RESEARCH ARTICLE

Effects of allergen-specific immunotherapy on functions of helper and regulatory T cells in patients with seasonal allergic rhinitis

Cengiz Kirmaz[1], Ozlem Ozenturk Kirgiz[2], Papatya Bayrak[1], Ozge Yilmaz[3], Seda Vatansever[4], Kemal Ozbilgin[4], Ece Onur[5], Onur Celik[6], Ayhan Sogut[3], Gungor Ay[7], Hasan Yuksel[3]

[1] Celal Bayar University, Medical Faculty, Department of Internal Medicine, Division of Immunology, Manisa
[2] Celal Bayar University, Medical Faculty, Department of Internal Medicine, Manisa
[3] Celal Bayar University, Medical Faculty, Department of Paediatric Allergy, Manisa
[4] Celal Bayar University, Medical Faculty, Department of Histology and Embryology, Manisa
[5] Celal Bayar University, Medical Faculty, Department of Biochemistry, Manisa
[6] Celal Bayar University, Medical Faculty, Department of Otolaryngology, Manisa
[7] Celal Bayar University, Science and Art Faculty, Department of Biology, Manisa, Turkey

Correspondence. C. Kirmaz, Assoc. of Prof., MD, 7445 sok No:10-H K:6 D:14, Saklibahce Evleri, Yenigirne, Karsiyaka, Izmir, Turkey
<ckirmaz@yahoo.com>

ABSTRACT. *Background.* **Seasonal allergic rhinitis (SAR) is characterized by a helper T (Th)2 cell-mediated immune response at the target site. There is a relative Th1 and/or regulatory T (Treg) cell insufficiency in patients with SAR. It has been demonstrated that there is a change in the balance between these cells after allergen-specific immunotherapy (SIT), which is a curative treatment modality for this disease. However, there are few studies that evaluate the number and function of these cells in the inflammatory area after SIT treatment.** *Objective.* **We aimed to investigate the distribution of Th1, Th2 and Treg cells in nasal biopsies and lavage fluid (NLF) specimens from patients with SAR, before and after SIT.** *Methods.* **Twenty-four, symptomatic SAR patients sensitized to *Olea europeae*, were enrolled in the study prior to treatment. Fifteen, non-allergic subjects with nasal septum deviation, who needed surgical treatment, served as the control group. NLF and inferior turbinate biopsies were obtained from both groups during the pollen season. Conventional, subcutaneous SIT with *Olea europeae* extract was initiated in patients with SAR. One year after the first biopsy, biopsies and NLF specimens were again obtained for re-evaluation. All biopsies were evaluated for Th1, Th2 and Treg cell counts by means of their transcription factors (T-bet, GATA-3 and FoxP3) using an immunohistochemical analysis method. Additionally, all NLF specimens were evaluated for the functions of these cells, by means of their specific cytokines, using an ELISA method.** *Results.* **When the basal status of those patients with SAR was evaluated based on transcription factors, prior to treatment, Th1 and Treg cells were found to be fewer than in non-allergic controls (p=0.001 for both T-bet and FoxP3). It was demonstrated that numbers of GATA-3-carrying cells, which are a marker for Th2, were not significantly different between the groups (p=0.276), but evaluation of the Th1/Th2 ratio revealed a relative Th2 dominance in patients with SAR prior to treatment. When evaluated on the basis of cytokine levels, it was observed that Th1-originated IFN-γ was lower in patients with SAR compared to the control group, both before and after treatment (p=0.012 for both comparisons), Th2-originated IL-4 levels were not significantly different between the groups either before or after treatment (p=0.649, p=0.855; respectively). Th2- and Treg cell-originated IL-10 levels were higher in patients with SAR before treatment (p=0.033), but this difference was not statistically signifact following treatment compared with controls (p=0.174). Treg cell-originated TGF-β levels were slightly lower in patients with SAR compared to the controls, although the difference was not statistically significant (p=0.178, p=0.296; respectively). None of the above mentioned cytokine levels changed significantly as a result of SIT.** *Conclusion.* **The results of our study indicate that although clinical findings improve after one year of SIT, this duration may not be sufficient to detect changes in cytokine patterns and transcription factors. Further studies that evaluate outcome over a longer duration of treatment would provide valuable information.**

Key words: allergen specific immunotherapy, allergic rhinitis, cytokines, nasal septal deviation, Th1, Th2, transcription factor, Treg cells

Atopic diseases are characterized by IgE-mediated allergic inflammation at the target site [1]. Release of T helper (Th)2-type cytokines by allergen-specific Th cells leads to an immune response in atopic individuals against "innocuous" antigens [2]. Th1 cells produce predominantly IL (interleukin)-2, IFN (interferon)-γ, and TNF (tumor necrosis factor)-β, but not IL-4, IL-5, IL-9, and IL-13. By contrast, Th2 cells are characterized by the predominant production of IL-4, IL-5, IL-9, and IL-13, but not IFN-γ and TNF-β [2]. Furthermore, in the past few years, a heterogeneous family of regulatory T (Treg) cells that are able to suppress the effector immune response by Th1 or Th2 cells has been described [3].

Different transcription factors play a role in the differentiation and cytokine production of these cell types. GATA-3, which inhibits the production of IFN-γ, increases transactivation of the IL-4 promoter, and directly regulates IL-5 and IL-13 expression, is an important transcription factor for Th2 differentiation [4]. T-bet (T-box expressed in T cells) is a member of the T-box family of transcription factors that regulates several developmental processes. T-bet expression strongly correlates with IFN-γ expression; it is specifically up-regulated in primary Th cells differentiated along the Th1, but not the Th2 pathway [5]. The transcription factor FoxP3 (forkhead/winged helix transcription factor) is critical for generating Treg cells, and has been described as the "master regulator" or "lineage-specification factor" for Treg cells [6, 7]. Transforming growth factor (TGF)-β that induces FoxP3 and suppressive Treg cells from naive T cells, is a cytokine generally secreted by Treg cells [6]. On the other hand, if Treg cells are activated, they begin to secrete large amounts of IL-10, which is a strongly inhibitor of effector immune responses [8].

