Enhancement of the Frequency and Function of IL-10-Secreting Type I T Regulatory Cells after 1 Year of Cluster Allergen-Specific Immunotherapy

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Key Words
Allergic rhinitis · Specific immunotherapy · Immunoglobulin G4 · IL-10-secreting type I T regulatory cells

Abstract
Background: Allergen-specific immunotherapy (SIT) is a highly effective treatment for allergic diseases, but the underlying mechanisms are unclear. In this study, we investigated the effects of cluster SIT with Dermatophagoides pteronyssinus (Der p) on IL-10-secreting type I T regulatory (Tr1) cells in Der p-sensitized patients with allergic rhinitis. Methods: This was a prospective randomized study involving 68 participants (aged 18–60 years) of whom 38 were patients with allergic rhinitis and received Der p-SIT for 1 year and 30 were nonallergic controls. IL-10+IL-4–CD4+ T cells were measured by flow cytometry for the patient group at baseline and at the end of 1 year of SIT, and for nonallergic controls. Similarly, IL-10 in supernatants from allergen-stimulated peripheral blood mononuclear cell (PBMC) cultures were measured by ELISA, and the suppressive effect of Tr1 cells on cell proliferation and cytokine release (IFN-γ and IL-4) in PBMCs was estimated in cultures from both groups. Allergen-specific serum IgE and IgG4 were also assessed by RAST and ELISA for the SIT group. Results: The levels of IL-10-secreting Tr1 cells, IgG4 and allergen-induced IL-10 synthesis from PBMC cultures were significantly increased and the function of Tr1 cells was enhanced after 1 year of SIT compared to baseline levels. In contrast, the level of IgE was not significantly changed. Conclusion: These data suggest that the cluster Der p-SIT may enhance the frequency and function of IL-10-secreting Tr1 cells.

Introduction

Type I allergy represents a major health problem worldwide, affecting more than 25% of the world’s population [1]. Allergic rhinitis (AR) is the most prevalent type I allergy, affecting 10–40% of the global population [2], and is characterized by an immunoglobulin (IgE)-mediated inflammation induced by antigens from different sources (e.g. pollen, mites and animal dander). Infiltrating cells, including T cells, eosinophils, mast cells and basophils, release several mediators, which give rise to
symptoms such as rhinorrhea, sneezing, nasal congestion and nasal pruritus. At present, the most common treatments for AR are mainly based on pharmacotherapy (e.g., antihistamines, nasal corticosteroids and decongestants) and allergen-specific immunotherapy (SIT). Drugs are generally effective in controlling symptoms but do not modify the immune process, and sometimes lead to impaired performance due to side effects [3, 4]. SIT is based on the administration of increasing doses of allergens in order to induce a state of desensitization in affected individuals. Unlike pharmacotherapy, allergen-SIT is recognized as a curative treatment for type I allergy, and in addition to controlling symptoms [5] it provides extended clinical effects, including prevention of new atopic sensitizations [6, 7] and a reduction in disease development from rhinitis to asthma [8].

Studies of SIT have demonstrated that its success is associated with the induction of peripheral tolerance. Although the mechanisms by which SIT induces the immunological tolerance are still unclear, the regulatory T cells (Tregs) are now recognized to play a fundamental role in the development and maintenance of immune tolerance [9]. In addition, studies have demonstrated that type I T regulatory (Tr1) cells may play an important role in healthy immune responses to environmental allergens [10–12]. To our knowledge, there has been no study of IL-10-secreting Tr1 cells after 1 year of cluster SIT with *Dermatophagoides pteronyssinus* (Der p). Thus, in this study we evaluated the effects of cluster SIT with Der p in the first year on the pool of circulating Tr1 cells.

