

Both allergen-specific CD4 and CD8 Type 2 T cells decreased in asthmatic children with immunotherapy

Fu C-L, Ye Y-L, Lee Y-L, Chiang B-L. Both allergen-specific CD4 and CD8 Type 2 T cells decreased in asthmatic children with immunotherapy.
Pediatr Allergy Immunol 2003; 14:284–291. © 2003 Blackwell Munksgaard

Allergen-specific immunotherapy (IT) has been used for the treatment of atopic diseases since the turn of this century. The precise working mechanisms, however, remain to be clarified. The aim of this study was to investigate the role of particular subsets of allergen-specific T cells in the non-atopic individuals, untreated asthmatic children and the asthmatic children receiving immunotherapy. We collected peripheral blood from 16 untreated asthmatic children and 17 asthmatic children receiving immunotherapy over one and half years. All the patients were sensitive to mite allergen. Peripheral blood mononuclear cells (PBMC) were isolated and, *in vitro*, stimulated with crude mite extract to enrich the mite-specific T-cell population. After 14 days, the enriched mite-specific T cells were stimulated with phorbol-12-myristate-13-acetate (PMA) and ionomycin for intracellular detection of cytokines such as IFN- γ , IL-4 in CD4⁺ and CD8⁺ T lymphocytes. The data here demonstrated that the levels of mite-specific IgG4 and IgA increased significantly in asthmatic children after immunotherapy. In addition, both IL-4 expressing CD4⁺ and CD8⁺ T cells were significantly lower in asthmatic children after immunotherapy compared with those of before treatment and the normal control ($p < 0.05$). In contrast, the frequency of IFN- γ expressing CD4⁺ and CD8⁺ T cells did not significantly differ between untreated and SIT-treated groups. All these data suggested that decreased Type 2 CD4⁺ and CD8⁺ T cells might be closely correlated with the regulatory mechanisms of immunotherapy.

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Key words: immunotherapy; intracellular staining; CD4⁺ T cells; CD8⁺ T cells

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Accepted 15 February 2003

Atopic diseases, such as bronchial asthma and allergic rhinitis, are characterized by IgE synthesis and the infiltration of eosinophils and basophils. The allergic inflammation has been demonstrated to be strictly dependent upon the production of cytokines such as interleukin-4 (IL-4), IL-5 and IL-13, which are secreted by Type 2 helper T cells (Th2). Th1 cells, the counterpart of Th2 cells, produce IL-2, TNF- α and IFN- γ , and thereby activate macrophages and trigger delayed-type hypersensitivity responses. Cytokine profiles of T cells have been suggested to play an important role in the immune response and the susceptibility to diseases.

Recent studies have shown that cytokine production is not limited to CD4⁺ T cells. CD8⁺ T cells have traditionally been regarded as only having cytotoxic properties and have the ability to lyse target cells sharing the MHC class 1 complex with fragments of specific antigen. It has been known for some time that CD8⁺ T cells produce cytokines such as TNF- β and IFN- γ (1, 2). Most CD8⁺ T cells produce IFN- γ , which limit viral replication, but there is increasing evidence that they may also elaborate Th2-type cytokines and therefore play a role in the regulation of immune responses. This subset of CD8⁺ T cells has been referred to as being 'Tc2' (3). Furthermore, IL-4 producing CD8⁺ T cells

also have the potential to both stimulate IgE class switching and promote allergic inflammation (4–6).

