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Allergen rush immunotherapy increases interleukin (IL)-12 production and IL-12 receptor β2 chain expression in patients with allergic asthma

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Abstract

Interleukin (IL)-12 production and IL-12 receptor (IL-12R) β2 chain expression were investigated in patients with allergic asthma successfully treated with rush immunotherapy (RIT) and control patients with mild allergic asthma. Peripheral blood mononuclear cells (PBMCs) were stimulated with *Dermatophagoides farinae* (Der f), and production of cytokines was measured. Furthermore, the effects of cytokines on IL-12R β2 chain expression on $CD4^+$ T cells were investigated. Production by PBMCs of IL-12 and IFN-γ was significantly higher and production of IL-4 was significantly lower after stimulation with Der f allergen in RIT-treated patients than in control patients. Significant increases in the expression of IL-12R β2 chain before and after stimulation of $CD4^+$ T cells with IL-12 or IFN-γ were observed in RIT-treated patients compared with that in control patients. Allergen RIT increases IL-12 production and IL-12R β2 chain expression and thus may convert cytokine production from Th2 to Th1 or Th0 type in allergic asthma.
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Keywords: Asthma; Immunotherapy; T cell; Interleukin-12; Interleukin-12 receptor; Interleukin-4; Interferon-γ

1. Introduction

Allergen-specific immunotherapy is effective against allergic diseases [1–3]. Meta-analysis of 20 double-blind and placebo-controlled studies has shown that immunotherapy significantly improves the control of asthma [4]. Our study of house dust mite (HDM)-specific T-cell lines and T-cell clones established before and after rush immunotherapy (RIT) in the same patients has demonstrated that RIT converts cytokine production from T helper (Th) 2 type to Th1 or Th0 type [5]. Furthermore, RIT decreases airway inflammation and airway hyperresponsiveness before and after bronchoprovocation with allergen in HDM-sensitized patients with asthma [6].

Interleukin (IL)-12 is a cytokine produced by dendritic cells, monocytes, and macrophages which plays an important role in the differentiation of naive T cells to Th1 cells [7,8]. IL-12 is a heterodimer composed of a 35-kDa peptide and a 40-kDa peptide. IL-12 p70 production and IL-12-dependent interferon (IFN)-γ release by whole blood cells after stimulation with *Staphylococus aureus* Cowan I strain is reduced in patients with allergic asthma [9]. In addition, expression of IL-12 p35 mRNA is decreased in bronchial biopsy specimens from patients with asthma [10].

IL-12 binds to IL-12 receptor (IL-12R), which is a heterodimer composed of β1 and β2 subunits [11,12]. IL-12R is expressed on activated T cells, natural killer (NK) cells, and NK T-cells. The β2 chain of IL-12R is essential for IL-12 signaling, which activates Stat4 and induces transcription of IFN-γ [13]. Expression of the IL-12R β2 chain is increased by stimulation with IL-12 and IFN-γ but is decreased by IL-4, IL-10, or tumor growth factor-β [14]. In addition, the IL-12R β2 chain is selectively expressed by Th1 cells but is not expressed by fully

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differentiated Th2 cells [15]. Therefore, IL-12R β2 chain expression may be a useful marker for the balance between Th1 and Th2. Because upregulation of IL-12R β2 chain expression enhances responsiveness to IL-12 and increases IFN-γ production by T cells, treatments that increase IL-12R β2 chain expression may inhibit Th2-mediated airway inflammation in asthma [16].

The purpose of the present study was to evaluate IL-12 production by peripheral blood mononuclear cells (PBMCs) obtained from patients with allergic asthma successfully treated with RIT or not treated with RIT and nonatopic healthy subjects after stimulation with *Dermatophagoides farinae* (Der f) allergen in the presence or absence of IL-4 or IFN-γ. In addition, expression of IL-12R β2 chain on $CD4^+$ T cells from these patients was analyzed after stimulation with several cytokines.

