

ORIGINAL ARTICLE

Detection of CD4⁺CD25⁺FOXP3⁺ regulatory T cells in peripheral blood of patients with chronic autoimmune urticaria

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ABSTRACT

Background/Objectives: Compelling evidence indicates a significant role for a population of CD4⁺ T regulatory cells in suppressing immune responses and in maintaining immunological homeostasis. This study aims to investigate the potential role of CD4⁺CD25^{HIGH}FOXP3⁺ T regulatory cells in patients with chronic autoimmune urticaria and to define the characteristics of CD4⁺CD25^{HIGH}FOXP3⁺ cells in chronic urticaria.

Methods: We used flow cytometry to assess the expression of CD4⁺CD25^{HIGH}FOXP3⁺ cells in the peripheral blood mononuclear cells of patients with chronic autoimmune urticaria.

Results: In this study, we found that patients with chronic autoimmune urticaria have a significantly reduced frequency of CD4⁺CD25^{HIGH}FOXP3⁺ cells ($1.39 \pm 0.27\%$ vs $2.09 \pm 0.54\%$; $P = 0.001$) in their peripheral blood, accompanied by a decreased intensity of FOXP3 expression (50.13 ± 9.79 vs 68.19 ± 6.40 ; $P < 0.001$). Notably, although patients with chronic idiopathic urticaria had a reduced frequency of CD4⁺CD25^{HIGH}FOXP3⁺ cells ($1.85 \pm 0.46\%$ vs $3.64 \pm 0.48\%$; $P < 0.001$), their FOXP3 expression levels did not differ from those in healthy controls.

Conclusions: Patients with chronic autoimmune urticaria displayed a reduced percentage of CD4⁺CD25⁺FOXP3⁺ regulatory T cells. The results imply CD4⁺CD25⁺FOXP3⁺ regulatory T cells may contribute to the autoimmune pathological process of chronic autoimmune urticaria.

Key words: chronic autoimmune urticaria, FOXP3, regulatory T cell.

INTRODUCTION

In 80–90% of patients with chronic urticaria (CU), no specific underlying cause is found, although there is a subset of these patients in whom autoantibodies to the high-affinity immunoglobulin E receptor FcεRI are found.¹ This subgroup is labelled chronic autoimmune urticaria (CAU).² It was later demonstrated that these antibodies stimulated the release of histamine. Antithyroid antibodies are reported to be more frequently found in patients with autoimmune urticaria.³

Examination of biopsies from urticaria patients demonstrated that most of the infiltrating T cells possessed a

Abbreviations:

CAU	chronic autoimmune urticaria
CIU	chronic idiopathic urticaria
CU	chronic urticaria
FOXP3	forkhead box P3
IgE	immunoglobulin E
mAb	monoclonal antibodies
MFI	mean fluorescence intensity
PBMC	peripheral blood mononuclear cells
Th	T helper
Treg	T regulatory

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CD4⁺ helper phenotype.⁴ The cytokine pattern reported elsewhere showed that skin biopsies of patients with CU resemble the T-helper cell type 0 (Th0) cytokine profile with increased levels of interleukin-4, interleukin-5 and interferon- γ messenger (m)RNA⁺ cells.⁵ Alternatively, it is also possible that there is a mixture of Th1/Th2 cell types in the skin of patients with CU. These observations support the notion that T lymphocytes and particularly CD4⁺ T cells are involved in the pathogenesis of CU. In the present study, we sought to determine whether autoimmunity in patients with CAU is associated with alterations in circulating CD4⁺CD25^{HIGH}FOXP3⁺ T regulatory (Treg) cells.

MATERIALS AND METHODS

Patients and controls

Thirty-six CU patients [18 men and 18 women, mean age 20 (range 8–40) years, mean time of disease 1.6 years (range 2 months–12 years)] and 28 healthy controls [16 men and 12 women, mean age 25 (range 20–40) years] participated in this study. We excluded patients with lesions lasting for more than 24 h and suspected of having urticarial vasculitis, patients with allergic [immunoglobulin E (IgE)-mediated] urticaria, patients with predominant physical urticaria and patients taking drugs that could exacerbate or suppress CU. All patients reported daily symptoms of urticaria lasting for at least 6 weeks. CAU was differentiated from CU by the basophil histamine-releasing assay and immunoblotting was undertaken to detect the anti-Fc ϵ R1 autoantibodies;⁶ of 36 cases, 12 were proven to be CAU and 24 chronic idiopathic urticaria (CIU). None of the subjects had ever taken oral corticosteroids or immunosuppressants before the study. Of the 36 CU patients, 29 patients had taken antihistamines orally. Skin testing and blood sampling were conducted at the seventh day or more after antihistamines were withdrawn. All patients hardly had symptoms when blood was taken.

