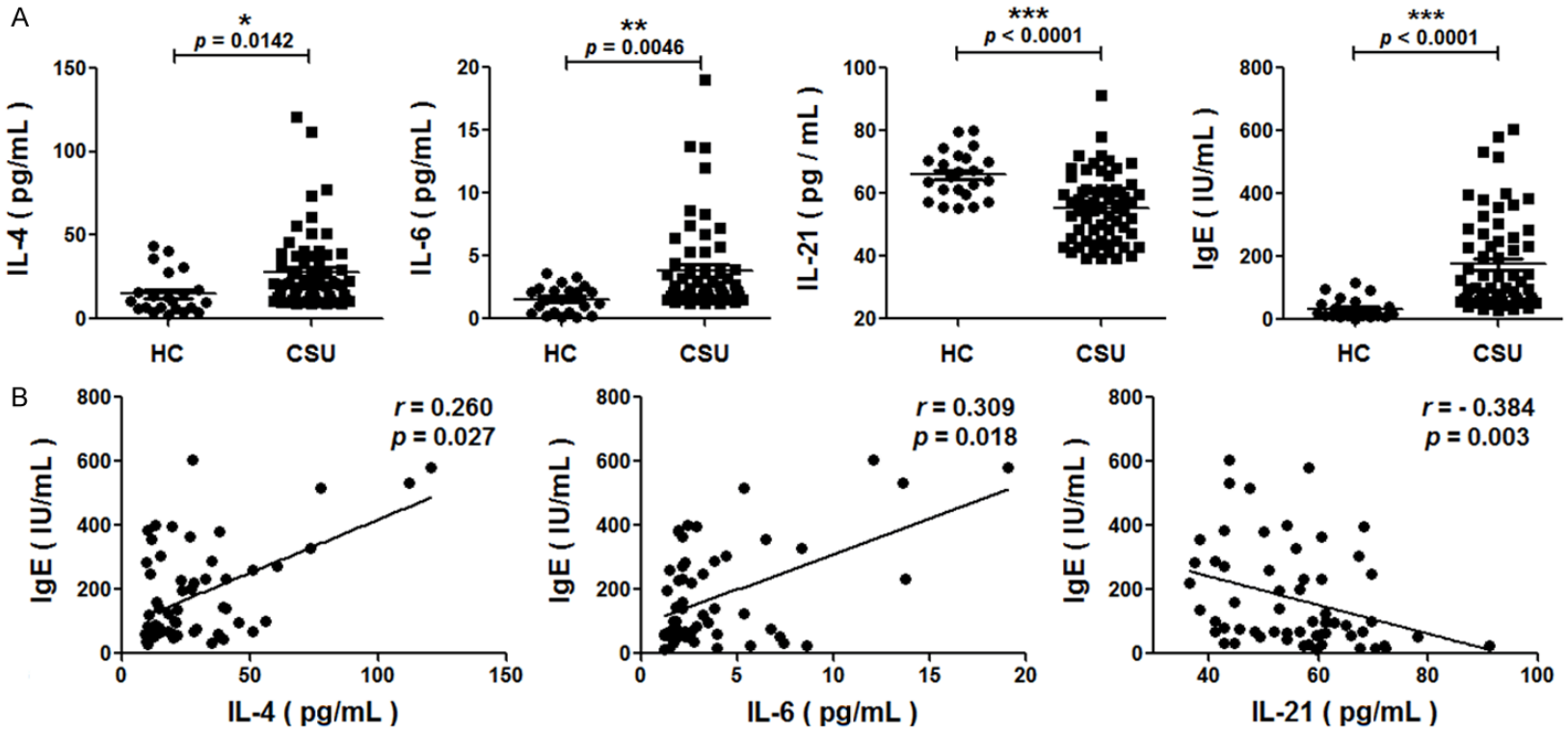

Figure 3. Relationship between plasma cytokines and total IgE in patients with chronic spontaneous urticaria (CSU). A. Concentrations of IL-4, IL-6, IL-21 and total IgE in the plasma of CSU patients and healthy controls (HC). Differences between CSU and HC were evaluated with a two-tailed Student's *t*-test. B. Correlation analysis between IL-4, IL-6, IL-21 and total IgE were conducted using Spearman's rank test. Each plot represented one sample. $P < 0.05$ indicated significant difference ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