2. Materials and methods

2.1. Subjects

Nine nonsmoking patients with mild intermittent allergic asthma were selected on the basis of: (a) positive cutaneous reactions to HDM extract, (b) high levels of house dust- and Der f-specific IgE in sera, (c) a baseline forced expiratory volume in 1 s greater than 70% of the predicted value, (d) increased bronchial responsiveness to inhaled histamine, and (e) no treatment other than rescue inhaled β2-agonists. No subjects had used inhaled or oral corticosteroids before the study period or had had a respiratory infection in the 4 weeks before enrollment. Inhaled β2-agonists were withheld for at least 12 h before each examination visit. Also enrolled were nine patients with allergic asthma who met the same criteria and had been successfully treated with RIT with Der f allergen for more than 6 months. In these patients RIT had prevented asthma symptoms and had maintained peak expiratory flow values within the green zone. Characteristics of patients enrolled into the present study are summarized in Table 1. Nine age-matched, nonatopic healthy subjects were also studied. All subjects gave written informed consent before entering into this study.

2.2. RIT

All patients were hospitalized during RIT with Der f allergen (Hollister-Stier, Spokane, WA) and the maximum dose was administered before day 7 according to the same protocol as described previously [6]. After patients had been discharged, they received the maintenance dose once every other week for 2 months and once a month thereafter. Mean duration of RIT was 25.7 ± 6.3 months.

2.3. Purification of PBMCs and $CD4^+$ T cells

PBMCs were isolated from heparinized venous blood on Ficoll-Hypaque gradients (Nacalai Tesque, Kyoto, Japan). A $CD4^+$ T-cell subset was purified from PBMCs. To deplete B cells, monocytes, NK cells, cytotoxic T cells, dendritic cells, early erythroid cells, platelets, and basophils, these cells were indirectly magnetically labeled with a cocktail of hapten-conjugated CD8, CD11b, CD16, CD19, CD36, and CD56 antibodies and magnetic cell separation system (MACS) microbeads coupled to an anti-hapten monoclonal antibody (Miltenyi Biotec, Auburn, CA). The magnetically labeled cells were depleted by retaining them on a MACS column in the magnetic field of the VarioMACS (Miltenyi Biotec). In all experiments, cells were cultured in RPMI-1640 supplemented with 10% v/v human AB serum, 100 U penicillin, and 100 μg/mL streptomycin in an atmosphere containing 5% CO_2 at 37 °C.

2.4. Cytokine production

For cytokine production, PBMCs (1×10^6 cells/mL) were either activated with 10 μg/mL of Der f or 5 μg/mL of PPD allergens for 24 h or not activated. Secretion of IL-4, IL-12 p40, IL-12 p70, and IFN-γ in the supernatant was measured with enzyme-linked immunosorbent assay (ELISA) kits (Biosource International, Camarillo, CA). In some experiments, PBMCs were stimulated with Der f (10 μg/mL) or PPD (5 μg/mL) allergens in the presence or absence of IL-4 (100 ng/mL), anti-IL-4 antibody (1 μg/mL), IFN-γ (500 ng/mL), or anti-IFN-γ antibody (5 μg/mL). The ELISA kits could detect IL-4, IL-12 p40, IL-12 p70, and IFN-γ at concentrations as low as 0.39, 15.0, 1.56, and 15.6 pg/mL, respectively. Although preliminary

Table 1
Subject characteristics

	Nonatopic healthy subjects	Control patients	RIT-treated patients
n	9	9	9
Sex, male/female	5/4	5/4	4/5
Age (yr)	24.8 ± 2.3	25.7 ± 1.2	28.0 ± 2.0
% Predicted FEV1 (%)	84.7 ± 2.9	82.1 ± 3.0	83.0 ± 2.4
IgE (IU/mL)	52.8 ± 32.3	552.0 ± 37.5	465.9 ± 45.7
Der f-IgE (IU/mL)	< 0.35	66.0 ± 4.8	47.1 ± 7.4
Duration of asthma (yr)		17.4 ± 1.2	20.4 ± 2.8

studies demonstrated that optimal time point for the production of IL-4 and IL-12 was 24 h and for IFN-γ production was 72 h after allergen stimulation, these cytokines were measured only 24 h after stimulation because of the limited number of cells obtained from patients.