As controls, 28 sex- and age-matched individuals with no history of CU and other autoimmune disease were enrolled. The study was approved by the institutional committee for

human experimentation of the Third Military Medical University and written informed consent was obtained from all studied subjects.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) of CU patients and control subjects were obtained from fresh heparinized venous blood by Ficoll-Paque density gradient centrifugation and suspended in DMEM supplemented with 5% fetal calf serum.

Flow cytometric analysis

To evaluate the *ex vivo* expression of cell surface markers on the PBMC of CU patients and healthy individuals, the monoclonal antibodies (mAb) used in this study were fluorescein isothiocyanate-conjugated anti-human CD4, phycoerythrin-conjugated anti-human CD25 and cychrome-conjugated anti-CD8. Single-cell suspensions (1×10^6 cells) were prepared and stained for 30 min at 4°C with optimal dilutions of each mAb, followed by two washes in phosphate-buffered saline, pH 7.4, containing 0.5% normal fetal calf serum. For intracellular staining of forkhead box P3 (FOXP3), cells were fixed and permeabilized according to the manufacturer's instructions before incubation with allophycocyanin-conjugated anti-human FOXP3 (PCH101) mAb for 30 min at 4°C. For all the markers evaluated in this study, appropriate isotype-matched control antibodies were used to determine non-specific staining. Samples analyses were performed on an FC500 flow cytometer. For phenotypic characterization and quantification of CD4⁺CD25^{HIGH}FOXP3⁺ cells, 100 000 events were recorded for each sample in the CD4⁺ lymphocytes gate. Next, the frequency of FOXP3⁺ cells expressing high levels of CD25 was calculated; the FOXP3 mean fluorescence intensity (MFI) in this population was assessed (Fig. 1). In all cases, non-specific background staining was below gate values

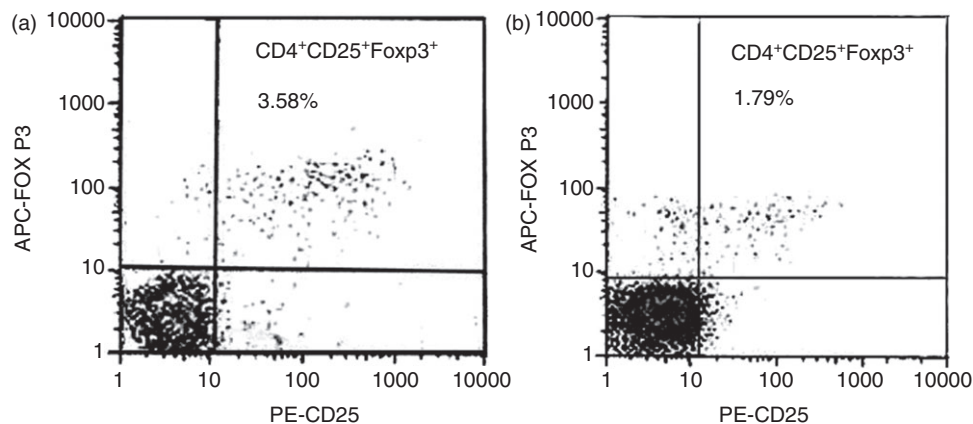


Figure 1 The frequency of CD25^{HIGH}FOXP3⁺ T cells within the CD4⁺ T cells gate within the lymphocytes population and their forkhead box P3 (FOXP3) mean fluorescence intensity were assessed for (a) healthy controls and (b) chronic urticaria patients.