Effective treatment for some allergic disorders can be achieved by immunotherapy with intact native allergens (7–9). Allergen-specific immunotherapy has been shown repeatedly to be effective in the treatment of choice for allergic rhinitis and asthma (10, 11). However, the mechanism by which allergen immunotherapy exerts its beneficial effect is still largely unclear. Among these proposed mechanisms, induction of blocking IgG antibodies, especially IgG4, was thought to be responsible for the protective effect of immunotherapy (12). Also, decreased production of IL-4 has been documented in patients receiving immunotherapy with a favorable clinical response. Recent reports indicated a shift from a more Th2-like type of immune response to Th1 after successful allergen-specific immunotherapy (9, 13). However, it is still unknown whether the cytokine shift is the result of changed capacity of cells to produce cytokines or changed percentages of allergen-specific cytokine-producing T-cell subpopulations. The aim of this study was to investigate the change of cytokine producing CD4⁺ and CD8⁺ allergen-specific T cells in asthmatic children before and after immunotherapy. IL-4 and IFN- γ production was measured at the single cell level using an intracytoplasmic cytokine staining technique with subsequent analysis by flow cytometry.

Materials and methods

Subjects

Peripheral blood was obtained from 4 normal volunteers and 16 newly diagnosed asthmatic children and 17 asthmatic children receiving immunotherapy over 1.5 years. The Human Research Committee of this Hospital approved this study, and informed consent was obtained from all study subjects. The patients were aged from 5 to 22 years (pre-IT group 13 males and 3 females, 10.0 ± 4.3 years; post-IT group 9 males and 8 females, 12.3 ± 3.8 years) and were sensitive to mites [*Dermatophagoides pteronyssinus* (Der p) and *D. farinae* (Der f)] with a positive history of exposure and a skin test response greater than 2+ (wheal >10 mm using the intracutaneous route with mite extract at a concentration of 10^{-6} $\mu\text{g/ml}$) and a positive mite RAST test (CAP system, Pharmacia Diagnostics, Uppsala, Sweden). IT-treated asthmatic children received their therapy according to a standardized protocol with crude mite extract as previ-

ously described (14). The patients of the both untreated group and immunotherapy groups were of moderate persistent or severe persistent clinical severity according to the Global Initiative for Asthma (GINA) guideline. Four age-matched non-allergic controls were included in the study. Blood from each group was separated and stored at -70°C until use.

Determination of allergen-specific antibodies

All sera were analyzed for concentrations of mite (Der p)-specific IgE and IgG4 antibodies titer by FEIA with the Pharmacia CAP-System (Kabi-Pharmacia, Uppsala, Sweden). The detection limit was 0.35 kU/l IgE. The concentration of mite-specific IgA antibodies was measured by Beckman Rate nephelometry.

Establishment of mite-specific T-cell lines

Peripheral mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll-Hypaque (Pharmacia) density gradient centrifugation. Crude mite extract was prepared as described according to the previous protocol (15). The cells were washed twice with Hank's balanced salt solution (HBSS) and resuspended in RPMI complete medium prior to use in cell culture. The T-cell lines were cultured according to the method of O'Hehir et al. (16). Briefly, PBMC ($2 \times 10^6/\text{ml}$) were stimulated with 5 $\mu\text{g/ml}$ of crude mite extract in RPMI-1640 medium supplemented with L-glutamine, HEPES, 50 $\mu\text{g/ml}$ streptomycin, 10 $\mu\text{g/ml}$ gentamycin, 2% TCH, and 2% human AB serum for 14 days. Viable cells were isolated ($5 \times 10^5/\text{ml}$) and re-stimulated with 5 $\mu\text{g/ml}$ crude mite extract in the presence of irradiated autologous MNCs ($5 \times 10^5/\text{ml}$) as antigen-presenting cells (APCs).

Re-stimulation of cells for analysis of intracellular cytokine production by flow cytometry

Intracellular cytokine was detected by flow cytometry using the method of Andersson et al. (17), with modifications (18, 19). Next, 10^6 mite-specific T cells were cultured for 6 h at 37°C and 5% CO_2 and stimulated with 50 ng/ml PMA and 1 $\mu\text{g/ml}$ ionomycin in the presence of 2 μM monensin (20). Monensin inhibits cytokine secretion, resulting in cytokine accumulation within the cells. Then, 10^6 cells were harvested and incubated for 30 min at 4°C with a cychrome-conjugated antibody specific to CD3 or CD4 or CD8 in 100 μl of staining buffer [PBS, 1% FCS and 0.1% sodium azide (NaN_3)],

