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Low frequency of $CD4^{+}CD25^{high}FOXP3^{+}$ T regulatory cells in Egyptian patients with chronic spontaneous urticaria

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Abstract

Chronic urticaria is a prevalent disabling dermatological disease. About 90%, are considered idiopathic and referred to as chronic spontaneous urticaria (CSU), and nearly half of them are likely to have autoimmune mechanisms. Regulatory T cells play a substantial role to prevent autoimmune diseases. Subsets of Tregs expressing the $CD4^{+}CD25^{high}$ and forkhead-box-P3 (FOXP3) transcription factor, crucial for their development and function, are best characterized in maintenance of self-tolerance. The objective of this study was the analysis of peripheral $CD4^{+}CD25^{high}FOXP3^{+}$(T regs) frequency in chronic spontaneous urticaria; and its possible association with autologous serum skin test (ASST). Fifty chronic spontaneous urticaria patients (25 with positive ASST and 25 with negative ASST) and 20 healthy controls were enrolled in this study. The frequency of $CD4^{+}CD25^{high}FOXP3^{+}$ (T regs) was analyzed by flow cytometry. A Significant decrease in peripheral blood $CD4^{+}CD25^{high}FOXP3^{+}$ T regs% was detected in CSU patients in comparison to healthy individuals (median [IQR], 1.47% [0.71–3.12] vs 1.79% [1.15–4.00]; *P* = 0.05). When ASST positive patients were compared with ASST negative patients, no significant difference was found in percentage of T regs, (P=0.112). In conclusion our data provided further insights into CSU pathogenesis. Reduced frequency of $CD4^{+}CD25^{high}FOXP3^{+}$(Tregs) in patients with urticaria, support the notion that CSU is an immune mediated disease and may help researchers to develop a novel immunotherapy strategy.

Keywords: Regulatory T cells: T regs, CD25, Forkhead-box-P3: FOXP3, chronic spontaneous urticaria: CSU, Autologous Serum Skin Test: ASST.

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Introduction

Chronic urticaria (CU) is one of the common diseases that impacts, in a negative way, the general well-being of individuals affected.[1] It is characterized by the occurrence of wheals spontaneously, mostly in middle age females, and continues for at least six weeks.[2] Most patients with CU remain undefined to a certain etiology and are classified as chronic idiopathic, in other words, spontaneous urticaria (CSU).[3] It

was found that chronic urticaria due to autoimmune causes was demonstrated in around 30-50% of CSU cases and are referred to as chronic autoimmune urticaria (CAU).[4] This autoimmunity is caused by a disorder in self-tolerance, which is normally preserved due to immune balance.[5,6]

One of the important components that is crucial in maintaining this balance is the regulatory T cells (Tregs).[7] The most widely identified T regulatory cell is a $CD4^+$ subpopulation expressing high levels of CD25 and the transcription factor FOXP3.[8,9] This $CD4^+CD25^{high}$ $FOXP3^+$Treg counteract the action of auto-aggressive T cells, therefore saving the body from autoimmune diseases.[10,11]

Any disorder in the expression of FOXP3 leads to devastating consequences ending in autoimmune diseases and even allergy.[12-14] Therefore, FOXP3 is the cornerstone element mandatory for proper development and function of $CD4^+CD25^+$ T cell.[5] Moreover, FOXP3 expression is specific for Treg and thereby allows easy and accurate quantification and detection of Treg.[15]

Autologous Serum Skin Test (ASST) is regarded as a test for autoreactivity rather than a specific test for autoimmune urticaria. CU patients with a positive ASST are more likely to be associated with HLADR4, to have autoimmune thyroid disease, a more prolonged disease course and may be less responsive to antihistamine treatment than those with a negative ASST.[16]

For a better understanding of CSU pathology, the aim of the present study was to measure the frequency of circulating $CD4^+CD25^{high}FOXP3^+$(Tregs) in patients with chronic spontaneous urticaria (CSU), compared to healthy controls, and its possible association with ASST.