Many cytokine-based studies, have demonstrated that the achievement of balance between the immunological response towards Th1 type and Th2 plays a key role in the effectiveness of allergen-specific immunotherapy (SIT) [9-12]. Treg cells are reviewed in a recent paper addressing this issue [13]. However, there seems to be a paucity of clinical, immunological research that evaluates the effects of SIT on all of these cells, particularly in inflamed areas.

We aimed to demonstrate the distribution of Th1, Th2 and Treg cells in nasal biopsies and lavage specimens from patients with seasonal AR (SAR), before and after SIT.

DONORS AND METHODS

Subjects

To obtain a standardized study population, twenty-four patients (19 F/5 M) with SAR, sensitized only to *Olea europeae* pollen, were enrolled in this study. Any patient who had active upper or lower respiratory infections or any other systemic disease such as a cancer, diabetes mellitus, chronic renal failure etc. and any who were receiving treatment such as antihistamines or corticosteroids for SAR or any other disease, were excluded from the study. Similarly, those patients in whom the nasal symptom score failed to improve after nine months of allergen-specific immunotherapy were excluded.

Fifteen patients (8 F/7 M) with nasal septum deviation (NSD), who underwent septoplasty, were enrolled as the non-allergic control group.

The mean age of the patients with SAR was 35.5±12 years. Diagnosis of SAR was based on history, physical examination and the laboratory findings. The nasal symptom scores (sneezing, nasal obstruction, nasal itching, watery nasal discharge; graded as 0=none, 1=mild, 2=moderate, 3=severe, up to a maximum of 12 points) were recorded for all patients during the pollen season, when their symptoms were at their worst *(table 1)*. Allergic sensitization was demonstrated by the skin prick test. Skin prick tests were performed in accordance with the EAACI guidelines [14]. All patients with SAR had a positive skin prick test reactivity only to *Olea europeae* pollen extract (Allergopharma Ltd, Reinbek, Germany).

The mean age of the control group was 29±10 years. These non-allergic, control patients had been diagnosed by an otolaryngologist based on history and physical examination accompanied by rhinoscopy and laboratory findings. The allergen skin prick test was negative in all non-allergic control patients.

Informed consent for the procedures described was obtained from all subjects. Approval for the study was given by the ethics committee of our hospital.

Study design

During the *Olea europeae* pollen season (19th-21st weeks of the year), when natural allergen exposure and the most severe symptoms of our patients were observed, nasal lavages were performed prior to nasal inferior turbinate biopsy. The subject, in the sitting position, was instructed to extend the neck approximately 30 degrees from the horizontal, and to refrain from breathing or swallowing. The nasal cavity was washed with 10 mL of physiological saline, warmed to 37°C; the patient refrained from breathing and swallowing. The subject flexed the neck and expelled the nasal lavage fluid (NLF) into a container 10 seconds after each lavage. All NLF was filtered through a 52 lm nylon filter to remove mucin, and the filtrate was centrifuged at 4°C for 10 min at 1,000*g*. The supernatant was stored at -80°C until assayed for cytokine levels (pre-treatment NLF of SAR). The same NLF collection procedure was performed for the control group prior to septoplasty during the same season as the patient group (NLF from the control group).

Inferior turbinate biopsies were obtained from the SAR patients using a cup forceps device under local anesthesia, followed by fixation with 10% formalin in phosphate-buffered saline (PBS) for a maximum duration of 24 hours. They were embedded in paraffin using a routine embedding procedure (pre-treatment biopsy of SAR). Biopsies from the control group were obtained during septoplasty by the same otolaryngologist (biopsy from the control group).

Following the pollen season, conventional subcutaneous SIT was initiated in our patients with SAR, using the appropriate SIT agent (*Olea europeae* 5-50-500-5,000 TU/mL, Code # 151, Allergopharma Novo-Helisen Depot, Allergopharma Ltd, Reinbek, Germany.). SIT courses involved a build-up phase (increasing allergen doses), and a maintenance phase (maximum dosage of the allergen) as previously described in the WHO position paper

Table 1
Pre- and post-treatment nasal symptom scores (NSS) of all patients; each one of the symptoms like sneezing, nasal obstruction, nasal itching, watery nasal discharge was graded as 0=none, 1=mild, 2=moderate, 3=severe with total 12 points.

	Pre-treatment NSS					Post-treatment NSS				
Patient	Sneezing	Nasal obstruction	Nasal itching	Watery nasal discharge	Total	Sneezing	Nasal obstruction	Nasal itching	Watery nasal discharge	Total
1	3	3	3	3	12	1	0	0	1	2
2	3	3	3	3	12	2	2	2	3	9
3	3	3	3	3	12	3	2	3	3	11
4	3	0	1	1	5	2	0	0	0	2
5	2	0	1	2	5	2	0	0	0	2
6	3	3	3	3	12	3	2	2	3	10
7	3	0	1	1	5	1	0	0	0	1
8	3	0	2	2	7	2	0	1	2	5
9	2	0	2	1	5	1	0	2	1	4
10	3	2	2	2	9	2	2	2	2	8
11	3	3	3	3	12	3	2	3	3	11
12	3	3	3	3	12	3	3	2	3	11
13	3	3	3	3	12	1	0	0	0	1
14	3	3	3	3	12	3	3	3	3	12
15	2	1	1	2	6	2	1	0	1	5
16	2	1	1	1	5	3	1	2	2	8
17	3	0	1	1	5	3	0	1	1	5
18	3	2	3	3	11	2	0	1	1	4
19	3	3	3	3	12	1	0	0	1	2
20	3	3	3	3	12	1	0	1	1	3
21	2	1	1	1	5	2	0	0	0	2
22	2	1	1	2	6	1	0	0	1	2
23	1	1	1	2	5	0	0	1	1	2
24	3	3	3	3	12	2	0	0	1	3
Mean±SEM					8.78±0.68					5.22±0.8

[15]. One year±five days after the first biopsies (during approximately the 9th month of SIT; same period for the natural allergen exposure), patients with SAR were re-evaluated, their symptoms were scored again according to the NSS *(table 1)*, and second NLF and biopsies were taken (post-treatment NLF and biopsy of SAR).