2.5. Immunohistochemical staining

To investigate the expression of IL-12R β2 chain, rat polyclonal anti-human IL-12R β2 antibody was produced and used for the immunohistochemistry as described previously [17]. Freshly isolated $CD4^+$ T cells were stimulated with IL-12 (5 ng/mL), IL-18 (500 ng/mL), IFN-γ (500 ng/mL), or IL-4 (100 ng/mL) for 24 h. Cells were spun so that they would adhere to glass slides. The specimens were fixed with cold acetone for 15 min. All subsequent procedures were performed with the Dako LSAB alkaline phosphatase kit (Dako, Carpinteria, CA). Endogenous alkaline phosphatase activity was exhausted by incubation with levamisole and then with goat serum for 30 min. Normal rat IgG or the rat anti-IL-12R β2 antibody was applied to the samples overnight at 4 °C. The reactivity was visualized with a streptavidin–biotin complex alkaline phosphatase system, and preparations were counterstained with hematoxylin. Control experiments with normal rat IgG showed no staining at all, and thus their results are not shown. To calculate the percentage of cells expressing the IL-12R β2 chain, 200 cells were counted for each preparation.

2.6. Statistical analysis

The significance of the differences between groups was calculated using the Mann–Whitney *U* test. We applied a Bonferroni correction for multiple comparisons. Data are expressed as mean ± standard error of mean (SEM), and a probability less than 0.05 was considered to indicate significance.

3. Results

3.1. Cytokine production by PBMCs

After stimulation with Der f allergen, IL-4 production in RIT-treated patients (1.91 ± 0.08 pg/mL) was significantly lower than that in control patients (2.94 ± 0.80 pg/mL, $p < 0.05$) but significantly higher than that in nonatopic healthy subjects (1.00 ± 0.15 pg/mL, $p < 0.01$, Fig. 1). IFN-γ production after stimulation with Der f allergen in RIT-treated patients (267.0 ± 23.3 pg/mL) was significantly higher than in control patients (51.4 ± 19.5 pg/mL, $p < 0.01$) and nonatopic healthy subjects (66.7 ± 15.0 pg/mL, $p < 0.01$, Fig. 1). However, IFN-γ (532.1 ± 113.6 pg/mL in RIT, 385.2 ± 19.1 pg/mL in controls, and 505.6 ± 87.8 pg/mL in nonatopic) and IL-12 p40 (470.3 ± 17.2 pg/mL in RIT, 514.0 ± 30.0 pg/mL in control, 510.0 ± 94.5 pg/mL in nonatopic) production after stimulation with PPD did not differ significantly between the three groups (Fig. 1). Production of IL-12 p40 after stimulation with Der f allergens was significantly higher in RIT-treated patients than in control patients (160.0 ± 29.7 vs 56.3 ± 30.5 pg/mL, $p < 0.01$) but did not differ significantly between RIT-treated patients and nonatopic healthy subjects (275.6 ± 43.8 pg/mL, Fig. 1). Although IL-12 p70 was also measured after stimulation with Der f allergen, it could be detected with ELISA in 5 of 9 samples from RIT-treated patients (2.13 ± 0.22 pg/mL) and 6

