

ENVIRONMENTAL DETERMINANTS

The Decreased CD4⁺CD25⁺ FoxP3⁺ T Cells in Nonstimulated Allergic Rhinitis Patients Sensitized to House Dust Mites

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Objective. Regulatory (CD4⁺CD25⁺) T cells have been shown to play an important role in the development of allergic diseases. This study aims to investigate CD4⁺CD25⁺ T cells, Forkhead box P3 (FoxP3⁺ cells), and T-helper 1/T-helper 2 (Th1/Th2) cytokines in newly diagnosed allergic rhinitis (AR) patients. **Methods.** Altogether, 10 subjects with AR and 12 age-matched nonallergic healthy subjects were included in this study. CD4⁺CD25⁺ T cells, FoxP3⁺ T cells in peripheral blood mononuclear cells (PBMCs) were evaluated by flow cytometry, and the Th1/Th2 cytokine levels were determined by cytometric bead array immunoassay in both PBMC supernatants and nasal lavage fluids. **Results.** The percentage of CD4⁺CD25⁺ T cells were significantly higher, whereas the percentage of FoxP3⁺ cells were lower in AR patients compared with healthy subjects. In PBMC culture supernatants, interleukin-10 (IL-10) levels were significantly lower ($p = .012$), whereas IL-4, IL-5, and tumor necrosis factor- α (TNF- α) levels in nasal lavage fluids were higher in AR patients compared with healthy subjects ($p = .026$, $p = .015$, $p = .03$, respectively). **Conclusions.** Our findings indicate that decrease in CD4⁺CD25⁺FoxP3⁺ T cell fraction and diminished levels of IL-10 are noteworthy without allergen stimulation in house dust mite AR patients.

Keywords allergic rhinitis, FoxP3, nasal lavage fluid, regulatory T cells, Th1/Th2 cytokines

INTRODUCTION

Allergic rhinitis (AR) is an inflammatory condition of the nasal mucosa induced by an immunoglobulin (Ig)-E-mediated response to a specific allergen. It affects 2–30% of the world's population with increasing prevalence over the last decade (1, 2).

Allergic reactions involve early- and late-phase responses. The early-phase response is initiated by the cross-linking of allergen-specific IgE molecules on the surface of mast cells and basophils, which can lead to immediate symptoms due to the release of histamine and other mediators, including substantial quantities of T-helper 2 (Th2) cytokines (3). Mast cell/basophil activation may be sufficient to create the Th2 cytokine milieu that causes Th2 T cell differentiation and expansion. In addition to immediate symptom generation, the mast cells also contribute to the eosinophilic mucosal inflammation and cytokine production including interleukin-4 (IL-4), IL-5, IL-6, and tumor necrosis factor- α (TNF- α) (4, 5). Late-phase response occurs several hours after immediate reaction and is due to cellular infiltration of eosinophils, basophils, monocytes, and lymphocytes to the sites of allergen exposure. Increased expression of cytokines such as IL-4, IL-5, IL-9, and IL-13 generated by T cells as well as by mast cells are involved in the generation and persistence of allergic inflammation (6).

Th2-type lymphocytes are crucial in the initiation, progression, and persistence of the allergic diseases. The disturbances in the balance of Th1- and Th2-mediated immune responses have been thought to be responsible for the development of allergic reactions to inhaled allergens. In recent years, regulatory or suppressive T cells have been presented to prevent immune responses against self-antigens and adaptive immune responses (7, 8), thereby controlling the development of autoimmune diseases, transplant rejection, and allergic diseases (9, 10). Minor fractions of CD4⁺ T cells in mice and humans constitutively express the IL-2 receptor α -chain (CD25) and are defined as CD4⁺CD25⁺ regulatory T cells (Treg), which actively control or suppress the function of the other cells (11).

Forkhead box P3 (FoxP3), which is expressed specifically in CD4⁺CD25⁺ T cells has been identified recently and appears to be a master transcription factor for the development of these cells. FoxP3 expression was shown to mediate the acquisition of regulatory and suppressive function of CD4⁺CD25⁺ T cells (12, 13).