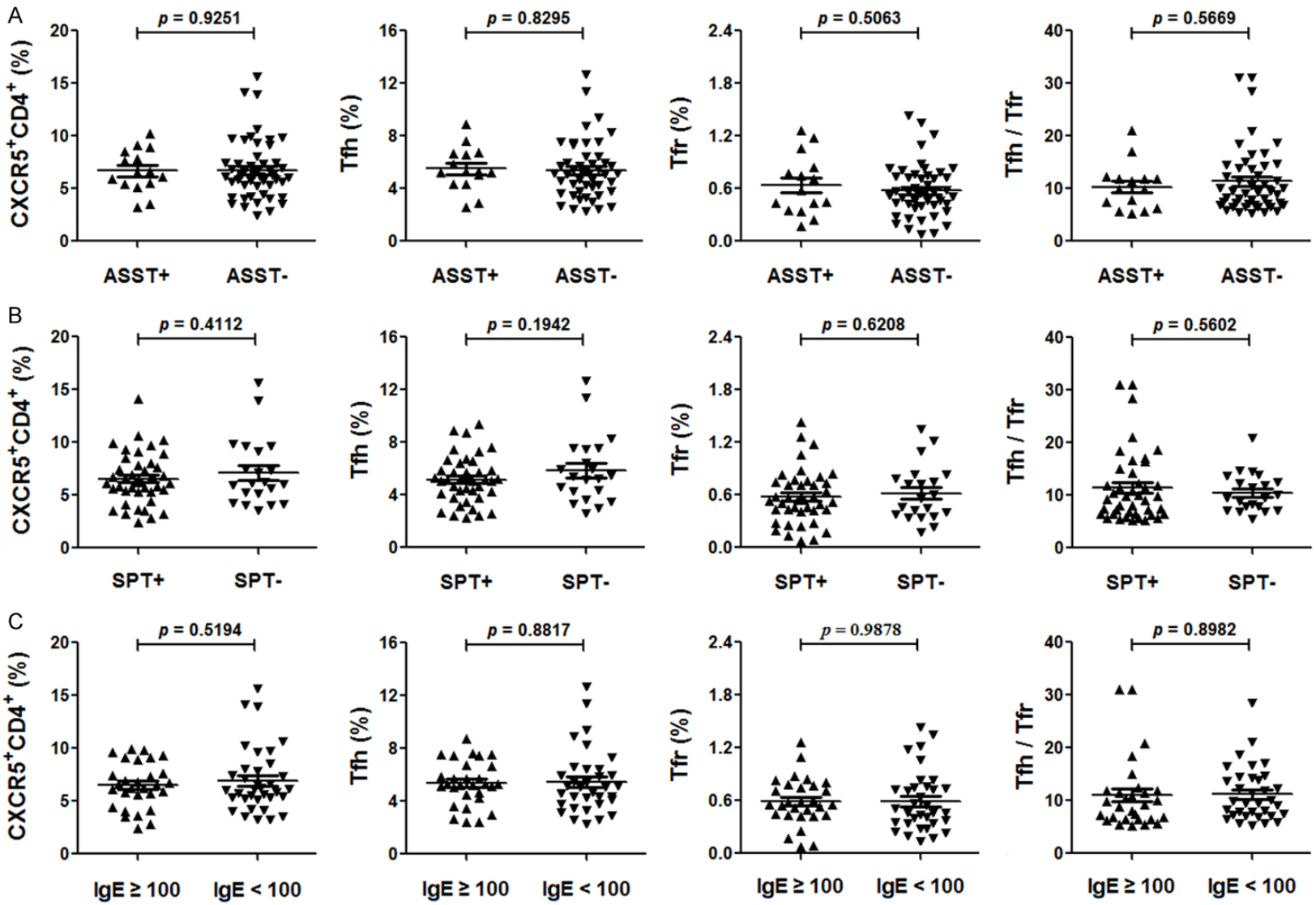

Figure 4. The status of circulating CD4⁺CXCR5⁺, Tfh, Tfr and Tfh/Tfr ratio in peripheral blood mononuclear cells (PBMCs) of different subgroups of patients with chronic spontaneous urticaria (CSU). Percentage of circulating CD4⁺CXCR5⁺, Tfh and Tfr cells in PBMCs and the Tfh/Tfr in ASST+ CSU and ASST- CSU patients (A), SPT+ CSU and SPT- CSU patients (B), the levels of total IgE ≥ 100 IU/ml CSU and IgE < 100 IU/ml CSU patients (C). Each plot represented individual subject. Horizontal bars indicated the mean and error bars the SEM. Significance between two groups was evaluated with a two-tailed Student's *t*-test and indicated by *p* value ($P < 0.05$ indicated significant difference).

