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Defective functions of circulating CD4⁺CD25⁺ and CD4⁺CD25[−] T cells in patients with chronic ordinary urticaria

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KEYWORDS

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FOXP3;
Suppressive function;
CD4⁺CD25[−] T cells;
Cytokines

Summary

Background: Patients with chronic ordinary urticaria (CU) are divided into two groups: 30–50% have chronic autoimmune urticaria, and the remainder have chronic idiopathic urticaria. CD4⁺CD25⁺ regulatory T (Treg) cells play critical roles in maintaining peripheral tolerance and preventing autoimmunity, but the characteristics of Treg cells have not yet been defined in CU.

Objective: To identify whether CD4⁺ T cells play an important immunoregulatory role in the etiology of CU, we determined the frequencies and functions of circulating CD4⁺CD25⁺ and CD4⁺CD25[−] T cells in CU patients and healthy control subjects, with special focus on the characteristics of CD4⁺CD25⁺ T cells.

Methods: Peripheral blood mononuclear cells (PBMCs) were obtained from CU and healthy controls in this study. The frequency of CD4⁺CD25⁺ T cells in PBMCs was detected by flow cytometry. The expression levels of forkhead box P3 (FOXP3) and transforming growth factor-β (TGF-β) in CD4⁺CD25⁺ T cells were detected by real-time PCR. Furthermore, the suppressive function of CD4⁺CD25⁺ T cells was analyzed. Additionally, the Th1/Th2 cytokine secretory profile in mitogen-stimulated CD4⁺CD25[−] T cells was measured by ELISA.

Abbreviations: CU, chronic ordinary urticaria; ASST, autologous serum skin test; Treg cells, regulatory T cells; FOXP3, forkhead box P3; TGF-β, transforming growth factor-β; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin.

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Results: An increased frequency of CD4⁺CD25⁺ T cells was observed in CU patients ($n = 19$) compared to control subjects ($n = 7$). No significant difference was detected in the expression levels of FOXP3 or TGF- β between CU patients ($n = 14$) and control subjects ($n = 7$). Strikingly, the suppressive capacity of CD4⁺CD25⁺ Treg cells from 2 of 5 CU patients was partially defective. We also found that cytokine production from CD4⁺CD25⁺ T cells was significantly reduced in CU patients ($n = 9$) compared to healthy donors ($n = 11$).

Conclusions: Our data demonstrate that CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells in PBMCs exhibit defective functions in CU patients.

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1. Introduction

Chronic ordinary urticaria (CU) is a rather common skin disorder characterized by recurrent, transitory, itchy wheals for more than 6 weeks. It is now known that up to half of patients with CU have circulating autoantibodies against the high-affinity immunoglobulin E (IgE) receptor (Fc ϵ RI) or, less commonly, against IgE [1]. These autoantibodies are believed to be the cause of the lesions in these patients, and their function has been demonstrated *in vivo* by inducing a wheal-and-flare response by an intradermal injection of autologous serum (through an autologous serum skin test, ASST) and *in vitro* by basophil and mast cell histamine release assays [1,2]. Plasma levels of these autoantibodies vary in proportion with the disease activity [3] and remission after removal of the cause [4]. The remaining 50% of patients with CU remain truly 'idiopathic'. These patients are often burdened with a voluminous file of irrelevant but expensive investigations. The clinical presentations of patients with and those without autoantibodies are surprisingly similar, although patients with autoantibodies tend to have more-severe disease compared to patients without autoantibodies [3,5].

The pathogenetic mechanisms of CU are not completely understood. Kikuchi et al. demonstrated an association with other autoimmune disease, reporting that antithyroid antibodies are more frequently found in patients with autoimmune urticaria [6]. Examination of biopsies from urticaria patients demonstrated that most of the infiltrating T cells possessed a CD4⁺ helper phenotype [7]. The cytokine pattern reported elsewhere showed that skin biopsies of patients with CU resembles the T helper cell type 0 (Th0) cytokine profile with increased levels of interleukin-4 (IL-4), IL-5, and interferon gamma (IFN- γ) messenger (m)RNA⁺ cells [8]. 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 especially CD4⁺ T cells are involved in

the pathogenesis of CU. Therefore, recruitment of these CD4⁺ T cells from the circulation to the site of inflammation might also play a central role in the development of urticaria that triggers mast cell degranulation.