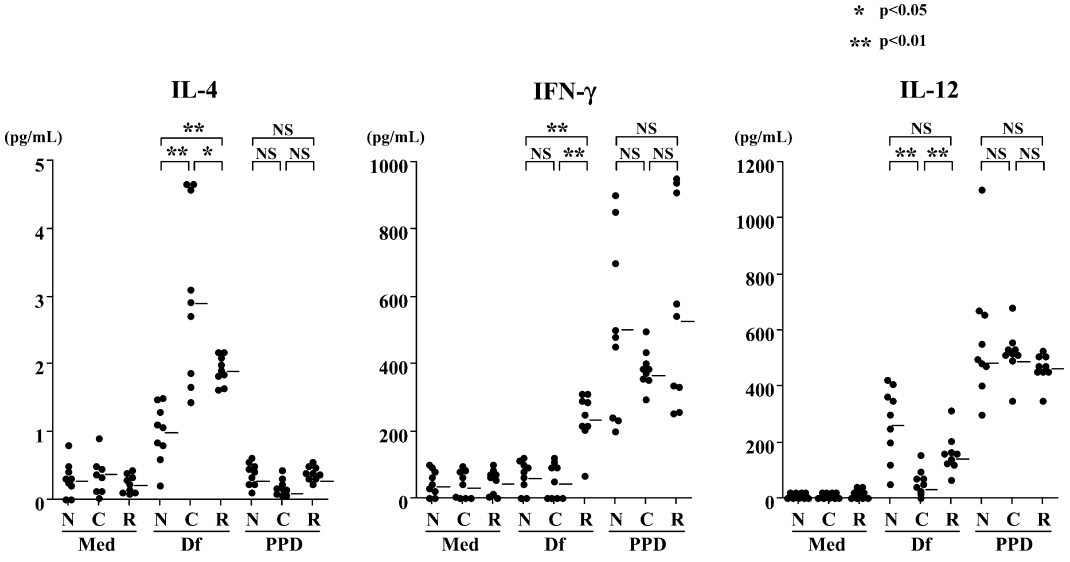

Fig. 1. Production of IL-4, IFN-γ, and IL-12 p40 by PBMCs from nine nonatopic healthy subjects (N), nine control patients (C), and 9 RIT-treated patients (R) after stimulation with Der f or PPD allergens. PBMCs (1×10^6 cells/mL) were stimulated with Der f (Df, 10 μg/mL) or PPD (5 μg/mL) allergens or were not stimulated. After 24 h of culture, the supernatants were collected and IL-4, IFN-γ, and IL-12 p40 were measured with ELISA. Horizontal bars indicate the mean of cytokine levels (NS, not significant).

of 9 samples from nonatopic healthy subjects (3.19 ± 0.51 pg/mL) but could not be detected in samples from any of the control patients with asthma.

3.2. Effect of IL-4 and IFN-γ on IL-12 production by PBMCs

Although stimulation of PBMCs with Der f allergen plus IL-4 significantly inhibited production of IL-12 p40 in control patients (56.3 ± 30.5–3.26 ± 3.26 pg/mL, $p < 0.01$) and nonatopic healthy subjects (275.6 ± 43.8–2.2 ± 0.74 pg/mL, $p < 0.01$), IL-12 p40 production by PBMCs from RIT-treated patients was resistant to this stimulation and there was no statistical significance (160.0 ± 29.7–96.7 ± 45.2 pg/mL, Fig. 2). Flow cytometric analysis of IL-4 receptors on PBMCs showed no significant differences between patients treated with RIT (78.3 ± 3.0%) and control patients (81.4 ± 1.4%). In contrast, addition of IFN-γ to Der f allergen significantly increased IL-12 p40 production in RIT-treated patients compared with that in control patients (610.4 ± 59.0 vs 338.0 ± 74.4 pg/mL, $p < 0.01$) but did not differ between RIT-treated patients and nonatopic healthy subjects (610.4 ± 59.0 vs 576.1 ± 32.1 pg/mL, Fig. 2). In some experiments, IL-12 p40 production was studied after stimulation of PBMCs with Der f allergen and anti-IL-4 antibody or anti-IFN-γ antibody. IL-12 p40 production was not affected by coincubation with either anti-IL-4 antibody (156.3 ± 12.7 pg/mL in RIT, 69.4 ± 16.1 pg/mL in control, and 25.4 ± 4.1 pg/mL in nonatopic) or anti-IFN-γ antibody (195.0 ± 24.7 pg/mL in RIT, 53.2 ± 11.2 pg/mL in control, and 225.2 ± 3.7 pg/mL in nonatopic) in either group of patients and nonatopic healthy subjects (Fig. 2).

3.3. Effect of RIT on IL-12R β2 chain expression

Expression of IL-12R β2 chain on $CD4^+$ T cells was significantly increased by the stimulation with IL-12 and IFN-γ in healthy nonatopic subjects (18.9 ± 3.0–45.3 ± 2.8%, $p < 0.01$, and 18.9 ± 3.0–31.3 ± 3.2%, $p <$ 0.01) and patients who received (16.0 ± 1.0–40.7 ± 3.2%, $p < 0.01$, and 16.0 ± 1.0–35.0 ± 2.5%, $p < 0.01$) or did not receive RIT (5.1 ± 1.2–9.7 ± 1.8%, $p < 0.05$, and 5.1 ± 1.2–10.8 ± 1.8%, $p < 0.01$, Fig. 3). In addition, expression of IL-12R β2 chain on $CD4^+$ T cells was significantly decreased by the stimulation with IL-4 in healthy nonatopic subjects (18.9 ± 3.0–7.8 ± 1.3%, $p < 0.01$) and patients with RIT (16.0 ± 1.0–6.0 ± 1.3%, $p < 0.01$, Fig. 3). Expression of IL-12R β2 chain on $CD4^+$ T cells was higher in healthy nonatopic subjects and patients