Subjects and Methods

Study participants

This cross-sectional case-control study included 70 participants, among whom 50 were patients with chronic spontaneous urticaria (7 males and 43 females; median age; 34.9 ± 11.8 years) and 20 were healthy controls (10 males and 10 females; median age 36.6 ± 11.6 years). The patients were recruited from the Allergy and Clinical Immunology clinic at Ain Shams University Hospitals. Inclusion criteria were patients complaining of spontaneous appearance of wheals, angioedema or both for > 6 weeks. They were diagnosed according to the EAACI/GA2LEN/EDF/WAO evidence and consensus-based guideline.[17] Patients with acute, allergic, physical or vasculitic urticaria, were excluded. None of them was pregnant, lactating or having major systemic illnesses. Antihistamines were stopped a week before enrollment, and systemic corticosteroid or immunosuppressive for at least 12 weeks, patients who suffered significant exacerbations or couldn't withstand stopping treatment were excluded from participation and received their treatment back. The control subjects were apparently healthy with negative history of allergy, urticaria or any other disease, besides they were age-matched and of the same social class as far as possible. An informed written consent was obtained from all participants. The study was approved by the institutional ethical committee board of Ain Shams University.

Study design

Assessment of participants included medical history, full clinical examination, and routine blood tests such as CBC, ESR, liver and renal function tests. Antithyroid antibodies, serum total IgE, autologous serum skin test (ASST) and $CD4^+CD25^{high}$ $FOXP3^+$Treg cell assay were measured for all patients. Based upon ASST results, the 50 CSU patients were categorized into 2 groups 25 patients with positive ASST and 25 patients with negative ASST.

Laboratory tests

-From each participant, 6 ml of venous blood were taken, where 3 ml were placed in an EDTA vacutainer tube (Becton Dickinson, Oxford, UK) for performing complete blood count (CBC) and $CD4^+CD25^{high}$ $FOXP3^+$Treg cells estimation by flow cytometry. The remaining of the blood was collected into a gel vacutainer tube (Becton Dickinson), and left to coagulate for half an hour. Serum was obtained by centrifugation at 1000 x g for quarter an hour and divided into

two aliquots; one aliquot for routine lab immediate assay Parameters (AST, ALT, creatinine, and BUN), while the remaining part of sera was aliquoted and stored at –20°C until used for the anti-thyroid antibodies, thyroid-stimulating hormone (TSH) and total serum immunoglobulin E (IgE) levels. All tests were performed at the Department of Clinical Pathology- Ain Shams University.

-CBC was done utilizing "Coulter counter" (T660) (Beckman. Coulter, California, USA).

-AST, ALT, creatinine, and BUN were measured on Synchron CX-9 autoanalyzer (Beckman Instruments Inc.; Scientific Instruments Division, Fullerton, CA, USA).

-Anti-thyroid antibodies (anti-thyroperoxidase and anti-microsomal antibodies) were performed by indirect immunofluorescence assay using commercially available kits (Inova Diagnostics, USA), according to the manufacturer's instructions.

-Total serum IgE levels (IU/ml) was estimated using an enzyme linked immunosorbent assay (ELISA) kit supplied by Biopharm (RIDASCREEN; R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions. This procedure has a sensitivity of 1IU/ml. The normal level of total IgE, in adults, is less than 100 IU/ml.

$CD4^+CD25^{high}$ $FOXP3^+$Treg flowcytometry analysis[18,19]

The test was done on lysed whole blood using three colors EPICS XL flow cytometer (Coulter Electronics, Florida, USA), by using the following monoclonal antibodies conjugated with different fluorescent dyes: Fluorescein isothiocyanate (FlTC)-conjugated anti-human CD4, Phycoerythrin (PE)-conjugated anti-human CD25, Phycoerythrin-Cyanine 5 (PE-Cy5)-conjugated anti-human FOXP3, PE-Cy5 isotype control, and PE isotype control, supplied by (eBioscience, USA). Two plastic test tubes were used: one for the test and the other for the isotypic control. According to the manufacturer's protocol, 50µL of EDTA treated whole blood were added to each tube, which contained 1 mL lysing solution, followed by a wash with phosphate-buffered saline (PBS). Then the cell pellet was stained with combinations of the following monoclonal antibodies (5 µL each): anti-CD4-FITC, anti-CD25-PE in the test tube; PE isotype control and anti-CD4-FITC in the control tube. Both tubes were then incubated at room temperature protected from light for 20 minutes, then washed once with PBS.