**Methods**

**Patients**

The study participants included 38 house dust mite-allergic patients (aged 18–60 years) and 30 nonallergic control subjects (aged 18–60 years), recruited from the Department of Otolaryngology Head and Neck Surgery allergy outpatient clinic at Beijing TongRen Hospital, Beijing, China. All patients had a documented history of house dust mite-induced moderate-to-severe rhinoconjunctivitis for at least 3 years and demonstrated a positive skin prick test result for Der p with a wheal diameter of at least 6 mm. Furthermore, all patients demonstrated a positive test for specific IgE to Der p with a RAST value of at least 0.7 kU/L. All patients rated the severity of the symptoms by the Total 5 Symptom Score (T5SS), including rhinorrhea, sneezing, nasal congestion, nasal and ocular pruritus on a 4-point scale of 0–3 (0 = none; 1 = slight; 2 = heavy; 3 = severe). None of the nonallergic controls had any symptoms of rhinitis, a positive skin prick test or specific IgE to Der p (total IgE levels <100 kU/L).

Patients with a history of asthma or atopic dermatitis were excluded and all participants avoided antihistamines or intranasal steroids for 1 week and oral steroids for 3 months prior to undergoing laboratory tests. The study protocol was approved by the review board for human studies of Beijing Institute of Otolaryngology and informed consent was obtained from all participants prior to entry into the study.

**Immunotherapy Protocol**

Patients undergoing immunotherapy were given Der p extract (Alutard SQ, ALK-Abello, Hørsholm, Denmark) for a total period of 1 year, according to a cluster protocol [13]. In the first 6 weeks, patients underwent visits for up-dosing, receiving two injections 1 hour apart with an increasing dose every week. The highest concentration, given in the sixth week, had an allergenic activity of 100,000 SQ/ml and contained 9.8 μg/ml Der p. After week 6, the dosing interval was increased to 1 month and maintained until the end of the first year. Blood samples were taken at the end of 1 year of SIT.

**Flow Cytometry**

Peripheral blood was obtained from each participant by venous puncture and collected into preservative-free heparin. Peripheral blood mononuclear cells (PBMCs) were isolated by means of Ficoll-Plaque Plus density gradient centrifugation (Amersham Biosciences, Piscataway, N.J., USA). For detection of intracellular cytokine, PBMCs (2 × 10⁶ cells/ml) were stimulated by incubation with 10 μg/ml Der p 1 (Der p major allergen 1, endotoxin ≤0.03 EU/μg. Indoor Biotechnologies, UK) for 24 h at 37°C. Twenty-five ng/ml phorbol 12-myristate 13-acetate (PMA), 1 μg/ml ionomycin and 1.7 μg/ml monensine (all from Sigma-Aldrich, St. Louis, Mo., USA) were also added for the final 4 h of the incubation period.

At the end of incubation, the Der p 1-stimulated PBMCs were surface stained with anti-CD3-APC and anti-CD8-PerCP, and fixed with 4% paraformaldehyde/PBS. After being permeabilized with FACS Permeabilizing Solution (BD Pharmingen, San Jose, Calif., USA), the cells were incubated for 30 min at room temperature with anti-IL-10-PE plus anti-IL-4-FITC, and each antibody...
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was matched with a respective isotype IgG1 as a control (all from BD Pharmingen). The Tr1 cells were characterized as being IL-10+/IL-4–/CD3+CD8– T cells, and analyzed by recording at least 20,000 events in the lymphocyte gate using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, N.J., USA).

**ELISA**

PBMCs obtained from 12 patients at baseline and at the end of 1 year of SIT, and from 12 nonallergic controls were resuspended at a concentration of 2 × 10^6 cells/ml in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 5% human sera, 100 U/ml penicillin-streptomycin (Gibco). Ten μg/ml Der p 1 was added to each sample and the PBMCs were incubated for 6 days at 37°C in a humidified incubator containing 5% CO₂. At the end of incubation, supernatants were collected from each culture and assayed for the presence of IL-10 by ELISA (R&D Systems, Minneapolis, Minn., USA).

**Analysis of Allergen-Specific Serum Antibodies (IgE and IgG4)**

Serum samples were collected from each patient at baseline and at the end of 1 year of SIT, and stored at −20°C until analysis. Der p-specific IgE was measured using the UniCAP 100 system (Pharmacia Diagnostics, Uppsala, Sweden) and Der p-specific IgG4 using commercial ELISA kits according to the supplier’s instructions (ALK-Abello).