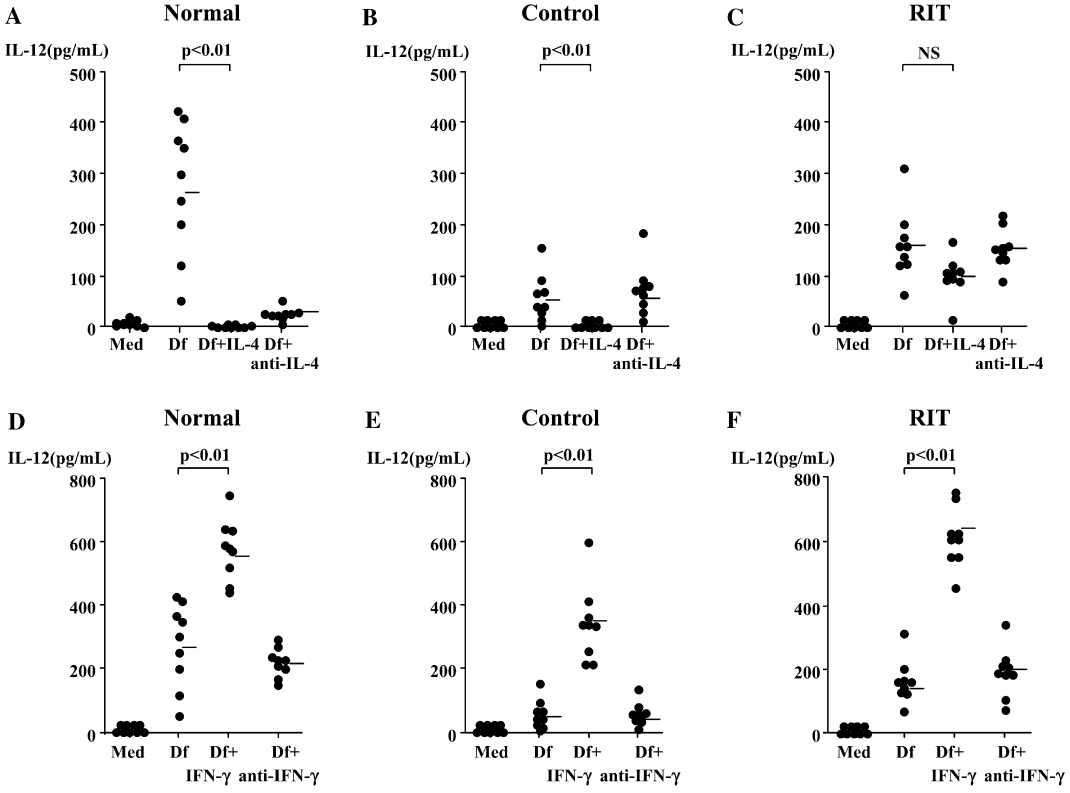

Fig. 2. Production of IL-12 p40 by PBMCs from nine nonatopic healthy subjects, nine control patients, and nine RIT-treated patients after stimulation with Der f allergen in the presence or absence of IL-4, anti-IL-4 antibody, IFN-γ, or anti-IFN-γ antibody. PBMCs (1×10^6 cells/mL) were stimulated with Der f (Df, 10 μg/mL) allergen in the presence or absence of IL-4 (100 ng/mL, A–C), anti-IL-4 antibody (1 μg/mL, A–C), IFN-γ (500 ng/mL, D–F), or anti-IFN-γ antibody (5 μg/mL, D–F) or not stimulated. After 24 h of culture, the supernatants were collected and IL-12 p40 was measured with ELISA. Horizontal bars indicate the means of IL-12 p40 levels (NS, not significant).