Measurements of cytokines

Levels of cytokines in NLF supernatant were measured using commercially available, human ELISA kits (IL-4, Catalog # ELH-IL4-001; IFN-γ Catalog # ELH-IFN gamma-001; IL-10, Catalog # ELH-IL10-001, TGF-β, Catalog # ELH-TGFbeta1-001. Ray Biotech, Inc., Norcross GA, USA) following the manufacturer's instructions. Levels of these mediators below the sensitivity of the assay were 5 pg/mL (intra-assay CV <10%; inter-assay CV 12%) for IL-4, 15 pg/mL (intra-assay CV 10%; inter-assay CV 12%) for IFN-γ, 1 pg/mL (intra-assay CV <10%; inter-assay CV 12%) for IL-10, 80 pg/mL (intra-assay CV <10%; inter-assay CV 12%) for TGF-β.
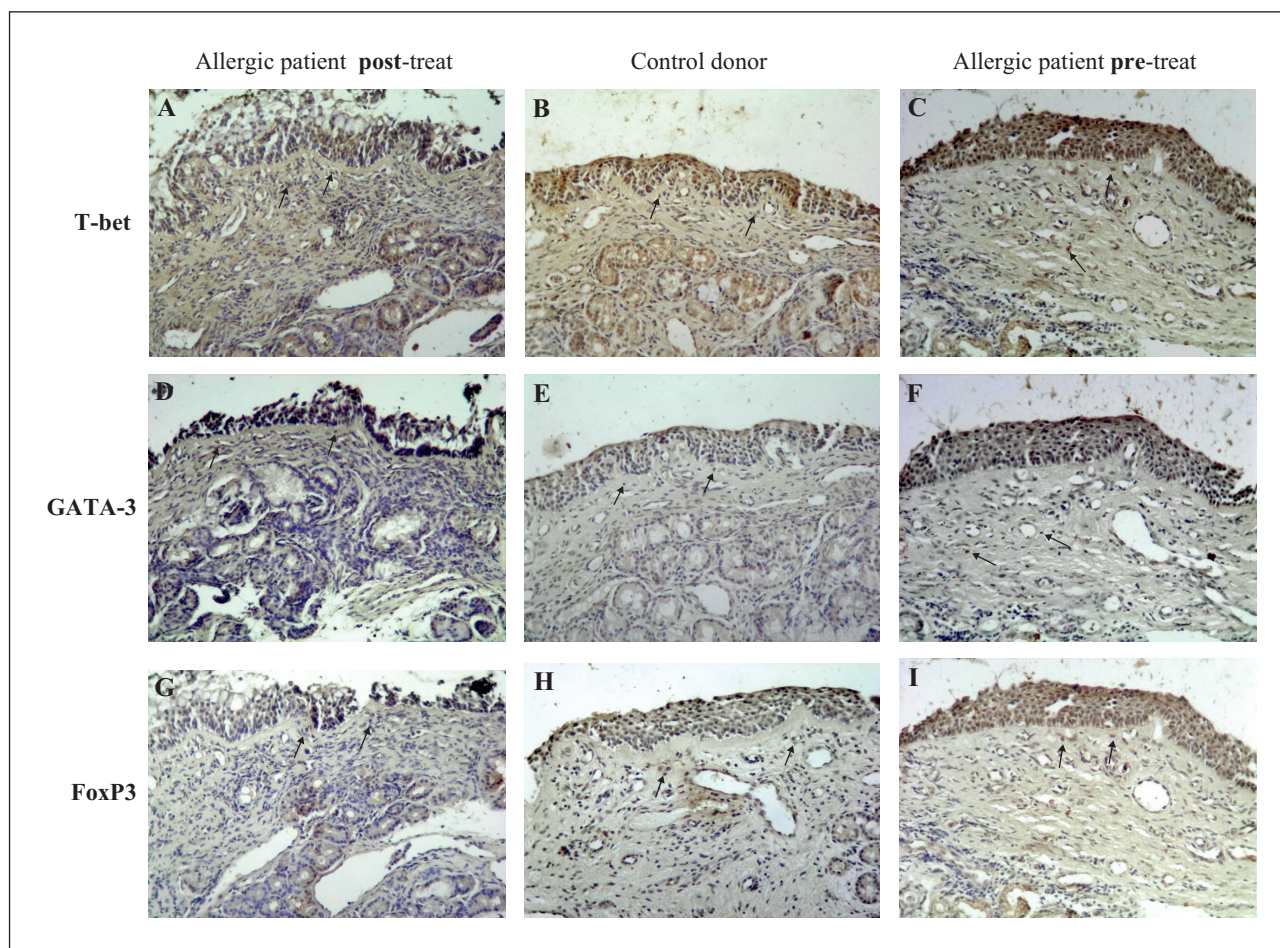
Immunohistochemistry

Antibodies
Monoclonal mouse antibodies were used to demonstrate GATA-3 (Catalog # sc-268, Santa Cruz Biotechnology, CA, USA) and FoxP3 (Catalog # sc-56680, Santa Cruz Biotechnology, CA, USA). Monoclonal rabbit antibodies were used to demonstrate T-bet (Catalog # sc-21003, Santa Cruz Biotechnology, CA, USA).