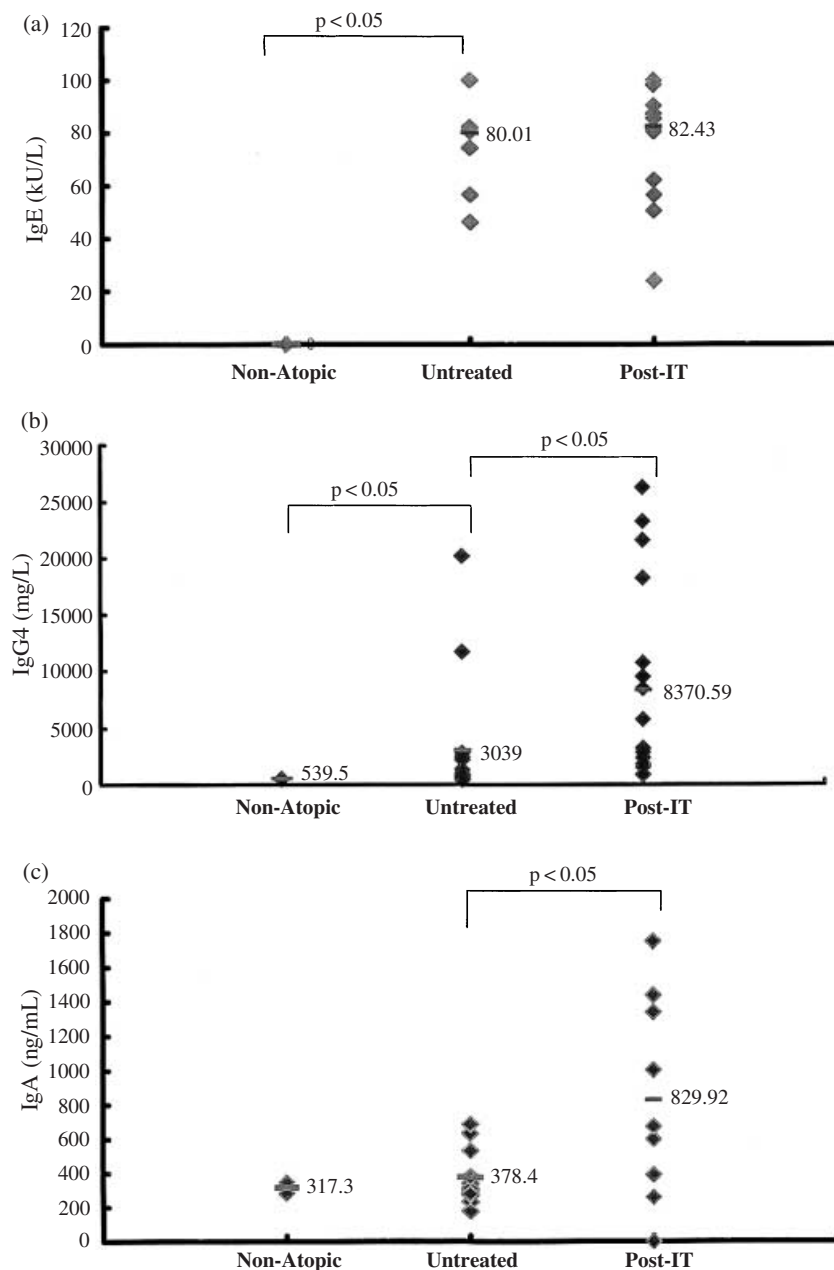


Fig. 1. Serum levels of mite-specific (a) IgE, (b) IgG₄ and (c) IgA antibodies in untreated asthmatic, post-IT asthmatic and non-allergic individuals. The data suggested both IgG₄ and IgA mite-specific antibodies increased after immunotherapy. Values represent means $\pm$ SD. * $p < 0.05$.