Xu et al. (14) have shown for the first time that CD4⁺CD25⁺FoxP3⁺ cells act at the nasal mucosa of AR patients. In that study, they found less Foxp3⁺ lymphocytes and decreased FoxP3 mRNA in AR patients when compared with the controls. Very recently, Skrinko et al. (15) have demonstrated the accumulation of FoxP3^{high} T cells in the nasal mucosa of pollen-allergic patients after the challenge with pollen extract.

In this study, peripheral blood CD4⁺CD25⁺ T cells, CD4⁺CD25⁺FoxP3⁺ T cells, and some cytokines related

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to allergy were investigated in AR patients and the results were compared with healthy individuals.

METHODS

Study Group

Altogether, 10 newly diagnosed patients with AR who had not received any therapy (mean age, 26 years) and 12 nonallergic healthy subjects (mean age, 26 years) were recruited for this study, following their informed consents. The study was approved by the ethical committee of Istanbul University, located in Istanbul. AR was diagnosed with the symptoms such as nasal itching, sneezing, watery rhinorrhea, and by positive skin-prick tests to major allergens of *Dermatophagoides pteronyssinus*; Der p1 and Der p2. A wheal size of 3 mm or greater than the negative control was accepted as positive skin-prick test. Non-atopic subjects did not have any of the allergic symptoms mentioned above. Specific IgE concentrations of the patients with AR and the healthy subjects were measured by two-step chemiluminescence immunoassay method. The patients were monosensitized to the house dust mites. The case and control groups were age- and sex-matched. Clinical and demographical data of both groups are shown in Table 1.

Twenty milliliters of heparinized blood was obtained from atopic subjects with AR and healthy non-atopic subjects by venopuncture. AR patients were free of any medication during blood sampling and nasal lavage specimens being taken. Nasal lavage specimens were taken upon endoscopic examination (16) and were stored at +4°C for a maximum of 2 hours until they were processed. After centrifugation, the supernatants were kept at -80°C until the cytokine assays were done.

Cell Purification and Flow Cytometric Analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient centrifugation over Ficoll (Sigma Chemicals Company, St. Louis, MO). CD4⁺ T cells were purified by autoMACS with CD4⁺ T cell isolation kit (MACS, Miltenyi Biotec AG, Bergisch, Gladbach, Germany), which provides isolation of highly pure, untouched CD4⁺ T cells by magnetically labeling of non-CD4⁺ T cells and with an antibody cocktail against CD8, CD14, CD16, CD19, CD36, CD56, CD123, T cell receptor (TCR)- γ/δ , and CD235a and

isolation of CD4⁺ T cells by negative-selection strategy. CD4⁺ T cell purity was determined as 96–99% by flow cytometry. T cell populations were analyzed by co-staining of intracellular Alexa-labeled anti-FoxP3 and phycoerythrin (PE)-labeled anti-CD25 with a BD FACSCalibur flow cytometry run by CellQuest Software (BD Bioscience, San Jose, CA).

Cell Cultures

Freshly purified PBMCs were washed twice with phosphate-buffered saline (PBS) solution and 1×10^6 cells/ml were cultured in RPMI-1640 medium for 72 hours without any stimulation. The culture supernatants were collected and stored at -80°C for the cytometric bead array (CBA) analysis.