**Isolation of IL-10-Secreting T Cells**

Heparinized blood (30 ml) was obtained from each of 8 recruited patients with AR at baseline and at the end of 1 year of SIT, and from 8 nonallergic controls, for cell isolation and functional analysis. For the isolation of allergen-specific IL-10-secreting Tr1 cells, 2 × 10^7 PBMCs from each donor were stimulated for 16 h with 10 μg/ml Der p 1 and IL-10-secreting Tr1 cells were isolated using the IL-10 Secretion Assay Cell Enrichment and Detection Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s protocol. Cells were harvested and labeled with IL-10 Catch Reagent for 5 min on ice and, after dilution with medium to a final concentration of 10^6 cells/ml, were incubated at 37°C for 45 min. At the end of incubation the cells were stained with PE-conjugated anti-IL-10 detection antibody for 10 min on ice. The cells were washed and resuspended, and then magnetically labeled with anti-PE-microbeads for 15 min at 4°C. After a further washing step, labeled cells were purified using an LD column (Miltenyi Biotec) and used further as required.

**Suppressive Capacity of IL-10-Secreting Tr1 Cells**

The suppressive capacity of IL-10-secreting T cells was assessed according to cell proliferation and cytokine production by PBMCs. PBMCs (5 × 10^5 cells/well) were cultured alone or as cocultures of PBMCs (5 × 10^5 cells/well) and Tr1 cells (1 × 10^4 cells/well) at a ratio of 50:1 in a final volume of 200 μl RPMI-1640 medium, and stimulated with 10 μg/ml Der p 1. Cultures were incubated for 3 days at 37°C in a humidified incubator with 5% CO₂, and triplicate medium wells were included as negative controls. At day 3, 100 μl of supernatant was removed for Th1 and Th2 cytokine (IFN-γ and IL-4, respectively) analysis using standard ELISA kits (R&D Systems). Cell proliferation of PBMCs was then assessed using a WST-8 (modified tetrazolium salt) cell proliferation kit (Cell Counting Kit-8, Dojindo, Japan) according to the manufacturer’s protocols. At the end of assay, the optical density (OD) of the culture medium was assessed by measuring absorbance at 450 nm and the suppression percentage (SP) of the PBMCs calculated according to the formula: SP = (OD_{PBMCs} - OD_{Tr1+PBMCs})/(OD_{PBMCs} - OD_{blank control}) × 100%.

**Statistics**

Data were expressed as mean ± standard deviation, unless otherwise specified. Statistical significance between groups was analyzed using a Mann-Whitney test. The significance of intragroup pre- and post-therapy changes were determined using the nonparametric Wilcoxon matched-pairs test. All analyses were performed using SPSS version 13.0 statistical software. A 5% significance level was used (α = 0.05), with power (1-β) equal to 90%. All tests were 2-tailed, and p values <0.05 were considered statistically significant.

**Results**

**Clinical Response**

The clinical efficacy of treatment was evaluated by the patients, based on the T5SS at baseline and at the end of 1 year of SIT. A reduction of >50% in T5SS at the end of SIT indicated an effective clinical effect according to the >30% reduction in symptoms score criterion of Malling [14] (fig. 1).

**Frequency of Tr1 Cells in PBMCs**

Figure 2a shows the CD3+/CD8– cells (considered to be equivalent to the CD4+ subset) and figure 2b the Tr1 (IL-10+/IL-4–/CD4+) cells, as detected by flow cytometry. Comparison of the frequencies of Tr1 cells among patients before and after 1 year of SIT with those of nonallergic controls indicated that the Tr1 cells were significantly decreased in the patients with AR at baseline compared with nonallergic controls (p < 0.0001), but significantly increased after 1 year of SIT compared with baseline and nonallergic controls (p < 0.0001 and p < 0.05; fig. 2c).