Maintaining a balance between activation and suppression of T-cell responses can be crucial in determining the outcomes of many immune-mediated diseases. In both mice and humans, functionally distinct T cell subsets are known as Th1, Th2, and regulatory T cells (Treg). Th1 cells secrete IL-2 and IFN- γ , while Th2 cells produce IL-4, IL-5, IL-13, and IL-10. A dominant Th1 cytokine profile is observed in organ-specific autoimmune diseases, whereas Th2 cell responses tend to predominate in allergic diseases [9]. Naturally occurring CD4⁺CD25⁺ Treg cells, which constitute 5–10% of peripheral CD4⁺ T cells in normal naïve mice and humans, were first described by Sakaguchi et al. [10]. These cells express constitutively high levels of CD25 and are produced in the thymus as a functionally mature T cell subpopulation. Other regulatory T-cell subsets have also been described. They can suppress the proliferation of naïve and memory CD4⁺ and CD8⁺ T cells through direct cell–cell contact, as well as secrete transforming growth factor- β (TGF- β) and IL-10 [11–13]. So far, the expression of forkhead box P3 (FOXP3) has been suggested to best identify populations of CD4⁺CD25⁺ T cells with regulatory function [14,15]. Recent studies suggested that allergic diseases and autoimmune disorders may be the result of impaired or deficient Treg responses [16,17]. In addition, a number of studies have indicated that Treg cells appear to control the development of autoimmune disease and also play critical roles in controlling asthma and allergies [18–20]. However, the characteristics and roles of Treg cells have not yet been defined in CU patients. It is possible that CU patients may have intrinsic abnormalities of Treg cells, and such deficient Treg cells may be unable to inhibit the ongoing inflammatory phenomenon.

In freshly prepared lymphocytes, a majority of CD4⁺ cells are CD25⁺. CD4⁺CD25⁺ T cells mainly

remain naïve and do not express FOXP3. However, no data are available regarding the proliferative capacity and Th1/Th2 cytokine secretory profile of stimulated CD4⁺CD25⁻ cells from CU patients.

In this study, we attempted to determine the frequencies and properties of circulating CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells in CU patients and healthy controls, in order to shed some light on the pathogenesis of CU. We also hope that the results may be of help to clinicians facing the issue of selecting an adequate therapeutic protocol for CU patients.

2. Materials and methods

2.1. Patients and controls

Thirty-four CU patients (17 males and 17 females, mean age 18 (range, 5–36) years) and 17 healthy controls (eight males and nine females, 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 (IgE-mediated) urticaria, patients with predominant physical urticaria, and those taking drugs that could exacerbate CU. All patients reported daily symptoms of urticaria lasting for at least 6 weeks. The autologous serum skin test (ASST), as defined by Grattan et al. [21], was performed in all CU patients. The urticaria of these patients was controlled with only antihistamines as needed. None of the subjects had taken oral corticosteroids or immunosuppressants before the study. Antihistamines were withdrawn from the patients 7 days before skin testing and blood sampling. An intracutaneous test with 0.05 ml of fresh, undiluted, autologous serum and a saline control was injected into the volar surface of the forearm and evaluated 30 min later. A difference of >1.5 mm between the wheal diameter elicited by the autologous serum and that by the saline control was considered a positive result. A positive response was detected in 22 of 34 subjects, whereas the test was negative in the other 12 individuals. Normal laboratory work-up included a complete blood cell count (CBC), complement levels (C3 and C4), anti-thyroglobulin antibodies, thyroid-stimulating hormone, antinuclear antibodies, and total serum IgE. The institutional committee for human experimentation approved this study.

2.2. Flow cytometric analysis

Phycoerythrin (PE)-conjugated anti-CD3, peridinin chlorophyll protein (PerCP)-conjugated anti-CD4, fluorescein isothiocyanate (FITC)-conjugated anti-

CD25, and an appropriate isotype control of mouse IgG1 were purchased from Becton-Dickinson Immunocytometry System (San Jose, CA). Briefly, a sample of whole blood was stained with monoclonal antibodies (mAbs) or an isotype control for 30 min in the dark. Stained cells were then washed twice, resuspended in cold buffer, and analyzed with the FACSCalibur system (Becton Dickinson). More than 1×10^4 cells were analyzed for each sample, and the results were processed using Cellquest software (Becton Dickinson).

2.3. Preparation of peripheral blood mononuclear cells (PBMCs)

Heparinized venous whole blood from each donor was layered on a Ficoll-Paque density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) and centrifuged for 30 min at $400 \times g$ and room temperature. The peripheral blood mononuclear cell layer was collected, washed, and resuspended in Hanks' balanced salt solution (Gibco BRL, Life Technologies, Eggenstein, Germany). The number of viable lymphocytes was determined by trypan blue exclusion and hemocytometry.

2.4. Isolation of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells

Human PBMCs were centrifuged and resuspended in RPMI-1640 medium (Bio-Whittaker, Boehringer Ingelheim, Belgium) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 25 mM HEPES. CD4⁺CD25⁺ Treg cells were purified using a CD4⁺CD25⁺ Treg cell isolation kit (Miltenyi Biotec, Bergisch, Gladbach, Germany) in a two-step procedure. First, non-CD4⁺ cells were indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ , glycophorin A, and anti-biotin microbeads. The labeled cells were subsequently depleted by separation over a MACS column. In the second step, CD4⁺CD25⁺ T cells were directly labeled with CD25 microbeads. Then CD4⁺CD25⁺ T and CD4⁺CD25⁻ T cells were separated by means of positive selection from the pre-enriched CD4⁺ T cell fraction. All steps were performed according to the manufacturer's instructions.