Immunohistochemical technique and assessment
In all cases, five μ-thick, paraffin-embedded sections of formalin-fixed tissues were used. Sections were de-paraffinated and dehydrated through a series of graded ethanol solutions. They were then incubated in distilled water containing 0.3% H_2O_2 to inhibit endogenous peroxidase activity. Sections were stained with primary antibodies: anti-T-bet, anti-GATA-3 and anti-FoxP3 with histostain-DS kit (Cat. no: 95-9999, Zymed Laboratories Inc., San Francisco, USA). After washing, application of the secondary antibody (biotinylated goat IgG anti-rabbit/mouse IgG for T-bet, GATA-3 and FoxP3) for 30 min was followed by three washes in PBS. The streptavidin-peroxidase complex (Universal Dako LSAB 2 System-K0675, Glostrup, Denmark) was added for 30 min. and washed with PBS three times. Slides were counter-stained with Mayer's hematoxylin, dehydrated, and cleared, and after the application of a cover-slip, they were analyzed on a light microscope with a BX 40 microscope (Olympus, Tokyo, Japan).
All histological analyses were performed by two investigators (S. Vatansever, K. Ozbilgin) who were blinded to all other information. A dark-blue precipitate on lymphocytic cells indicated the presence of T-bet, GATA-3 or FoxP3 molecules. In unbiased, randomized 10 fields of the X200 magnification, stained lymphocytic cells in the epithelium and in the mucosa beneath the basement membrane of the epithelium, were counted and expressed as cells per square millimeter *(figure 1A-F)*. To assess the reproducibility of the cell count, 10 randomly selected classes were counted blind, twice, and a good correlation between assays was obtained (correlation coefficient 0.9).

Figure 1
A) Immunohistochemical T-bet stained lymphocytic cells in submucosal epithelium of patients with seasonal allergic rhinitis (pre-treatment biopsy of SAR).
B) Immunohistochemical T-bet stained lymphocytic cells in submucosal epithelium of treated patients with seasonal allergic rhinitis (post-treatment biopsy of SAR).
C) Immunohistochemical T-bet stained lymphocytic cells in submucosal epithelium of control subjects (biopsy of control group).
D) Immunohistochemical GATA-3 stained lymphocytic cells in submucosal epithelium of patients with seasonal allergic rhinitis (pre-treatment biopsy of SAR).
E) Immunohistochemical GATA-3 stained lymphocytic cells in submucosal epithelium of treated patients with seasonal allergic rhinitis (post-treatment biopsy of SAR).
F) Immunohistochemical GATA-3 stained lymphocytic cells in submucosal epithelium of control subjects (biopsy of control group).
G) Immunohistochemical FoxP3 stained lymphocytic cells in submucosal epithelium of patients with seasonal allergic rhinitis (pre-treatment biopsy of SAR).
H) Immunohistochemical FoxP3 stained lymphocytic cells in submucosal epithelium of treated patients with seasonal allergic rhinitis (post-treatment biopsy of SAR).
I) Immunohistochemical FoxP3 stained lymphocytic cells in submucosal epithelium of control subjects (biopsy of control group).
Small arrows show the specific antibody-stained lymphocytic cell samples.

Pollen count

Atmospheric pollen surveys were conducted at the Celal Bayar University, Morris Sinasi Hospital Campus and Ulu Park Public Garden, Manisa, Turkiye ($38°36'$ N, $27°26'$ E). Samples of airborne pollen grains were collected weekly during two *Olea europeae* pollen season periods, from March 2007 to July 2007 and March 2007 to July 2008, by the gravimetric method, using a Durham sampler [16]. The slides, coated with white Vaseline, were collected each Monday at 9:00 a.m. and prepared using glycerine jelly containing 0.002% methyl violet (Merck, Germany). Pollen grains on the slide were counted under a light micro-scope. The *Olea europeae* pollen count was expressed as pollen per square centimeter.

Statistical analysis

The average of the immunohistochemical results from the two investigators was used for statistical analy-sis. Morphometric data for the immunohistochemistry and cytokine levels were expressed as mean ($\pm$SEM) and analyzed using the Mann-Whitney U test for pre-, post-treatment biopsies and NLF from SAR patients *versus* biopsy and NLF from the control group. The significance of the differences of within-group (pre-treatment NSS *versus* post-treatment NSS and pre-treatment biopsy and NLF of SAR *versus* post-treatment biopsy and NLF of SAR) comparisons was determined using Wilcoxon's signed-rank test. The cor-relation between the *Olea europeae* pollen counts in
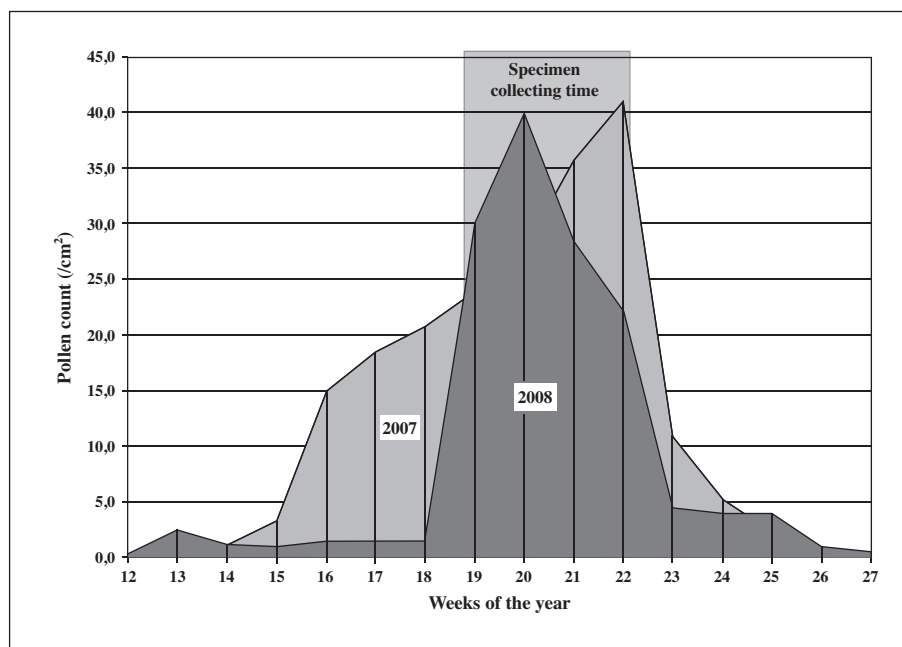
Figure 2

Olea europeae pollen count in expected pollination season of 2007 and 2008. Each year's 19th-21st weeks shows same pollen count. In the same way, all specimens were collected during these weeks.

the two pollen seasons were analyzed using Pearson's correlation test. P-values less than 0.05 were accepted as statistically significant.

