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Correlation between regulatory T cells and autologous serum skin test among chronic spontaneous urticaria patients

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Abstract

Chronic spontaneous urticaria (CSU) is considered as an autoimmune disorder in around 30-50% of cases and referred to as chronic autoimmune urticaria. T regulatory cells (Tregs) may be involved in the pathogenesis of CSU. However, their exact role has not yet been fully defined in those patients. This study aimed to investigate the possible role of Tregs cells subsets in CSU patients. In a case-control study, conducted at dermatology clinics, Suez- Canal University Hospitals, Ismailia, Egypt; 25 CSU patients and 25 apparently healthy control blood donors with matched age and gender were recruited. CSU patients were subjected to history taking, physical examination, assessment of urticaria activity score (UAS) and autologous serum skin test (ASST). Blood samples were obtained from both groups to estimate the number of Tregs cells by flow cytometric analysis. The mean values of $CD4^+$ Fox $P3^+$ and $CD4^+$ $FoxP3^+$ $CD25^+$ Tregs in the study cases were increased significantly compared to the controls (Mean ± SD, **[**73.51±26.63**]** vs **[**4.68±2.98**]**; *P*= 0.001 and **[**0.97±0.59**]** vs **[**0.56±0.36**]** ; *P* =0.003), respectively. However, the mean values of $CD4^+$ $CD25^+$ Tregs were decreased significantly in CSU patients compared to the controls (0.27±0.38 vs 3.63±1.44, *P*= 0.001). Mean levels of $CD4^+CD25^+$ and $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs cells decreased in positive ASST group in comparison to the ASST negative group, but this decrease did not reach statistical insignificance. In conclusion, our data indicated that CSU was associated with alterations in circulating Tregs cells subsets supporting the autoimmune theory.

Keywords: Chronic spontaneous urticaria, T regulatory cells, autologous serum skin test, autoimmunity.

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Introduction

In the United States, the rate of chronic urticaria is estimated to be 1.4% every year. It has two types: chronic spontaneous urticaria (CSU) and inducible urticaria. CSU is a common disease in Egypt, accounting for 1.13% of our patients.[1] CSU patients suffer from pruritic wheals and flare-type skin reactions with or without

angioedema that usually persist for less than 24 hours. Some patients show angioedema only without other signs.[2] This chronic skin disease causes a decrease in quality of life of the patients and affects their performance at work and school.[3]

Several hypotheses have been proposed to explain CSU pathogenesis; the autoimmune hypothesis is the most accepted one.[4,5] This may be due to histamine-releasing autoantibodies against the α subunit of high-affinity IgE-receptor (FcεRI) or IgE in about 45% of CSU patients.[6-8] Also, several autoantibodies against autoantigens involved in the pathophysiology of this disease, including IgE autoantibodies against thyroperoxidase, antinuclear autoantibodies, anti-endothelial autoantibodies, antiparietal autoantibodies, autoantibodies against FcεRII receptors on the surface of eosinophils are recently reported.[9,10] IgE autoantibodies against double-stranded (ds) DNA have been described to have histamine-releasing activity towards basophils in CSU patients.[11-13]

T regulatory cells (Tregs) are a crucial "self-check" built into the immune system that suppresses immune responses of the other cells, which could prevent further excessive reactions. Tregs express the biomarkers.[14]

A primary cause of autoimmune diseases, allergy and inflammatory disorders can be abnormalities in the function or number of Tregs. Therefore, maintaining a balance between activation and suppression of T-cell responses is crucial in the outcome of many immune-mediated diseases.[15,16]

Autologous serum skin test (ASST) is a simple in-vivo clinical test that detects basophil histamine release. It is considered more as an autoreactivity test than a specialized test for autoimmune urticaria. Patients with a positive ASST are more likely to have HLA-DR4, autoimmune thyroid illness, a longer disease course, and less responsive to antihistamine treatment than those with a negative ASST. [1]

Tregs cells may be involved in the pathogenesis of CSU. However, their exact role has not yet been fully defined in those patients. This study aimed to estimate the count of Tregs cells subsets in the peripheral CSU patients' blood and to determine whether there is a correlation between it and the severity of CSU and results of ASST.