-Intracellular FOXP3 staining:

The cell pellet was re-suspended in 0.5 ml of freshly prepared fixation/permeabilization working solution and incubated for 30 minutes in the dark at 4°C. This was followed by washing once with PBS, then washing once again with 1 ml of 1x permeabilization buffer. Ten µL of anti-FOXP3-PE-Cy5 monoclonal and PE-Cy5 isotype control were added in the test tube and control tube, respectively, and incubated for 30 minutes at 4°C with minimal exposure to light. This was followed by washing once with PBS, then the stained cell pellet is re-suspended in 0.5 mL PBS sheath fluid solution for analysis by flow cytometer.

-Data acquisition and analysis were performed by EPICS XL flowcytometry, using SYSTEM II version 3 software with a standard three-color filter configuration. Isotype-matched controls were used to set up the threshold for gating.

-Lymphocytes were firstly identified according their size and complexity on the forward/side scatter plot (figure 1-A). $CD4^+$T cells were identified based on $CD4^+$ expression (figure 1-B). PE isotype control was used to set gates separating $CD4^+CD25^+$ cells from $CD4^+CD25^-$ cells. Then according to the intensity of positive expression of CD25 on $CD4^+$ cells, two areas on the plot were noticed: the dim area ($CD4^+CD25^{low}$) (figure 1-D region F), and the bright one ($CD4^+CD25^{high}$) (figure 1-D region E). PE-Cy5 isotype control was used to set the gates for separating $FOXP3^+$ cells from $FOXP3^-$ cells. Tregs were identified by the combined expression of $CD4^+$, $CD25^{high}$ and FOXP3.

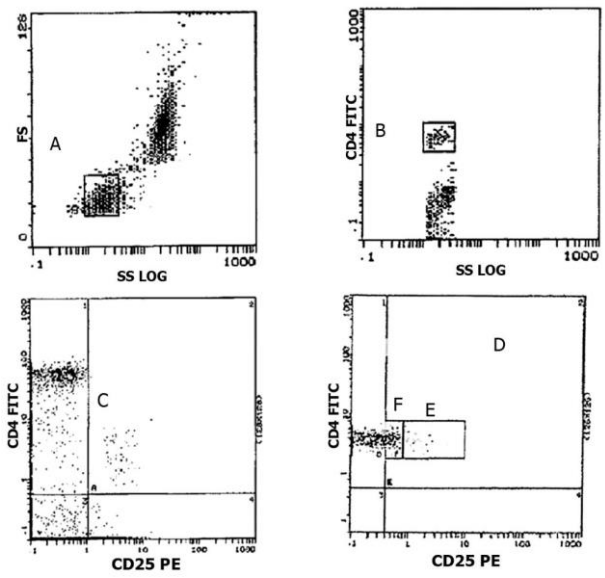

Figure 1. Gating strategy of the flowcytometric analysis of $CD4^{+}CD25^{high}$cells in peripheral blood. Representative dot plots of flow cytometry and the gating strategy used (A to D). (A) Shows forward and side scatter to gate lymphocytes. (B) Shows that $CD4^{+}$cells were acquired after gating the lymphocyte population by forward and side scatter properties. (C) and (D) show the gating approach for $CD25^{+}$cells and for discrimination between $CD25^{high}$ (figure 1-D region E) and $CD25^{low}$ (figure 1-D region F) on $CD4^{+}$cells. The gates for the $CD25^{high}$ and $CD25^{low}$ populations were set by comparing the CD25 expression levels of $CD4^{+}$ cells.