**Production of IL-10 in PBMC Der p 1-Stimulated Cultures**

Supernatants of Der p-stimulated PBMCs were analyzed to determine the effect of SIT on cytokine production after 6 days in culture. The level of IL-10 was significantly decreased in the patients with AR at baseline compared to nonallergic controls (p < 0.05), but significantly enhanced after 1 year of Der p-SIT compared with baseline and higher than in nonallergic controls (p < 0.0001 and p < 0.05; fig. 3).
Changes in Allergen-Specific IgE and IgG4

Assessment of allergen-specific serum IgE and IgG4 demonstrated that the concentration of allergen-specific IgE was not significantly altered in the patients with AR at baseline compared to that after 1 year of Der p-SIT (p > 0.05; fig. 4a). In contrast, the concentration of IgG4 was significantly increased after 1 year of SIT (p < 0.001, fig. 4b).

Suppressive Capacity of Allergen-Specific IL-10-Secreting Tr1 Cells

Figure 5 demonstrates the suppressive effect of allergen-specific IL-10-secreting Tr1 cells on PBMCs of patients with AR, prior to and at the end of 1 year of Der p-SIT, and nonallergic controls (fig. 5). Prior to SIT, PBMCs from patients with AR displayed enhanced proliferation (p < 0.05; fig. 5a), and production of both Th1 (IFN-γ, p < 0.05; fig. 5b) and Th2 (IL-4, p < 0.0001; fig. 5c) cytokines, compared with PBMCs from nonallergic controls. Although coculture of PBMCs with allergen-specific Tr1 cells led to a decreased suppressive effect on proliferation (fig. 5d) and production of Th1 (fig. 5b) and Th2 cytokine (fig. 5c) by PBMCs of the patients with AR prior to SIT compared with nonallergic controls, this suppressive effect was significantly enhanced after 1 year of SIT.
Discussion

In the current study, we focused on the frequency and function of allergen-specific Tr1 cells in the first year of cluster Der p-SIT and found that successful Der p-SIT, as indicated by >50% decrease in TSSS, was associated with a markedly increased frequency of allergen-specific Tr1 cells, higher levels of IL-10 and IgG4, and an enhancement of the suppressive effect of allergen-specific IL-10-secreting Tr1 cells on cell proliferation and cytokine production (IL-4 and IFN-γ).

Allergic diseases are caused by an aberrant immune response mediated through the Th2 cells, associated cytokine patterns, including IL-4, IL-5 and IL-13 [15], and induction of IgE production by B cells. Consequently, the most direct findings with potential relevance to effective SIT are related directly to the control of these Th2 immune effectors and the production of IgE. In the past decade there has been strong evidence that peripheral Treg cells play a crucial role in the control of these Th2 immune effectors and the production of IgE. In the past decade there has been strong evidence that peripheral Treg cells play a crucial role in the control of harmful T cell responses [16–18]. Absence or defective function of Treg cells has also been correlated with hyper IgE syndrome, hypereosinophilia and autoimmunity in humans. However, their presence and induction have been associated with immune tolerance [17, 19]. Among the known subpopulations of Treg cells, there are two subgroups of Treg cells that have received special attention. The CD4+ CD25+Foxp3+ Treg cells are thought to represent naturally occurring Treg cells evolving in the thymus or adaptive Treg cells developing in the periphery [20]. Another subgroup generated outside the thymus is the Tr1 cells, characterized by the secretion of IL-10 [21]. There has been one study on the mechanisms of immune responses to allergens which has demonstrated that Treg cells are dominant in healthy individuals [10]. However, if a detectable immune response is mounted, Tr1 cells specific for common environmental allergens consistently represent the dominant subset. In contrast, there are several reports showing that nonallergic individuals do not have increased numbers of allergen-specific Tregs [22, 23], which may suggest that there is no need for these cells, since these people have no Th2-biased immune response against the allergens. However, in a previous study, we
found that the frequency of the IL-10-secreting Tr1 cells was decreased in patients with allergic-house dust mite rhinitis [24], whereas other studies have demonstrated that IL-10-secreting Tr1 cells can be induced by allergen-SIT in humans [25–28]. Although data have shown that allergen-specific IL-10-secreting Tr1 cells were induced during the induction phase of allergen-SIT [28, 29], data on the persistence of these cells during the maintenance phase of Der p-SIT are rare. In the present study, our data demonstrate that the Der p-specific, IL-10-secreting Tr1 cells were induced after 1 year of SIT compared with baseline values. Moreover, we also observed increased amounts of IL-10 secreted in PBMC culture from patients after 1 year of Der p-SIT compared with pretreatment, which is in line with a previous study by Francis et al. [27]. However, another study reported enhanced IL-10-secret-
ing CD3+T cells in patients allergic to birch pollen on sublingual immunotherapy (SLIT) during the induction phase but not after the first year of SLIT [29]. This discrepancy may be explained by different treatment protocols or different allergens.

