



Allergen immunotherapy in intermittent allergic rhinitis reduces the intracellular expression of IL-4 by CD8⁺ T cells

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Summary

Background: T helper subset dysregulation is evident in allergic disorders. The role of T cytotoxic subsets is less understood. We investigated whether allergen immunotherapy in intermittent allergic rhinitis influences the intracellular expression of IL-4 and IFN-gamma by CD3⁺CD8[−] and CD3⁺CD8⁺ cells.

Methods: Nineteen adult patients with intermittent allergic rhinitis were evaluated before the pollen season, and then after one preseasonal course of subcutaneous allergen immunotherapy. Twelve healthy nonatopic patients matched for age and sex served as controls. Intracellular expression of IFN-gamma and IL-4 by CD3⁺CD8[−] (Th1 and Th2, respectively) and CD3⁺CD8⁺ (Tc1 and Tc2, respectively) was estimated by flow cytometry in peripheral blood cells after stimulation with PMA and ionomycin.

Results: Before immunotherapy the percentages of Th1, Th2, Tc1 and Tc2 did not significantly differ between the patients and the controls. After immunotherapy the percentage of Tc2 was lower in the rhinitic patients than in the controls (0.38% vs. 0.45%, $p=0.04$). The percentage of Tc2 cells decreased significantly after immunotherapy in the intermittent allergic rhinitis group (0.64% vs. 0.38%, $p=0.02$) with tendency to decrease in ratios of Tc2/Tc1 ($p=0.059$) and with no changes in ratios of Th2/Th1. The percentages of Th1, Th2 and Tc1 were comparable before and after immunotherapy within the rhinitic patient group.

Conclusions: The preseasonal allergen subcutaneous immunotherapy applied to intermittent allergic rhinitis patients suppressed the percentage of IL-4 producing CD3⁺CD8⁺ cells. Decreased number of CD3⁺CD8⁺IL-4⁺ cells may participate in the regulatory mechanisms of immunotherapy. © 2007 Elsevier Ltd. All rights reserved.

Introduction

After the discovery of two functionally distinct subsets of Th cells in mice by Mossmann et al. in 1986 [1], Th1/Th2

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imbalance has been proved to play a key role in allergic inflammation. Recently it has been shown that naïve CD8 T cytotoxic/suppressor cells similarly to CD4 cells secrete not only proinflammatory cytokines such as IFN- γ or TNF- β , but they may also produce Th2-type cytokines, e.g. IL-4, IL-5 and IL-10 [2,3]. Thus T cytotoxic/suppressor cells may differentiate into two subsets of effector cells accordingly to the profile of secreted cytokines: "Tc1" and "Tc2". The role of Tc1 and Tc2 cells in pathomechanism of allergic reactions, including anaphylactic and delayed hypersensitivity, has not been definitely established, and data are equivocal. IL-4 producing Tc2 subset has been assumed to play a role in the regulation of allergic response because of its potential to stimulate IgE class switching [4]. Moreover, it has been reported that IL-4 promotes the development of a CD8 T-lymphocyte phenotype with low perforin and granzyme expression, so of lower cytotoxic properties [5]. On the other hand inhibition of IgE responses by both Tc1 and Tc2 cells has been found [6]. There are also some data coming from animal [7,8] and human studies [9] suggesting the role of T cytotoxic cells and Tc2 cells in allergic asthma.

Intermittent allergic rhinitis (IAR) belongs to a group of atopic diseases and is characterized by enhanced IgE synthesis and the involvement of eosinophils, mast cells and T cells in allergic inflammation in the nasal mucosa. The most effective method of treatment of IAR is an avoidance of culprit allergen but sometimes it is not possible. The alternative method of disease modifying treatment may be allergen-specific immunotherapy that has a profound potential to change the natural history of the respiratory allergy [10]. This method has been recommended by EAACI and WHO as an important part of allergy treatment, particularly in cases of severe disease [11,12].