Table 2. Comparison between different subgroups of patients with chronic spontaneous urticaria (CSU)

	ASST		*p* value	SPT		*p* value	total IgE (IU/ml)		*p* value
	positive	negative		positive	negative		≥ 100	< 100	
Number	15	48		42	21		29	34	
Female gender (%)	12 (80%)	28 (58.3%)	0.132	24 (57.1%)	16 (76.2%)	0.143	19 (65.5%)	21 (61.8%)	0.762
Age (years), mean (range)	33.2 (13-52)	39.5 (13-70)	0.172	37.6 (13-70)	39.5 (13-70)	0.654	38.5 (13-64)	38 (13-70)	0.897
UAS, mean (range)	5.13 (2-6)	4.9 (2-6)	0.486	5.02 (2-6)	4.82 (2-6)	0.499	5.07 (2-6)	4.85 (2-6)	0.459

Note: CSU: chronic spontaneous urticaria; ASST: autologous serum skin testing; SPT: skin prick testing; UAS: urticaria activity score. Significance between two groups was evaluated with a two-tailed Student's *t*-test and indicated by *p* value. $p < 0.05$ was indicated as significant.

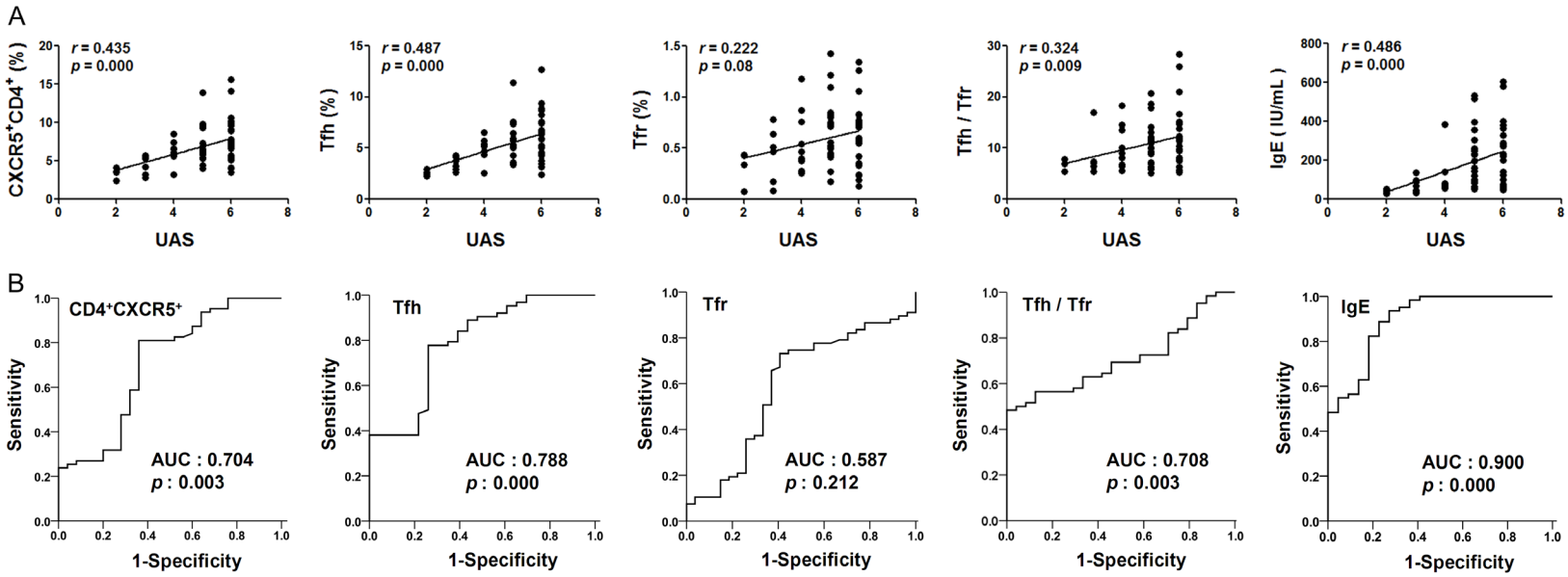

Figure 5. Correlation between CD4⁺CXCR5⁺ cells, Tfh cells, the Tfh/Tfr ratio, total IgE in the peripheral blood of chronic spontaneous urticaria (CSU) and disease. A. Correlation between CD4⁺CXCR5⁺, Tfh, Tfr, Tfh/Tfr, total IgE and urticaria activity score (UAS) in patients with CSU. Each plot represented one sample. Correlation analysis were conducted using Spearman's rank test. B. Receiver operating characteristic (ROC) curves for association of CSU disease (versus healthy controls) based on the CD4⁺CXCR5⁺, Tfh, Tfr, Tfh/Tfr and total IgE. Statistical results of area under the curve (AUC) and *p* value in ROC for association of CSU disease were shown in the graph. $0.5 < AUC < 1$ and $P < 0.05$ indicated that they were associated with the disease activity.

IgE and total IgE in allergic immune response to airborne allergens [27]. Our results that total IgE in plasma of patients with CSU was elevated and associated with UAS confirmed earlier reports [28, 29]. Taken together, the formation of germinal centers and the increased frequencies of $CD4^+CXCR5^+$ cells and Tfh cells in peripheral blood may be involved in the production of total IgE and contributing to the pathogenesis in CSU patients.