pH 7.4–7.6]. Then cells were washed with staining buffer and fixed for 20 min at 4°C with 100 μ l of PBS containing 4% paraformaldehyde. During fixation, the samples were vigorously vortexed to avoid cell clumping. All subsequent washes and incubations were done in PBS containing 0.1% NaN₃ and 0.1% saponin (Sigma, St. Louis, MO) for cell membrane permeabilization. For intracellular staining, fixed cells were incubated for 30 min at 4°C with 50 μ l of saponin-PBS buffer, 0.1 μ g of PE-mouse anti-human IL-4 antibody (PharMingen, San Diego, CA) and 0.25 μ g of FITC-mouse anti-human IFN- γ antibody (PharMingen). After washing twice with saponin-PBS buffer, they were

re-suspended in staining buffer and analyzed by flow cytometry.

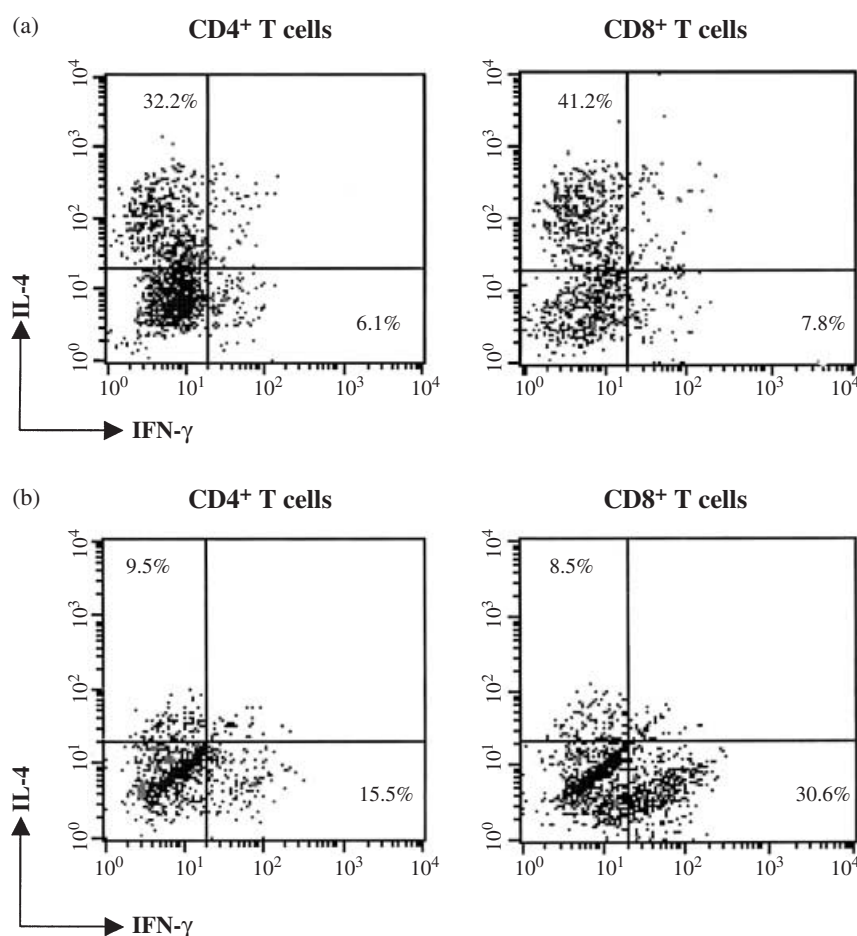
Statistical analyses

Data were expressed as mean $\pm$ SEM for each group. Two-tailed unpaired Student's *t*-test was used to compare changes between the three study groups. A *p*-value of less than 0.05 was considered to be significant.