Allergen immunotherapy is a therapeutic modality applied to allergic patients for more than a century [13]. Despite its apparent efficacy, in most properly managed patients its mechanism of action remains not completely understood. Recently a thesis of decrease in production of IL-4, IL-5 and IL-13 by CD4⁺ T cells has been proposed [14]. Neutralisation of cytokine activity is parallel with the induction of activity of CD4⁺CD25⁺ T cells and T cell tolerance. There are some data that percentage of CD4 and CD8 type 2 T cells decreased in asthmatic children treated with immunotherapy [15]. However the data about the role of CD8⁺ T cell subsets in immunotherapy of intermittent allergic rhinitis is unclear. So we decided to assess the effect of preseasonal immunotherapy on the secretion of IL-4 and IFN- γ by T CD4⁺ and T CD8⁺ cells on a single cell level in patients with intermittent allergic rhinitis.

Materials and methods

Nineteen patients with intermittent allergic rhinitis (IAR group, $n=19$, 11 females, mean age 26 ± 7.53 years) sensitive to tree pollen (3 patients) or grass and rye pollen (16 patients) were included into the study. The patients were recruited from the District Outpatient Clinic of Allergology, Zabrze. Diagnosis of intermittent allergic rhinitis was based on clinical history and skin prick test results. Rhinitis was stated as intermittent according ARIA criteria. In some cases serum level of allergen-specific IgE against the culprit

allergens was estimated to confirm the diagnosis. None of the patients had asthma diagnosed. All patients were examined outside the pollen season, in autumn or early winter before starting the immunotherapy. Then 13 subjects from this group were estimated once again after a course of pre-seasonal immunotherapy. The other patients who were seen earlier were not followed-up because of: one case of pregnancy, one case of cessation of immunotherapy due to a large local adverse reaction, in one case because of moving to another part of the country and the other three subjects refused the second blood sampling.

Twelve healthy nonatopic subjects (eight females, mean age 24 ± 4.7 years) formed a control group (CON). Nonatopic state of the control subjects was confirmed by anamnesis and skin prick test results. None of the participants was taking antihistamines or corticosteroids, nor any other medications before blood sampling.

The study was approved by the Local Ethics Committee at the Medical University of Silesia, Katowice and written informed consent was obtained.

Immunotherapy

Immunotherapy was applied according to a preseasonal schedule comprising the up-building dose and the maintenance dose phases. Preparations containing modified allergen were used (Allergovit®; Allergopharma, Reinbeck, Germany). Patients were given injections of Allergovit® according to a standard protocol, beginning at a dose 0.1 mL of 1000 TE/mL extract (bottle A) and increasing at weekly intervals for 7 weeks until a maintenance dose of 0/6 mL of 10,000 TE/mL extract (bottle B) was reached and then continued monthly until the pollen season started.

Antibodies and reagents

CD3 PerCP, mouse IgG, CD69 PE, anti-IL-4 and anti-IFN- γ conjugated with PE, CD8 FITC and FACS lysing solution and FACS permeabilizing solution were purchased from Becton Dickinson, phorbol-12-myristate acetate (PMA) and ionomycin from Sigma, RPMI from BioWhittaker.

Intracellular cytokine expression

Flow cytometric evaluation of intracellular expression of IL-4 and IFN- γ was performed according to the manufacturer's instruction (Becton Dickinson, Application Note: Detecting Intracellular Cytokines in Activated Lymphocytes). Briefly, whole blood was collected into tubes containing sodium heparin and diluted 1:1 in RPMI 1640 and was assayed within 6 h of collection. All activation procedures were performed in capped polystyrene test tubes. The cells were activated with PMA (10 ng/mL) and ionomycin (1 mcg/mL) in the presence of monensine and then incubated for 4 h at 37°C in a water bath. Surface staining with 20 mcL of CD3 PerCP (BD Biosciences) and 20 mcL of CD8 FITC was then performed. After mixing and 15 min incubation at room temperature in the dark 2 mL of FACS lysing solution was added followed by 10 min incubation. Then the sample was centrifugated for 5 min and the supernatant was