It has been demonstrated that IL-10 decreased IL-4-induced IgE switching but increased IL-4-induced C4 transcript expression and IgG4 production [30]. In this study, we found that Der p-SIT induced the production of IL-10 and increased the number of IL-10-secreting Tr1 cells. However, Der p-SIT did not significantly change specific IgE levels after 1 year of treatment; although a significant increase in specific IgG4 was observed. This is in accordance with the findings of Jutel et al. [28]. Previous studies [31, 32] also demonstrated that SIT induced a transient increase in serum specific IgE, followed by a gradual decrease over a period of months or years of treatment. Although we did not analyze other serum antibodies or IgG isotypes in this study, these might also be induced by SIT. In some previous studies, allergen-specific IgA, IgG1 and IgG2 inductions were reported in patients successfully treated by allergen-SIT [33] and IgG4 was increased by IL-10 secreted from Tr1 cells and CD4+CD25+ Treg cells [34]. Thus, it is clear that further studies are needed to determine the changes in different serum antibodies at different phases of SIT.

Our study demonstrated that in addition to inducing increased IL-10-secreting Tr1 cells, allergen-SIT also enhanced the suppressive effect on cell proliferation and cytokine production. In previous studies, we and others have demonstrated that the suppressive effects of Tr1 cells are important in nonallergic individuals [10, 24]. However, in the present study, our data showed that the role of IL-10-secreting Tr1 cells on suppressing cell proliferation and cytokine productions was decreased in AR patients prior to SIT compared with nonallergic controls, but enhanced after 1 year of allergen-SIT. These results suggest that the capacity of IL-10-secreting Tr1 cells from AR patients to suppress cell proliferation and cytokine production is restored after 1 year of SIT. Furthermore, our study shows that IL-10-secreting Tr1 cells play an important role in healthy immune responses to environmental allergens and in the induction immune tolerance in AR-accepted SIT. Although it was encouraging to record this result, it was necessary to analyze it carefully. As there are no known specific cell-surface markers of Tr1 cells, it is difficult to purify and analyze these cells in vitro. In the present study, we isolated the IL-10-secreting Tr1 cells using the IL-10 Secretion Assay Cell Enrichment and Detection Kit (Miltenyi Biotec). However, as several T cell types, including Th2 cells, are known to secrete IL-10, we cannot exclude the possibility that the isolated IL-10-secreting T cells preparation contained some contaminating Th2 cells. Nevertheless, the frequency of Tr1 cells among CD4+ T cells was increased after 1 year of SIT and the proportion of Tr1 cells was enhanced in the isolated IL-10-secreting T cells. Thus the enhanced suppressive effect may be ascribed to the enhancement of the proportion of Tr1 cells in the isolated IL-10-secreting T cells. Future studies could shed more light on these aspects, as well as the role of Tr1 cells observed in different phases of SIT.

In summary, this study has demonstrated that cluster Der p-SIT induced immune tolerance, as characterized by the induction of IL-10-secreting Tr1 cells, accompanied by the increased production of allergen-specific IgG4, a higher level of IL-10 and an enhanced suppressive function of allergen-specific IL-10-secreting Tr1 cells. Further studies are needed to better understand the potential role that IL-10-secreting Tr1 cells play in induction and maintenance of allergen immune tolerance in cluster Der p-SIT. Similarly, a precise connection between Der p-SIT-induced IL-10 and the production of IgG4 needs to be confirmed.

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