

Insufficient Increment of CD4+CD25+ Regulatory T Cells after Stimulation in vitro with Allergen in Allergic Asthma

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Key Words

Allergic asthma · Allergen · Regulatory T cell · Interleukin 10 · Interferon- γ

Abstract

Background: Allergen exposure can lead to the development of allergic asthma, sensitized asymptomatic or nonallergic responses in different conditions. Regulatory T (T_{reg}) cells are involved in controlling the immune response direction. The aim of this study was to determine whether allergic asthmatics had defects in CD4+CD25+ T_{reg} cells and CD4+IL-10+ T_{reg} cells after specific allergen exposure.

Methods: Peripheral-blood mononuclear cells (PBMCs) were isolated from 20 patients with asthma sensitized to house dust mite, 24 asymptomatic subjects sensitized to house dust mite and 22 nonallergic subjects. Cells were cultured without stimulation and with rDer p 1. Frequencies and proliferations of CD4+CD25+ T cells and CD4+IL-10+ T cells were measured by flow cytometry. Concentrations of IL-10, IL-4, interferon (IFN)- γ and tumor growth factor β_1 in culture supernatants and sera were determined by ELISA. **Results:** PBMCs derived from sensitized asthmatic patients had the lowest percentage of CD4+CD25+ T_{reg} cells and the highest IL-4 level in response to allergen among the 3 groups. PBMCs derived from sensitized asymptomatic subjects had higher

percentages of both CD4+CD25+ T_{reg} cells and CD4+IL-10+ T cells than those from sensitized asthmatic patients in rDer p-1-stimulated cultures, and they had the highest levels of IL-10 and IFN- γ responses to allergen among the 3 groups.

Conclusions: Patients with allergic asthma had an insufficient CD4+CD25+ T_{reg} cell response and an excessive Th2 response to specific allergen. The high levels of T_{reg} cell response and IL-10 and IFN- γ responses to specific allergen can partly explain the immunological characteristics associated with sensitized asymptomatic subjects.

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Introduction

Allergic asthma is characterized by airway hyperreactivity to a variety of allergens, chronic airway inflammation with pulmonary eosinophilia, mucus hypersecretion and the presence of specific IgE to allergens [1, 2]. The occurrence of asthma is due to an aberrant immune response mediated by T helper 2 (Th2) cells and an associated cytokine pattern including interleukin (IL)-4, IL-5 and IL-13 [3, 4]. Many allergens implicated in asthma such as house dust mite, cat dander and *Aspergillus* mold, are widely distributed in the environment. The interaction of environmental and genetic factors can lead to the

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development of allergic disorders in some individuals but not in others following allergen exposure. T cell proliferation in response to allergen can be detected in primary cultures of peripheral-blood mononuclear cells (PBMCs) from both allergic and nonallergic individuals. Only PBMCs from allergic donors show increased production of Th2 cytokines [5–7]. There are several mechanisms that may explain this phenomenon. One possibility is that regulatory T (T_{reg}) cells prevent Th2-type responses to specific allergens in nonallergic and sensitized asymptomatic individuals.

The expression 'regulatory T lymphocytes' refers to cells that actively control or suppress the function of other cells, generally in an inhibitory fashion. These T_{reg} cells appear to be important for the development of autoimmune diseases and the rejection of transplants, and may also play a critical role in controlling allergic diseases, including allergic asthma. Two broad categories of T_{reg} cells have been described: the thymus-derived and naturally occurring CD4+CD25+ T_{reg} cells, which have been identified in human thymus, cord and adult blood [8–10], and inducible allergen-specific populations such as IL-10-secreting CD4+ T_{reg} cells, which have been described following different regimens of antigen administration [11, 12]. Studies from several separate groups have provided evidence for impaired CD4+CD25+ T_{reg} -mediated inhibition of allergen-specific Th2 responses in allergic patients during the hay fever season [13, 14] or in individuals who mount vigorous Th2 responses to allergen [15]. The inhibition of anti-CD3-driven plus anti-CD28-driven CD4+CD25– T-cell proliferation by CD4+CD25+ T_{reg} cells does not differ between nonallergic and allergic donors [13], implying that differences of naturally occurring CD4+CD25+ T_{reg} cells between these two groups are restricted to allergen-specific regulation. Depletion of CD4+CD25+ T cells from the PBMCs of healthy nonallergic individuals reveals enhanced Th2 responses to various allergens, including milk, nickel and grass *in vitro* [13, 16, 17]. In addition to CD4+CD25+ T_{reg} cells, allergen-specific IL-10-secreting CD4+ T_{reg} cells also play an important role in immune regulation. IL-10-secreting CD4+ T_{reg} cells specific to various food or inhalant allergens are detectable in PBMCs from both nonallergic and allergic individuals. They inhibit the proliferation of PBMCs activated by the specific allergen. A decreased frequency of allergen-specific IL-10-secreting CD4+ T_{reg} cells has been observed in allergic patients [18]. Successful venom and aeroallergen immunotherapy has been found to be associated with the generation of allergen-specific T_{reg} cells that secrete suppressive cytokines such