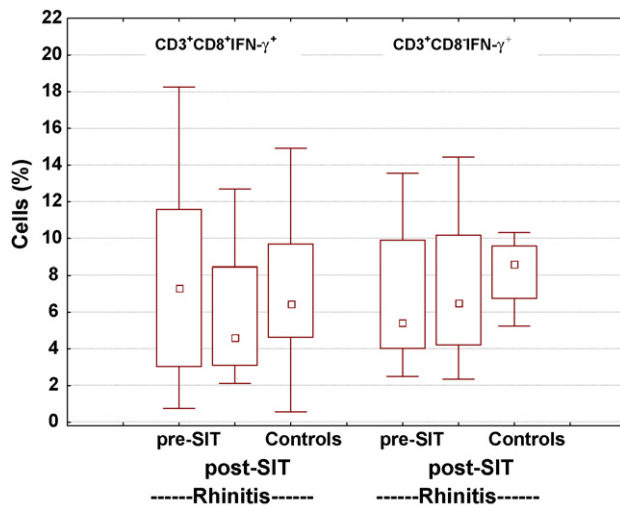


Figure 1 Percentage of $CD3^+CD8^+ IFN-\gamma^+$ and $CD3^+CD8^- IFN-\gamma^+$ cells in the group of patients with intermittent allergic rhinitis evaluated before season (pre-SIT) and after the course of immunotherapy (post-SIT) and in the control group. The comparison within rhinitis group was performed by Wilcoxon test and comparison between subjects and controls by Mann–Whitney *U* test. Squares indicate median, boxes—interquartile range and whiskers—minimal and maximal values. Values were comparable in all groups (statistically nonsignificant).

removed. 500 μ L of FACS permeabilizing solution (BD Biosciences) was added and incubated for 10 min. After two to three times washing 20 μ g/L of PE-conjugated anti-IL-4 or anti-IFN- γ monoclonal antibodies was added, mixed and incubated for another 30 min. Analysis was performed with FACScalibur flow cytometer equipped with an argon laser. Acquisition and analysis were performed with CellQuest Pro software package (Becton Dickinson).

Statistical analysis

Results were expressed as median and interquartile ranges. Because of not-normally distributed data nonparametric tests were used (Mann–Whitney rank sum test and Wilcoxon signed rank test). All analyses were performed with a software package (The Quick STATISTICA PL.). *p* Values less than 0.05 were considered significant.

Results

In the group of patients with intermittent allergic rhinitis the percentage of $CD3^+CD8^-$ producing IL-4 (1.25% [0.53–2.01] vs. .91% [0.65–1.25]), $CD3^+CD8^-$ producing IFN- γ (5.42% [4.02–9.9] vs. 6.52% [4.21–10.18]) and $CD3^+CD8^+$ producing IFN- γ cells (7.3% [3.04–11.58] vs. 4.6% [3.11–8.44]) were comparable before and after one course of immunotherapy (Figs. 1 and 2). The percentage of $CD3^+CD8^+$ producing IL-4 cells decreased significantly after immunotherapy (0.64% [0.41–1.34] vs. (0.38% [0.12–0.61], *p* = 0.02, Wilcoxon signed rank test). The percentage of IL-4 producing $CD3^+CD8^-$, IL-4 producing $CD3^+CD8^+$ cells, IFN- γ