RESULTS

Demographic findings for the patients (age, sex, ethnic origin) with SAR and the control group cases were similar. A statistically significant reduction was detected in the NSS obtained during the post-treatment period when compared to those obtained during the pre-treatment period (5.22±0.8, 8.78±0.68; respectively, p<0.0001) (*table 1*). Pollen counts were similar and there was a good correlation between the two defined pollen seasons (19th-21st weeks of the years, both 2007 and 2008) (correlation coefficient=0.524, p=0.008) (*figure 2*).

Results for the cytokines

Results for all cytokines are summarized in *table 2* as mean±SEM.

IFN-γ levels in both pre- and post-treatment NLF of SAR patients were significantly lower than the values found in the control group (p=0.012 for both comparisons). However, the IFN-γ levels in pre-treatment NLF of SAR and in post-treatment NLF of SAR patients did not differ (p=0.597) (*figure 3A*).

Comparison of IL-4 levels within the pre-treatment NLF of SAR patients and the control group; pre- and post-treatment NLF of SAR; post-treatment and control group did not express any difference (p=0.649, p=0.799, p=0.855; respectively) (*figure 3B*).

We observed that the IL-10 levels in pre-treatment NLF from SAR patients were significantly higher than those in the NLF of control group (p=0.033). Although, post-treatment NLF IL-10 levels were also higher than those in the NLF from the control group, this difference was not statistically significant (p=0.174). In addition, we found that IL-10 levels in pre- and post-treatment NLF from SAR patients did not change significantly (p=0.707) (*figure 3C*).

TGF-β levels in both pre- and post-treatment NLF from SAR patients were slightly lower than those in the NLF from the control group; but these differences were not statistically significant (p=0.178, p=0.296; respectively). In addition, we did not observe any changes in TGF-β levels in pre- and post-treatment NLF from SAR patients (p=0.704) (*figure 3D*).

Results for the transcription factors

T-bet-expressing lymphocyte counts in pre- and post-treatment biopsies from SAR patients were significantly lower than those found in biopsies of the control group

Table 2

Mean±SEM cytokine levels in nasal lavage fluid of the patients with seasonal allergic rhinitis and control group at pre-treatment evaluation.

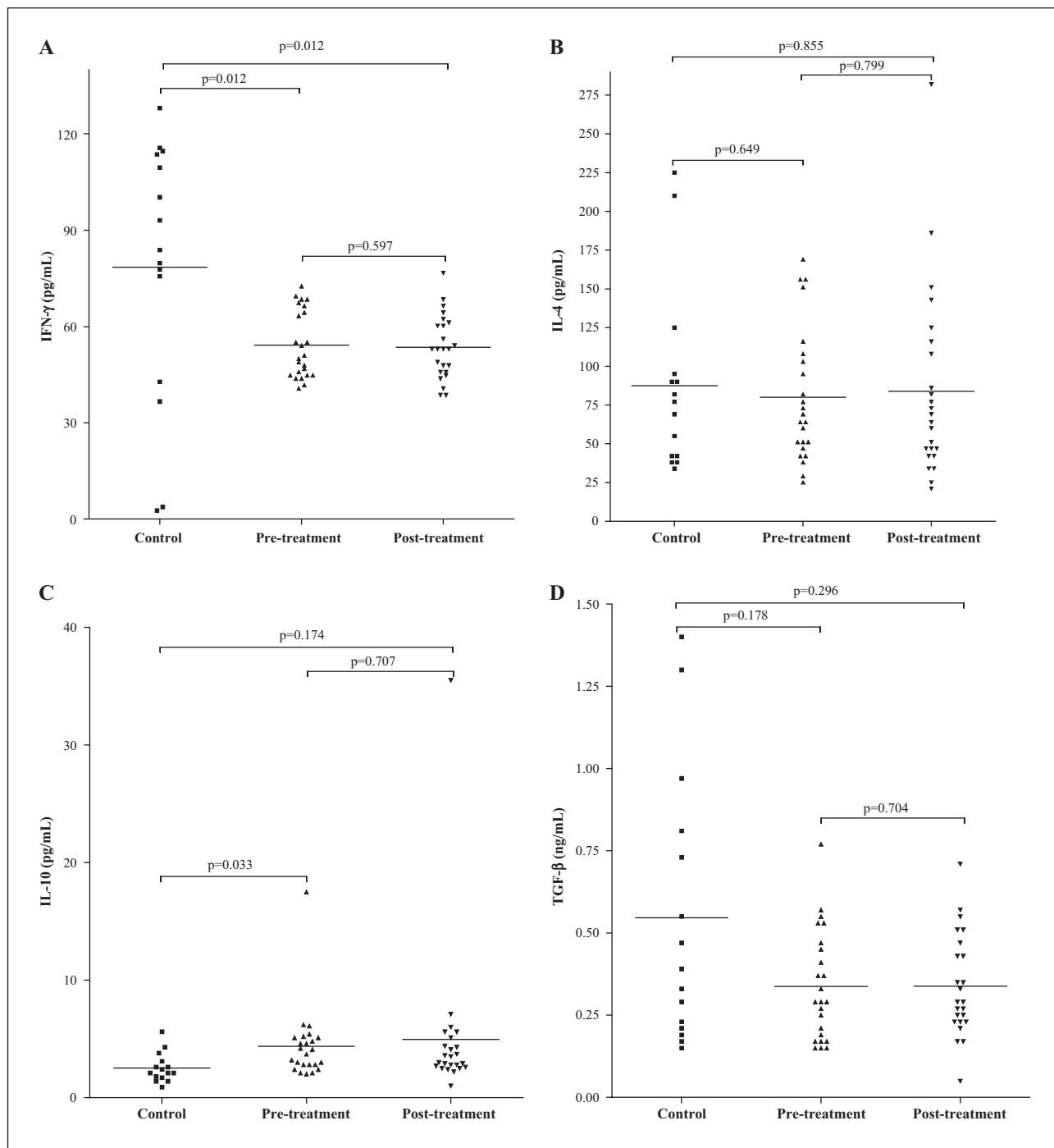|				p-value		
				Within group	Intergroup	
	Pre-treatment	Post-treatment	Control	Pre-treatment *versus* post-treatment	Pre-treatment *versus* control	Post-treatment *versus* control
IL-4 (pg/mL)	79.96±8.76	83.83±12.29	87.47±15.25	0.799	0.649	0.855
IFN-γ (pg/mL)	54.13±2.14	53.53±2.02	78.47±10.33	0.597	0.012*	0.012*
IL-10 (pg/mL)	4.4±0.6	5±1.4	3.4±0.8	0.707	0.033*	0.174
TGF-β (ng/mL)	0.34±0.03	0.34±0.03	0.55±0.11	0.704	0.178	0.296

Figure 3
Mean±SEM cytokine levels in control, pre- and post-treatment nasal lavage fluid. IFN-γ **(A)**, IL-4 **(B)**, IL-10 **(C)**, TGF-β **(D)**.