2.5. RNA extraction, first-strand cDNA synthesis, and quantitative real-time polymerase chain reaction (PCR)

Real-time PCR was used to study FOXP3 and TGF- β mRNA expressions. Total mRNA was extracted using

the GeneStrips mRNA isolation kit (RNAure, Irvine, CA) according to the manufacturer's protocol. In brief, isolated subsets of T cells were placed in 40 μ l of lysis buffer, transferred to hybridization tubes, shaken for 60–90 min, and finally washed with 100 μ l of wash buffer. Then, 50–500 ng of mRNA was reverse-transcribed with random hexamer primers (Promega, Madison, WI), SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), and RNaseOUT ribonuclease inhibitor (Invitrogen) in the same hybridization tube. The primers and probes used in the real-time PCR for FOXP3 and TGF- β were purchased from Assays-on-Demand Gene Expression Products (Applied Biosystems, Foster City, CA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Each real-time PCR system contained 1–6 μ l of cDNA and 10 μ l of TaqMan MasterMix (Applied Biosystems); diethyl pyrocarbonate (DEPC)-treated H₂O was added to a final desired volume of 20 μ l. Real-time PCRs for each individual gene were performed in triplicate, and the results were analyzed by means of the dual-labeled fluorogenic probe method with a sequence detector (ABI Prism 7700; Applied Biosystems). The mRNA expression levels of each gene were calculated using the $2^{-\Delta\Delta C_T}$ (comparative threshold cycle, or C_T) method, as detailed by the manufacturer (Technical Bulletin 2; Applied Biosystems). The efficiencies of the targets (FOXP3 and TGF- β) and reference (GAPDH) were approximately equal, as proven in individual validation experiments. Quantities of target gene expressions in the test samples were normalized to the corresponding GAPDH mRNA levels of a healthy control subject (calibrator).

2.6. *In vitro* proliferation assay

To analyze the T-cell proliferative capacity, indicated numbers of CD4⁺CD25[−] T cells were cultured with irradiated antigen-presenting cells in 96-well, round-bottomed plates and stimulated with 2.5 μ g/ml phytohemagglutinin (PHA, Sigma, St. Louis, MO). To analyze the suppressive capacity of CD4⁺CD25⁺ T cells, CD4⁺CD25⁺ (suppressors) and CD4⁺CD25[−] (responders) T cells (2×10^4 cells/well) were stimulated with 2.5 μ g/ml PHA in round-bottomed 96-well plates. CD4⁺CD25[−] T cells were cocultured with various numbers of CD4⁺CD25⁺ T cells (responder/suppressor ratios of 1:1, 1:1/2, and 1:0). All cells were cultured in a final volume of 200 μ l in the presence of irradiated 4×10^4 CD4⁺ T cell-depleted accessory cells/well. Cultures were incubated for 72 h at 37 °C in a humidified, 5% CO₂-containing incubator. Eighteen hours before harvesting, [³H]-thymidine was added at a final concentration of

1 μ Ci/well. Cells were then harvested, and the incorporated radioactivity was counted as an index of proliferation.

2.7. *In vitro* cytokine determination

For cytokine production, CD4⁺CD25[−] T cells were suspended at 2×10^6 cells/ml in RPMI-1640 complete medium and then stimulated with 10 μ g/ml PHA at 37 °C in a humidified, 5% CO₂-containing incubator. Supernatants were harvested after 48 h. Productions of IL-2, TNF- α , IFN- γ , IL-4, IL-5, and IL-10 by CD4⁺CD25[−] T cells were evaluated with commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN). The levels of cytokines were determined from standard curves of purified recombinant cytokines. All tests kits were used according to the procedures suggested by the manufacturer.

2.8. Statistical analysis

Data are expressed as the mean $\pm$ S.E.M. and were compared statistically among groups using the Mann–Whitney *U*-test. The correlation coefficient, *r*, was generated using Spearman's rank correlation. A *p* value of <0.05 was considered statistically significant.

3. Results

3.1. Percentages of CD3⁺CD4⁺ and CD4⁺CD25^{hi} T cells in CU patients

The frequency of CD4⁺CD25⁺ T cells among peripheral CD4⁺ T cells has become an important parameter for characterizing populations of Treg cells. First, PBMCs from 19 CU patients and seven healthy control subjects were examined for the frequency of CD3⁺CD4⁺ T cells by flow cytometry. Our data indicated that CU patients had a significantly higher number of CD4⁺ T cells in PBMCs compared with control subjects ($23.46 \pm 1.05\%$ vs. $17.20 \pm 1.34\%$; *p* = 0.006) (Fig. 1A). When patients were divided based on the presence (p+) or absence (p−) of a positive response to the ASST, the percentages of CD4⁺ T cells in PBMCs were also significantly higher in both groups of CU patients compared to control subjects (p + CU vs. the control, *p* = 0.015; p − CU vs. the control, *p* = 0.021). CD4⁺CD25^{hi} T cells are CD4⁺ T cell subsets with high or bright CD25 surface expression and are considered to be a phenotypic marker for Treg cells. We found that CU patients had more CD4⁺CD25^{hi} T cells than control subjects ($4.47 \pm 0.34\%$ vs. $2.34 \pm 0.41\%$; *p* = 0.001) (Fig. 1B).