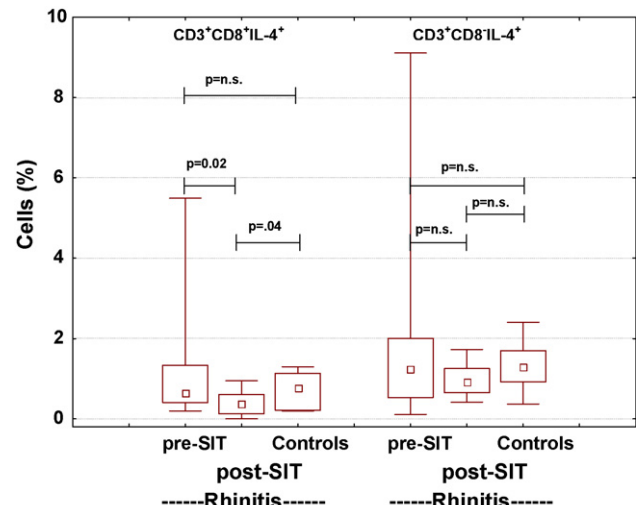


Figure 2 Percentage of $CD3^+CD8^+ IL-4^+$ and $CD3^+CD8^- IL-4^+$ cells in the group of patients with intermittent allergic rhinitis evaluated before season (pre-SIT) and after the course of immunotherapy (post-SIT) and in the control group. The comparison within rhinitis group was performed by Wilcoxon test and comparison between subjects and controls by Mann–Whitney *U* test. Squares indicate median, boxes—interquartile range and whiskers—minimal and maximal values.

producing $CD3^+CD8^-$ and IFN- γ producing $CD3^+CD8^+$ cells did not differ significantly between the patients before immunotherapy and the control group. After one course of immunotherapy the percentage of IL-4 producing $CD8^+$ cells in the group of rhinitic patients was lower than in the controls (0.38% [0.12–0.61] vs. 0.45% [0.02–0.63], *p* = 0.04), the percentage of other cell subsets were comparable in both groups (Figs. 1 and 2).

Ratios between type 2 (IL-4) and type 1 (IFN- γ) cytokines producing $CD3^+CD8^-$ or $CD3^+CD8^+$ cells were calculated both for the values obtained before and after immunotherapy treatment, and in the control group as well. Ratios of $CD3^+CD8^+ IL-4^+ / CD3^+CD8^+ IFN-\gamma^+$ cells tended to decrease after the course of immunotherapy (*p* = 0.059), ratios of $CD3^+CD8^- IL-4^+ / CD3^+CD8^- IFN-\gamma^+$ cells did not change after the treatment.

The ratios $CD3^+CD8^+ IL-4^+ / CD3^+CD8^+ IFN-\gamma^+$ and $CD3^+CD8^- IL-4^+ / CD3^+CD8^- IFN-\gamma^+$ were comparable in the controls and in allergic rhinitic patients before and after immunotherapy (Table 1).

Discussion

This study was addressed to evaluate the effect of preseasonal immunotherapy on T helper and T cytotoxic/suppressor cells in patients with intermittent allergic rhinitis.

In the present study the intracellular expression of IFN- γ in $CD4^+$ and $CD8^+$ T cells did not change after the course of preseasonal subcutaneous immunotherapy and was still comparable both with values outside the season and with those obtained in the control subjects. However, we observed a significant reduction in the percentage of $CD3^+CD8^+$ expressing IL-4 cells after one course of

Table 1 Tc2/Tc1 and Th2/Th1 ratios before and after immunotherapy in the group of patients with intermittent allergic rhinitis (IAR group) and in the control group (CON group)

	Pre-SIT rhinitic group (n = 19)	Post-SIT rhinitic group (n = 13)	Control group (n = 12)
Tc2/Tc1	0.13 (0.08–0.21)	0.06 (0.02–0.11) [†]	0.07 (0.04–0.17)
Th2/Th1	0.12 (0.09–0.37)	0.13 (0.09–0.19)	0.16 (0.11–0.21)

Values were comparable in all groups (Mann–Whitney *U* test and Wilcoxon paired test).