as IL-10 and tumor growth factor (TGF)- β [19–21]. In situations where tolerance is 'naturally' induced, for example in children who grow out of their allergies to cow's milk or in bee keepers who received multiple stings, associated increases in IL-10-secreting CD4+ T_{reg} cells and CD4+CD25+ T_{reg} cells have been reported [19, 22]. All these studies suggest: (1) both CD4+CD25+ T_{reg} cells and IL-10-secreting CD4+ T_{reg} cells actively control immune responses to allergens in healthy individuals, and (2) their quantities and functions might be impaired in allergic diseases, particularly during chronic allergen exposure.

As a result, it is hypothesized that there may be some defects in the quantity of CD4+CD25+ T_{reg} cells and CD4+IL-10+ T_{reg} cells in response to allergen stimulation in patients with allergic asthma. The aim of this study was to determine the number of CD4+CD25+ T cells and CD4+IL-10+ T cells and the levels of cytokines in cultured PBMCs derived from patients sensitized to house dust mite with asthma and the effect of house dust mite allergen on them. Data were compared with those of asymptomatic subjects sensitized to house dust mite and of healthy nonallergic subjects.

Materials and Methods

Subjects

Twenty patients sensitized to house dust mite with asthma during an acute exacerbation period were selected from the Section of Respiratory Diseases of Renmin Hospital of Wuhan University (Wuhan, China) and Zhongnan Hospital of Wuhan University (Wuhan, China). Twenty-four asymptomatic volunteers sensitized to house dust mite and 22 healthy nonallergic volunteers were recruited by advertisement and from medical students of Wuhan University. All subjects completed a standard questionnaire about allergic symptoms and underwent an examination including chest X-ray examination, lung function test, methacholine challenge test, skin prick test (SPT) and measurement of serum allergen-specific IgE. Patients with asthma sensitized to house dust mite were chosen on the basis of (1) meeting the criteria of the Global Initiative for Asthma [23] and having no other allergic symptoms; (2) a clear history of asthma symptoms, which is made worse by sweeping the floor, making the bed or being in a dusty atmosphere, together with a record of a significant decrease in peak expiratory flow in those conditions (less than 80% of the patient's own previous best measurements using the same peak flow meter); (3) being monosensitized to house dust mite allergen among those common allergens, and (4) having not received immunotherapy in the previous 5 years, having not received oral steroids or theophylline preparations in the previous 3 months, and having not received inhaled steroids in the previous 2 weeks. Asymptomatic subjects sensitized to house dust mite were monosensitized to house dust mite allergen and had no allergic symptoms such as eczema, anaphylactic conjunctivitis, allergic rhinitis or asthma. Nonallergic subjects were asymptom-

atic, had negative skin responses to those common aeroallergens and negative radioallergosorbent tests (RAST) for the same panel of allergen extracts. All subjects were life-long nonsmokers and had no record of recent infection in the preceding 4 weeks or of any current medical illness other than allergic asthma.

The study was approved by the institutional review board of the Medical School of Wuhan University for Human Studies, and all participants gave written informed consent.

Assessment of Monosensitization to House Dust Mite

An SPT was done using stock solutions for common airborne allergens including *Dermatophagoides pteronyssinus*, mixed grass pollen, *Cladosporium herbarum* and *Alternaria tenuis*, dog dander and cat fur (Soluprick; ALK, Horsholm, Denmark) by the same skilled nurse. Serum allergen-specific IgE was measured via RAST (Phadebas RAST, Pharmacia, Milton Keynes, UK) for the same panel of allergens. Monosensitization to house dust mite was defined by a strongly positive SPT response only with *D. pteronyssinus* of at least 4 mm greater than that elicited by the negative diluent control and positive RAST for the same allergen extract [24].