(34.58±0.87 *versus* 61.20±1.34; 36.83±2.29 *versus* 61.20±1.34; respectively, p=0.001 for both comparisons). In addition, T-bet-expressing lymphocyte numbers seen in biopsies from SAR patients showed an increasing trend, although this was not statistically significant at the post-treatment evaluation compared to the pre-treatment evaluation (p=0.398) *(figure 4A)*.

Both GATA-3-expressing lymphocyte counts in pre- and post-treatment biopsies from SAR patients did not differ from the counts found in the biopsies from the control group (32.58±0.69 *versus* 32.80±1.50; p=0.276;

31.75±0.94 *versus* 32.80±1.50; p=0.147; respectively). Additionally, GATA-3-expressing lymphocyte counts were similar in pre- and post-treatment biopsies from SAR patients (p=0.429) *(figure 4B)*.

Nevertheless, FoxP3-expressing lymphocyte counts did not differ in pre- and post-treatment biopsies of SAR patients (25.92±0.66 *versus* 26.00±1.28, p=0.848), both of them were significantly lower than that in biopsies from the control group (25.92±0.66 *versus* 59.20±1.39; 26.00±1.28 *versus* 59.20±1.39; p=0.001 for both comparisons) *(figure 4C)*.
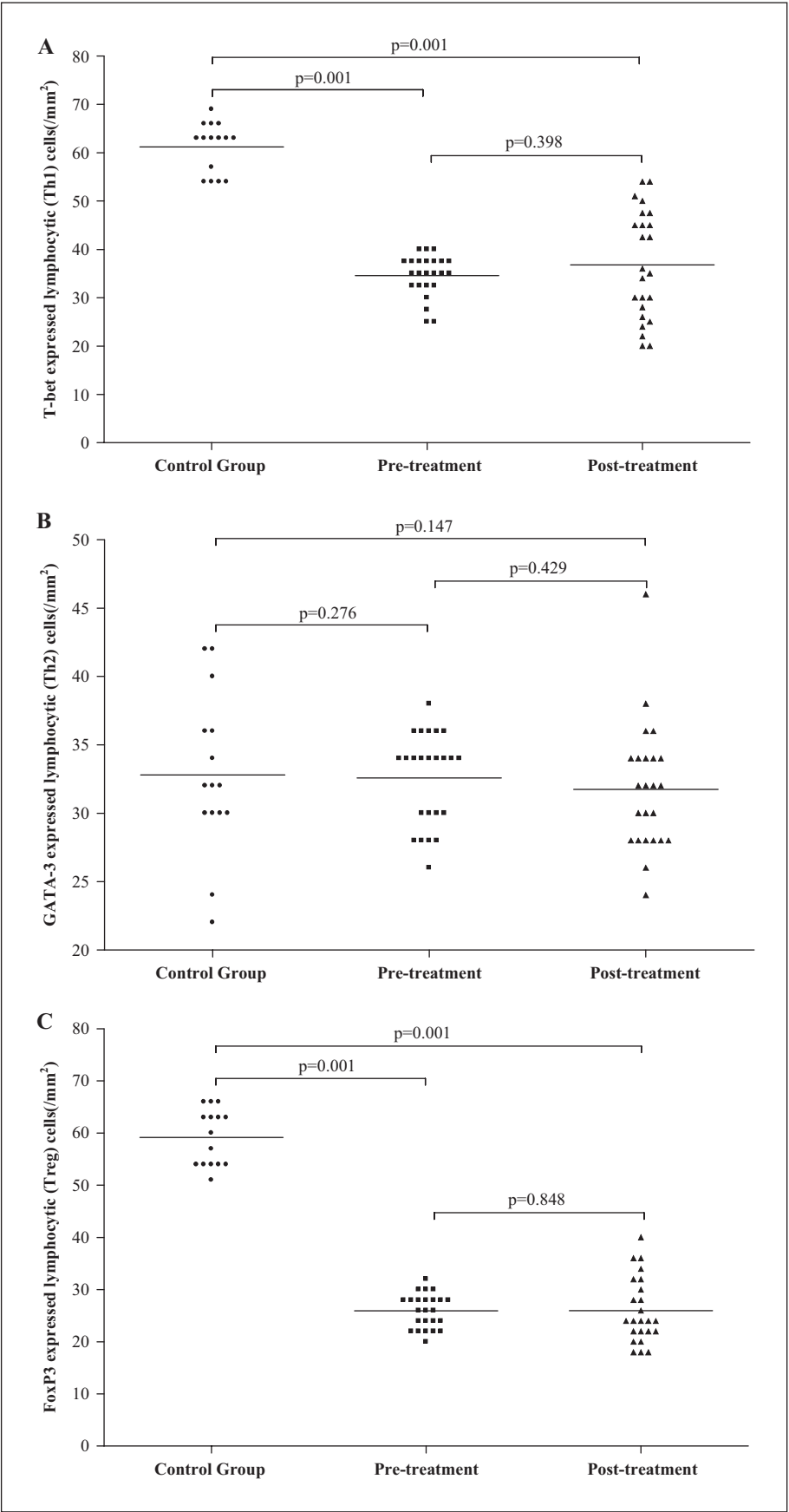
Figure 4
Mean±SEM transcription factor expressing lymphocyte cells per square millimeter in control, pre- and post-treatment nasal biopsy specimens.
T-bet **(A)**, GATA-3 **(B)**, FoxP3 **(C)**.