Subjects and Methods

A total of 25 CSU patients and 25 blood donor volunteers, as a control group, were included in the study. The two groups were matched for age and gender. The protocol, of this case-control study, was reviewed and approved by the Research Ethics Committee, Faculty of Medicine, Suez Canal University (approval No. 2376 dated May 2015). All participants signed an informed consent before taking any data or doing any investigations. CSU patients were recruited from dermatology clinics, Suez Canal University Hospitals and diagnosed in accordance with the EAACI/GA^{2}LEN/EDF/WAO guideline.[2] The demographic information of the patients and disease-related parameters, duration of urticaria, ASST and weekly urticaria activity score (UAS7) were evaluated.

Patients with the following conditions: lesions lasting for more than 24 hrs, suspected of having urticarial vasculitis, predominant physical urticaria, other autoimmune diseases, receiving corticosteroids or other immunosuppressive drugs, and inability to discontinue antihistamines for one week before blood sampling were excluded from our study.

Urticaria Activity Score (UAS) Determination

UAS was calculated according to EAACI/GA^2 LEN/EDF/WAO Guidelines. [(2)] UAS was estimated every week using the number of wheals and pruritus intensity. These wheals and pruritus appeared during a week before the day of blood sampling.

Patients were asked to document 24-h self-evaluation scores for 7 days by applying the following scheme (0= no wheals, 1= <20 wheals/24 h, 2= 20–50 wheals/24 h and, 3= >50 wheals/24 h). Furthermore, pruritus intensity was determined according to the score (no = 0, mild = 1, moderate = 2, severe = 3). Thus, the sum score of 7 consecutive days was within a minimum score of 0 and a maximum score of 42. Accordingly, weekly UAS was scored as

follows: 0–14 (mild), 15–29 (moderate) and 30–42 (severe).

Autologous Serum Skin Test (ASST) evaluation

ASST was performed according to EACCI/GA^{2}LEN/EDF/WAO.[2] To avoid false-negative results, patients were required to stop antihistamines (2nd generation) for at least 3 days and corticosteroids and other immunosuppressive medicines for at least 4 weeks prior to testing. All patients were injected with 0.05 mL of fresh autologous serum, then the wheal and flare reaction was read after 30 min. Saline solution (0.9% weight/volume NaCl) intradermal injection was performed as a negative control. Patients showing a red wheal with a mean diameter at least 1.5 mm greater than controls (saline solution treatment) were considered positive.

Collection of Blood Samples

A whole blood sample was taken from each patient and control, and peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized venous blood using Ficoll-Paque density gradient centrifugation (eBioscience, San Diego, California, USA).

Flow cytometric analysis

-Surface staining with monoclonal antibodies:

Monoclonal antibodies with conjugated dyes were used to assess the cell markers. In a separate tube, 100 µl of PBMC, isolated from a patient or a control subject, were mixed with the fluorescein isothiocyanate-conjugated (FITC), anti-human CD4, and phycoerythrin conjugated (PE), anti-human CD25 monoclonal antibodies (mAb) supplied by (eBioscience, San Diego, California, USA). The tubes were incubated at 4°C for 20 minutes, then washed twice with phosphate-buffered saline, containing 0.5% normal fetal calf serum at pH 7.4, according to the manufacturer's instructions. Cells were fixed and permeabilized with all ophycocyanin-conjugated anti-human FOXP3 (PCH101) mAb (eBioscience, San Diego, California, USA) for 30 min at 4°C for intracellular staining of forkhead box P3 (FOXP3). Appropriate isotype-matched control antibodies were used to detect any non-specific staining.[17]

-Data Analysis and interpretation:

The cells acquisition and samples analyses were performed on an FC500 flow cytometer (Beckman Coulter Life Sciences, UK). For each sample, 100,000 events were recorded. The frequency and quantification of $CD4^+CD25^+FOXP3^+$ cells were calculated in $CD4^+$ gate. Then, the frequency of $FOXP3^+$cells expressing CD25 were calculated and the FOXP3 mean fluorescence intensity (MFI) in this population were assessed. In all cases, non-specific background staining below gate value was considered in the FOXP3 MFI calculation. Analysis of flowcytometric data were performed using Flow Jo software. [(17)]

Statistical analysis

Data were managed using the Statistical Package of Social Sciences (SPSS) version 20. Data description was primarily based on means and standard deviations (SD, normal data) for continuous endpoints and on frequencies for categorical endpoints. Unadjusted comparisons between patients and control group were made using the t-test or Mann-Whitney U test for continuous endpoints and the Chi-Square test for categorical endpoints.