Measurement of Lung Function and Methacholine Challenge Test

Flow volume curves were obtained through a dry spirometer (Vmax 229, United States Sensor-Medics, Yorba Linda, Calif., USA) and the best volume of the 3 manoeuvres was expressed as the percentage of predicted normal and used for analysis. Peak expiratory flow was monitored by asthma patients with a Personal peak flow meter (Respironics, Nantes, France) twice daily or several times each day over periods of suspected exposure to house dust mite and during periods of nonexposure. Each measurement consisted of 3 attempts, of which the highest value was used for further analysis.

The provocative concentration of methacholine causing a 20% fall in FEV₁ was measured by inhalation methacholine challenge with a doubling concentration of methacholine (0.03–32 mg/ml) delivered by a dosimeter (Mefar, Bovezzo, Italy) with an output of 100 µl. Spirometric and methacholine challenge test results were recorded by the same lung function technician.

Major Reagents

Carboxyfluorescein diacetate succinimidyl diester (CFSE; Molecular Probes, USA) was dissolved in dimethylsulfoxide at a concentration of 5 mmol/l. Recombinant *D. pteronyssinus* allergen 1 (rDer p 1, Indoor Biotechnologies, Charlottesville, Va., USA) was stored at –20°C. At the concentration of 1 µg/µl, which was the concentration used in our study, the allergen had undetectable endotoxin using a limulus amebocyte lysate (LAL) gel-clot assay (Chinese Horseshoe Crab Reagent Manufactory Co. Ltd., Xiamen, China) with a sensitivity of 0.06 EU/ml. The allergen was >99% pure. Phytohemagglutinin (PHA) was purchased from Gibco (Paisley, UK). Human T_{reg} cell staining kit (w/PE Foxp3 PCHI01, FITC CD4, APC-CD25; T_{reg} Kit) was purchased from eBioscience Inc. (San Diego, Calif., USA). All antibodies used in flow cytometry assays including phycoerythrin-Cy5 (PE-Cy5) antihuman CD4, PE antihuman CD25, PE antihuman IL-10, PE mouse IgG1, K isotype control, and PE-Cy5 mouse IgG1, K isotype control, monensin solution (1 mol/l), flow cytometry staining buffer, intracellular fixation buffer, and permeabiliza-

tion buffer were also purchased from eBioscience Inc. ELISA kits for the human cytokines IL-10, TGF-β₁, IL-4 and interferon (IFN)-γ were purchased from BD PharMingen (San Diego, Calif., USA).

Isolations of PBMCs

Peripheral venous blood samples (6 ml/sample) were collected from 10 of the healthy nonallergic subjects for the determination of Foxp3 expression in CD4+CD25+ T cells. Peripheral venous blood samples (15 ml/sample) were then obtained from all participants. Samples were collected in heparinized tubes and not in cold preservations. PBMCs were isolated by density gradient centrifugation within 1.5 h of collection. Briefly, blood samples diluted with an equal volume of phosphate buffer solution (PBS) were layered over the Ficoll-Paque Premium (GE Healthcare BioSciences AB, Uppsala, Sweden) and centrifuged at 400 g for 30 min at 18–20°C. PBMCs were collected from the interface, then washed twice in PBS and adjusted to a concentration of 1 × 10⁷ cells/ml. Cell viabilities were more than 95% as examined by trypan blue exclusion assays.

CFSE Staining

Freshly isolated PBMCs that were used for proliferation analysis of T_{reg} cells were labeled with CFSE according to the manufacturer's instructions. Briefly, they were incubated with 5 µmol/l CFSE in serum-free PBS at 37°C in 5% carbon dioxide for 10 min. Labeling was terminated by adding a 40% volume of fetal bovine serum (Sigma) to quench the free CFSE. The labeled cells were then washed 3 times in PBS containing 5% fetal bovine serum (Sigma). After this step, greater than 99% of cells were positively stained and the fluorescence intensity ranged between 10³ and 10⁴ (data not shown) as measured by flow cytometry in a FACS Calibur instrument (Becton Dickinson, San Jose, Calif., USA).

