



Increased circulating pro-inflammatory cytokines and imbalanced regulatory T-cell cytokines production in chronic idiopathic urticaria

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Received 30 March 2008; received in revised form 29 May 2008; accepted 29 May 2008

KEYWORDS

Chronic idiopathic urticaria;
Pro-inflammatory cytokines;
T-cell cytokines;
Lymphoproliferation;
mRNA

Abstract

The immunologic characterization of chronic idiopathic urticaria (CIU), mainly regarding cytokine profile needs more investigation. We examined circulating inflammatory cytokine levels, T-cell induced secretion, and cytokine mRNA expression in patients with CIU subjected to the intradermal autologous serum skin test (ASST). Increased levels of circulating pro-inflammatory cytokines, such as TNF- α , IL-1 β , IL-12p70, and IL-6 have been observed in most of patients with CIU, together with an enhancement of IL-2 secretion following T-cell stimulation. Highlighting the inflammatory profile in CIU found in ASST positive, is the enhanced B-cell proliferative responsiveness and increased IL-17 secretion levels. ASST-positive patients also exhibited impaired IL-4 secretion associated with increased IL-10 production. Altered cytokine expression in patients with ASST-negative, was the down-modulation of spontaneous IL-10 mRNA expression levels in peripheral blood mononuclear cells. Our findings support the concept of immunologic dysregulation in CIU, revealing a systemic inflammatory profile associated with disturbed cytokine production by T cells, mainly related to IL-17 and IL-10 production.

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Abbreviations: CIU, Chronic Idiopathic Urticaria; PBMC, peripheral blood mononuclear cells; ASST, autologous serum skin test; HC, healthy control; PHA, phytohemagglutinin; PWM, pokeweed mitogen; SEA, staphylococcal enterotoxin A.

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1. Introduction

Chronic urticaria is a common disabling disorder occurring in 0.1% of the population, with an average episode length of 3–5 years in adults [1]. Chronic urticaria is considered idiopathic (CIU) in a substantial percentage of cases, whereas approximately 35% to 40% of patients appears to be of autoimmune origin [2,3]. Since they have circulating IgG antibodies directed against the α -subunit of the high-affinity IgE receptor (Fc ϵ RI α) or less commonly, against IgE itself. Circulating autoantibodies initiate a urticarial eruption by cross-linking the IgE receptor, thereby activating cutaneous mast cells [4]. The autoimmune subgroup represents the most severe form of chronic urticaria [5,6]. However, the pathogenic mechanism in the 55% to 60% of patients with CIU remains unknown. Other circulating mediators, such as cytokines produced by peripheral blood mononuclear cells (PBMCs) that may modulate secretion of cytokines of various other innate and adaptive immune cells may also function as histamine-releasing factors [7,8]. A positive autologous serum skin test (ASST) is a marker of CIU, together with an *in vitro* basophil and mast cell histamine-release assay. Although the ASST is a test of autoreactivity rather than a specific test of autoimmunity, it is not known if positivity to the ASST may identify an immunologic subset of CIU patients.

Activated mast cells may release both preformed and newly synthesized mediators, as well as chemotactic factors and cytokines that recruit and activate various inflammatory cells, such as polymorphonuclear cells (PMNs) and lymphocytes. Cytokines and chemokines promote immune cell recruitment and activation, and thus play an integral role in the coordination and persistence of chronic inflammation. Therefore, circulating cytokines have been described to facilitate the perpetuation of cellular and humoral autoimmune processes [9,10]. The inflammatory response exemplified by spontaneous wheals demonstrates pro-inflammatory features, involving a prominent role for lymphocytes with a mixed Th1/Th2 response [11], and neutrophils, adhesion molecules, IL-8 and chemokine receptors exerting main role in the inflammation [12]. In contrast, ASST induced wheals seem to imply different activities mainly represented by basophil granulocytes and IL-4 [12].

Furthermore, inflammatory etiopathogenesis in CIU has been described as related to increased IL-4, IL-13, and IL-18 serum levels [13–15]. Cytokines in the circulation are a feature of amplified inflammation in chronic urticaria that may be helpful in understanding the immunopathogenesis of the idiopathic variety of chronic urticaria.