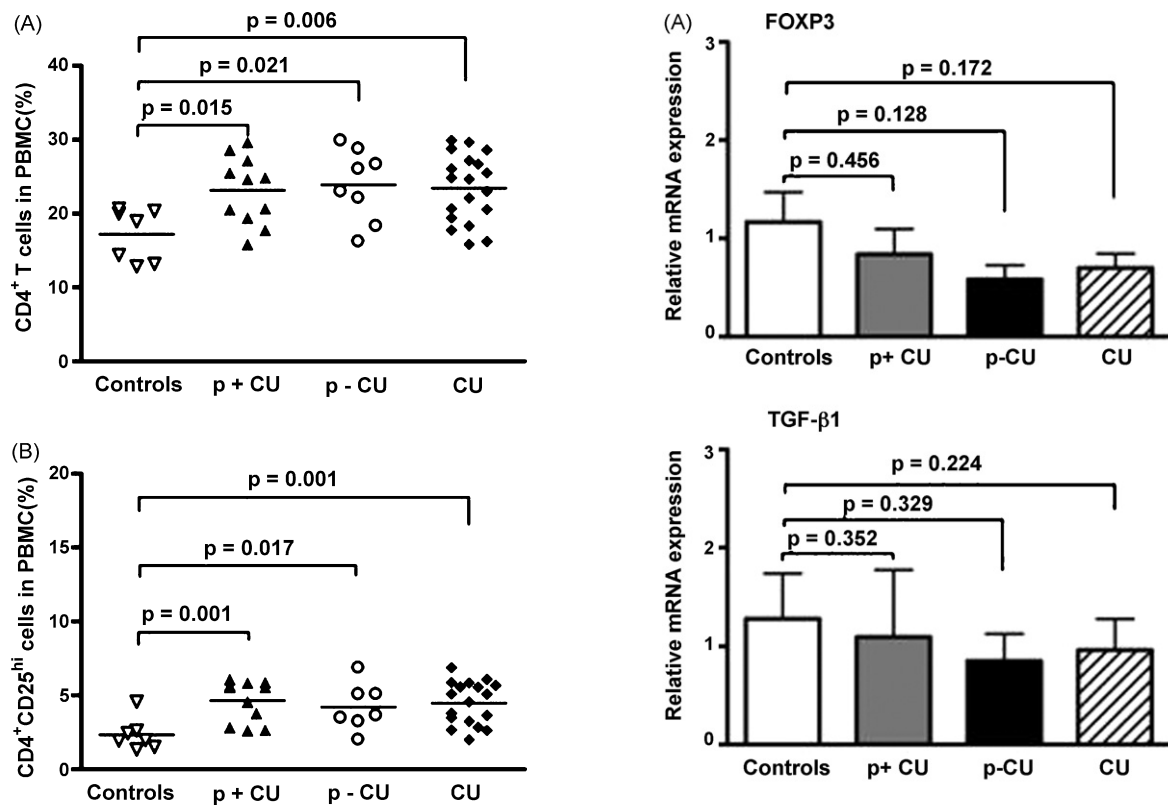


Fig. 1 Comparison of the frequencies of CD3⁺CD4⁺ (A) and CD4⁺CD25^{hi} (B) T cells in peripheral blood mononuclear cells (PBMCs) between CU patients ($n = 19$) and control subjects ($n = 7$). The CU patients were divided into two groups based on the presence (p + CU) or absence (p - CU) of a response to the autologous serum skin test (ASST). Peripheral blood was stained with PE-conjugated anti-CD3, PerCP-conjugated anti-CD4, and FITC-conjugated anti-CD25 antibodies. The percentages shown in (A) are the percentages of CD4⁺ T cells in PBMCs; the percentages shown in (B) are the percentages of CD25^{hi} in CD4⁺ T cells. Distributions, means, and statistical differences are shown.

Similarly, numbers of CD4⁺CD25^{hi} T cells were also significantly higher in both groups of CU patients compared to control subjects (p + CU vs. the control, $p = 0.001$; p - CU vs. the control, $p = 0.017$).

3.2. Expression of FOXP3 and TGF- β mRNAs by CU patients

CD4⁺ T cells were purified by means of negative selection from 14 CU patients and seven healthy control subjects to examine the expressions of FOXP3 and TGF- β in human cells. Peripheral CD4⁺ T cells were separated using immunomagnetic beads to obtain CD4⁺CD25⁺ T cells and CD4⁺CD25⁻ T cells with a CD4⁺CD25⁺ T-cell isolation kit. FOXP3 and TGF- β gene expressions were detected by means of real-time PCR in purified CD4⁺CD25⁺ T cells. The