Results

This study included 25 CSU patients and 25 control subjects matched for age and gender. Patients and controls mean age (± SD) were 38±11 and 43±8 years, respectively. Of the CSU cases, seven (28%) were males and 18 (72%) females. The control group included 9 (36%) male subjects and 16 (64%) females **(**Table 1).

The mean duration of the disease was 6.72±9.45 months. While the mean of UAS was 24.92±11.9 months. ASST showed positive results in eight (36%) patients. Out of the study group, 12 (48%) patients had angioedema (Table 2).

Table 1. Demographic data of Study subjects

Demographic data		Patients (N=25)	Control (N=25)	*P* value
Age (years)	Mean±SD	37.76±10.93	43.16±8.42	NS
	Median (IQR)	37 (30-46)	44 (38-49)	
Gender	Male No (%)	7 (28%)	9 (36%)	NS
	Female No (%)	18 (72%)	16 (64%)	

P >0.05 is not significant (NS) SD: Standard Deviation, IQR: Inter Quartile Ratio.

Table 2. Clinical characteristics of the 25 study patients.

Clinical Characteristics		No	%
Angioedema	Present	12	48%
	Absent	13	52%
Disease duration (months)	Mean±SD	6.72±9.45	
UAS	Mean±SD	24.92±11.9	
ASST	Negative	17	64%
	Positive	8	36%

UAS: Urticaria Activity Score, ASST: Autologous Serum Skin Test.

Comparison of regulatory T cells subsets among case and control groups

The mean values of $CD4^+$ Fox $P3^+$ and $CD4^+$ $FoxP3^+$ $CD25^+$ Tregs in the study cases were increased significantly compared to the controls (Mean±SD, **[**73.51±26.63**]** vs [4.68±2.98]; *P*=0.001 and **[**0.97±0.59**]** vs [0.56±0.36]; *P*=0.003), respectively. However, the mean values of $CD4^+$ $CD25^+$ Tregs were decreased significantly in CSU patients compared to the controls (**[**0.27±0.38**]** vs **[**3.63±1.44**]**; *P*= 0.001) (Table 3 and Figure 1).

Table 3. Comparison of $CD4^+$Fox $P3^+$, $CD4^+CD25^+$, $CD4^+FoxP3^+$ $CD25^+$ regulatory T cells between case and control groups.

Regulatory T cells		Case	Control	*P* value
$CD4^+$ Fox $P3^+$	Mean ± SD	73.51±26.63	4.68±2.98	0.001
	Median (IQR)	82.2 (65.5-93.8)	4 (2.5-5.81)	
$CD4^+$ $CD25^+$	Mean ± SD	0.27±0.38	3.63±1.44	0.001
	Median (IQR)	0.05 (0-0.6)	3.33 (2.5-4.6)	
$CD4^+FoxP3^+$ $CD25^+$	Mean ± SD	0.97±0.59	0.56±0.36	0.003
	Median (IQR)	0.79 (0.61-1.23)	0.43 (0.31-0.62)	

P <0.05 is significant SD: Standard Deviation, IQR: Means Inter Quartile Ratio.