Cytokines are the dominant factors guiding the development, differentiation, and regulation of immune cells. Nevertheless, a dependency on the type of *in vitro* stimulus has been shown in CIU patients with respect to different patterns of cytokine production. High levels of IL-4 and IFN- γ produced by activated CD4⁺ T cells upon phorbol myristate acetate stimulation [13] and down-regulation of both Th1 and Th2 cytokines upon phytohemagglutinin (PHA) stimulation, regardless of the ASST response, have both been described in CIU [16]. Moreover, high levels of IL-10 induced by mitogen have been verified in patients with CIU, again without regard to a positive or negative ASST response [17].

Inflammatory cytokines, such as IL-17, have been related with a number of human autoimmune and inflammatory dis-

eases [18,19], though this has not yet been evaluated in CIU. In humans, IL-17 production is generally thought to be restricted to T cells, secreted by activated CD4⁺ T cells (Th1 and Th0 cells, but not Th2) [20]. A new CD4⁺ T effector lineage, known as Th17, in which IL-17 is the major cytokine factor, have been shown to be significantly related to immune regulatory effects, such as the regulation of tissue inflammation [21]. Th17 cells are likely a mechanism through which the adaptive immune system communicates with the innate immune system to promote inflammation.

In the present study, we focused our attention on cytokine production, examining in particular the circulating levels and T-cell induced secretion. We also estimated peripheral immune status by assessing cytokine mRNA synthesis by PBMCs from CIU patients subjected to the ASST. We therefore tested the immunological competence of patients with CIU through examining the *in vitro* polyclonal B and T-cells proliferative response.

2. Materials and methods

2.1. Study subjects

All patients with continuous CIU were selected from the Dermatological Outpatient Clinic of the Hospital das Clínicas de São Paulo (HC-FMUSP). These patients had wheals daily for 6 weeks or longer, occurring at least 4 times a week. Patients with physical urticaria, urticarial vasculitis, or food allergies were excluded, as previously described [22]. Antihistamine medications were withdrawn 48 h before the study, and none of the patients was taking corticosteroids or immunosuppressive drugs at the time of venipuncture. Samples were obtained from 29 patients with CIU (5 males and 24 females; median age 42.5 years, aged 20–66 years) and 33 health controls (7 males and 26 females, median age 35.5 years, aged 23–57 years). All subjects gave written informed consent, and the study protocol was approved by the institutional ethics committee of the HC-FMUSP.

2.2. Autologous serum skin test

All patients were subjected to an ASST performed according to standard methods using fresh autologous serum injected intradermally into the volar forearm skin. A positive skin test was defined as the appearance of a red serum-induced weal with a diameter ≥ 1.5 mm than that of the saline-induced response at 30 min.

2.3. Lymphoproliferative response

PBMCs were isolated from heparinized venous blood by Ficoll-Hypaque gradient (Pharmacia, Sweden) centrifugation and were diluted in RPMI medium supplemented with gentamicin (40 μ g/mL) and 10% pooled AB human serum (Sigma, St Louis, MO, USA). Cells (2.0×10^5 cells/well) were cultivated in 96-well flat bottom microplates (Costar, Cambridge, MA, USA) with medium only, 2.5 μ g/mL PHA (Gibco-BRL, NY, USA), pokeweed mitogen (PWM, 5 μ g/mL; Sigma, St. Louis, MO, USA), or staphylococcal enterotoxin A (SEA, 0.04 μ g/mL; Sigma), at 37 °C and 5% CO₂. Cells were cultured in triplicate for 3 d or 6 d, then pulsed with 1 μ Ci/well of ³H-Thymidine (New England