Th1/Th2 Cytokine Determination by Cytometric Bead Array

The levels of Th1 and Th2 cytokines; IL-2, IL-4, IL-5, IL-10, IFN- γ , and TNF- α from cell culture supernatants; and nasal lavage fluids were determined by a human CBA kit (Th1/Th2 kit I; BD Pharmingen, San Diego, CA). The assay kit provided a mixture of six microbead populations with distinct fluorescent intensities (FL-3) and was precoated with capture antibodies specific for each cytokine. A 50-ml portion of samples or the standard cytokines provided were added to the premixed microbeads in 12×75 mm Falcon tubes (BD Bioscience). Following the addition of 50 ml of a mixture of PE-conjugated antibodies against the cytokines, the mixture was incubated for 3 hours in the dark at room temperature. This mixture was washed and centrifuged at 500g for 5 min and the pellet was resuspended in 300 ml of wash buffer. Two-color cytometric analysis was carried out using FACSCalibur flow cytometry (BD Bioscience) and 3000 events were acquired for each sample. Individual cytokine concentrations were indicated by their fluorescent intensities (FL-2) and were computed using the standard reference curve of CellQuest and CBA software (BD Bioscience). Data were obtained and analyzed by CBA software. Units for assessment were protein expression in supernatants of stimulated whole blood cultures (pg/ml). The limits of detections for individual cytokines were as follows: IL-2, 2.6 pg/ml; TNF- α , 2.8 pg/ml; IL-10, 2.8 pg/ml; IFN- γ , 7.1 pg/ml; IL-4, 2.6 pg/ml; and IL-5, 2.4 pg/ml.

Statistical Interpretation

The data were expressed as means $\pm$ standard deviation (SD). Statistical analyses were performed by using SPSS 15.0 version (SPSS, Chicago, IL, USA), and Mann-Whitney U-test was used for comparisons.

RESULTS

Increased CD4⁺CD25⁺ but Decreased FoxP3 Expression in Allergic Rhinitis

Clinical and demographic characteristics of AR patients and healthy subjects are given in Table 1. The mean

TABLE 1.—Clinical and demographic data of AR patients and healthy subjects.

	Allergic rhinitis patients (n = 10)	Non-atopic subjects (n = 12)
Sex (female/male)	5/5	6/6
Age (years)	26 (17–48)	26 (22–35)
Skin-prick test (Der p1) (mm)	12.8 $\pm$ 1.5	<3
Skin-prick test (Der p2) (mm)	12 $\pm$ 1.5	<3
Specific IgE (Der p1) (IU/l)	27.5 $\pm$ 8.2	0.78 $\pm$ 0.03
Specific IgE (Der p2) (IU/l)	21.1 $\pm$ 5.9	0.63 $\pm$ 0.2

specific IgE (sIgE) levels of allergic patients were 27.5 ± 8.2 IU/l for Der p1 and 21.1 ± 5.9 IU/ml for Der p2, and the mean Der p1 sIgE level of the healthy subjects was 0.78 ± 0.03 IU/l and Der p2 sIgE level was 0.63 ± 0.2 IU/l. Wheal sizes of allergic patients were 12.8 ± 1.5 mm for Der p1 and 12.0 ± 1.5 mm for Der p2, in skin-prick tests.

Purified CD4⁺ T cells from PBMCs of healthy donors and AR patients were co-stained with anti-CD25 and anti-FoxP3. The median level of CD25⁺ T cells was significantly higher in AR patients compared with healthy non-atopic controls ($5.34 \pm 0.84\%$ vs $1.92 \pm 0.44\%$; $p = .002$, respectively). In contrast, expression of FoxP3 was significantly reduced in AR patients ($p = .002$; Figure 1).

