

Report

Circulating level of CD4⁺ CD25⁺ FOXP3⁺ T cells in patients with chronic urticaria

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Abstract

Background CD4⁺ CD25⁺ T-regulatory (Treg) cells play critical roles in maintaining peripheral tolerance and preventing autoimmunity. As characteristics of Treg cells have not been precisely investigated in chronic urticaria (CU) yet, this study was performed.

Objective To determine the frequencies of circulating CD4⁺ CD25⁺ FOXP3⁺ T cells and serum levels of interleukin (IL)-10, transforming growth factor (TGF)- β , and IL-17 in patients with chronic autoimmune urticaria and chronic idiopathic urticaria compared to healthy controls.

Methods Peripheral blood mononuclear cells (PBMCs) were obtained from patients with CU and healthy controls. The frequency of CD4⁺ CD25⁺ T cells in PBMCs and expression levels of FOXP3 were detected by flow cytometry. The serum levels of IL-10, TGF- β , and IL-17 were measured by enzyme-linked immunosorbent assay.

Results A significant decrease in the percentage of circulating CD4⁺ CD25⁺ FOXP3⁺ T cells was detected in patients with CU, compared to control subjects. However, no significant difference was detected on the serum levels of IL-10, TGF- β , and IL-17 between patients with CU and control subjects.

Conclusions This study demonstrated that the frequency of Treg cells in PBMCs was decreased in patients with CU. Further studies are needed to clarify the exact role of Treg cells in the pathogenesis of CU and factors regulating their function.

Introduction

Chronic urticaria (CU) is a rather common skin disorder, characterized by recurrent, transitory, itchy wheals for more than six weeks. Lesions recur at least twice a week for more than six weeks and should not be physical or recurrent acute urticaria with repeated exposures to a specific agent.¹ Circulating antibody against anti-IgE receptor (Fc ϵ RI) antibodies could be detected in about 30–40% of patients with CU, while anti-IgE antibodies could be found in an additional 10% of patients. Thyroid autoantibodies are also present in about 24% of patients with CU.^{2,3} The impression of these autoantibodies can

be followed through an autologous serum skin test (ASST) or basophil and mast cell histamine release assays.^{2,3} Therefore, CU is considered autoimmune in 40–45% of patients with CU, whereas it appears to be idiopathic in 55–60% of cases. In the latter subgroup, the autoimmune parameters are negative.⁴ Patients with autoantibodies tend to have more severe disease than patients without autoantibodies are. Although the exact pathogenic mechanisms of CU are not completely understood, infiltration of CD4⁺ T-helper (Th) cells in biopsy specimens from patients with CU suggest that the recruitment of these T cells from the circulation to the site of inflammation might also play a central role in triggering

mast cell degranulation.⁵ On the other hand, introducing the naturally occurring CD4⁺ T-regulatory (Treg) cells⁶ has shed new light on the mechanisms of immunological disorders. Several types of T cells with regulatory activity have been described, while their role in the control of a variety of autoimmune diseases has been delineated.⁷

In humans, approximately 30% of CD4⁺ T cells express CD25, but only a small fraction of these cells (2–4%) expresses forkhead box P3 (FOXP3) protein. It seems that high expression of FOXP3 correlates with high expression of CD25, which represents cells with a suppressive function. Populations of CD4⁺ CD25⁺ T cells with regulatory function have been best identified by the expression of FOXP3.^{8,9} Tregs can inhibit the effector T cells by different mechanisms, including direct cell–cell contact or secretion of transforming growth factor (TGF)- β and interleukin (IL)-10.^{10,11}

Anomalies in the function or number of Tregs can be a primary cause of autoimmune disease, allergy, and inflammatory disorders in humans. This association became apparent mainly when the immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome was elucidated. The IPEX syndrome is linked to the dysfunction of the transcriptional activator FOXP3, and patients with this condition have significant autoimmune manifestations. Therefore, maintaining a balance between activation and suppression of T-cell responses is crucial in the outcomes of many immune-mediated diseases. Some studies suggested that the impaired or deficient Treg responses may result in autoimmune disorders and allergic diseases such as asthma.^{12,13} However, the exact role of Treg cells has not yet been defined in patients with CU.

In the present study, we aimed to determine the frequencies of circulating CD4⁺ CD25⁺ FOXP3⁺ T cells in patients with CU and healthy controls.