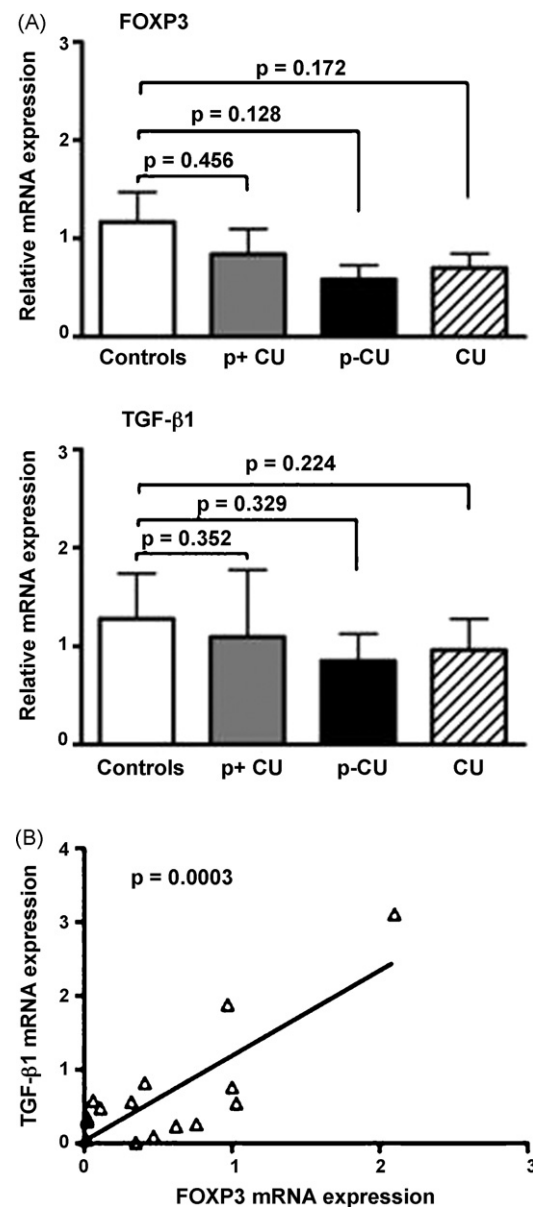


Fig. 2 Comparison of messenger (m)RNA expressions of FOXP3 (A) and TGF- β (B) between CU patients ($n = 14$) and control subjects ($n = 7$). The CU patients were divided into two groups based on the presence (p + CU) or absence (p - CU) of a response to the autologous serum skin test (ASST). Relative expressions of FOXP3 and TGF- β mRNA in CD4⁺CD25⁺ T cells were determined by real-time quantitative polymerase chain reaction (PCR). GAPDH was used as a reference gene. Normalized FOXP3 and TGF- β mRNA abundances were determined from the ratio of FOXP3 mRNA or TGF- β mRNA to GAPDH mRNA expression, respectively. Mean values, standard deviations, and statistical differences are shown.

mRNA expression of FOXP3 did not significantly differ between CU patients and control subjects (p + CU vs. the control $p = 0.456$; p - CU vs. the control $p = 0.128$) (Fig. 2A). In addition, mRNA expression of TGF- β did not differ between CU

patients and control subjects ($p + \text{CU}$ vs. the control $p = 0.352$; $p - \text{CU}$ vs. the control $p = 0.329$) (Fig. 2A). Similar data were also observed for IL-10 gene expression (data not shown). Furthermore, levels of mRNA expression by FOXP3 were significantly correlated with levels of mRNA expression by TGF- β ($r = 0.8$, $p = 0.0003$) (Fig. 2B).

3.3. Suppression of proliferation of $\text{CD4}^+\text{CD25}^-$ T cells by $\text{CD4}^+\text{CD25}^+$ T cells in CU patients

To assess the function of $\text{CD4}^+\text{CD25}^+$ T cells in CU patients, we separated $\text{CD4}^+\text{CD25}^+$ and $\text{CD4}^+\text{CD25}^-$ cells from five CU patients and four healthy control subjects, and determined the effect of $\text{CD4}^+\text{CD25}^+$ cells on $\text{CD4}^+\text{CD25}^-$ cell proliferation. To quantitate their regulatory function, $\text{CD4}^+\text{CD25}^+$ T cells were cocultured with autologous $\text{CD4}^+\text{CD25}^-$ responder cells at different ratios (responder/suppressor ratios of 1:0, 1:0.5, and 1:1). As shown in Fig. 3A, the presence of $\text{CD4}^+\text{CD25}^+$ T cells clearly reduced the proliferative response of $\text{CD4}^+\text{CD25}^-$ T cells in the four control subjects tested. However, the $\text{CD4}^+\text{CD25}^+$ T cells isolated from two of five patients poorly inhibited responder $\text{CD4}^+\text{CD25}^-$ T cell proliferation. Furthermore, comparing percentages of inhibition of proliferation from each study subject indicated that cocultured cells from some CU patients even induced proliferation instead of inhibition (Fig. 3B), reflecting a complete loss of suppressor function.