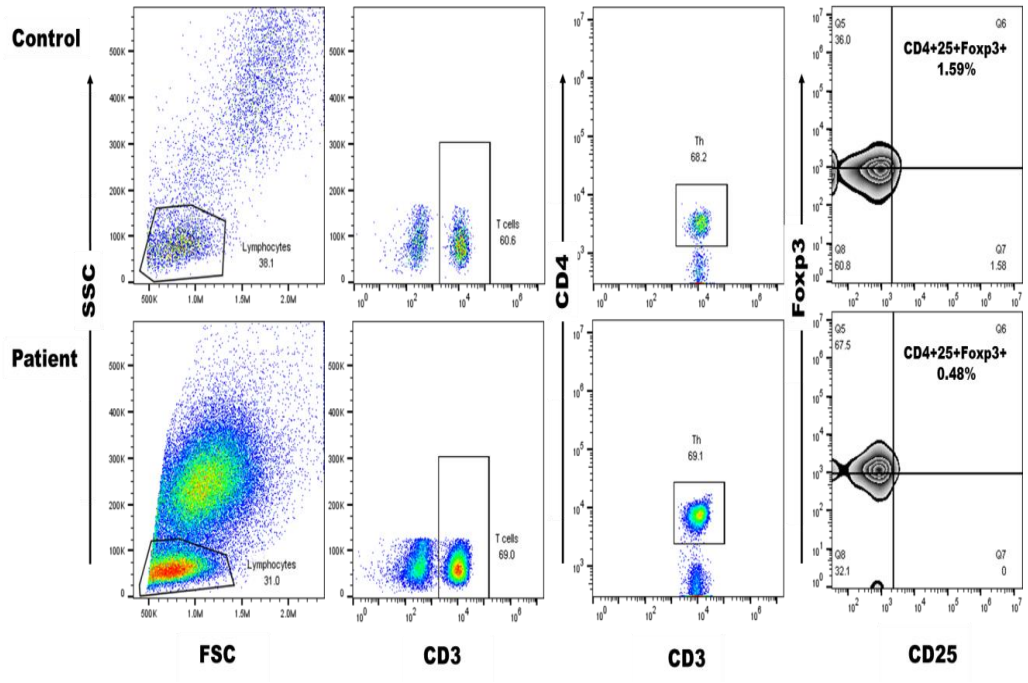

Figure 1. Flow cytometric analysis of Tregs cells in patient and control groups.

Peripheral blood sample cells were surface stained using monoclonal anti-human antibodies against CD3, CD4, and CD25. Surface staining was followed by intercellular staining using anti-human monoclonal antibodies against Foxp3. The pseudo color plot on the left shows the gating of lymphocytes using FSC (Forwared SCatter) versus SSC (Side SCatter), followed by detection of T cells using SSC versus CD3, and the gating of T cells. The following pseudo color plot shows the gating of CD4+ T cells using CD3 versus CD4. Finally, the Treg CD4+CD25+Foxp3+ cells percentage on the contour plot using CD25 versus Foxp3.

Comparison of Tregs cells subsets among study patients according to ASST

Although the median and IQR of $CD4^+CD25^+$ and $CD4^+$ $FoxP3^+$ $CD25^+$ Tregs % were decreased in the ASST positive group **[**0% (0-0.11)**]** and **[**0.7% (0.43-1.12) than in the ASST negative group [0.17% (0-0.61)**]** and [0.84% (061-1.26)], but this decrease did not reach statistical insignificance (*P* =0.21 and 0.2, respectively). Also, the median and IQR of $CD4^+Fox\ P3^+$ Tregs % were increased in the ASST positive group **[**86.95% (76.8-94.9)] compared to the ASST negative group **[**73.4% (63.8-93.5)], however, the difference did not reach statistical significance (*P*= 0.34) (Table 4 and Figure 2). Finally, Table 5 shows that there was no correlations between UAS or duration of the disease and CD4+ Fox P3+, CD4+ CD25+, CD4+ FoxP3+ CD25+ Tregs subsets (*P* >0.005).

Table 4. Comparison of Tregs cells subsets among CSU patients according to ASST

Regulatory T Cells	ASST Negative	ASST Positive	*P* value
$CD4^+FoxP3^+$			
Mean±SD	71.61±25.44	77.57±30.41	NS
Median (IQR)	73.4 (63.8-93.5)	86.95 (76.8-94.9)	
$CD4^+$ $CD25^+$			
Mean±SD	0.34±0.41	0.135±0.3	NS
Median (IQR)	0.17 (0-0.61)	0 (0-0.11)	
$CD4^+FoxP3^+CD25^+$			
Mean±SD	1.08±0.63	0.74±0.41	NS
Median (IQR)	0.84 (061-1.26)	0.7 (0.43-1.12)	

P >0.05 is not significant (NS) ASST: Autologous Serum Skin Test, IQR: Inter Quartile Ratio.