Cell Culture

PBMCs were seeded at the density of 1 × 10⁶ cells/ml in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Sigma) in 6-well round-bottom cell culture plates. PBMCs derived from 10 nonallergic subjects were cultured without stimulation for 7 days, with 1 µg/ml rDer p 1 for 7 days, and with 10 µg/ml PHA for 48 h. CFSE-labeled PBMCs were cultured without stimulation and with 1 µg/ml rDer p 1 in duplicates for 7 days. Cultures were maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide. Culture medium was half-changed every 4 days with addition of rDer p 1 to the previous concentration.

Flow Cytometry Assay

To determine the percentage of Foxp3+ T cells in CD4+CD25+ T cells, cultured PBMCs were harvested 48 h later (PHA-stimulated cultures) or on day 7 (unstimulated and rDer-p-1-stimulated cultures) for flow cytometry assays. Cells were stained with a human regulatory staining kit according to the manufacturer's protocols. Briefly, cells were incubated with a cocktail of FITC antihuman CD4 and APC antihuman CD25 in the dark at 4°C for 30 min and washed with staining buffer for further intranuclear staining of Foxp3. Cells were then fixed and permeabilized with fixation/permeabilization buffer in the dark at 4°C for 1 h, washed twice with permeabilization buffer, blocked with normal rat serum (2% final volume) for 15 min, incubated with PE antihuman Foxp3 (PCHI01) in the dark at 4°C for 45 min, and washed twice with

Table 1. Subjects' clinical characteristic

	Sensitized asthmatic patients (n = 20)	Sensitized asymptomatic subjects (n = 24)	Nonallergic subjects (n = 22)
Sex (female/male)	12/8	14/10	10/12
Age, years	28 (18–39)	23 (19–30)	24 (18–33)
SPT to house dust mite	+	+	–
Specific IgE, IU/l	58 (0.78–101) ^a	41.65 (0.78–101) ^a	<0.34 (0–0)
FEV ₁ , % predicted	70.5 (48.0–81.0) ^b	94.0 (90–100)	94.2 (90–100)
PC ₂₀ Mch, mg/ml	1.21 (0.1–6.7) ^b	>32.0	>32.0

Data are medians, with ranges in parentheses, or numbers of subjects. PC₂₀ Mch = Provocative concentration of methacholine causing a 20% fall in FEV₁. ^a p = 0.000 versus nonallergic subjects; ^b p = 0.000 versus sensitized asymptomatic subjects and nonallergic subjects.

permeabilization buffer. Cocktails of FITC and APC mouse IgG1 isotype control and PE rat IgG2a isotype control were used.

To detect the percentages of CD4+CD25+ T cells and CD4+IL-10+ T cells in CD4+ T cells and their proliferations, CFSE-labeled PBMCs were harvested on day 7 for flow cytometry assays. For CD4 and CD25 stainings, cells washed and suspended with staining buffer were incubated with PE-Cy5 antihuman CD4 and PE antihuman CD25 in the dark at 4°C for 30 min and then washed twice with staining buffer. For intracellular cytokine IL-10 staining, cells were cultured with monensin (3 µmol/l in the medium) for the final 4 h and stained with PE-Cy5 antihuman CD4 as described above, then after fixation in intracellular fixation buffer in the dark at 18–20°C for 20 min and washing twice with permeabilization buffer, cells were incubated with PE antihuman IL-10 at 4°C for 30 min and washed twice with permeabilization buffer. PE mouse IgG1, K isotype control and PE-Cy5 mouse IgG1, K isotype control were used.

All the labeled cells were resuspended in 200 µl staining buffer and analyzed by flow cytometry using a FACS Calibur instrument (Becton Dickinson) with Cell Quest software package (Becton Dickinson). Lymphocytes were gated for analysis. The CFSE diluent analysis was performed using the Proliferation Wizard module of the Modfit software (Mac3.1 SP2, Verity Software House Inc.) within the gated CD4+CD25+ T cells and CD4+IL-10+ T cells.

Cytokine Assay

Concentrations of IL-10, IL-4, IFN-γ and TGF-β₁ in culture supernatants and sera were determined by ELISA kits according to the manufacturer's protocols. Culture supernatants were harvested on day 7 and stored cell free in aliquots at –70°C until analysis. The detection limits of assays were 1.1 pg/ml for IL-4, 7 pg/ml for TGF-β₁ and 12.5 pg/ml for IFN-γ and IL-10.