[†] Comparison between pre-SIT and post-SIT values in the patients, *p* = .059.

preseasonal subcutaneous immunotherapy. The number of CD3⁺CD8⁺ expressing IL-4 was lower not only as compared to values before immunotherapy, but also lower than in the control healthy group. At present few data are available on the role of T cytotoxic-suppressor cells producing a distinct cytokine profile similar to Th2 cell, in pathomechanism of subcutaneous immunotherapy. The first attempts to evaluate the role of Tc2 cells in allergic rhinitic patients sensitive to house dust mites and treated with conventional immunotherapy were undertaken by O'Brien et al. who failed to measure Der p 1-specific release of significant amounts by CD8 [16].

Fu et al. found that the levels of mite-specific Th2 (CD4⁺IL-4⁺) and Tc2 (CD8⁺IL-4⁺) cells were significantly lower in a group of children with allergic asthma treated with immunotherapy than in untreated children with allergic asthma [15]. The study by Fu et al. is in some aspects different from our design, as in Fu's study there was not a direct comparison within one group before and after immunotherapy, but the two different groups of patients were included into the analysis, and flow cytometric estimation was performed in isolated peripheral blood mononuclear cells stimulated *in vitro* with crude mite extract. The material comprised children with perennial asthma. Nevertheless they also reported decrease in Tc2 cells as a consequence of immunotherapy. The similar results were reported by Gardner et al. [17] who explored surface expression of adhesion molecules on cytokine (IL-4, IL-10 and IFN- γ)-positive circulating T cell following SIT in allergic rhinitis with sensitization to house dust mite and found that the proportions of IL-4⁺CD4⁺ and CD8⁺ T cells were significantly decreased at 9 months of SIT in comparison with pre-SIT.

Contradictory results were obtained by Majori et al. [18] who did not observe significant changes in interferon- γ /IL-4 ratio in peripheral blood CD8⁺ T cells after building-up phase of immunotherapy, nor after 1 year of treatment of patients with mite-sensitive asthma. He found an increased interferon- γ /IL-4 ratio in peripheral blood CD4⁺ T cells as a result of immunotherapy. Previously, it had been suggested that allergen immunotherapy results in enhanced role of IFN- γ producing "suppressor" CD8⁺ cells, however the results of our study and aforementioned studies do not confirm that hypothesis, as the percentage of CD8⁺ cells expressing IFN- γ did not change after the immunotherapy.

The important role for effector CD8 (+) T cells in the development of airway hyperresponsiveness and airway inflammation have been suggested also by Miyahara et al. [8] who evaluated two distinct populations of antigen-primed CD8⁺ T cells, namely effector (T(EFF)) and central memory (T(CM)) cells. T(EFF) cells produced IL-13, as demonstrated at a single cell level, and indicated Tc2-type cytokine pro-

duction by this T cell population. There are some other data concerning the role of CD8⁺ cells expressing type 2 cytokines in asthma. In naïve mice to which Tc2 cells were adoptively transferred and then were challenged with allergens, nonspecific bronchial hyperresponsiveness developed with inflammatory response characterized by increased number of eosinophils. Inflammatory response was quantitatively similar to Th2 driven response [7]. Interestingly, Tc1 cells were shown in asthmatics independently of atopy [19] suggesting that T cytotoxic cells producing IFN- γ may reflect their role in an additional inflammation, different from allergic inflammation.