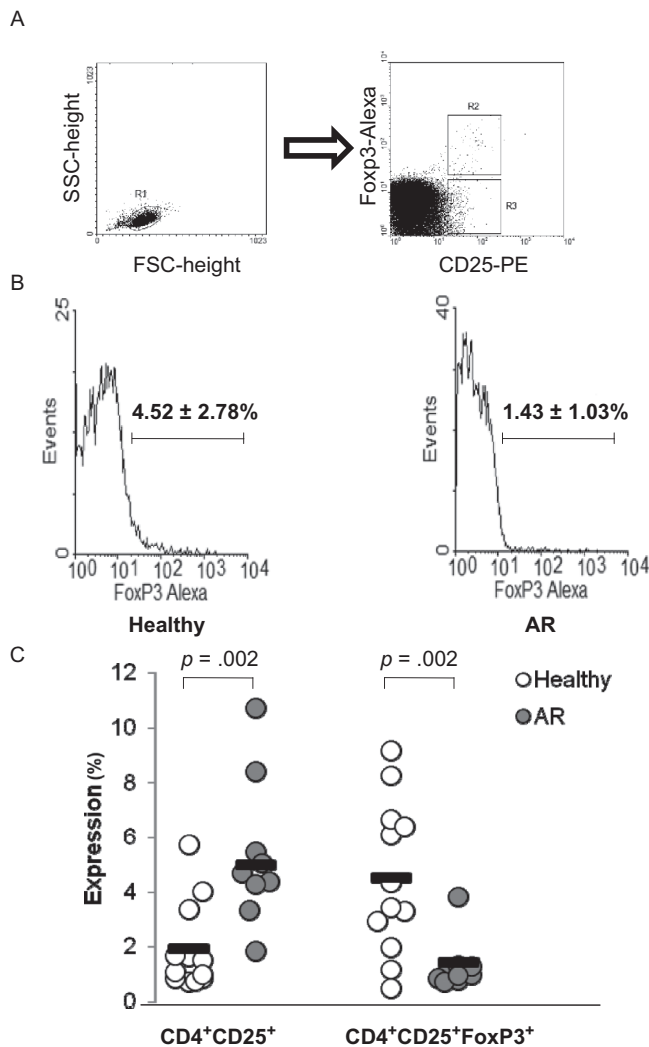


FIGURE 1.—Increased CD4⁺CD25⁺ but decreased FoxP3 expression in AR. (A) Gating strategy for CD4⁺CD25⁺FoxP3⁺ Treg cells are shown. R1 represents the lymphocyte gate on FSC/SSC graph, R2 represents the gate for CD4⁺CD25⁺FoxP3⁺ Treg cells, and R3 represents the gate for CD4⁺CD25⁺ activated T cells. (B) Histograms show FoxP3 expression levels of AR patients and healthy subjects, data represent mean $\pm$ SD of FoxP3 expression percent. (C) CD25 expression of purified CD4⁺ T cells from AR patients and healthy subjects was determined by staining with PE-labeled anti-CD25, and FoxP3 expression was determined by intracellular staining with Alexa-labeled anti-FoxP3 monoclonal antibody and analyzed by flow cytometry. $p < .05$ was considered to be statistically significant.

Differences of Cytokine Contents between Allergic Rhinitis Patients and Healthy Subjects

Cell-culture supernatants collected from PBMC as well as nasal lavage fluids, both from healthy and AR subjects, were analyzed by CBA method for the detection of cytokines.

The IL-10 levels of allergic patients in PBMC culture supernatants were significantly lower than those of non-atopic patients (16.9 ± 16.5 vs 56.7 ± 41.6 pg/ml; $p = .012$). Although TNF- α concentrations displayed great variability in allergic patients, they were not found significantly different compared with control group (73.7 ± 120 vs 6.5 ± 6.6 pg/ml). The median levels of IL-4 and IL-5 in two groups were also similar (0.78 vs 0.79 pg/ml for IL-4 and 1.45 vs 3.0 pg/ml for IL-5). Other cytokines such as IFN- γ and IL-2 were not found to be measurable in PBMC culture supernatants (Figure 2A).

When the cytokine levels of nasal lavage fluids were investigated, the median levels of IL-4, IL-5, and TNF- α in AR patients were significantly higher compared with healthy subjects ($p = .026$, $p = .015$, and $p = .03$, respectively; Figure 2B). There were no significant alterations in the other cytokine levels of nasal lavage fluids of allergic patients.