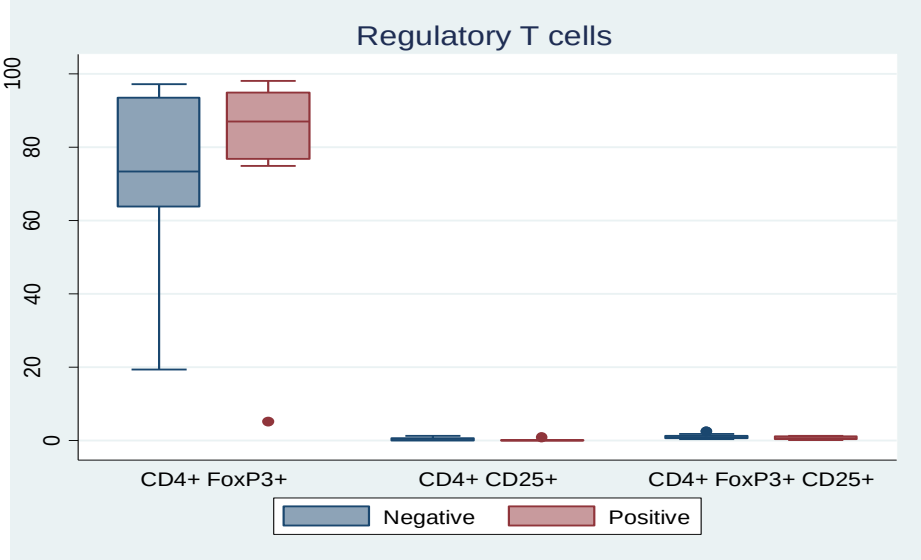

Figure 2. Comparison of Tregs cells subsets among CSU patients according to ASST positive (red color) and ASST negative (blue color).

Table 5. Correlations between disease duration, UAS and Tregs cells subsets in CSU patients.

Tregs cells subsets	UAS	Duration of disease	
$CD4^+$ Fox $P3^+$	-0.103	0.100	Rho
	0.625	NS	*P* value
$CD4^+$ $CD25^+$	0.225	-0.196	Rho
	0.279	NS	*P* value
$CD4^+FoxP3^+$ $CD25^+$	0.227	0.099	Rho
	0.276	NS	*P* value

P >0.05 is not significant (NS) UAS: Urticaria Activity Score, Rho: Spearman correlation coefficient.

Discussion

This study aimed to estimate the count of Tregs cells subsets in the peripheral CSU patients' blood and to determine whether there is a correlation between it and the severity of CSU and results of ASST.

Clinically, urticarial is characterized by sudden appearance of wheals and/or angioedema.[2] Out of the CUS studied group, 48% of patients had angioedema. Our results agreed with most previous revisions which found that around 30–50% of patients suffer from angioedema with or without wheals.[18,19]

In our study, the mean values of $CD4^+$ Fox $P3^+$ and $CD4^+CD25^+$ $FoxP3^+$ Tregs cells in CSU patients were increased compared to the controls. While the mean levels of $CD4^+$ $CD25^+$ Tregs cells were decreased in CSU patients compared to the controls. These results disagree with some studies that observed a reduced frequency of $CD4^+CD25^+FOXP3^+$ cells in chronic urticaria patients when compared to control individuals.[3,17,20] Such discrepancy in study findings may be because those studies included only patients with chronic autoimmune urticaria not all CSU patients, including autoimmune and idiopathic cases, as done in our study. Those studies showed that patients exhibited a reduced percentage of $CD4^+CD25^+FOXP3^+$ regulatory T cells which could imply that $CD4^+CD25^+FOXP3^+$ regulatory T cells may contribute to the autoimmune pathological process of chronic autoimmune urticaria.

In the present study, ASST was performed, and the results were positive in 36% of patients. This agreed with various previous studies, showed that the prevalence of positive ASST results in CSU patients varied from 34% to 67%.[21,22] Also, we tried to find out the

correlation between $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells and ASST. We found that the mean levels of $CD4^+CD25^+$, and $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells were decreased in the ASST positive group compared to the ASST negative group, which confirm the autoimmune pathogenesis of CSU. According to previous studies ASST is considered as a bedside clinical test which can detect the presence of autoimmunity in patients with CSU.[1,21,23,24]

Our results agreed with a study, reported that 56% of CSU patients had negative ASST, and pointed out to lack of functional circulating autoantibodies in these patients.[25] However, since 44% of their patients had positive ASST, they suggested that this observation may indicate the presence of functional circulating autoantibodies and an autoimmune basis for their disease.[25]

In conclusion, our data indicated that CSU was associated with alterations in circulating Treg cells subsets supporting the autoimmune theory. However, more studies are needed to help understanding the mechanisms underlying the pathogenesis of CSU.

Author Contributions

AM, ST; performed the laboratory work. AM; made the statistical analysis. AM, AA, MA; examined the patients. AM, ST, AA, MA; collected samples. All authors participated in writing and reviewing the paper

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The protocol, of this case-control study, was reviewed and approved by the Research Ethics Committee, Faculty of Medicine, Suez Canal University (approval No. 2376 dated May 2015)..

Informed consent

All participants signed an informed consent before taking any data or doing any investigations.

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