Multiple factors, including cytokines, such as IL-4, IL-6, TGF-β, IL-12 and IL-21, are involved in the differentiation and function of Tfh cells [30]. In OVA plus alum-sensitized mice, anti-OVA and total IgE antibodies were induced and promoted by IL-4-secreting Tfh cells [20]. The specific block of IL-6R signaling on T cells can suppress the number of Tfh cells and the increase in the concentration of total IgE, Derp 1-specific IgE or IgG1 in B cells after sensitization in $CD4^{Cre}xgp130^{-/-}$ mice [19]. The IL-6/STAT3 signaling pathway has been shown to regulate the frequency of Tfh cells in the peripheral blood of patients with rheumatoid arthritis [18]. IL-21, a cytokine produced by Tfh cells, is essential for the immunomodulation of humoral and cellular immune responses. The role of IL-21 in the production of IgE remains controversial, regarding its promotion or inhibition of specific immune functions [31-34]. Our results indicated that the total IgE was related to IL-4, IL-6, and IL-21, which suggested that Tfh cells might promote the production of total IgE through IL-4 and IL-6, and induced the class switching of B cells in CSU patients, while IL-21 inhibited the production of IgE from B cells, as demonstrated in previous studies [4, 21, 35-38].

In the current study, the positive correlation between $CD4^+CXCR5^+$ cells, Tfh cells, Tfh/Tfr ratio and the UAS, and the higher AUCs of $CD4^+CXCR5^+$ and Tfh cells frequencies and Tfh/Tfr ratio indicated their potential use as serological markers in clinical diagnosis and follow-up of therapeutic response in patients with CSU.

There are some limitations in the current study. The lack of dynamic observations, such as the changes of the above parameters during the active versus and remission period of CSU, make it impossible to verify the time-course change of the immunological parameters. The relationship between the changes of the above parameters and the therapeutic efficacy is not clear. It remains to be confirmed whether increase of IL-4 and IL-6 and decrease of IL-21 can induce $CD4^+CXCR5^+$ cells and Tfh cells to help B cells to produce IgE in patients with CSU.

In conclusion, the positive correlation between $CD4^+CXCR5^+$ cells, Tfh cells and the UAS, and the higher AUCs of $CD4^+CXCR5^+$ and Tfh cells frequencies in CSU indicated their potential use as serological markers in clinical diagnosis and follow-up of therapeutic response in patients with CSU. Our results provided the primary evidence that the regulation of class switching of B cells may be associated with immunopathogenesis and disease severity of CSU.

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Disclosure of conflict of interest

None.

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