Autologous serum skin test (ASST)

ASST was performed according to the recommended methodology of an expert panel, representing the Dermatology section of the European Academy of Allergy and Clinical Immunology (EAACI).[20] Patients were instructed to stop Antihistamines a week before enrollment, and systemic corticosteroid or immunosuppressive for at least 12 weeks as mentioned above. Venous blood was collected into sterile glass tubes without accelerator or anticoagulant. Blood was allowed to clot at room temperature for 30 min before separation. Separation was done with a bench centrifugation of 450–500 x g for 10 minutes. The volar forearm skin was cleaned with antiseptic, avoiding the wrist and skin areas known to have had spontaneous wheals in the previous 48 h.

The ASST was performed with a 0.5–1 ml sterile syringe and needle 27G. 0.05 ml of fresh undiluted serum was injected intradermally in by introducing the bevel of the needle uppermost and aiming to raise a palpable bleb of fluid within the papillary dermis. The ASST response was validated by performing a positive histamine control, by intradermal injection of 0.5–1 µg histamine (50 µl of 10–20 µg/ml histamine solution). As a negative control skin test, 50 µl sterile physiological (normal) saline was injected intradermally using the same method as for serum. leaving 3–5 cm gaps between each of the three injections. After half an hour, a clean transparent ruler was pressed lightly onto the skin surface of a positive ASST to demonstrate the oedema within a skin test site more easily by blanching the erythema. ASST is positive if ASST mean wheal is about 1.5 mm in diameter, or more than the response induced by saline.

Statistical analysis

We analyzed our data using IBM© SPSS© Statistics version 24 (IBM© Corp., Armonk, NY) and MedCalc© version 14 (MedCalc© Software bvba, Ostend, Belgium). To examine the normality of numerical data distribution we applied Shapiro-Wilk test. Then we presented normally distributed numerical variables as mean ± SD and used unpaired t-test to compare inter-group differences. Median and interquartile represent non-normally distributed numerical data and Mann-Whitney test was applied to compare intergroup differences. Categorical variables were presented as ratio or number and percentage and we used Fisher's exact test to compare differences. *P*-values ≤ 0.05 were considered statistically significant.

Results

A total of 50 cases and 20 healthy controls were included in the study. Both groups were comparable for age and sex. Demographic and clinical characteristics of CSU group are shown in table 1.

Table 1. Demographic and clinical characteristics of CSU patients.

Variable	Cases (n=50)
Age (years): mean±SD	34.9 ±11.8
Gender(M/F)	7/43
Disease duration (years)	2 (0.67 - 6)
Family history of atopy: n (%)	
Negative	43 (86.0%)
Positive	7 (14.0%)
Frequency of attacks: n(%)	
Daily	33 (66.0%)
Every other day	8 (16.0%)
Weekly	6 (12.0%)
Monthly or Occasionally	3 (6.0%)
Associated Angioedema	28 (56.0%)
Positive antithyroid antibodies	11 (22.0%)

Frequency of $CD4^+CD25^{high}$ $FOXP3^+$ (Tregs)

As regards the frequency of $CD4^+CD25^{high}$ $FOXP3^+$ (Tregs) in both cases and controls, the percentages of these cells were significantly reduced in CSU patients [1.47 (0.71 - 3.12) %] compared to healthy controls [1.79 (1.15 - 4.00) %] (*P*=0.05) (figure 2).

No significant correlation was detected between both T-cell subpopulation and age of the cases or disease duration (data not shown).