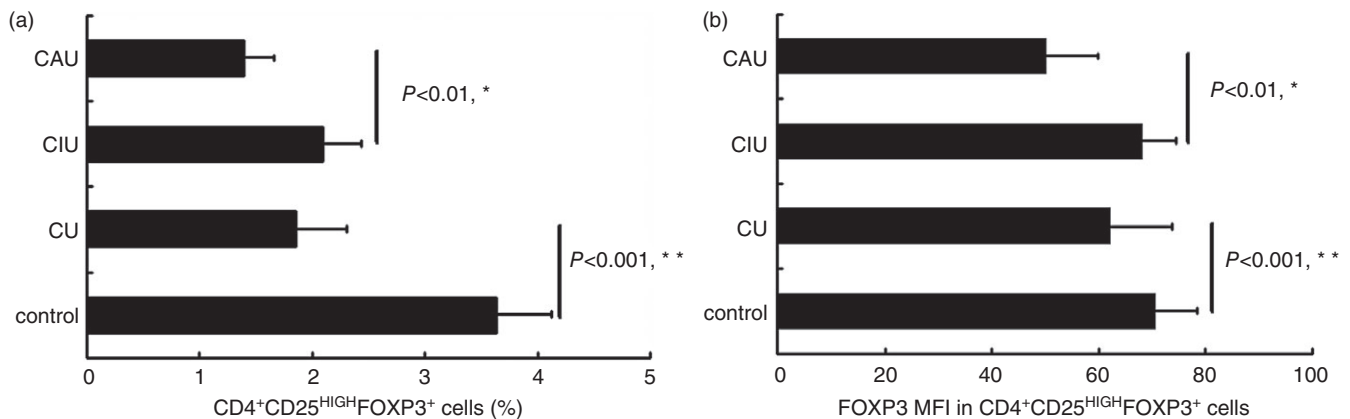


Figure 2 The frequency of CD4⁺CD25^{HIGH}FOXP3⁺ cells and forkhead box P3 (FOXP3) mean fluorescence intensity in healthy subjects and chronic urticaria (CU) patients. Peripheral blood mononuclear cells of 28 healthy controls (Control) and 36 CU patients were stained for CD4, CD25 and FOXP3. CU patients were classified as chronic idiopathic urticaria (CIU; *n* = 24) and chronic autoimmune urticaria (CAU; *n* = 12). (a) Frequency of CD4⁺CD25^{HIGH}FOXP3⁺ cells. The horizontal bars indicate the mean frequency of T regulatory (Treg) cells in each group. (b) FOXP3 expression in CD4⁺CD25^{HIGH}FOXP3⁺ cells. The horizontal bars indicate the mean FOXP3 mean fluorescence intensity in the Treg population for each of the study groups.

considered in the FOXP3 MFI calculation. Analysis of flow cytometry data was performed using FlowJo software.

Statistical analysis

Statistical analyses were performed using ANOVA. Results are expressed as mean ± standard error of the mean. Differences between groups were determined by a two-tailed Student's *t*-test. Statistical significance was assigned when *P* < 0.05.

RESULTS

The frequency of CD4⁺CD25^{HIGH}FOXP3⁺ cells in patients with CAU and CIU

The PBMC from CU patients and healthy individuals were analysed by flow cytometry to determine the frequency of CD25^{HIGH}FOXP3⁺ cells within the CD4⁺ T-lymphocyte population. We observed a reduced frequency of CD4⁺CD25^{HIGH}FOXP3⁺ cells in CU patients when compared with healthy individuals ($1.85 \pm 0.46\%$ vs $3.64 \pm 0.48\%$; *P* < 0.001; Fig. 2a). Furthermore, our results demonstrated for the first time that CAU patients have a lower frequency of CD4⁺CD25^{HIGH}FOXP3⁺ cells in their peripheral blood than patients with CIU ($1.39 \pm 0.27\%$ vs $2.09 \pm 0.34\%$; *P* = 0.001; Fig. 2a).