DISCUSSION

Allergic rhinitis, characterized by allergic inflammation of the nasal mucosa, is associated with an increased Th2 type immunological reaction [1]. Currently, SIT is the only treatment of allergic rhinitis with the potential to modify the course of the disease [17, 18].

There are many cytokine-based studies involving the nasal microenvironment and peripheral blood mononuclear cells (PBMCs). These studies have demonstrated that a Th2-type immunological reaction is responsible for the inflammatory processes in atopic/allergic patients [1, 12, 19]. However, we have not found very much immunological research that shows exactly how Th1, Th2 and Treg cells are involved or the responses to the SIT of these cells in nasal tissue.

Allergic rhinitis has been shown to reduce quality of life, sleep quality etc. to an extent that correlates with the symptom score [20]; SIT has been reported to improve symptom scores [1, 21, 22]. We too detected a symptomatic relief with allergen-specific immunotherapy. This was reflected in the NSS recorded during the pollen season, and in the pre- and post-treatment evaluations of NSS that were performed when pollen counts were at very similar levels. Improvements in the NSS might thus be attributable to SIT. When pre-treatment values were evaluated, it was shown that IL-4 levels and cells expressing GATA-3 transcription factor were very similar in control and SAR cases. After treatment, IL-4 levels were similar between the groups, but lymphocytes expressing GATA-3 transcription factor showed a decreasing trend compared to both pre-treatment SAR patients and control cases although this was not statistically significant. It was a surprise to find IL-4, a Th2 cytokine, was present at similar levels in SAR and control patients in our study that evaluated the nasal microenvironment during a period of active disease; it is well known that the T lymphocyte profile veers towards a Th2-type response in atopic diseases such as SAR [1, 2] Similarly, it was unexpected to find similar lymphocyte numbers expressing Th2-specific transcription factor in SAR and control cases during the pre-treatment period.

GATA-3 stabilizes the Th2 phenotype by two methods; it shuts down Th1 development through repression of IL-12 receptor β2-chain expression, and augments its own expression by positive feedback auto-regulation [23]. In our study, when IFN-γ levels and lymphocytes expressing T-bet transcription factor as Th1 indicators were evaluated before treatment, both were lower in SAR patients compared to the control cases. In other words, the Th1 cytokine profile and Th1 type cell number in the inflammatory microenvironment were significantly lower in SAR patients when compared to the control cases. These data are concordant with previous literature [1, 2]. Moreover, according to the commonly known "hygiene hypothesis", the Th1-type cytokine profile (especially IFN-γ) that does not develop at young ages, is present in atopic cases [24]. This leads to the relative absence of inhibition of a Th2 immune response. When both cell types and cytokine profiles are considered, the Th2/Th1 ratio demonstrates a Th2 predominance in SAR patients. When Th1 cells and cytokine patterns were evaluated in the SAR patients after nine months of SIT, they were lower than those found in the control cases. Additionally, observation of increased T-bet transcription factor-expressing lymphocytes in the inflammatory microenvironment following treatment, as compared to the pre-treatment period, might be an evidence of an interruption of Th2-cell predominance. Thus, all previous and recent data have shown that Th1 and Th2 cells function cross-reactively in the immune system. They inhibit each other during development, and demonstrate a shift from a Th2- to Th1-type cytokine profile with SIT [1, 2, 9-11, 21, 22]. Therefore low levels of Th1 indicators in SAR patients were expected to be found initially, and then an increase in T-bet expressing cells following nine months of SIT treatment. Although Th1 cells are the major source of IFN-γ, other cells can also produce this cytokine [25]. Therefore, the absence of any change in IFN-γ levels paralleled by the change in T-bet-expressing cells was not regarded as abnormal.

At pre-treatment assessment, it was observed that FoxP3, which is a Treg cell transcription factor, and IL-10, an immunosuppressive cytokine secreted by these cells, were decreased in the inflammatory area in SAR patients compared to the control cases. Treg cells have the ability to control and modify the development of allergic diseases, altering the ongoing sensitization and effector phases. They directly inhibit the activation of allergen-specific Th2 cells via a minimization of the production of IL-4, IL-5, IL-13 and IL-9 [26-28]. On the other hand, Treg cells also inhibit the induction of Th0/Th1 cell development and their effector functions in Th1-predominant diseases [29]. They are also noted for their ability to suppress Th1-mediated pathologies by a process that is dependent upon IL-10 [1, 2, 30, 31]. Taken together, the inhibitory and regulatory functions of Treg cells exert actions on both Th1 and Th2 cells. Thus, if Treg cell activity is impaired, this results in increased Th1 and Th2 responses (reduced immune suppression), accounting for the observed increment in the prevalence not only of Th2-mediated allergic diseases, but also of Th1-mediated autoimmune disorders [32]. Therefore, low numbers of lymphocytes expressing FoxP3 transcription factor and hence low IL-10 levels, and though insignificant low levels of TGF-β in SAR patients at pretreatment assessment compared to the control cases support these data. We did not detect any statistically significant increase in the number or function of Treg cells in SAR patients at the post-treatment assessment compared to the pre-treatment assessment. However, we observed an increasing trend in IL-10 levels. Moreover, at the post-treatment assessment, SAR patients displayed low FoxP3 transcription factor-expressing lymphocyte numbers; although there were low levels of IL-10 and TGF-β in the inflammatory micro-environment compared to the control cases, despite receiving nine months of SIT, these differences were not statistically significantly.

We were not able to detect any improvement in the number and function of Treg cells, or any suppression of Th2-type cell function in patients with SAR exposed to natural allergen after SIT as we had hypothesized at the beginning of our study. Although, these changes have been reported to occur after SIT in previous literature [13, 18], it was demonstrated to vary according to allergen type, allergen dose and atopy status of the patient [33]. It might be concluded that the allergen type administered, the atopic status of patients, and/or the allergen dose administered, are not hugely relevant, as we detected an improvement in NSS values following nine months of treatment and because we have a classical conventional subcutaneous

SIT regime. However, it may be inferred that the duration of the SIT was not sufficient to induce significant improvements in immunological parameters as we had hypothesized, despite improvements in clinical well-being parameters.