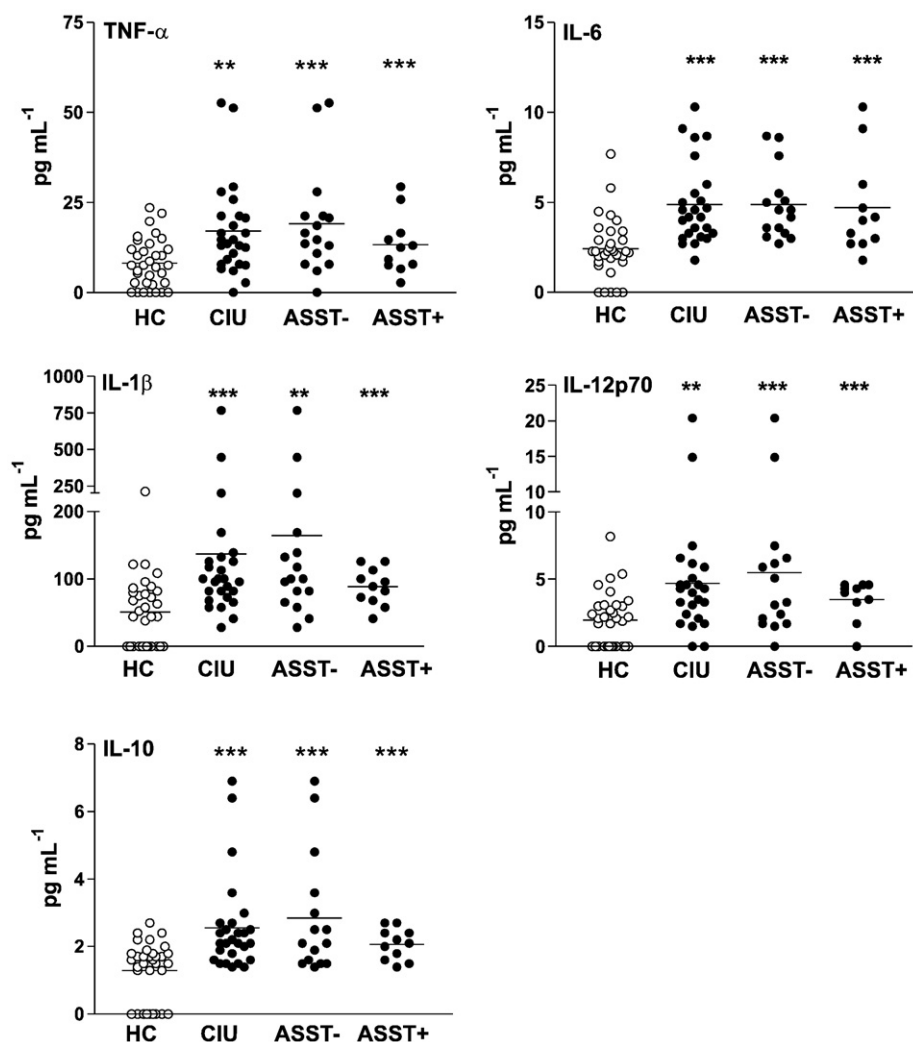


Figure 1 Circulating cytokine levels in patients with CIU were evaluated by cytometric bead array. HC, sera from healthy controls; CIU, sera patients; ASST-, CIU with negative ASST; ASST+, CIU with positive ASST. The horizontal line represents the means. *** $P<.001$, ** $P<0.01$ compared with the HC group.

Nuclear, USA) per well for an additional 18 h before harvest. Cell-bound radioactivity was measured by liquid scintillation counting (1205 Betaplate, Wallac, USA). Proliferation was quantified by stimulation index (SI), which is calculated as the ratio of mean counts per minute (cpm) of stimulated to unstimulated replicates.

2.4. Cytokine measurements

Cultures of PBMC (3.0×10^6 cells/well) in RPMI medium supplemented with 10% AB human serum (Sigma) were incubated in 24-well plates (Costar) with medium or PHA ($2.5 \mu\text{g/mL}$) for 24 and 48 h at 37°C and 5% CO_2 . Cell-free supernatants were

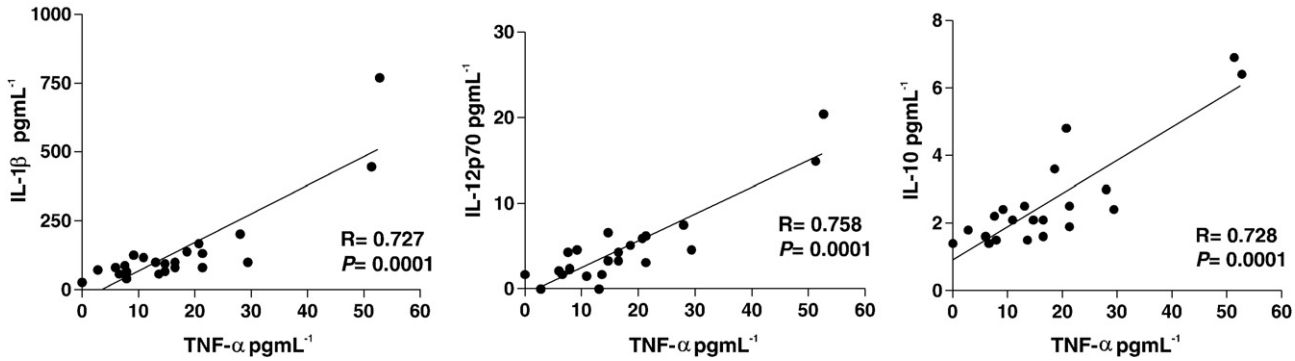


Figure 2 Correlation of TNF-α with IL-1β IL12p70 and IL-10 serum levels from patients with CIU.