Another finding from our study is that at the baseline, outside the pollen season the intracellular expression of IL-4 and IFN- γ by CD4⁺ and CD8⁺ T cells was similar in allergic patients sensitized to tree or grass pollen and in the control subjects. This finding is on the contrary to Th2 paradigm in allergy, but it may be explained by the fact that only patients with intermittent allergic rhinitis sensitive to seasonal allergens were included. In our previous study [20] we found a comparable intracellular expression of IL-4 and IFN- γ by CD3⁺ T cells in intermittent allergic rhinitis patients and in healthy nonatopics outside the pollen season, and higher expression of type 2 cytokine, i.e. IL-4 by CD3⁺ T cells in the pollen season. Ohta et al. [21] who used the same method of evaluation of intracellular expression of cytokines in T cells confirmed the predominance of Th2 in symptomatic patients with allergic rhinitis as compared with nonatopic control. The studied group was not homogenous in terms of type of allergic rhinitis as the authors included into the study patients either with seasonal (ragweed, Japanese cedar) or perennial (house dust mites) sensitization and only in the symptomatic period. They did not evaluate the intermittent allergic rhinitic patients outside the season. In a study performed by Wosinska-Becler et al. [22] comparable production of IL-5, IL-10, IL-13 and GM-CSF by lymphocytes T helper (CD3⁺CD8[−]) outside the pollen season and in healthy controls was found. These results together may indicate that outside the pollen season T cells come back to their "non-allergic" status and after natural exposure to sensitizing allergens become of Th2 type.

The protocol of activation of cytokine producing cells and kind of cell activator is a critical parameter for intracellular cytokine staining. In our study we used polyclonal activators, PMA and ionomycin. T helper cells were indicated by CD3⁺CD8[−] phenotype so as to avoid a transient loss of CD4 expression from the surface of T cells, induced by PMA. Other polyclonal activators, such as Concanavalin A, phytohemagglutinin (PHA), Staphylococcus enterotoxin B and monoclonal antibodies directed against subunits of the TCR/CD3 complex (with or without antibodies directed

against costimulatory receptors such as CD28) are also useful. Cell stimulation with polyclonal activators is an effective and easy to use process, yet nonspecific. Stimulation with clinically significant allergens is the most specific one, however the production of some cytokines may be too low to detect. In some experiments performed in PBMC culture it was found that non-specific polyclonal activators (PMA and PHA) were necessary to obtain cytokine production by T cells detectable on a single cell level [23]. Thus we decided to use PMA and ionomycin in our study.

In conclusion, we found that the preseasonal allergen subcutaneous immunotherapy applied to intermittent allergic rhinitis patients suppressed the percentage of IL-4 producing CD3⁺CD8⁺ cells. We evaluated changes in intracellular expression of IL-4 and IFN- γ by CD4⁺ and CD8⁺ T cells after only one course of preseasonal allergen immunotherapy that has limited the complete interpretation of our results. Further studies are warranted to assess how long-term immunotherapy affects these cytokine production. Moreover, the understanding of the clinical importance of the decrease in IL-4 expression by T cytotoxic cells as a consequence of allergen-specific immunotherapy would be helpful in development of further therapeutic strategies.