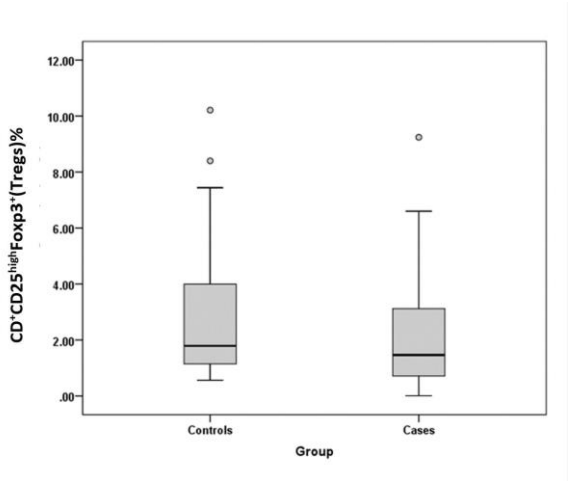

Figure 2. Box plot showing the $CD4^+CD25^{high}$ $FOXP3^+$ (Tregs) % in in peripheral blood of patients with CSU and controls. The T reg cells were significantly lower in patients with CSU than controls.

Frequency of $CD4^+CD25^{high}$ $FOXP3^+$ (Tregs) in patients; classified based on the results of antithyroid antibody tests

$CD4^+CD25^{high}FOXP3^+$(Tregs) % was lower in CSU patients with positive antithyroid antibodies (n=11), compared to those with negative antithyroid antibodies (n=39); 0.95 (0.56 - 1.75) vs 1.55 (0.78 - 3.17) % respectively. Still this difference did not reach statistical significance.

Frequency of $CD4^+CD25^{high}FOXP3^+$(Tregs) in patients; classified based on the results of ASST

The CSU patients were selected as two groups; ASST positive and ASST negative groups. Although median and IQR of $CD4^+CD25^{high}FOXP3^+$ (Tregs) % was lower in ASST positive group [0.95 %(0.64 - 2.46)] than in ASST negative group [1.58 (1.09 - 3.36)], yet this reduction did not reach statistical significance (Figure 3).

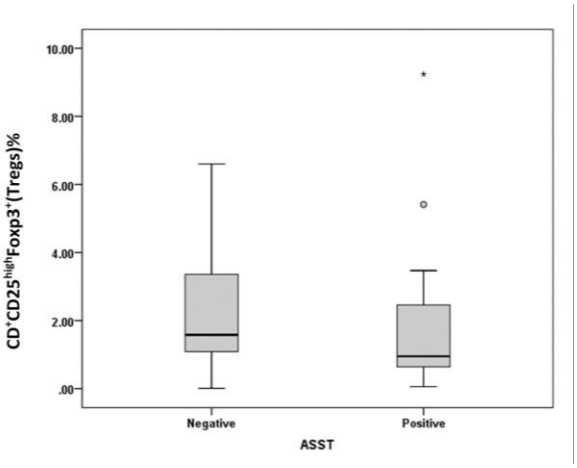

Figure 3. Comparison between CSU patients with positive ASST and negative ASST. As regards percentage of $CD4^+CD25^{high}$ $FOXP3^+$ Treg cells in peripheral blood showing lower percentage in cases with positive ASST.

Discussion

Chronic urticaria is complex in pathogenesis and therefore it is not utterly understood.[21] Our aim mainly in this study was to determine if CSU is associated with altered peripheral Treg cell homeostasis. We investigated the proportion of $CD4^+CD25^{high}FOXP3^+$ (Tregs) in the peripheral blood of CSU patients.

T regulatory cells (Tregs) have been recently discovered as a unique subpopulation of T cells, which crucially function on the prevention of

autoimmunity and regulate transplantation tolerance.[24] In humans, $CD4^+CD25^+$ T cells are stratified into two populations; suppressive $CD4^+CD25^{high}$ T cells and non-suppressive $CD4^+CD25^{low}$ T cells.[23] A fundamental marker of regulatory T cells is FOXP3, which is a transcription factor essential for proper operation of $CD4^+CD25^+$ Tregs.[24] The fraction of $FOXP3^+$ cells is present more within the $CD25^{high}$ compared with the total $CD25^+$ T cell subset.[25]

In this study, we first demonstrated that CSU patients compared to healthy controls showed statistically significant reduction in peripheral frequencies of $CD4^+CD25^{high}FOXP3^+$(Tregs). On comparing ASST positive with ASST negative patients we found $CD4^+CD25^{high}FOXP3^+$ (Tregs) % more reduced in ASST positive group, although this reduction did not reach a statistical significance. Deficiency of regulatory T cells (Treg) in CSU further suggests a significant role played by these cells in suppressing autoimmune processes.