3.4. Th1/Th2 cytokine production in $\text{CD4}^+\text{CD25}^-$ T cells of CU patients

The proliferative response of isolated $\text{CD4}^+\text{CD25}^-$ T cells from six CU patients and seven healthy control subjects was investigated after stimulation with PHA in the presence of irradiated antigen-presenting cells. When $\text{CD4}^+\text{CD25}^-$ T cells were cultured in low numbers of cells per well, these cells had a slightly poor response in CU patients compared to control subjects (Fig. 4A, 2.5×10^4 cells/well, $p = 0.067$; 5×10^4 cells/well, $p = 0.262$). However, the degree of proliferation did not differ between patients with CU and control subjects when $\text{CD4}^+\text{CD25}^-$ T cells were cultured at a high density of 1×10^5 cells/well. Taken together, these data indicate a somewhat poor immune response of naïve CD4^+ T cells in CU patients.

To examine the ability of cytokine production by $\text{CD4}^+\text{CD25}^-$ T cells in PBMCs, 4×10^5 $\text{CD4}^+\text{CD25}^-$ T cells/well from nine CU patients and 11 control subjects were stimulated with PHA, and Th1 (IFN- γ , TNF- α , and IL-2)/Th2 (IL-4, IL-5, and IL-10) cytokine production was assayed by ELISA. The pro-

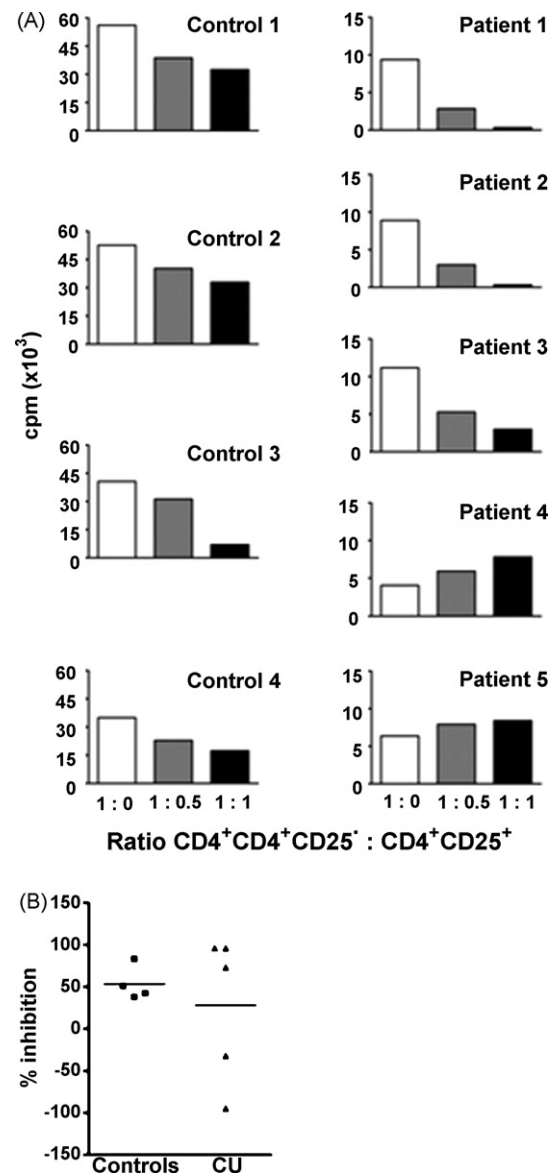


Fig. 3 (A) Dose-related suppression of proliferation of $\text{CD4}^+\text{CD25}^-$ T cells by different ratios of $\text{CD4}^+\text{CD25}^+$ T cells from CU patients ($n = 5$) and control subjects ($n = 4$). (B) Percentages of inhibition of proliferation of $\text{CD4}^+\text{CD25}^-$ T cells by $\text{CD4}^+\text{CD25}^+$ T cells at a 1:1 ratio from CU patients ($n = 5$) and healthy controls ($n = 4$). Values were calculated from proliferation assays with phytohemagglutinin (PHA) stimulation.

ductions of IFN- γ , TNF- α , and IL-2 were significantly reduced in CU patients compared to control subjects (IFN- γ , $p = 0.038$; TNF- α , $p = 0.008$; IL-2, $p = 0.031$) (Fig. 4B). Similarly, CU patients were characterized by lower productions of IL-4, IL-5, and IL-10 compared to control subjects (IL-4, $p = 0.036$; IL-5, $p = 0.028$; IL-10, $p = 0.021$) (Fig. 4B). Taken together, these findings indicate a generalized downregulation of both Th1 and Th2 cytokine production in CU patients.