Table 1 Profile of *in vitro* lymphoproliferative mitogens responsiveness in CIU patient

Stimulus	Groups			
	Healthy control	CIU	ASST–	ASST+
PHA#	98.95 ± 59.54	119 ± 92.42	105.3 ± 63.39	133.7 ± 116.7
SEA	63.22 ± 37.49	79.84 ± 57.32	66.46 ± 43.98	90.13 ± 65.64
PWM	63.75 ± 52.49	96.50 ± 60.91*	90.72 ± 64.86	102.3 ± 58.73**

Data represent the mean ± SD of stimulation index obtained from PBMC proliferative response of healthy control individuals (HC, *N*=29), CIU patients (CIU, *N*=29), and patients divided according to ASST result, negative (*N*=15) or positive (*N*=14). **P*<0.026, ***P*<0.01 vs. HC individuals.

harvested and stored at -70°C . Cytokines from appropriately diluted culture supernatant and undiluted serum were measured using Th1/Th2 and inflammatory cytokine bead array (CBA) kits (Becton Dickinson, San Diego, CA, USA). Briefly, latex particles with 6 different fluorescent intensities coated with antibodies against specific cytokines were incubated with the samples. The particles were then labeled with a mixture of PE conjugated cytokine-specific antibodies and analyzed by flow cytometry (FACSCalibur, BD, San Jose, CA). IL-17A measurement of the culture supernatants was performed by ELISA (R&D Systems, Minneapolis, MN), and reaction was read at 450 nm in an ELISA microplate reader (Molecular Devices).

2.5. RNA isolation from PBMC and real-time polymerase chain reaction

RNA was isolated from PBMC (1×10^6 cells) using the QIAamp RNA Blood Mini kit (Qiagen, Valencia, CA). Reverse transcription was performed using a cDNA synthesis kit, Sensiscript

Reverse Transcriptase (Qiagen). Real-time polymerase chain reaction (RT-PCR) was performed on 3.0 μL cDNA, with 20 μL Platinum SYBR® Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA), 0.8 μL ROX Reference Dye, 14.6 μL Rnase/DNase-free water, and 0.8 μL (10 μM) of gene-specific primers (Invitrogen, Carlsbad, CA). RT-PCR was carried out in an iCycler (BioRad, EUA) based on specific cytokine primers and general SYBR green fluorescence detection for 45 cycles of: denaturation at 95°C for 15 s; annealing at 62°C for 5 s, and extension at 72°C for 15 s. For TNF- α the annealing and extension were performed at 60°C for 1 min. Primers sets were as follows: albumin sense: 5'-GCTGTCATCTCTTG TGGGCTGT-3', albumin antisense: 5'-AAA-CTCATGGGAGCT GCTGGTT-3', melting temperature (*T_m*) 70°C ; IFN- γ sense: 5'-GTTTTGGGTTCTCTTGGCTGTTA-3', IFN- γ antisense: 5'-AAAA-GAGTTCCATTATCCGCTACATC-3'; IL-4 sense: 5'-ACAGCCTCACAG AGCAGAAGACT-3', IL-4 antisense: 5'-TGTT-CTTGGAGGCAGCAAAGA-3'; IL-10 sense: 5'-GCTGG-AGGACTTTAAGGGTTACCT-3', IL-10 antisense: 5'-CTTGA-