In support of this hypothesis, Sun et al.[26] previously published data, showed decreased frequency of $CD4^+CD25^{high}FOXP3^+$cells in CU patients when compared to healthy control group. Additionally, there was lower expression of FOXP3 rather than number of cells in chronic autoimmune urticaria opposed to chronic idiopathic urticaria in general. This decreased expression of transcription factor FOXP3 in $CD4^+CD25^{high}$Treg cells could explain their altered function, and consequently justifies the occurrence of autoimmune phenomenon. The authors however owed reduced frequency of $CD4^+CD25^{high}$ $FOXP3^+$cells in CU patients to their preferable shift to the sites of active inflammation, thus leaving the circulation.[26] Opposite to this assumption, a decreased proportion of $CD4^+FOXP3^+$Tregs, was detected in the inflammatory infiltrate in skin of patients with cutaneous lupus erythematosus, and on the other hand no difference in the peripheral frequency of circulating $CD25^{high}$ and $FOXP3^+$cells between the same patients and controls.[27] Interestingly, Antiga et al.[28] revealed a significantly reduced frequency of $CD25b^{right}FOXP3^+$cells in peripheral blood of scleroderma and morphoea patients when compared with healthy controls and inflammatory dermatoses patients. Also, in the same patients, and when measured in cutaneous infiltrate, FOXP3 expression and $FOXP3^+/CD4^+$ cell ratios were significantly reduced. These findings propose that diminished Tregs within the circulation of patients suffering from autoimmune pathology was not necessarily the result of their favorable homing into the skin, or the reverse, it rather denotes a real down regulation of these cells, which subsequently participate in the pathogenesis of autoimmune diseases. In addition, this highly raises the needs to study more deeply the kinetics of Tregs in these conditions.

Furthermore Arshi et al.[29] in agreement with our findings, reported a significantly lower percentage of circulating $CD4^+CD25^+$ $FOXP3^+$ T cells in CU patients compared to control subjects, however after classifying their patients according to the response to ASST, there were no significant differences in the level of circulating Tregs between patients with ASST-positive and ASST-negative CU. This could might indicate an underlying immune dysregulation in CSU both ASST positive and ASST negative.

On the other hand, Chen et al.[30] surprisingly, found that patients with CU had more $CD4^+CD25^{high}$Treg cells in peripheral blood mononuclear cells than did healthy control subjects. No difference was identified between ASST positive and ASST negative patients, both showed significantly more numbers of $CD4^+CD25^{high}$ T cells compared to control subjects, while FOXP3 expression in $CD4^+CD25^+$Treg cells from patients and controls, did not significantly differ. Somehow in line with this, Ou et al.[31] observed significantly higher $CD4^+CD25^+$ T cells in atopic dermatitis patients. Chen et al.[30] data on the function of $CD4^+CD25^+$ cells demonstrated that some patients with CU have disturbed regulatory activity, supporting the possible role of a deficient immunoregulatory function in the pathogenesis of CU. The increase in frequency of Treg cells reported in this study can be explained by being a compensatory mechanism for their defective function in CU patients or just expanded proportion of functionally deranged Tregs. The connection between CU and autoimmune