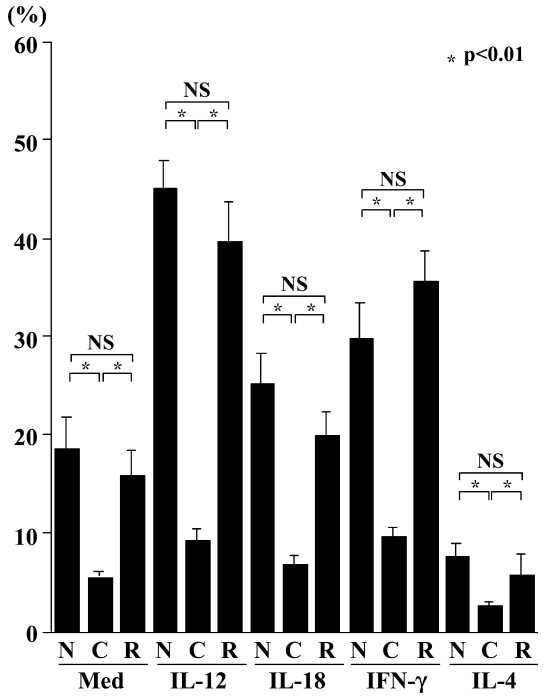

Fig. 3. Expression of IL-12R β2 chain after stimulation of $CD4^+$ T cells with several cytokines in nine nonatopic healthy subjects (N), nine control patients (C), and 9 RIT-treated patients (R). Immunocytochemical staining of the IL-12R β2 chain with rat polyclonal antibodies after stimulation of $CD4^+$ T cells with IL-12 (5 ng/mL), IL-18 (500 ng/mL), IFN-γ (500 ng/mL), or IL-4 (100 ng/mL) for 24 h. To calculate the percentage of $CD4^+$ T cells expressing the IL-12R β2 chain, 200 cells were counted for each preparation (NS, not significant, $^*P < 0.01$).

with RIT than that in control patients at baseline ($18.9 \pm 3.0\%$ in nonatopic, $16.0 \pm 1.0\%$ in RIT, $5.1 \pm 1.2\%$ in control) and after stimulation with IL-12 ($45.3 \pm 2.8\%$ in nonatopic, $40.7 \pm 3.2\%$ in RIT, $9.7 \pm 1.8\%$ in control), IL-18 ($25.4 \pm 3.1\%$ in nonatopic, $20.0 \pm 2.3\%$ in RIT, $7.0 \pm 1.3\%$ in control), IFN-γ ($31.3 \pm 3.2\%$ in nonatopic, $35.0 \pm 2.5\%$ in RIT, and $10.8 \pm 1.8\%$ in control), and IL-4 ($7.8 \pm 1.3\%$ in nonatopic, $6.0 \pm 1.3\%$ in RIT, $2.8 \pm 0.3\%$ in control, Fig. 3). Levels of IL-12R β2 chain on $CD4^+$ T cells were not significantly different between healthy nonatopic subjects ($18.9 \pm 3.0\%$) and patients treated with RIT ($16.0 \pm 1.0\%$, Fig. 3).

4. Discussion

We have previously demonstrated that RIT is an effective anti-inflammatory treatment in patients with allergic asthma by inducing changes in cytokine production from Th2 type to Th1 or Th0 type [5,6]. In the present study, we have found an increased production of IL-12 by PBMCs after stimulation with Der f allergen and IL-12 production was resistant to IL-4 in RIT-treated patients with asthma. In addition, IL-12R β2 chain expression on $CD4^+$ T cells were increased after stimulation with IL-12 or IFN-γ in patients with RIT and their levels were significantly elevated compared with that in control patients not treated with RIT. Therefore, increases in both IL-12 production and IL-12R β2 chain expression may contribute to the conversion of cytokine production from Th2 type to Th1 or Th0 type by successful allergen RIT.

The mechanisms of allergen immunotherapy are extremely complex and may depend on the type, route, or dose of allergens. The beneficial effects of immunotherapy are unlikely to include the production of allergen-specific IgG4 that interacts with allergens before they can bind to IgE, because clinical effects precede the increase in IgG4 levels [18]. A more likely mechanism is the induction of tolerance in Th2 cells, possibly initiated by autocrine action of IL-10 leading to decrease in the production of the Th2 cytokines IL-4 and IL-5 [5,19,20]. The effect on Th1 cytokine production was less consistent. Both increases and decreases in IFN-γ production have been reported [20,21]. In the present study, we found decreased Th2 cytokine production and increased Th1 cytokine production. Therefore, both mechanisms are important for the clinical efficacy of RIT with Der f allergen in patients with allergic asthma.

IL-12 is an important cytokine for the differentiation of naive T cells to Th1 cells. Administration of IL-12 to mice by inhalation or gene delivery into epithelial cells inhibits Th2 cytokine production, eosinophilic airway inflammation, and airway hyperresponsiveness in the mouse model of asthma [22–26]. In patients with allergic asthma, expression of IL-12 p35 mRNA is decreased in bronchial biopsy specimens [10]. Moreover, IL-12 p70 and IL-12-dependent IFN-γ production are decreased in patients with allergic asthma [9], a finding that suggests IL-12 production by antigen-presenting cells is defective.