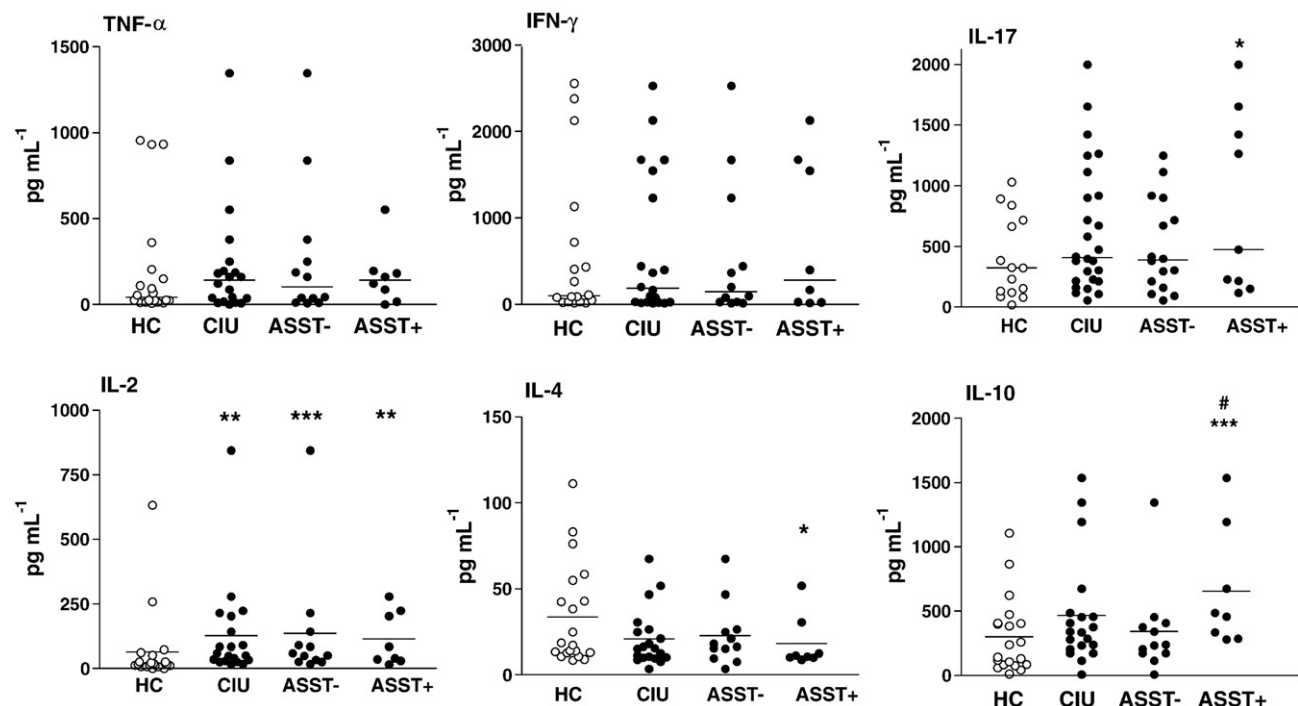


Figure 3 Profile of cytokines secretion induced by PHA stimulation in patients with CIU. Measurements of TNF- α , IFN- γ , IL-2, IL-4 and IL-10 in supernatants of PBMC cultures from health control individuals (HC), patients with CIU, ASST negative (ASST–) or positive (ASST+) upon PHA stimulus for 48 h were performed by cytometric bead array. Determination of IL-17 was carried out by enzyme-linked immunosorbent assay (ELISA). Horizontal line represents means. ****P*<.001, ***P*<.01 and **P*<.05 compared with the HC group; # *P*<.05 compared with the ASST– group.

TGTCTGGGTCTTGGTTGT-3'; TNF- α sense: 5'-CCCAGGCAGT-CAGATCATC TTC-3', TNF- α antisense: 5'-GCTTGAGGGTTTGTA-CAACATG-3' (Tm 55 °C) [23–25]. The copy number of cytokine message was normalized to the housekeeping gene, albumin, and presented as a ratio of cytokine to albumin copy number.

2.6. Statistical analysis

The Mann–Whitney *U* test was used to compare variables between patients with CIU and healthy subjects. The Kruskal–Wallis test with Dunn's posttest was used to compare non-paired data between 3 sets of data. Correlations of data were analyzed by the non-parametric Spearman test. *P* values less than .05 were considered significant.

3. Results

3.1. Circulating cytokines in chronic idiopathic urticaria

Typical inflammatory cytokines, such as TNF, IL-1 β , IL-6, as well as IL-12p70 and IL-10 were significantly increased in sera from patients with CIU compared to healthy control (HC) individuals (Fig. 1). Alteration of cytokine levels was verified to occur in CIU patients with both positive and negative ASSTs.