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References

- [1] Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986;136:2348–57.
- [2] Sad S, Marcotte R, Mosmann TR. Cytokine-induced differentiation of precursor mouse CD8⁺ T cells into cytotoxic CD8⁺ T cells secreting Th1 or Th2 cytokines. *Immunity* 1995;2: 271–9.
- [3] Li L, Sad S, Kagi D, Mosmann TR. CD8Tc1 and Tc2 cells secrete distinct cytokine patterns in vitro and in vivo but induce similar inflammatory reactions. *J Immunol* 1997;158:4152–61.
- [4] Erard F, Wild MT, Garcia-Sanz JA, Le Gros G. Switch of CD8⁺ T cells to noncytolytic CD8⁺CD4⁺ cells that make Th2 cytokines and help B cells. *Science* 1993;260:1802–5.
- [5] Kienzie N, Buttigieg K, Groves P, Kawula T, Kelso A. A clonal culture system demonstrates that IL-4 induces a subpopulation of noncytolytic T cells with low CD8, perforin, and granzyme expression. *J Immunol* 2002;168:1672–81.
- [6] Thomas MJ, MacAry PA, Noble A, Askenase PW, Kemeny DM. T cytotoxic 1 and T cytotoxic 2 CD8 T cells both inhibit IgE responses. *Int Arch Allergy Immunol* 2001;124:187–9.
- [7] Sawicka E, Noble A, Walker C, Kemeny DM. Tc2 cells respond to soluble antigen in the respiratory tract and induce lung eosinophilia and bronchial hyperresponsiveness. *Eur J Immunol* 2004;34:2599–608.
- [8] Miyahara N, Swanson BJ, Takeda K, Taube C, Miyahara S, Kodama T, et al. Effector CD8⁺ T cells mediate inflammation and airway hyperresponsiveness. *Nat Med* 2004;10:865–9.
- [9] Bratke K, Haupt F, Kuepper M, Bade B, Faehndrich S, Luttmann W, et al. Decrease of cytotoxic cells in allergic asthma correlates with total serum immunoglobulin E. *Allergy* 2006;61:1351–7.
- [10] Möller C, Dreborg S, Ferdoushi HA, et al. Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study). *J Allergy Clin Immunol* 2002;109:251–6.
- [11] Malling HJ, Weeke B. EAACI immunotherapy position papers. *Allergy* 1993;48(Suppl. 14):9–35.
- [12] Bousquet J, Lockley RF, Malling HJ. WHO position paper: allergen immunotherapy: therapeutic vaccines for allergic diseases. *Allergy* 1998;53(Suppl. 44):1–42.
- [13] Noon L, Cantab BO. Prophylactic inoculation against hay fever. *Lancet* 1911;1:1572–3.
- [14] Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiadinnidis C, Crameri R, et al. Immune response in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 2004;199:1567–75.
- [15] Fu CL, Ye YL, Lee YL, Chiang BL. Both allergen-specific CD4 and CD8 type 2 T cells decreased in asthmatic children with immunotherapy. *Pediatr Allergy Immunol* 2003;14:284–91.
- [16] O'Brien RM, Xu H, Rolland J, Byron KA, Thomas WR. Allergen-specific production of interferon-gamma by peripheral blood mononuclear cells in allergic disease and following immunotherapy. *Clin Exp Allergy* 2000;30:333–40.
- [17] Gardner LM, Thien FC, Douglass JA, Rolland JM, O'Hehir RE. Induction of T "regulatory" cells by standardized house dust mite immunotherapy: an increase in CD4⁺CD25⁺ interleukin-10⁺ T cells expressing peripheral tissue trafficking markers. *Clin Exp Allergy* 2004;34:1209–19.
- [18] Majori M, Caminati A, Corradi M, Brianti E, Skarpa S, Perci A. T-cell cytokine pattern at three time points during specific immunotherapy for mite sensitive asthma. *Clin Exp Allergy* 2000;30:341–7.
- [19] Magnan AO, Mely L.G., Kamilla CA, Badier MM, Monteo-Julian FA, Guillot CM, et al. Assessment of Th1/Th2 paradigm in whole blood in atopy and asthma. Increased IFN-gamma producing CD8 (+) T cells in asthma. *Am J Respir Crit Care Med* 2000;161:1790–6.
- [20] Glück J, Rogala B, Mazur B. Intracellular production of IL-2, IL-4 and IFN-gamma by peripheral blood CD3⁺ cells in intermittent allergic rhinitis. *Inflamm Res* 2005;54:91–5.
- [21] Ohta N, Samuray S, Yoshitake H, Aoyagi M. Analysis of Th1, Th2, Tc1 and Tc2 cells in patients with allergic rhinitis. *Clin Exp Allergy Rev* 2005;5:68–71.
- [22] Wosinka-Becler K, Plewako H, Hakansson L, Rak S. Cytokine production in peripheral blood cells during and outside the pollen season in birch allergic patients and non-allergic controls. *Clin Exp Med* 2004;34:123–30.
- [23] Koscher V, Milhe F, El Biase M, Vervloet D, Magnan A. Variation of T-cell activation in allergic subjects during natural pollen exposure. *Allergy* 2006;61:35–42.