Results

We examined the changes of mite-specific antibodies in untreated asthmatic, IT-treated

Fig. 2. Representative FACS profiles of mite-specific T cells in (a) untreated and (b) post-IT asthmatic individuals. CD4⁺ and CD8⁺ mite-specific T cells were gated and stained with mAb to intracellular IFN- γ and IL-4.



asthmatic and non-atopic individuals. The value of mite-specific IgE in the untreated asthmatic group (61.7 ± 35.9 kU/l; 12.3–100 kU/l) (median $\pm$ SD; min.–max.) was significantly higher than non-atopic groups (0 kU/l) ($p < 0.0001$). On the other hand, in the post-IT treatment group, the level of mite-specific IgE (81.1 ± 23.0 kU/l; 23.9–100 kU/l) was not significantly different from that of the untreated asthmatic group ($p = 0.136$) (Fig. 1a). In the post-IT treatment group, the level of mite-specific IgG4 (8370.6 ± 8631.0 μ g/l; 873–26,241 μ g/l) was significantly higher than the untreated asthmatic group (3039.0 ± 5322.0 μ g/l; 500–20,208 μ g/l) ($p = 0.042$) (Fig. 1b). The level of mite-specific IgA did not differ between the non-atopic group (317.3 ± 45.9 ng/l; 284.9–349.7 ng/l) and the untreated asthmatic group (378.4 ± 159.8 ng/l; 177.5–688.3 ng/l). But, the level of mite-specific IgA was significantly higher in the post-IT treatment group (829.9 ± 590.0 ng/l; 0–1750.1 ng/l) than in the untreated asthmatic group ($p = 0.053$) (Fig. 1c).

In order to analyze the effect of allergen-specific immunotherapy (IT) on allergen-specific

T cells, we compared the cytokine production of mite-specific T-cell lines from 16 asthmatic children, 17 asthmatic children receiving mite-specific immunotherapy, and 4 non-allergic individuals. All the mite-specific T cells were examined for intracellular accumulation of IL-4 and IFN- γ using the immunofluorescence staining technique and subsequent analysis by flow cytometry. To discriminate cytokine producing CD4⁺ and CD8⁺ T cells, intracellular cytokine staining was performed in combination with cell surface marker analysis. Figure 2 represents FACS profiles of mite-specific T cells in untreated asthmatic individuals stained with mAb to surface marker and intracellular cytokine. The results are summarized in Fig. 3. In untreated asthmatic children, the percentages of CD4⁺IL-4⁺ (Th2) ($28.0 \pm 15.5\%$; 3.4–55.0%) and CD8⁺IL-4⁺ (Tc2) ($27.6 \pm 19.0\%$; 4.8–50.1%) mite-specific T cells were higher compared with that of non-allergic individuals ($p < 0.05$) (Fig. 3). In addition, mite-specific Th2 ($12.6 \pm 9.0\%$; 0.21–27.1%) and Tc2 cells ($5.7 \pm 4.89\%$; 0.01–12.8%) in IT-treated asthmatic children were significantly lower than those of untreated asthmatic