We were unable to detect cytokines in a large percentage of HC sera samples. These negative percentages were 21% for TNF- α (7 of 33), 36.4% for IL-12p70 and IL-1 β (12/33); 57.6% for IL-6

(19 of 33), and 100% for IL-10. The frequency of undetectable cytokines in the sera of CIU patients was lower than seen in HCs, 14% IL-12p70 (4/29), 6.9% TNF- α (2/29), only 1 negative case for IL-6, and none were negative for IL-1 β . There was one exception, IL-10, which was detectable in only 5 patients, all of which belonged to the negative ASST group. As shown in Fig. 2, TNF- α serum levels of CIU patients displayed a strong positive correlation with IL-1 β ($R=0.727$, $P=0.0001$), IL-12p70 ($R=0.758$, $P=0.0001$), and IL-10 levels ($R=0.728$, $P=0.0001$). We observed a weak correlation between TNF- α serum levels from HC individuals and IL-12p70 levels ($R=0.468$, $P=0.012$).

3.2. *In vitro* proliferative response to mitogen and T-cell cytokine production in patients with CIU

To verify the cytokine secretion response to T-cell mitogenic stimulus, we first analyzed the *in vitro* PBMC ^3H -thymidine incorporation after PHA, PWM, or the bacterial superantigen, SEA. The lymphoproliferative response of patients with CIU to both PHA and SEA was comparable to the HC group (Table 1). However, patients with CIU displayed a significantly increased PBMC proliferative response to PWM, due to the enhanced response of positive ASST patients compared to HC individuals.

Fig. 3 shows that PHA-stimulated IFN- γ and TNF- α levels were similar between patients and controls. A significant increase in IL-2 secretion was observed in patients with CIU compared to HC individuals, regardless of ASST response. A similar pattern of IL-

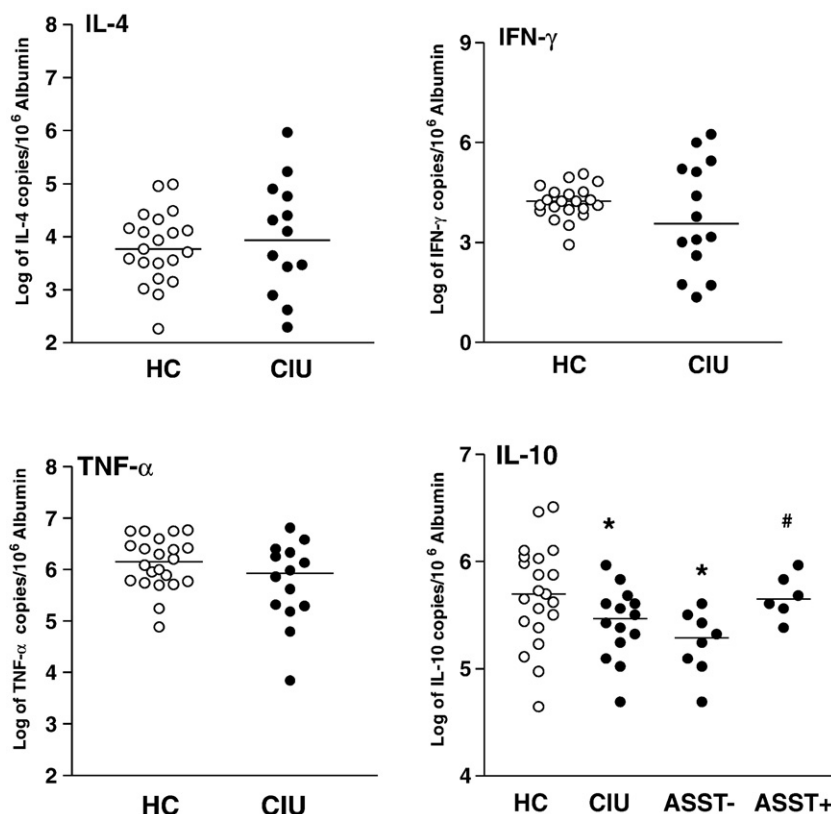


Figure 4 Spontaneous cytokine mRNA expression by PBMCs from patients with CIU. HC, unstimulated PBMC from healthy individuals; CIU, patients with CIU; ASST– negative for ASST; ASST+, positive for ASST. IFN- γ , IL-4, TNF- α , IL-10, and albumin mRNA expression levels were measured by quantitative reverse transcriptase polymerase chain reaction. Horizontal line represents means. * $P<0.05$ compared with the HC group; # $P<0.05$ compared with the ASST– group.