Statistical Analysis

Statistical analysis was performed with SPSS 10.0 software. Groups of 3 data sets were compared by using a Kruskal-Wallis test with a Dunn multiple comparison posttest. Groups of paired data were analyzed by using the Wilcoxon matched-pair signed-rank test. p values of less than 0.05 were considered to be statistically significant.

Results

Clinical Characteristics of Subjects

We recruited 20 patients with asthma sensitized to house dust mite during an acute exacerbation period, 24 asymptomatic donors sensitized to house dust mite and 22 healthy nonallergic subjects. Subjects' clinical characteristics are summarized in table 1.

Percentage of Foxp3+ T Cells among CD4+CD25+ T Cells in Cultured PBMCs

Because CD25 is upregulated during T cell activation, we compared the expression of Foxp3 in CD4+CD25+ T cells in unstimulated, rDer-p-1-stimulated and PHA-stimulated cultures of PBMCs derived from 10 nonallergic subjects. Increased percentages of CD4+CD25+ T cells were observed in rDer-p-1- and PHA-stimulated cultures compared with unstimulated ones. However, Foxp3+ T cells represented 79.8% (78.9–80.8%) of CD4+CD25+ T cells in unstimulated cultures, 76.5% (75.5–77.1%) in rDer-p-1-stimulated cultures and 14.5% (9.9–17.3%) in PHA-stimulated cultures on average. That is to say, most of the CD4+CD25+ T cells in unstimulated and rDer-p-1-stimulated cultures of human PBMCs were CD4+CD25+Foxp3+ T_{reg} cells. The increment of CD4+CD25+ T cells after stimulation with rDer p 1 was mainly due to the increment of CD4+CD25+Foxp3+ T_{reg} cells, while the increment of CD4+CD25+ T cells after stimulation with PHA was mainly due to T cell activation. Analysis of Foxp3 fluorescence for 1 representative donor gating on CD4+CD25+ T cells was shown in figure 1.