Materials and methods

Patients and controls

All patients with continuous CU were selected from the Allergy Outpatient Clinic of Hazrat-e-Rasool Hospital. These patients had a wheal for six weeks or longer, occurring at least twice a week. Patients with physical urticaria, urticarial vasculitis, or food allergies were excluded. The questionnaire was filled by a trained fellow of immunology and allergy for all of the patients, while the disease activity score was calculated by patients for one week and then collected.

Basic laboratory tests, including a complete differential blood cell count, erythrocyte sedimentation rate, C-reactive protein, liver function tests, thyroid function tests, thyroid autoantibodies, antinuclear antibodies, total serum IgE, stool exam, and urinalysis were done. Patients were free of antihistamines for

seven days before skin testing and blood sampling. None of the patients had used corticosteroids or immunosuppressive drugs.

Samples were obtained from 41 patients with CU in two subgroups (seven males and 34 females; mean age 39.7 years, aged 17–65 years) and 20 healthy controls (four males and 16 females; mean age 36 years, aged 22–48 years).

All the subjects signed written informed consent forms, and the study protocol was approved by the Institutional Ethics Committee of Tehran University of Medical Sciences.

Autologous serum skin test

The ASST was performed on all patients with CU according to standard methods, using 0.05 ml of fresh autologous serum injected intradermally into the volar forearm skin. Histamine skin prick testing (10 μ g/ml) and 0.05 ml of saline were used as positive and negative controls, respectively. A positive skin test was defined as the appearance of a red serum-induced wheal with a diameter of ≥ 1.5 mm than that of the saline-induced response at 30 minutes. A positive response was detected in 21 of 41 subjects, whereas the test was negative in the remaining 20 individuals.

Preparation of peripheral blood mononuclear cells

Approximately 4 ml of venous blood was collected from each subject in heparinized tubes, and the peripheral blood mononuclear cells were separated using *Ficoll*-Paque gradient centrifugation (Baharafshan company, Tehran, Iran). Surface expression of CD4 and CD25 were analyzed with specific antihuman monoclonal antibodies for CD4 and CD25 (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's protocol. For intracellular staining of FOXP3, the cells were fixed in formaldehyde (5%), permeabilized with saponin buffer (0.5%), and then incubated at room temperature in the dark for 20 minutes. The specific antihuman FOXP3 monoclonal antibody was added to the tube, after washing with 2 ml of wash buffer (phosphate-buffered saline containing 0.01% sodium azide and 0.5% bovine serum albumin), and then incubated at room temperature in the dark for 30 minutes. After final washing, the cells were resuspended in 0.5 ml staining buffer and then analyzed with PAS III PARTEC flow cytometer (Germany).

Cytokine measurements

Concentrations of IL-10, TGF- β , and IL-17 were measured in the serum samples of patients and healthy controls with commercial enzyme-linked immunosorbent assay tests (Bender Med system, C.E, Vienna, Austria) according to the manufacturer's protocol.

Statistical analysis

The Mann–Whitney *U*-test was used to compare variables between patients with CU and healthy subjects as well as groups subjected to ASST vs. controls. Correlations of data

were analyzed by the non-parametric Spearman test. $P < 0.05$ was considered statistically significant.

Results

Samples were obtained from 41 patients with CU and 20 healthy controls. The median duration of the disease was 12 months (range 2–240 months), which was associated with angioedema in about 49% (20 cases). The disease activity score was calculated, and the median score was 6 (range 3–6).

Thyroid function tests and thyroid autoantibodies were measured in all of the patients; 39 cases (95%) were euthyroid, while only two patients (5%) were subclinically hypothyroid. Meanwhile antithyroid peroxidase was positive in 29.3% (12 patients), positive antithyroglobulin was detected in 19.6% (eight cases) (Table 1). Patients were subdivided into two groups, based on the results of the ASST (Table 2).