IL-10 and IL-4 secretion was found in both patient and control groups; however, paradoxical production of IL-10, concomitant with decreased IL-4 was detected in patients with a positive ASST. Furthermore, patients with positive ASST revealed highly variable, but significantly increased IL-17 secretion compared to healthy control individuals.

Together, these findings demonstrate the presence of circulating pro-inflammatory cytokines in the majority of patients with CIU, as well as altered cytokine production by T cells, particularly in patients with a positive ASST response.

3.3. Spontaneous cytokines mRNA expression in PBMC from CIU patients

To verify the steady-state levels of cytokine mRNAs, unstimulated PBMC were evaluated by Real Time PCR, as shown in Fig. 4. Spontaneous mRNA expression of IFN- γ , IL-4, TNF- α , as well as IL-2 and IL-6 (data not shown) were found at similar levels in both patients with CIU and controls. We did not observe differential expression of these cytokines among patients according to the ASST response. Unexpectedly, IL-10 mRNA expression levels were significantly decreased in patients with CIU, due to the negative ASST patients, compared to both the HC group and ASST positive patients.

4. Discussion

The inflammatory etiopathogenesis of CIU was characterized in this study by increased circulating levels of pro-inflammatory cytokines, such as IL-1 β , IL-12p70, TNF- α and IL-6.

Systemic circulation of cytokines may reflect production by many cell types, and is likely produced mainly at the site of inflammation, therefore it is rarely measurable in healthy individuals. Contrary to healthy individuals, more than 90% of the patients with CIU had detectable levels of TNF- α , IL-6, and IL-1 β , and 84% for IL-12p70. These results also demonstrate that the use of sensitive multiplex bead-based flow cytometry assay for circulating cytokines may be useful in the diagnosis/prognosis of diseases with basis in immune function disorder. The data further reinforces a possible role for inflammatory cytokines in idiopathic urticaria, which represents the majority of chronic urticaria patients [1,2]. However, it has been showed that the TNF- α levels are not altered in blood of patients with CIU [26]. One reason for the circulating TNF- α levels found in our study is probably the use of cytometric bead array system for measuring serum cytokine concentrations.

The typical inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, as well as IL-12p70 detected in the sera of CIU patients may be implicated as factor(s) contributing to the skin lesions seen in CIU, as well as to influencing the behavior and properties of immune and other cells. In a previous report, we showed that peripheral blood basophils of CIU patients have an activated status *ex vivo*, displaying up-regulation of degranulation/activation surface markers together with IL-3-induced hyperresponsiveness for anti-IgE cross-linking histamine release [22]. This emphasizes that, besides the possibility of an intrinsic basophil defect, some circulating factors may favor the maintenance of activated basophils in the peripheral blood. Circulating cytokines, such as IL-18, have been suggested to be histamine-releasing factors, since IL-18 has been

found at high levels in CIU patients with positive ASST in parallel with clinical severity scores [15].

With regard of cytokine secretion induced by T-cell stimulation, we observed that IL-2, an essential cytokine for T-cell activation, was increased in CIU patients, despite the observation of a normal polyclonal T-cell proliferative response. It has been described in CIU, a disturbance in the p21Ras pathway in lymphocytes [27], which proto-oncogene is involved in T-cell receptor activation, as well as in IL-2 receptor signaling [28]. Also they showed that patients retained a normal *in vitro* proliferative response to T-cell mitogen [27]. Although we found an activated T-cell profile in CIU patients, this did not seem to interfere with the *in vitro* T-cell mitogen proliferative response. Nevertheless, we found that patients positive for ASST had an increased B-cell responsiveness toward pokeweed mitogen, indicating that B-cell polyclonal activation is further enhanced when requiring T-cell activation and/or cytokine production. However, we did not performed *in vitro* assays for histamine-releasing factors. It should be an important parameter for chronic autoimmune urticaria, since ASSTs and *in vitro* histamine-release assays seem to look into distinct aspects of the disease, and the specificity of ASST has been questioned [29].