DISCUSSION

In this study, CD4⁺CD25⁺FoxP3⁺ T cells were lower when compared with healthy subjects, whereas an increased percentage of CD4⁺CD25⁺ T cells was found. Previous investigations reported that the frequency of CD4⁺CD25⁺ Treg cells in peripheral blood from allergics might be decreased, similar to, or increased compared with those of nonallergics. It has been shown that the frequencies of CD4⁺CD25⁺FoxP3⁺ T cells were similar in AR patients compared with that of healthy subjects (17). In that study, CD4⁺CD25^{high} cells retained suppressive activity on allergen-stimulated cell proliferation and cytokine production of Th1 but not Th2 cells in both groups. However, the frequencies of allergen-specific IL-10⁺IL-4⁺CD4⁺ T cells were reduced in AR patients, and correlated inversely to clinical symptom scores. On the other hand, some animal studies demonstrated that FoxP3⁺ Treg cells accumulate in the airway mucosa during aeroallergen challenge and exert their suppressive function locally (18, 19). In an experimentally induced human AR model, Skrinko et al. (15) observed similar findings and proposed that Treg cells are operative in allergic upper airway inflammation. However, in one of the first human model studies, less FoxP3⁺ lymphocytes and decreased FoxP3⁺ mRNA were found in nasal mucosa of AR patients without allergen stimulation (14). A very recent study conducted during the spring season confirmed these results in pollen allergic patients (20). The decreased peripheral FoxP3⁺ T cells found in our study, which was conducted without allergen stimulation, are in concordance with these studies that demonstrate decreased FoxP3⁺ cells in either nasal mucosa or peripheral blood

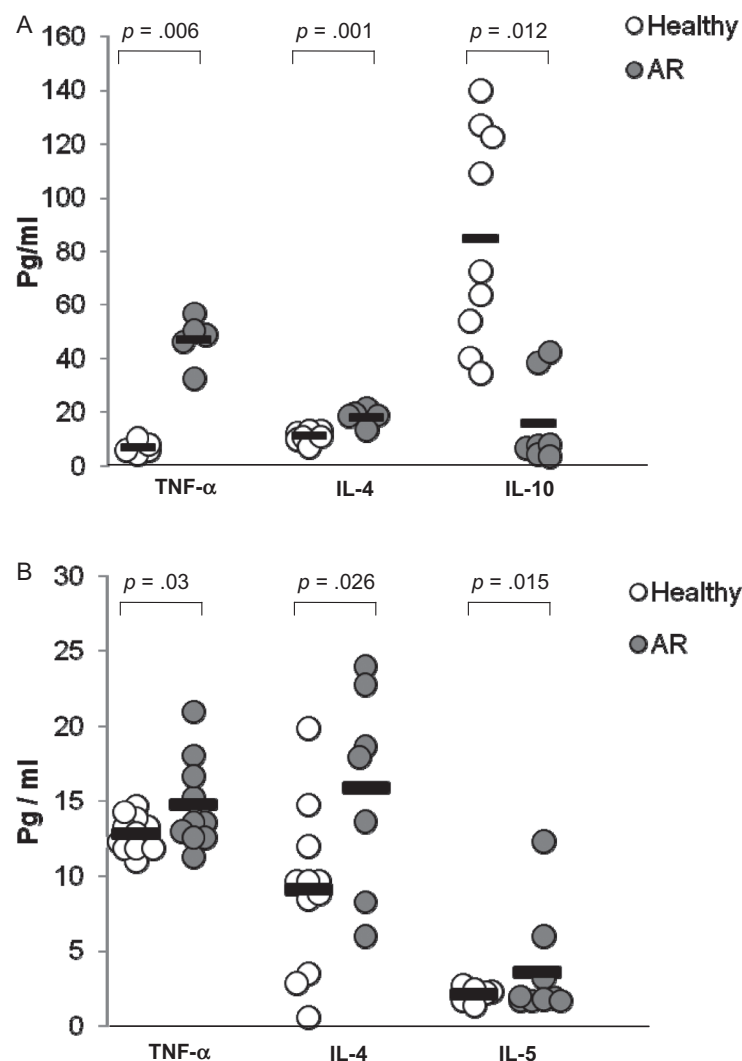


FIGURE 2.—Differences of cytokine contents between AR patients and healthy subjects. (A) Freshly purified PBMCs from AR patients and non-atopic subjects were cultured in RPMI-1640 medium for 72 hours. The Th1 and Th2 cytokine levels were measured in the (A) culture supernatants and in (B) nasal lavage specimens. $p < .05$ was considered to be statistically significant.