Percentages of CD4⁺ CD25⁺ FOXP3⁺ T cells in patients with chronic urticaria

Patients with CU had a significantly lower percentage of circulating CD4⁺ CD25⁺ FOXP3⁺ T cells, compared to control subjects ($P = 0.020$). After classifying patients based on the response to the ASST, the percentages of circulating CD4⁺ CD25⁺ FOXP3⁺ T cells were also significantly lower in both groups of patients with CU, compared to control subjects: patients with ASST-negative CU vs. controls ($P = 0.040$), and patients with ASST-positive CU vs. controls ($P = 0.032$; Fig. 1). There were no significant differences in the level of circulating Tregs

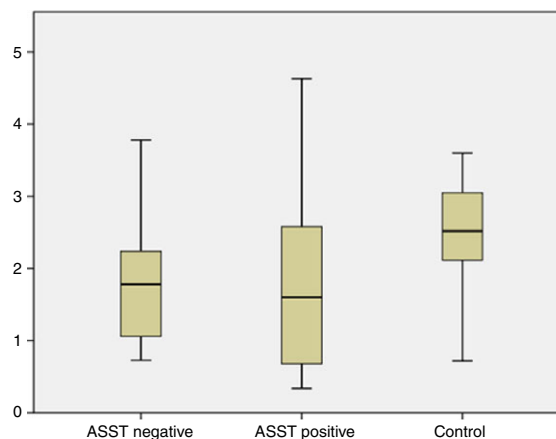


Figure 1 Percentage of circulating CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells in patients with ASST-negative and ASST-positive chronic urticaria in comparison with healthy controls

between patients with ASST-positive and ASST-negative CU ($P = 0.782$).

Circulating level of cytokines in chronic urticaria

Circulating cytokines, such as IL-10 and TGF- β , as well as IL-17 were measured in sera of the subjects. Alteration of cytokine levels in patients with CU were verified with both positive and negative ASST. In a large percentage of control sera samples, the serum levels of IL-10 and IL-17 were undetectable. These negative percentages were 70% for IL-10 (14 of 20) and 90% for IL-17 (18 of 20). The frequencies of these undetectable cytokines were similar in sera of the two subgroups of patients with CU.

Although there was no significant difference in the serum levels of IL-17 and IL-10 from patients with CU and the control group ($P = 0.20$ and $P = 0.84$, respectively), IL-17 serum levels of patients with CU displayed a strong positive correlation with serum levels of IL-10 ($R = 0.369$, $P = 0.018$); this correlation was not found in healthy controls. Furthermore, the correlation was more significant in ASST-positive patients ($R = 0.494$, $P = 0.023$).

The serum concentration of TGF- β from patients with CU (neither ASST positive nor ASST negative patients) showed no significant difference in comparison with control subjects ($P = 0.28$ and $P = 0.13$, respectively). Interestingly, we observed a correlation between the circulating percentage of Tregs and the TGF- β serum levels from control individuals ($R = 0.520$, $P = 0.019$; Fig. 2a), but it did not appear in patients with CU (Fig. 2b,c).

Discussion

CU seems to be due to an autoimmune process in more than one-third of patients. It is believed that thyroid

Table 1 Antithyroid antibodies: results and thyroid status in patients with chronic urticaria

	Statistic	Results	
Thyroid function test	41	Euthyroid	39 (95.1%)
		Subclinical hypothyroidism	2 (4.9%)
Antithyroid peroxidase	41	Positive	12 (29.3%)
		Negative	29 (70.7%)
Antithyroglobulin	41	Positive	8 (19.6%)
		Negative	33 (80.4%)

Table 2 Autologous serum skin test results in patients with chronic urticaria

	wheal negative	wheal positive
Autologous serum skin test	20 (49%)	21 (51%)