thyroid disease is unquestioned. About 25% of patients with CIU have antithyroid antibodies, regardless of thyroid functional status.[32] In the present study, we showed reduced both cell subtypes more in CSU patients with positive antithyroid antibodies (n=11), compared to those with negative antithyroid antibodies (n=39), although no statistically significant difference was obtained. In this regard Bossowski et al.[33] found out $CD4^+CD25^{high}$ T lymphocytes and $CD4^+$ $FOXP3^+$ significantly decreased in patients with Graves' disease and Hashimoto thyroiditis (untreated), when compared to healthy control subjects, pointing to their role in triggering and may be the ongoing autoimmune process in thyroid diseases. Also, a report by Qin et al.[34] studying patients with new-onset Graves' Disease, observed a significantly decreased proportion of circulating $CD4^+FOXP3^+$Treg cells and reduced mRNA expression of the transcription factor FOXP3. They also found an inverse correlation between the proportion of $CD4^+FOXP3^+$Treg cells and thyroid-stimulating antibody activity, proposing that these deficient Treg cells in Graves' Disease patients likely lose their capacity to prevent B cells from producing autoantibodies.

Earlier studies[35] revealed that animal models lacking $CD4^+CD25^+$Tcells developed different autoimmune disease, including autoimmune gastritis, thyroiditis, and type 1 diabetes. Besides, it has been shown in transgenic mice, in which endogenous FOXP3 gene expression is reduced in Th cells, that decreased expression of FOXP3 caused immune disease by impairing the suppressive capacity of these cells and converting them into a type of cells that initiated, rather than inhibited, inflammation.[36] In human, several reports have shown a relationship between various autoimmune diseases and the deficiency of the number or function of Treg cells. In Systemic Lupus Erythematosus patients, Liu et al. and Crispin et al. detected a low number of $CD4^+CD25^+$Tregs,[37,38] while a reversible defect in the suppressive function of $CD4^+CD25^+$Tregs from active SLE patients was reported by Valencia et al.[39] Miyara et al.[40] however, demonstrated that the number rather than the function of $CD4^+CD25^+$Treg in active SLE patients was reduced. These inconsistent results might be related to the therapeutic modalities given to SLE patients before being enrolled into the studies. Zhang et al.[41] studied new-onset SLE patients before receiving treatment, to rule out the effect of various medications, and found significantly decreased percentage of $CD4^+CD25^+$ T cells and $CD4^+CD25^{high}$ subpopulation within $CD4^+$ T cells in the new-onset active SLE patients when compared to inactive patients.

In active Vogt-Koyanagi-Harada syndrome (VKH) which is an autoimmune ocular disease, a significantly decreased percentage of $CD4^+CD25^{high}$ Tregs and $CD4^+CD25^{high}$ $FOXP3^+$cells were observed, compared with healthy controls and patients with inactive VKH. This result suggested a definite correlation between decreased frequency of these cells and active autoimmune uveitis in VKH patients.[42]

"How much FOXP3 expressing natural Treg is reduced, will define the phenotype and severity of autoimmune disease based upon the hierarchical genetic predisposition of each autoimmune disease".[43] Recent evidence showed that $CD4^+CD25^+$Tregs may even cure certain autoimmune diseases, for example, inflammatory bowel disease in animal models.[44]

In conclusion, we provided evidence for a decreased frequency of $CD4^+CD25^{high}FOXP3^+$ (Tregs) % in CSU patients regardless of the other autoreactivity available investigations such as ASST or antithyroid antibodies. In the light of our own data and other different studies, deficiency of these cells could be a sign of immune dysregulation, that plays a role in the pathogenesis of CSU with potential for therapeutic manipulation to produce increases in Tregs numbers, noting such treatment remains speculative since another possibility is that reduced numbers of these cells may be a consequence, i.e., epiphenomenon, rather than a cause of CSU.

Limitation of the study was the relatively small sized sample. This may be explained by the strict selection of patients. Detection of other cytokines and correlation with Treg cells frequency would have been useful to determine the exact role in CSU.

Author Contributions

MAE: Conceptualization, supervision, MNF: Methodology, statistical analysis. NAEM writing original draft. SSG and ASM: Data curation, SSG: Resources. NAM: Investigation. EEA: Writing, Reviewing and Editing.

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 study was approved by the institutional ethical committee board of Ain Shams University.

Informed consent

A signed consent form was obtained from each study participant.

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