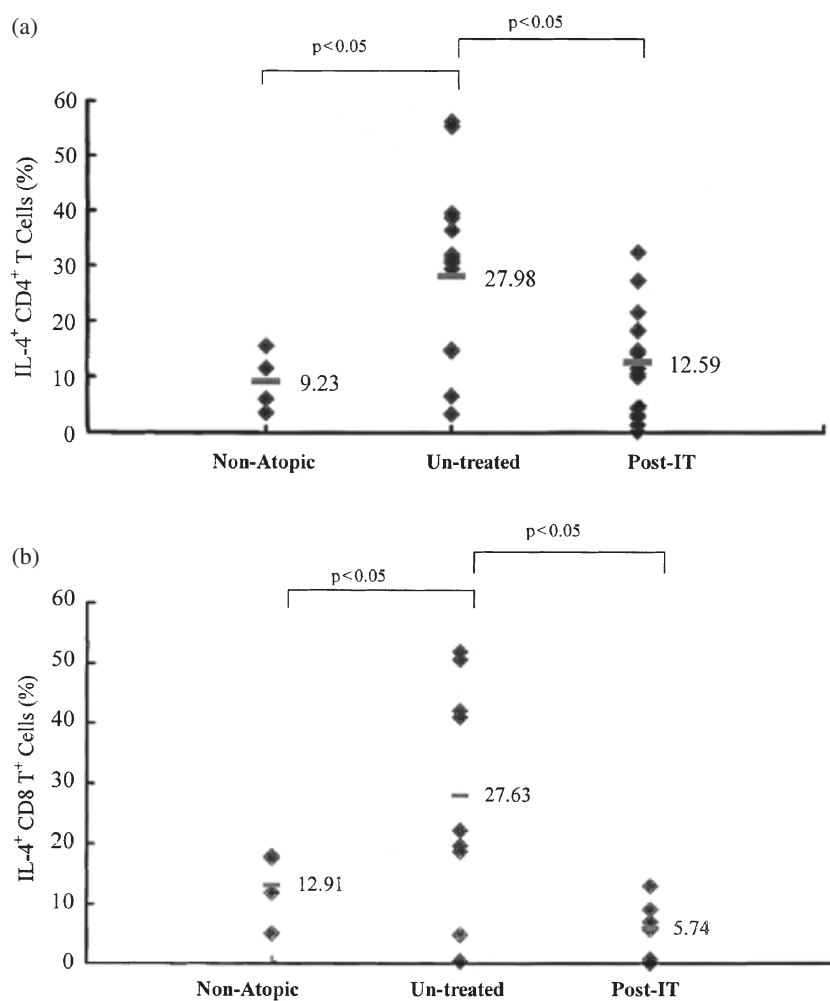


Fig. 3. Percentage of (a) CD4⁺ IL-4⁺ and (b) CD8⁺ IL-4⁺ mite-specific T cells in untreated asthmatic, post-SIT asthmatic and non-allergic individuals. The frequency of cells is given as a percentage of total CD4⁺ or CD8⁺ T cells (= 100%) producing IL-4. Both CD4⁺ and CD8⁺ IL-4⁺ mite-specific T cells were higher in atopic children compared with those of the control group. However, the percentage of both populations decreased after immunotherapy. Values represent means $\pm$ SD. * $p < 0.05$.

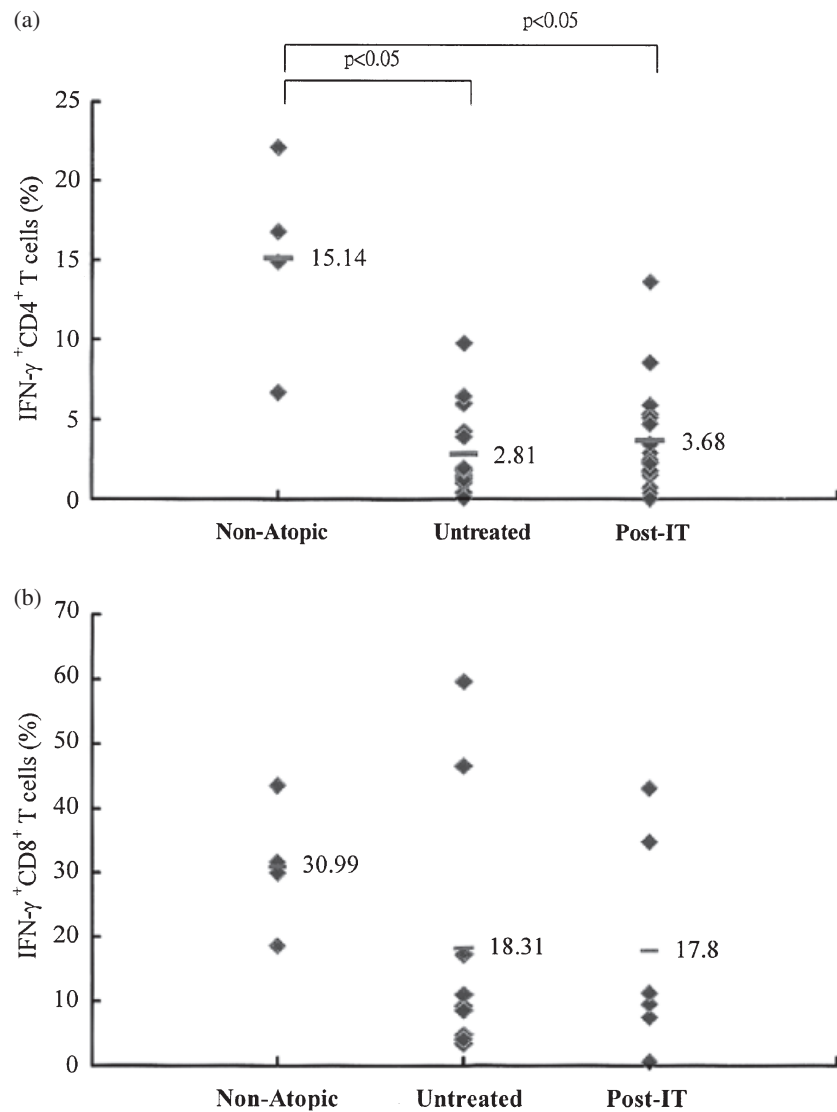
children ($p < 0.05$). Thus, our results indicate that not only CD4⁺ but also CD8⁺ mite-specific T cells that produce IL-4 have an increased frequency in allergic patients. Furthermore, the frequency of IL-4 producing T cells showed the tendency to decrease when allergic patients were treated using allergen-specific immunotherapy.