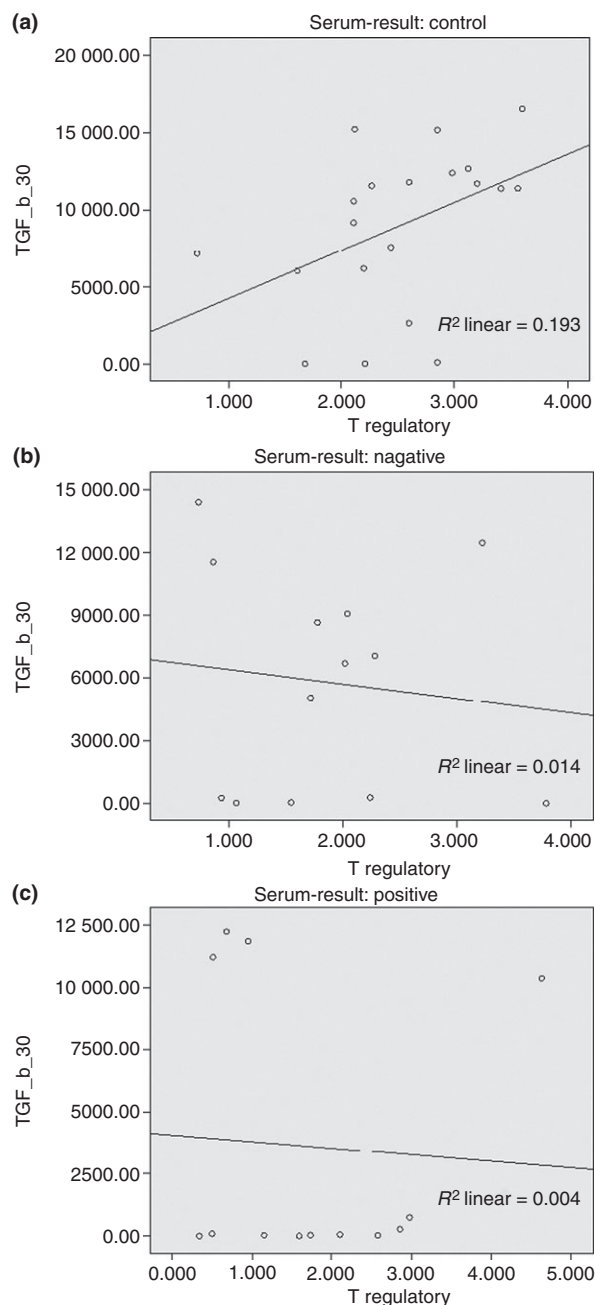


Figure 2 (a) Correlation between circulating percentage of Tregs and serum level of TGF- β in healthy controls. (b) Correlation between circulating percentage of Tregs and serum level of TGF- β in patients with ASST-negative chronic urticaria. (c) Correlation between circulating percentage of Tregs and serum level of TGF- β in patients with ASST-positive chronic urticaria. TGF, transforming growth factor; Tregs, T-regulatory cells

disease and CU are frequently associated, but thyroid status is not related to the occurrence of urticaria. As we found in this study, thyroid autoantibodies are positive in

some patients with CU,^{14,15} but the presence of antithyroid antibodies seems to be correlated with other autoimmune determinations, rather than being a direct causative agent in chronic idiopathic urticaria.¹⁶ Therefore, looking for autoimmune markers other than antinuclear antibodies may be useful.

The role of Tregs and TGF- β from naturally occurring Treg and FOXP₃-expressing Tregs has been amplified in maintaining self-tolerance and immune homeostasis, autoimmunity, and inflammatory disorders.¹⁰ In this study, we demonstrated that the frequency of circulating CD4⁺ CD25⁺ FOXP₃⁺ T cells in peripheral blood mononuclear cells of patients with CU were significantly decreased in comparison to healthy controls. Reduced frequency of CD4⁺ CD25⁺ FOXP₃⁺ cells in the peripheral blood of patients with chronic autoimmune urticaria was shown previously in a study by Sun *et al.*,¹⁷ and they reported a decreased intensity of FOXP₃ expression.

Although Chen *et al.*,⁵ reported an increased frequency of CD4⁺ CD25⁺ T cells in patients with CU, compared to controls, no significant differences were detected in the expression levels of FOXP₃ or TGF- β between patients with CU and control subjects; therefore, they suggested the functions of circulating CD4⁺ CD25⁺ and CD4⁺ CD25⁻ T cells in patients with chronic ordinary urticaria were defective. The results of this study support the paradigm of deficiency or dysfunction of the CD4⁺ CD25⁺ FOXP₃⁺ Treg cells in the development of CU, but as FOXP₃ is expressed on non-Treg cells, analysis of Treg requires functional analyses.¹⁰

Among the few studies on cytokine profiles of patients with CU there is a study that showed all Th1 and Th2 cytokines in both patients and controls were normal, except the IL-12 concentration, which was higher in patients.¹⁸ Dos Santos *et al.*,¹⁹ demonstrated enhanced IL-17 secretion levels in patients with CU with a positive skin test, by inducing T-cell stimulus. Our finding did not indicate any significant difference in the serum level of IL-17 between the two groups of patients and controls. We also found no significant difference between the serum levels of IL-10 and TGF- β in patients with CU and healthy controls.