FOXP3 expression in patients with CAU and CIU

As FOXP3 is an important molecule related to the differentiation and function of Treg cells,⁷ we analysed FOXP3 expression in healthy individuals and CU patients. The MFI of FOXP3 was assessed in CD4⁺CD25^{HIGH}FOXP3⁺ cells and a significant decrease in FOXP3 expression was observed in CU patients compared with control subjects (62.17 ± 11.47 vs 70.62 ± 7.91 , *P* = 0.001; Fig. 2b). We therefore further

analysed the FOXP3 MFI in CD4⁺CD25^{HIGH}FOXP3⁺ cells in both subgroups of CU patients. We found that FOXP3 expression was greatly reduced in the CD4⁺CD25^{HIGH}FOXP3⁺ cells of the CAU subgroup when compared with those from CIU patients (50.13 ± 9.79 vs 68.19 ± 6.40 , *P* < 0.001) and control subjects (50.13 ± 9.79 vs 70.62 ± 7.91 , *P* < 0.001; Fig. 2b). In contrast, when compared with healthy subjects, the CIU subgroup of patients did not differ in FOXP3 MFI (68.19 ± 6.40 vs 70.62 ± 7.91 , *P* > 0.05; Fig. 2b).

DISCUSSION

Our principal aim in this study was to investigate whether CAU correlates with altered peripheral Treg cell homeostasis. We demonstrate that CAU patients show reduced frequencies of CD4⁺CD25^{HIGH}FOXP3⁺ cells in the peripheral blood, accompanied by decreased FOXP3 expression. Our findings also suggest that it would be of interest to explore whether alterations in the differentiation and/or function of B lymphocytes could impact the development and/or peripheral homeostasis of CD4⁺CD25^{HIGH}FOXP3⁺ Treg cells.

There were a number of different mechanisms that may account for the reduced frequency of CD4⁺CD25^{HIGH}FOXP3⁺ cells observed in CAU patients. Considering that our observations are based on analysis of circulating blood leukocytes, they may not be representative of the dynamics of Treg cells in lymphoid tissues, particularly in the case of inflammatory conditions such as autoimmune disorders. It could be possible that the reduced frequency of CD4⁺CD25^{HIGH}FOXP3⁺ cells in CAU patients may result from the preferential migration of these lymphocytes to sites of inflammation, thus leaving the circulation.

It has been reported that CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells in PBMC exhibited defective functions in CU patients.⁸ In our study, CU patients were classified as CAU patients and CIU patients. We further explored whether

these defective functions in CU patients could result from decreased FOXP3 expression in CAU patients. We found reduced frequencies of CD4⁺CD25^{HIGH}FOXP3⁺ cells and decreased FOXP3 expression in CAU patients. Our observations may be relevant in the understanding of the aetiology of CAU and the important role of FOXP3 in the functions of T lymphocytes.

There is evidence from other autoimmune diseases that an altered frequency and/or decreased migratory capacity of regulatory T cells might be relevant, as shown recently for systemic lupus erythematosus.⁹ Defects in the naive T-cell compartments in the thymus have been observed;^{10,11} this defect in thymopoiesis may contribute to the low frequency of CD4⁺CD25^{HIGH}FOXP3⁺ cells and ultimately autoimmune disorders.

A reduced expression of FOXP3 in the peripheral blood of patients with CAU could result from either its lower expression in all FOXP3⁺ cells or selective elimination of FOXP3^{HIGH} cells. Because in this small study of CU patients those without autoimmunity had FOXP3 MFI values similar to those of healthy controls, analysis of FOXP3 MFI may be a useful diagnostic tool to identify and follow individuals at risk for developing CAU. Notably, the functional consequences of reduced FOXP3⁺ cells may be amplified in individuals with inverted CD4/CD8 ratios because in these patients the total numbers of FOXP3⁺ cell would be further decreased.

Although the mean frequency of Treg cells in our CAU patients was lower than that observed in our CIU subgroup, Figure 2a shows that there is a partial overlap of this parameter between patients with and without autoantibodies. The major difference between CAU and CIU patients was the intensity of FOXP3 expression rather than the number of these cells. It has been shown in transgenic mice in which endogenous FOXP3 gene expression is attenuated in Th cells that decreased expression of FOXP3 caused immune disease by subverting the suppressive function of these cells and converting them into effector cells that contributed to, rather than inhibited, inflammation.¹²

In conclusion, our findings might offer a way to improve CU patients' classification and prompt the development of therapies targeting regulatory T cells in CAU autoimmunity; all the results may provide another valuable insight into the mechanisms underlying the pathogenesis of CU.

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