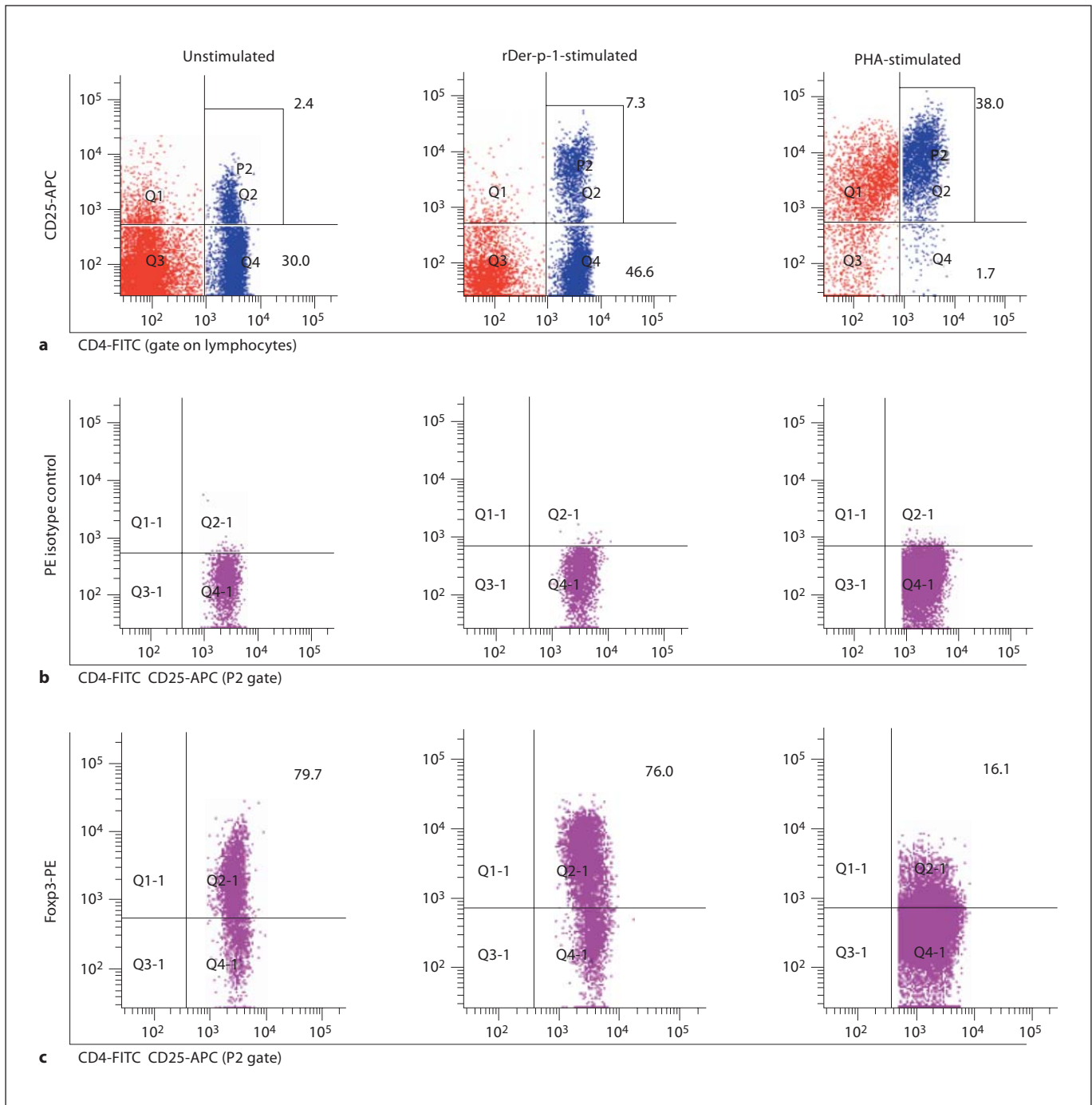


Fig. 1. Analysis of Foxp3 expression in CD4+CD25+ T cells in cultured PBMCs from nonallergic subjects. After culture without stimulation for 7 days, with 1 μ g/ml rDer p 1 for 7 days and with 10 μ g/ml PHA for 48 h, PBMCs were analyzed for the expression of CD4, CD25 and intranuclear Foxp3 by flow cytometry. A rep-

resentative analysis of CD4+CD25+ T cells in lymphocytes (**a**) and Foxp3+ T cells in the gated CD4+CD25+ T cells (**c**) in unstimulated, rDer-p-1-stimulated and PHA-stimulated cultures is shown. **b** PE rat IgG2a isotype control for PE-antihuman Foxp3 PCH101.

Table 2. Relative percentages of CD4+CD25+ T cells and CD4+IL-10+ T cells in cultured PBMCs derived from sensitized asthmatic patients, sensitized asymptomatic subjects and nonallergic subjects

Group	CD4+CD25+ T cells/CD4+ T cells, %		CD4+IL-10+ T cells/CD4+ T cells, %	
	unstimulated	rDer-p-1-stimulated	unstimulated	rDer-p-1-stimulated
Sensitized asthmatic	3.58 (1.52–13.39) ^a	4.71 (1.55–17.62) ^{b, d}	2.37 (0.47–10.02)	3.99 (1.34–15.04) ^c
Sensitized asymptomatic	13.47 (4.30–25.95)	16.60 (11.42–42.83)	8.62 (0.80–11.68)	10.21 (2.80–29.05)
Nonallergic	9.66 (4.28–27.02)	18.33 (5.64–28.12)	5.86 (0.68–14.98)	9.24 (2.92–23.18)

Data are median (range in parentheses) percentages of CD4+CD25+ T cells or of CD4+IL-10+ T cells in the population of CD4+ cells. ^a $p = 0.004$, ^b $p = 0.002$, ^c $p = 0.038$ versus sensitized asymptomatic subjects; ^d $p = 0.013$ versus nonallergic subjects.

Relative Percentages and Division Indexes of CD4+CD25+ T Cells and CD4+IL-10+ T Cells in Cultured PBMCs

Both CD4+CD25+ T cells and CD4+IL-10+ T cells were present in unstimulated and rDer-p-1-stimulated cultures of human PBMCs as shown in table 2.