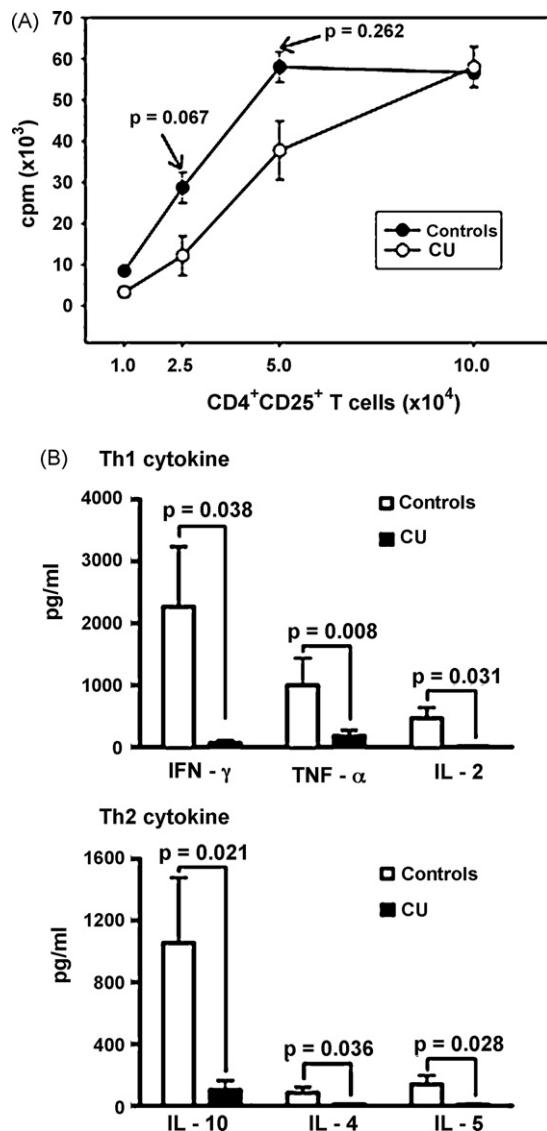


Fig. 4 (A) Comparison of the proliferative capacity of CD4⁺CD25⁻ T cells alone between CU patients ($n = 6$) and control subjects ($n = 7$). Proliferation responses were determined by [³H]-thymidine uptake, as represented by counts per minute (cpm). (B) Cytokine expression of CD4⁺CD25⁻ T cells of CU patients ($n = 9$) and healthy controls ($n = 11$). CD4⁺CD25⁻ T cells were isolated and stimulated with phytohemagglutinin (PHA). The total Th1 cytokine secretion of interferon (IFN)- γ , tumor necrosis factor (TNF)- α , and interleukin (IL)-2, and Th2 cytokine secretion of IL-4, IL-5, and IL-10 were assayed by ELISA. Mean values, standard deviations, and statistical differences are shown.

4. Discussion

The major advance in our understanding of CU in recent years has been the discovery that CU in 30–50% of patients is due to an autoimmune process and therefore is not strictly “idiopathic”. Although mast cell degranulation certainly initiates the inflamma-

tory process in autoimmune chronic urticaria and is assumed to do so in idiopathic chronic urticaria, no alternative mechanism for mast cell degranulation in the idiopathic groups has been suggested to date. Thyroid disease and chronic urticaria are frequently associated, but there is no evidence that the thyroid autoantibodies are pathogenic in the context of chronic urticaria. Several anecdotal accounts have implicated *Helicobacter pylori* in the etiology of CU [22,23], but treatment of the infection has no influence on CU [24,25]. In this study, our data demonstrated that the number of CD4⁺ T cells in PBMCs of CU patients significantly increased. Therefore, we assume that CD4⁺ T cells have a wider role and are not merely confined to lesional skin. We even suggest a systemic activation of CD4⁺ T cells may be able to trigger the onset of wheals.

As a minor fraction of approximately 5–10% of CD4⁺ T cells in unmanipulated animals and healthy humans, CD4⁺CD25⁺ Treg cells play a critical role in preventing organ-specific autoimmunity and allograft rejection and in maintaining self-tolerance [10]. These cells show a constitutively high expression of CD25 and exert an immune regulatory function. Since these cells arise directly from the thymus, they have been called natural regulatory T cells. It remains to be investigated whether a deficiency or abnormality of the CD4⁺CD25⁺ Treg cell population is responsible for or is involved in CU disease. Surprisingly, we found that patients with CU had more CD4⁺CD25^{hi} Treg cells in PBMCs than did healthy control subjects. Findings of some other investigations are consistent with our results. It was reported that Treg cells seem to be enriched in inflamed rheumatoid synovial membranes and synovial fluid from patients with rheumatoid arthritis [26,27]. In addition, Robinson et al. reported that CD4⁺CD25⁺ cells in bronchoalveolar lavage fluid increased after allergen challenge [28]. Ou et al. also observed that CD4⁺CD25⁺ T cells were significantly higher in patients with atopic dermatitis [29]. However, further research should include identification of these Treg cells with a specific intracellular anti-FOXP3 mAb to determine what proportion of CD4⁺CD25^{hi} T cells are effective Treg cells.

FOXP3 is considered to be the most specific marker for Treg cells. FOXP3 is both necessary and sufficient for the development and function of CD4⁺CD25⁺ Treg cells. In addition, it is well documented that subsets of Treg produce TGF- β and IL-10, including naturally occurring Treg cells. Nakamura et al. demonstrated that stimulated CD4⁺CD25⁺ T cells may be a preferential source of secreted TGF- β , and under certain circumstances, may produce mainly surface-bound TGF- β [30]. In this study, we showed that FOXP3 and TGF- β tran-

scripts were detectable, but the levels of mRNA expression were similar in CD4⁺CD25⁺ Treg cells from patients and controls. However, it has been reported that both normal and mutated FOXP3 alleles are equally expressed in T cells of healthy carriers of immune dysregulation polyendocrinopathy X-linked syndrome [31,32]. Therefore, it is possible that functional compensation of mutated FOXP3 might be present in the increased numbers of CD4⁺CD25⁺ Treg cells in an effort to regain immune control in patients with CU.