Altered production of the IL-10 was observed in CIU at 3 levels: 1) in the circulation, 2) spontaneous mRNA expression, and 3) protein secreted upon T-cell stimulus. A small percentage of CIU patients with negative ASST exhibited increased IL-10 serum levels, probably produced by macrophages or keratinocytes, other than T cells, B cells, or monocytes, since, in the steady-state condition, peripheral mononuclear cells from these patients have shown down-modulation of IL-10 mRNA levels. However, unsorted peripheral mononuclear cells do not determine which cell population was responsible for the decreased IL-10 expression, allowing only verification of the peripheral immune status.

Moreover, patients with positive ASST displayed increased IL-10 secretion upon T-cell stimulus. In CIU, high levels of IL-10 were previously shown to be induced by mitogen, accompanied by decreased IFN- γ , in patients with or without positive response to ASST [17]. Our findings indicate that alteration of IL-10 secretion is not related with modulation of IFN- γ expression or secretion. Indeed, dysregulation of IL-10, occurred to different degrees according to the ASST response of CIU patients. IL-10 is well known to down-regulate specific effector function in monocytes, dendritic cells, natural killer cells, B cells, Th1 cells, and PMNs [30–32], which may result in diminished production of pro-inflammatory cytokines and chemokines. Moreover, IL-10 may exert potent immune stimulatory properties, as a cofactor for B-cell survival, proliferation, differentiation, and Ig secretion, as well as inducing the proliferation of CD8 T+ cell cytotoxic activity and enhancing T-cell responsiveness to IL-2 [33]. The increased IL-10 levels detected in patients presenting CIU, who were ASST positive, could be a consequence of an increase in systemic inflammatory cytokines, such as TNF- α and IL-12. This observation was supported by the positive correlation between IL-10 and TNF- α , IL-1 β , IL-12 verified only in CIU patients, but not in healthy individuals. These pro-inflammatory cytokines may lead to an up-regulation of IL-10 expression, as observed in certain inflammatory disorders [34,35].

Interestingly, our results highlight the inflammatory profile found in CIU patients with a positive skin test, demonstrating enhanced IL-17 secretion levels induced by T-cell stimulus. IL-17 is known to possess broad inflammatory function; it induces expression of many innate inflammatory mediators, including IL-6, acute phase proteins, G-CSF, and prostaglandin E2 [36], and may synergize with TNF- α . The specific lineage of CD4⁺ T cells that secrete IL-17 (T helper 17, Th17), has emerged though mounting evidence to play a central role in inflammation and autoimmunity [18,19]. Furthermore, in humans, Th17 cells require T-cell receptor ligation by cell–cell contact with Toll-like receptor-activated monocytes [37], and unlike IFN- γ , IL-17 expression is not down-regulated by the presence of FOXP3⁺ regulatory CD4⁺ T cells [37]. Of note, patients with CIU and ASST positivity, show a similar pattern of IL-10 and IL-17 secretion upon T-cell stimulus, in parallel to increased circulating levels of TNF- α , IL-1 β and IL-6. These pro-inflammatory cytokines, in turn, are known to be induced by IL-17, which may contribute to the inflammatory profile founded in CIU. A possible role of IL-17 in the pathology of inflammatory skin disorders, such as psoriasis, contact hypersensitivity and atopic dermatitis has been described, which indicates that IL-17 is an important mediator of tissue inflammation [38]. In chronic idiopathic urticaria, it might be relevant to verify whether Th17 cells are involved in its pathogenesis and the extent of their involvement.

Altogether the findings of this report support the concept of immunologic dysregulation in CIU, reflected by the systemic inflammatory profile in association with a disturbance of T-cell induced cytokine production. The common immunological feature in CIU was the presence of circulating pro-inflammatory cytokines, which were found in the majority of patients regardless of their ASST status. Others observed immunological features were primarily related to patients with positive ASST, including enhanced B-cell proliferative response, and increased IL-17 and IL-10 secretion levels, together with impaired IL-4 secretion. Understanding the mechanism through which the signals from regulatory T cells and/or IL-10 are integrated to influence the generation of the pro-inflammatory effects of IL-17 and other typical pro-inflammatory cytokines will be critical knowledge for the comprehension of the inflammatory etiopathogenesis in chronic idiopathic urticaria.

Acknowledgments

This research was supported by FAPESP (no. 2004/06937-5), CAPES and LIM 56-HC/FMUSP.

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