Figure 4 summarizes the percentage of IFN- γ production within the CD4⁺ or CD8⁺ T subsets. In non-allergic individuals, CD4⁺ IFN- γ ⁺ mite-specific T cells (Th1) ($15.1 \pm 5.6\%$; 6.7–22.2%) were higher than those in untreated asthmatic children ($2.8 \pm 2.5\%$; 0.4–9.8%) ($p < 0.0001$). In contrast, the frequency of IFN- γ secreting CD4⁺ T cells did not significantly differ between untreated ($2.8 \pm 2.6\%$; 0.4–9.8%) and post-IT treatment groups ($3.7 \pm 3.4\%$; 0.1–13.6%). The relative distribution of CD4⁺ and CD8⁺ mite-specific T cells producing IFN- γ did not also differ significantly between study groups. Thus, our results indicate that IFN- γ expressing T cells were not affected by allergen-specific immunotherapy.

Discussion

Effective treatment of allergic patients by immunotherapy consists of immunization with increasing doses of allergen up to a maintenance dose, which is then administered at regular intervals for at least 3 years, and eventually resulting in the amelioration of allergic symptoms (7,21). The precise mechanisms of hyposensitization are still not well defined; blocking antibodies, allergen-specific regulatory cells and change of cytokines pattern all have been suggested (22–24). Most studies have demonstrated that one of the characteristics of successful immunotherapy is the induction of allergen-specific IgG4 antibodies, even with the persistence of allergen-specific IgE (25). The results here confirmed that the IgG4 mite-specific antibody increased in asthmatic children who received immunotherapy compared with those of the normal or untreated asthmatic group. Our data also showed that mite-specific IgE in the serum was not significantly different between the untreated and

Fig. 4. Percentage of (a) $CD4^+$ $IFN-\gamma^+$ and (b) $CD8^+$ $IFN-\gamma^+$ mite-specific T cells in untreated asthmatic, post-IT asthmatic and non-allergic individuals. The frequency of cells is given as a percentage of total $CD4^+$ or $CD8^+$ T cells (= 100%) producing $IFN-\gamma$. $CD4^+$ $IFN-\gamma^+$ mite-specific T cells were lower in asthmatic children compared with those of the control group. No significant differences were noted in both $CD4^+$ and $CD8^+$ $IFN-\gamma^+$ mite-specific T cells after immunotherapy. Values represent means $\pm$ SD. * $p < 0.05$.