The percentage of CD4+CD25+ T cells among CD4+ T cells in the sensitized asthmatic group was lower than that in the sensitized asymptomatic group without stimulation ($p = 0.004$). Although it was also lower than that in the nonallergic group, no significant difference was found between them without stimulation ($p = 0.06$). An increase in the percentage of CD4+CD25+ T cells after rDer p 1 stimulation was seen in all 3 groups (all $p < 0.05$). Interestingly, the increment in CD4+CD25+ T cell percentages in the sensitized asthmatic group was less than that in the sensitized asymptomatic group and the nonallergic group (fig. 2a). Therefore, the percentage of CD4+CD25+ T cells in the sensitized asthmatic group was lower than that in the sensitized asymptomatic group ($p = 0.002$) and the nonallergic group ($p = 0.013$) in rDer-p-1-stimulated cultures.

The percentage of CD4+IL-10+ T cells among CD4+T cells was the lowest in the sensitized asthmatic group and the highest in the sensitized asymptomatic group without stimulation, but this difference did not reach statistical significance ($p = 0.071$). The percentage of CD4+IL-10+ T cells increased after stimulation with rDer p 1 in all these 3 groups (fig. 2b). A significant difference in percentage of CD4+IL-10+ T cells was found only between the sensitized asthmatic group and the sensitized asymptomatic group in rDer-p-1-stimulated cultures ($p = 0.038$).

The percentages of CD4+CD25+ T cells and CD4+IL-10+ T cells among CD4+ T cells in the sensitized asymptomatic group were similar to those in the nonallergic

group both in unstimulated and rDer-p-1-stimulated cultures.

CFSE diluent analysis was performed on the gated CD4+CD25+ T cells and CD4+IL-10+ T cells in rDer-p-1-stimulated cultures. The division index, which is the average number of cell divisions that the responding cells underwent, was used for analysis. Analysis of CFSE fluorescence for 1 representative donor gating on CD4+IL-10+ T cells is shown in figure 3. The statistic 'precursor frequency' that the result provides is the same as the 'percent divided'. Division index = (proliferation index) (percent divided). On average, the division index of CD4+CD25+ T cells was 0.20 (0.04–0.31) in the sensitized asthmatic group, 0.82 (0.05–2.33) in the sensitized asymptomatic group and 0.74 (0.03–1.98) in the nonallergic group. No statistical difference was found among them ($p = 0.067$). The average division index of CD4+IL-10+ T cells was 0.20 (0.00–0.31) in the sensitized asthmatic group, 0.52 (0.12–1.86) in the sensitized asymptomatic group and 0.20 (0.01–0.43) in the nonallergic group. The p value was 0.058 as compared with the division index of CD4+IL-10+ T cells among stimulated cultures of these 3 groups.

Cytokine Levels in Culture Supernatants and Sera

We determined the concentrations of IL-10, IL-4, IFN- γ and TGF- β_1 in supernatants of cultured PBMCs from sensitized asthmatic patients, sensitized asymptomatic subjects and nonallergic subjects on day 7 by ELISA; data are shown in table 3 and figure 4.

Although the baseline concentration of IL-10 was the lowest in supernatants from the sensitized asthmatic group and the highest in those from the sensitized asymptomatic group, no statistical difference was found in unstimulated conditions among these 3 groups ($p = 0.057$). After stimulation with rDer p 1, the concentration