Allergen immunotherapy also affects IL-12 expression. Grass pollen immunotherapy increases the number of IL-12 p35 mRNA+ cells in cutaneous biopsy specimens 24 h after allergen injection [27]. In addition, the number of IL-12 p35 mRNA+ cells correlates positively with the number of IFN-γ mRNA+ cells and negatively with the number of IL-4 mRNA+ cells. In the present study, we found that production of both IL-12 p40 and IL-12 p70 by PBMCs after stimulation with Der f allergen was higher in patients successfully treated with RIT than in control patients not receiving RIT. Therefore, these results suggest that RIT increases production of IL-12 by PBMCs in patients with allergic asthma.

Because IL-12 production was inhibited by IL-4, PBMCs were stimulated with both Der f allergen and IL-4. Interestingly, IL-12 p40 production by PBMCs was completely inhibited by the addition of IL-4 to Der f allergen in control patients but was not significantly affected in patients treated with RIT. Expression of IL-4 receptor on PBMCs did not different significantly between RIT-treated patients and control patients.

Therefore, these results suggest that monocytes from RIT-treated patients are already primed to produce IL-12 in vivo and may become resistant to IL-4. Further studies are necessary to elucidate the precise mechanisms of these effects.

Production of IL-12 p40 may be 100 times greater than production of IL-12 p70, the potentially bioactive form of IL-12 [28]. In the present study, IL-12 p70 was detected in only 5 of 9 samples from patients receiving RIT but in none of the samples from control patients. A possible reason why IL-12 p70 was not detected in all samples may be that IL-12 p70 was produced by PBMCs in only small amounts after stimulation with Der f allergen. However, the fact that IL-12 p70 was detected in 5 of 9 samples only from RIT-treated patients indicates that RIT increases IL-12 p70 production.

IL-12 p40 can be covalently linked to the p35-related protein p19; the resulting heterodimer is known as IL-23 [29]. Murine IL-23 induces strong proliferation of memory T cells but not of naive T cells, whereas IL-12 has no effect on memory T cells [29]. Although RIT significantly increased the production of IL-12 p40 from PBMCs after stimulation with Der f, how RIT affects IL-23 production is unknown. Further studies are needed to investigate the effect of RIT on IL-23 production.

The IL-12R β2 chain is selectively expressed on Th1 cells but not on fully differentiated Th2 cells [15]. Expression of IL-12R β2 chain mRNA is increased in so-called Th1 diseases, such as tuberculosis and sarcoidosis [30]. Therefore, the IL-12R β2 chain is a useful marker for the activity of Th1-type diseases. We have reported that expression of IL-12R β2 chain is decreased in patients with allergic asthma [17]. Moreover, PBMCs from atopic children with asthma express less IL-12R β2 chain mRNA than that in PBMCs from nonatopic children after stimulation via the CD2 pathway by phytohemagglutinin [31]. Indeed, upregulation of the IL-12R β2 chain enhances responsiveness to IL-12 and increases IFN-γ production by T cells. In the present study, we found that elevated level of the expression of IL-12R β2 chain on $CD4^+$ T cells were observed before and after stimulation with IL-12 and IFN-γ in RIT-treated patients. Therefore, present study demonstrates that RIT increases expression of the IL-12R β2 chain on $CD4^+$ T cells and thus may increase IFN-γ production by stimulation with Der f allergen in RIT-treated patients with asthma.

Studies in a murine asthma model suggest that administration of IFN-γ decreases IgE production, airway inflammation, and airway hyperresponsiveness [32,33]. Therefore, effective counterbalancing of the cytokine profiles from Th2 to Th1 may be an effective treatment for asthma. However, administration of IFN-γ through inhalation or subcutaneous injection failed to improve asthma in human patients [34,35]. In addition, transfer of OVA-specific Th1 cells into severe combined immune deficiency mice did not attenuate Th2 cell-induced airway hyperresponsiveness but did cause severe airway inflammation [36]. These findings suggest that induction of IFN-γ by Th1 cells alone may not be able to prevent or protect against Th2-mediated allergic responses in asthma.

In conclusion, we have demonstrated that allergen RIT increases IL-12 production by PBMCs and expression of the IL-12R β2 chain on $CD4^+$ T cells in patients with allergic asthma. Therefore, RIT may convert cytokine production from Th2 type to Th1 or Th0 type and inhibit airway inflammation in allergic asthma.

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