IT-treated groups. A recent report showed that a high exposure to cat allergens results in high IgG4 titers and decreased allergic symptoms, supporting a role for IgG4 antibodies in down-regulating allergic responses (26). The results of our study revealed that the serum level of mite-specific IgA increased without significant change of total IgA after immunotherapy. The previous results also suggested that allergen-specific IgA increased in sera or nasal secretions after immunotherapy in patients with allergic rhinitis (27). A significant rise in specific IgM and IgA-secreting cells after immunotherapy of asthma patients has been demonstrated (28). They speculated that the protective function of these partly differentiated plasma cells was mediated by allergen-specific secretory IgA. These results are consistent with ours. However, the hypothesis has to be verified. Because the serum level of these antibodies cannot reflect the real inflammatory status of

the lung, further studies to demonstrate the secretory IgA of bronchial alveolar lavage in asthmatic patients and to clarify whether the clinical improvement following the treatment correlated with the increase in the specific IgA are needed.

The difference and interaction between Type 1 and Type 2 T helper cells has become a major subject of research in the past few years. These data suggested that local concentration or types of cytokines might determine the fate of the T cells *in vivo* (29). The data here suggested regulation of TH_1 and TH_2 balance might play a critical role in the influence of allergen-specific hyposensitization on alleviation of symptoms. We confirmed these data using the flow cytometric method of intracellular cytokine detection that permits the simultaneous detection of cytokine protein and T-cell surface markers in non-separated samples at the single cell level and thus

provides an insight into the frequency of T cells capable of producing different cytokines. However, in order to stain the intracellular cytokine of one cell, we have to stimulate cells before staining by a strong stimulator such as PMA and ionomycin, but most cell clones irrespective of antigen specificity will be activated. But, the aim of our study was to investigate the allergen-specific T-cell subpopulation. Thus, we *in vitro* stimulated T cells with mite to enrich the population of mite-specific T cells. That is may be a reason why the percentage of IL-4⁺CD8⁺ cells observed in this study seems higher than previous study.

In contrast with previous studies, which examined the cytokine profile of peripheral T cells, we applied this technique to study cytokine profiles in mite-specific T cells of children with mite-sensitive asthma. In this study, we found that the level of mite-specific Th2 (CD4⁺IL-4⁺) and Tc2 (CD8⁺IL-4⁺) cells in asthmatic children was higher than normal individuals. Furthermore, these cells are significantly decreased in the IT-treated children. A study of Majori et al. revealed a significant rise in the IFN γ /IL-4 ratio among peripheral blood CD4⁺ T cells in patients with HDM-sensitive asthma during the course of IT, but there were no significant changes in the IFN γ /IL-4 ratio in peripheral blood CD8⁺ T cells three times during the study (before SIT, after 3 months and after 1 years of treatment) (30). This is different from our study, which may be due to the fact that the change of mite-specific Tc2 cells cannot affect the population of peripheral blood CD8⁺ T cells. In the study, we compared the changes of allergen-specific T cells between untreated and treated asthmatic children. Small numbers of non-allergic controls, expressing higher allergen-specific Th1 activity, were collected for the reference data in the study. More studies about the change of mite-specific T cells during the course of immunotherapy in the same patients and also more normal controls may be needed to clarify this point.

Although many reports have reported that allergen-specific immunotherapy affects the cytokine production profiles of allergen-specific T cells. To date, few studies examining the effect of IT on the number of allergen-specific T cells in relation to clinical improvement and bronchial inflammation before and after immunotherapy have been published. We revealed that a different cytokine production pattern of allergen-specific T cells resulted from the different levels mite-specific Th2 and Tc2. Our finding indicated that allergen-specific immunotherapy resulted in mite-specific Th2 cells deletion, which may explain the

clinical efficacy of IT. However, further research into the precise mechanisms of specific IT is clearly required.

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