in AR. However, the increased number of $CD4^+CD25^+$ T cells in spite of lower FoxP3 $^+$ cells in this study seems to be in conflict. Similarly, Lin et al. had shown that although the percentage of natural Treg (nTreg) in peripheral blood mononuclear cells was increased, the expression of FoxP3 and its cell-induced suppressive activity was significantly lower in Der p-sensitive asthmatic children when compared with non-atopic controls (21). It may be speculated that Treg cells of our subjects, who had relatively severe disease, may contain fewer Treg and more activated effector T cells during allergic inflammation, as recently activated effector T cells also express CD25 (22). It was shown by Grindebacke et al. that the $CD4^+CD25^+$ T cell population may contain significant numbers of allergen-specific effector T cells (23). Similarly, Lee et al. reported that the patients with severe allergic airway disease had more $CD4^+CD25^+$ T cells and $CD4^+CD25^{high}$ T cells than the patients with less severely affected disease (24). In fact, the suppressive phenotype of $CD4^+CD25^+$ Treg cells is associated with the expression of FoxP3, which is a specific

marker for Treg cells. Roncador et al. showed that less than 30% of $CD4^+CD25^+$ T cells express FoxP3 protein both in patients with AR and healthy donors, which is consistent with the findings of a previous study that showed approximately half of the whole $CD4^+CD25^+$ population in peripheral blood from healthy individuals express the FoxP3 protein (25). Therefore, our results, indicating decreased FoxP3 $^+$ cells in spite of increased $CD4^+CD25^+$ population, may be reflecting the dysregulation in the pathogenesis of allergic disease.

We also found decreased IL-10 levels in the PBMC culture supernatants of allergic patients when compared with healthy subjects. The most important subset of inducible Treg (iTreg) cells is T regulatory type I (Tr1) cells, which suppress immune function by secretion of predominantly IL-10 and transforming growth factor- β (TGF- β) (26). It has been emphasized that IL-10 has a critical role in initiating the development of Th2-polarized responses, but also has an important function by downmodulating Th2-driven inflammation in late phase (27). In mice, IL-10

administration before allergen treatment induced antigen-specific T cell unresponsiveness and demonstrated the pivotal role of IL-10 in the establishment of peripheral T cell tolerance (28). Studies with bronchoalveolar lavage fluid from asthmatic patients have revealed lower IL-10 levels than in healthy controls, and the T cells from children suffering from asthma have been shown to produce less IL-10 mRNA than T cells from healthy children (29, 30). Together, these findings indicate an association between increased IL-10 production and decreased allergic reactions (31). As Treg cells are a major source of IL-10, it has been concluded that Treg cells secreting IL-10 are involved in the suppression of allergic Th2 cell responses in humans, which is supported by several human allergen-specific immunotherapy studies (31).

Several cytokines are considered to play a pivotal role in allergic responses, particularly IL-4, IL-5, IL-6, and TNF- α (20). In this study, although IL-4, IL-5, and TNF- α levels of PBMCs did not show any differences between AR patients and non-atopic subjects, the levels of nasal lavage fluids were higher in AR patients than in controls. In particular, TNF- α is claimed to impair the regulatory activity of Tregs by downregulating the FoxP3 expression via the TNF- α receptor-2 (TNFR2) signaling pathway (32, 33).

It is known that local and systemic responses may differ. This is possibly revealed by the cytokine contents of nasal lavage fluid and cell-culture supernatants, representing local allergic responses and systemic status, respectively. The TNF- α levels were lower, whereas IL-4 were similar in culture supernatants when compared with nasal lavage fluids of AR patients. Decreased TNF- α may be an indicator of a Th2-polarized local immune response in nasal mucosa. In addition, decreased Treg content in peripheral blood together with decreased IL-10 levels may be the reflection of a local disease in peripheral blood, so that local immune responses can be observed systemically.

CONCLUSION

Our findings indicate that decreased CD4⁺CD25⁺FoxP3⁺ T cell fraction and diminished IL-10 probably reflecting Tr1 cell inhibition is noteworthy without allergen stimulation in house dust mite AR patients.

ACKNOWLEDGMENTS

The authors thank Feyza Tuncer, M.Sc., for critically reading and correcting the article.

DECLARATION OF INTEREST

This study was supported by the Research Fund of the Istanbul University with the project number 1471. The authors declare no conflicts of interest.

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