In conclusion, there is a relative increase in Treg cell numbers and function and a decrease in Th2 after one year of SIT compared to the pre-treatment values. However absolute changes were not observed. These results suggest that although clinical findings improve after one year of SIT, this duration may not be sufficient for detecting changes in cytokine patterns and transcription factors. Further studies that evaluate the outcome over a longer treatment period would provide valuable information.

Disclosure. *None of the authors has any conflict of interest or financial support to disclose.*

REFERENCES

1. Bousquet J, Van Cauwenberge P, Khaltaev N. Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001; 108(5 Suppl.): 147.

2. Romagnani S. Immunologic influences on allergy and the Th1/Th2 balance. *J Allergy Clin Immunol* 2004; 113: 395.

3. Bluestone JA, Abbas AK. Natural *versus* adaptive regulatory T cells. *Nat Rev Immunol* 2003; 3: 253.

4. Rengarajan J, Szabo SJ, Glimcher LH. Transcriptional regulation of Th1/Th2 polarization *Immunol Today* 2000; 21: 479.

5. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000; 100: 655.

6. Dardalhon V, Awasthi A, Kwon H, *et al*. IL-4 inhibits TGF-(-induced FoxP3+ T cells and, together with TGF-(, generates IL-9+ IL-10+ FoxP3- effector T cells. *Nat Immunol* 2008; 9: 1347. Erratum in: *Nat Immunol* 2009; 10: 551.

7. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor FoxP3. *Immunity* 2005; 22: 329.

8. Maynard CL, Harrington LE, Janowski KM, *et al*. Regulatory T cells expressing interleukin 10 develop from FoxP3+ and FoxP3- precursor cells in the absence of interleukin 10. *Nat Immunol* 2007; 8: 931.

9. Durham SR. New insights into the mechanisms of immunotherapy. *Eur Arch Otorhinolaryngol* 1995; 252(1 Suppl.): 64.

10. Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Muller UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN-gamma secretion in specific allergen-stimulated T cell cultures. *J Immunol* 1995; 154: 4187.

11. McHugh SM, Deighton J, Stewart AG, Lachmann PJ, Ewan PW. Bee venom immunotherapy induces a shift in cytokine responses from a Th2-2 to a Th1 dominant pattern: comparison of rush and conventional immunotherapy. *Clin Exp Allergy* 1995; 25: 828.

12. Soderlund A, Gabrielsson S, Paulie S, Hammarstrom ML, Rak S, Troye-Blomberg M. Allergen induced cytokine profiles in type I allergic individuals before and after immunotherapy. *Immunol Lett* 1997; 57: 177.

13. Jutel M, Akdis C. T-cell regulatory mechanisms in specific immunotherapy. *Chem Immunol Allergy* 2008; 94: 158.

14. Dreborg S, Frew A. EAACI Subcommittee on Skin Tests. Position paper Allergen standardization and skin tests. *Allergy* 1993; 48(14 Suppl.): 49.

15. Bousquet J, Lockley R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol* 1998; 102: 558.

16. Durham OC. A proposed standard method gravity sampling, counting and volumetric interpolation of results. *Journal of Allergy* 1946; 17: 79.

17. Palomares O, Yaman G, Azkur A K, Akkoc T, Akdis M, Akdis C A. Role of Treg in immune regulation of allergic diseases. *Eur J Immunol* 2010; 40: 1232.

18. Larche M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. *Nat Rev Immunol* 2006; 6: 761.

19. Benson M, Strannegard IL, Wennergren G, Strannegard O. Low levels of interferon-gamma in nasal fluid accompany raised levels of T-helper 2 cytokines in children with ongoing allergic rhinitis. *Pediatr Allergy Immunol* 2000; 11: 20.

20. Yuksel H, Sogut A, Yilmaz H, Yilmaz O, Dinc G. Sleep actigraphy evidence of improved sleep after treatment of allergic rhinitis. *Ann Allergy Asthma Immunol* 2009; 103: 290.

21. Frew AJ. Immunotherapy of allergic disease. *J Allergy Clin Immunol* 2003; 111(2 Suppl.): 712.

22. Malling HJ. Immunotherapy for rhinitis. *Curr Allergy Asthma Rep* 2003; 3: 204.

23. Zhou M, Ouyang W. The function role of GATA-3 in Th1 and Th2 differentiation. *Immunol Res* 2003; 28: 25.

24. Holgate ST. The epidemic of allergy and asthma. *Nature* 1999; 402 (6760 Suppl.): B2-4.

25. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-γ. *Annu Rev Immunol* 1997; 15: 749.

26. Akdis M, Verhagen J, Taylor A, *et al*. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory and T helper 2 cells. *J Exp Med* 2004; 199: 1567.

27. Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998; 102: 98.

28. Jutel M, Akdis M, Budak F, *et al*. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* 2003; 33: 1205.

29. Wolf D, Hochegger K, Wolf AM, *et al*. CD4$^+$CD25$^+$ regulatory T cells inhibit experimental anti-glomerular basement membrane glomerulonephritis in mice. *J Am Soc Nephrol* 2005; 16: 1360.

30. Singh B, Read S, Asseman C, *et al*. Control of intestinal inflammation by regulatory T cells. *Immunol Rev* 2001; 182: 190.

31. Martin E, O'Sullivan B, Low P, Thomas R. Antigen-specific suppression of a primed immune response by dendritic cells mediated by regulatory T cells secreting interleukin-10. *Immunity* 2003; 18: 155.

32. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 2002; 347: 911.

33. Bellinghausen I, König B, Böttcher I, Knop J, Saloga J. Regulatory activity of human CD4$^+$ CD25$^+$ T cells depends on allergen concentration, type of allergen and atopy status of the donor. *Immunology* 2005; 116: 103.