In this study, the positive correlation between the circulating percentages of Tregs and TGF- β serum levels is detected only in control individuals. Therefore, there may be an immune dysregulation in patients with CU (ASST positive or ASST negative) resulting from decreased circulating percentages of Tregs or some additional Treg dysfunction causing this lack of correlation. Therefore, *in vitro* functional analysis of Tregs in patients with CU is needed.

On the other hand, positive correlation between serum levels of IL-17 and IL-10, which only appear in patients with CU (particularly in the ASST-positive group), as well

as lack of correlation between circulating levels of Treg cells and IL-10, support the concept of a possible additional immune dysregulation in the level or function of Th17 in patients with CU.

Increased systemic inflammatory cytokines may induce immune dysregulation.¹⁹ In inflamed areas of patients with rheumatoid arthritis,^{20,21} in bronchoalveolar lavage fluid after allergen challenge,²² and in patients with atopic dermatitis,²³ Treg cells seem to be enriched.

IL-17 is known to possess broad inflammatory functions. The specific lineage of CD4⁺ T cells that secrete IL-17 (Th17) are thought to play a central role in inflammation and autoimmunity.^{24,25} In humans, Th17 cells require T-cell receptor ligation by cell-cell contact with Toll-like receptor activated monocytes;²⁶ however, further research is necessary to differentiate the relationship between Th17 and Treg.

High serum levels of IL-17, IL-23, and TNF- α among patients with chronic spontaneous urticaria were found in one study;²⁷ these cytokines were significantly higher in ASST-positive patients than in ASST-negative patients. Moreover, there was a significant positive correlation between serum IL-17, IL-23, TNF- α , and disease activity score.²⁸ A similar increasing level of IL-10 and IL-17 secretion upon the T-cell stimulus in patients with CU and ASST positivity was seen in another study.¹⁹ In addition, a possible role of IL-17 in some inflammatory skin disorders, such as psoriasis, contact hypersensitivity, and atopic dermatitis has been described.²⁷ In CU it may be helpful to verify whether Th17 cells are involved in its pathogenesis and the extent of their involvement as well as the role of Th17 in orchestrating tissue inflammation and autoimmunity.

Altogether, our findings suggest the concept of immunologic dysregulation in CU, which is supported by a decreased percentage of circulating Tregs. Understanding the mechanism through which the signals from Treg cells and/or Th17 are integrated to influence the generation of cytokines will be critical knowledge for the comprehension of the pathogenesis in CU. On the other hand, a short half-life of cytokines and chemokines and their consumption in lesional tissues may explain controversy in the results of serum concentrations of cytokines. Therefore, focusing on *in vitro* stimulation of T cells and their cytokine production may be helpful.

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References

- Atkins D, Frank MM, Dreskin SC. Urticaria and angioedema. In: Kliegman RM, Stanton BF, St. Geme JW, *et al.*, eds. *Nelson Textbook of Pediatrics*, 19th edn. Philadelphia: Elsevier Saunders, 2011: 811–815.
- Niimi N, Francis DM, Kermani F, *et al.* Dermal mast cell activation by autoantibodies against the high affinity IgE receptor in chronic urticaria. *J Invest Dermatol* 1996; 106: 1001–1006.
- Hide M, Francis DM, Grattan CE, *et al.* Autoantibodies against the high-affinity IgE receptor as a cause of histamine release in chronic urticaria. *N Engl J Med* 1993; 328: 1599–1604.
- Kaplan AP. Urticaria and angioedema. In: Bochner BS, Busse WW, Holgate ST, *et al.*, eds. *Middleton's Allergy: Principle and Practice*, 7th edn. Philadelphia: Mosby Elsevier, 2008: 998–1016.
- Chen WC, Ciang BL, Liu HE, *et al.* Defective functions of circulating CD4⁺ CD25⁺ and CD4⁺ CD25[−] T cells in patients with chronic ordinary urticaria. *J Dermatol Sci* 2008; 51: 121–130.
- Sakaguchi S, Sakaguchi N, Asano M, *et al.* Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α -chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; 155: 1151–1164.
- Bach JF. Regulatory T cells under scrutiny. *Nat Rev Immunol* 2003; 3: 189–198.
- Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; 299: 1057–1061.
- Zheng Y, Rudensky AY. Foxp3 in control of the regulatory T cell lineage. *Nat Immunol* 2007; 8: 457–462.
- Randolph DA, Fathman CG. CD4⁺ CD25⁺ regulatory T cells and their therapeutic potential. *Annu Rev Med* 2006; 57: 381–402.
- Jonuleit H, Schmitt E, Stassen M, *et al.* Identification and functional characterization of human CD4⁺ CD25⁺ T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001; 193: 1285–1294.
- Ling EM, Smith T, Nguyen XD, *et al.* Relation of CD4⁺ CD25⁺ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004; 363: 608–615.
- Kriegel MA, Lohmann T, Gabler C, *et al.* Defective suppressor function of human CD4⁺ CD25⁺ regulatory T cells in autoimmune polyglandular syndrome type II. *J Exp Med* 2004; 199: 1285–1291.
- Leznoff A, Sussman GL. Syndrome of idiopathic chronic urticaria and angioedema with thyroid autoimmunity: a study of 90 patients. *J Allergy Clin Immunol* 1989; 84: 66–71.
- Leznoff A, Josse RG, Denburg J, *et al.* Association of chronic urticaria and angioedema with thyroid autoimmunity. *Arch Dermatol* 1983; 119: 636–640.