To investigate the direct suppressive capacity of CD4⁺CD25⁺ cells from CU patients and healthy control subjects, we cocultured CD4⁺CD25⁺ cells with CD4⁺CD25⁺ cells and stimulated them with PHA. Our data on the function of CD4⁺CD25⁺ cells demonstrate that some patients with CU have defective regulatory activity, and this result supports the possible role of a deficient immunoregulatory function in the pathogenesis of CU. In this regard, we propose that Treg cells might increase in frequency to compensate for their defective function in CU patients. Furthermore, if such impaired function of Treg cells is on a "per cell" basis, it is possible that the net effects of Treg can be balanced in these patients. Of course, further studies are needed to establish whether CD4⁺CD25⁺ Treg cells act normally at sites of CU disease, such as lesional skin. However, it should be noted that even though we demonstrated that the function of CD4⁺CD25⁺ cells is normal in some CU patients, this suppressive effect might not be sufficient to prevent the development of CU and might be able to affect the disease severity. However, the factors that determine that Treg cells in some CU patients show diminished function remain to be elucidated.

To date, few studies have been carried out to define the cytokine profiles in PBMCs or CD4⁺ T cells in CU patients. In addition, no data are available regarding the Th1/Th2 cytokine secretory profile in naïve CD4⁺CD25⁺ T cells of CU patients. In this work, we examined the secretory pattern of Th1/Th2 cytokines in stimulated CD4⁺CD25⁺ T cells of patients with CU. IL-4, IL-5, and IL-10 represent Th2 cytokines, whereas IFN- γ , TNF- α , and IL-2 represent the Th1 profile. IL-4 is the principal cytokine that induces naïve T cells to differentiate into Th2 cells. In this study, we observed that the stimulated Th2 cytokine (IL-4, IL-5, and IL-10) secretion was significantly reduced in CU patients. It is possible that IFN- γ suppresses Th2 cytokine secretion. However, in our patients, that was unlikely, since IFN- γ levels did not increase. These findings suggest the concept of immune dysregulation resulting in a cytokine imbalance underlying or being associated with the development of CU. Compar-

able findings were also reported by other groups. Szegedi and co-workers found that the frequency of IFN- γ -producing CD4⁺ peripheral T cells from CU patients was significantly lower than that of control subjects [33]. Piconi et al. found that mitogen-stimulated production of IL-2 and IFN- γ by PBMCs was significantly reduced in CU patients compared to control subjects [34]. Moreover, Rapoport and co-workers reported that IL-4 secretion from stimulated PBMCs was reduced in CU patients, and this was associated with some reductions in both IL-2 and IFN- γ secretions [35]. However, these data are in contrast to the cytokine pattern of lesional skin shown in CU patients. Further study is necessary in the future to clarify whether it is a difference in ability for cytokine production of individual CD4⁺CD25⁺ cells or in the number of cytokine-producing cells between patients and controls. Moreover, in the inflammatory scenario, the reduced Th1 and Th2 cytokine secretion seen in CU patients is not easily understandable. Evidence for probable T cell dysregulation was reported by Confino-Cohen et al., who demonstrated disturbed signaling via the p21Ras pathway in lymphocytes of CU patients [36]. The p21Ras protein is involved in both T cell receptor activation and in IL-2 receptor activation, suggesting a pivotal role of this protein in mediating the immunologic response. In addition, it was reported that genes predisposed towards autoimmunity which affect immunoreactivity might not necessarily act by making the immune system more reactive [37]. Thus, we speculate that aberrant p21Ras signaling of activated CD4⁺ T cells from CU patients is induced by a primary unknown environmental or genetic trigger, which results in this generalized suppression of cytokine secretion. We also found aberrant cytokine secretion in our patients irrespective of their ASST results. This possibly indicates that aberrant cytokine secretion is a more-sensitive indicator of immune dysregulation than an autologous serum injection in CU patients. Moreover, future studies are needed to clarify the relationship between cytokine secretion patterns of CD4⁺CD25⁺ T cells and the suppressive capacity of CD4⁺CD25⁺ T cells in patients with CU.

In summary, we found increased numbers of CD4⁺CD25⁺ T cells in the PBMCs of patients with CU. No significant difference was observed in the FOXP3 or TGF- β transcripts of CD4⁺CD25⁺ T cells in PBMCs from patients and controls. However, the suppressive function of CD4⁺CD25⁺ T cells was defective in a significant proportion of these CU patients. Further studies are needed to clarify the exact role of CD4⁺CD25⁺ T cells in the pathogenesis of CU and identify factors regulating their function in CU. Furthermore, our data demonstrated downregula-

tion of both Th1 and Th2 cytokine secretions in stimulated CD4⁺CD25⁻ T cells from patients with CU, and these results may provide another valuable insight into the mechanisms underlying the pathogenesis of CU.

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Conflict of interest

None.

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