- 16 Kikuchi Y, Fann T, Kaplan A. Antithyroid antibodies in chronic urticaria and angioedema. *J Allergy Clin Immunol* 2003; **112**: 218.
- 17 Sun RS, Sui JF, Chen XH, *et al.* Detection of CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells in peripheral blood of patients with chronic autoimmune urticaria. *Australas J Dermatol* 2011; **52**: e15–e18.
- 18 Chacón de Petrola MR, Quintero V, Ruiz NB, *et al.* Serum Th1 and Th2 cytokines in chronic urticaria patients. *J Allergy Clin Immunol* 2009; **123**: S105.
- 19 dos Santos JC, Azor MH, Nojima VY, *et al.* Increased circulating pro-inflammatory cytokines and imbalanced regulatory T-cell cytokines production in chronic idiopathic urticaria. *Int Immunopharmacol* 2008; **8**: 1433–1440.
- 20 Möttönen M, Heikkinen J, Mustonen L, *et al.* CD4⁺ CD25⁺ T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis. *Clin Exp Immunol* 2005; **140**: 360–367.
- 21 Cao D, Malmström V, Baecher-Allan C, *et al.* Isolation and functional characterization of regulatory CD25 brightCD4⁺ T cells from the target organ of patients with rheumatoid arthritis. *Eur J Immunol* 2003; **33**: 215–223.
- 22 Robinson D, Hamid Q, Bentley A, *et al.* Activation of CD4⁺ T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *J Allergy Clin Immunol* 1993; **92**: 313–324.
- 23 Ou LS, Goleva E, Hall C, *et al.* T regulatory cells in atopic dermatitis and subversion of their activity by superantigens. *J Allergy Clin Immunol* 2004; **113**: 756–763.
- 24 Miossec P. Interleukin-17 in rheumatoid arthritis: if T cells were to contribute to inflammation and destruction through synergy. *Arthritis Rheum* 2003; **48**: 594–601.
- 25 Afzali B, Lombardi G, Lechler RI, *et al.* The role of T helper17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. *Clin Exp Immunol* 2007; **148**: 32–46.
- 26 Zanin-Zhorov A, Cahalon L, Tal G, *et al.* Heat shock protein 60 enhances CD4⁺ CD25⁺ regulatory T cell function via innate TLR2 signaling. *J Clin Invest* 2006; **116**: 2022–2032.
- 27 van Beelen AJ, Teunissen MB, Kapsenberg ML, *et al.* Interleukin-17 in inflammatory skin disorders. *Curr Opin Allergy Clin Immunol* 2007; **7**: 374–381.
- 28 Atwa MA, Emara AS, Youssef N, *et al.* Serum concentration of IL-17, IL-23 and TNF- α among patients with chronic spontaneous urticaria: association with disease activity and autologous serum skin test. *J Eur Acad Dermatol Venereol* 2013; **28**: 469–474.