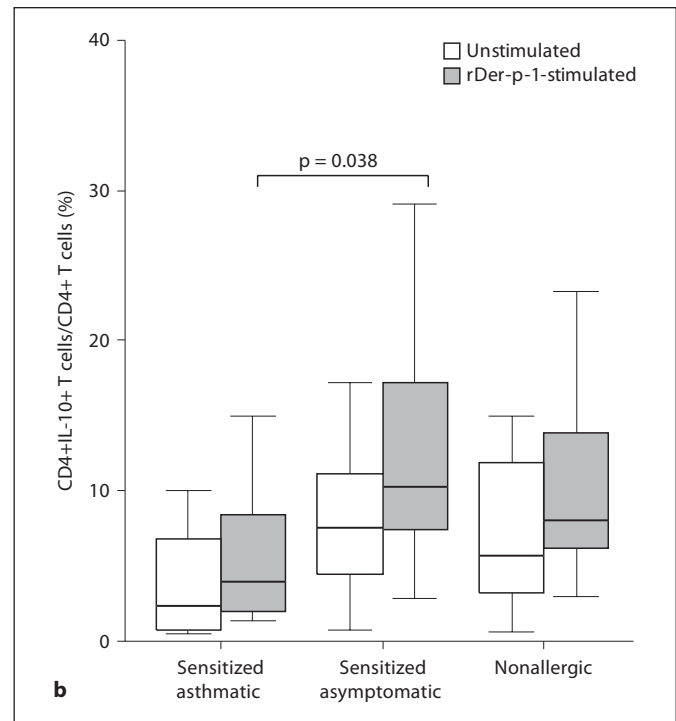
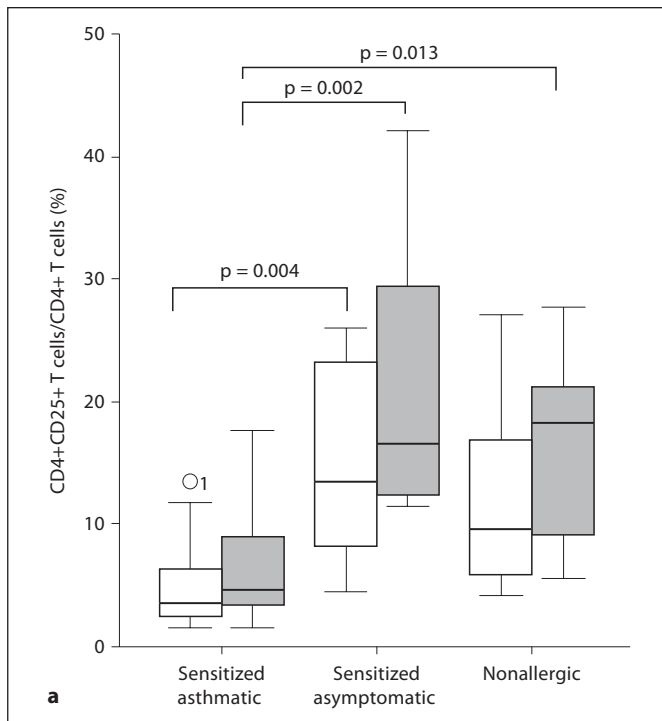
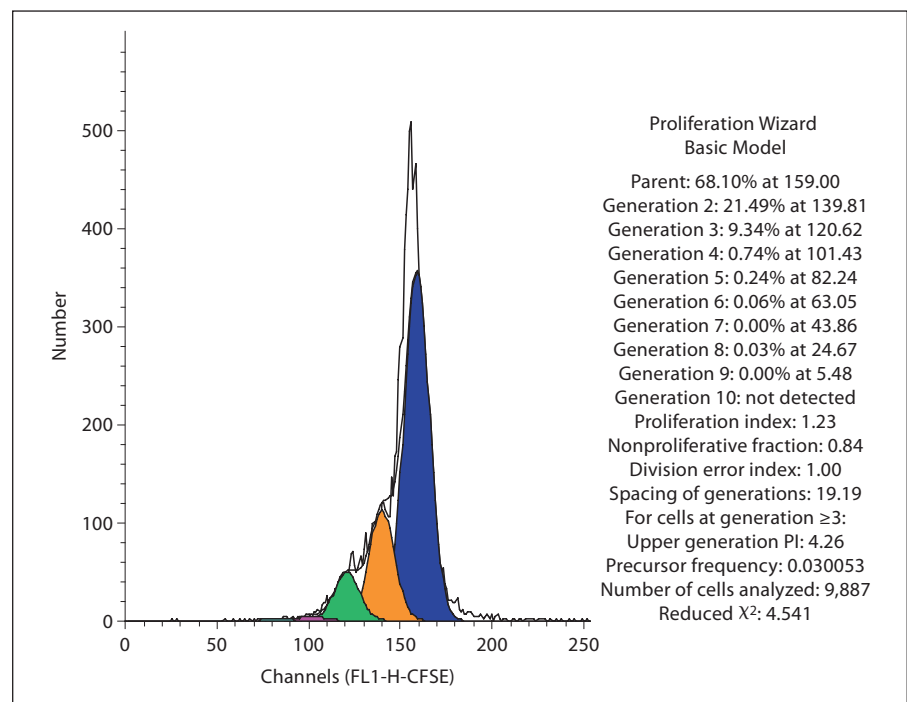


Fig. 2. Relative percentages of CD4+CD25+ and CD4+IL-10+ T cells in cultured PBMCs derived from 20 patients sensitized to house dust mite with asthma, 24 asymptomatic subjects sensitized to house dust mite and 22 nonallergic subjects. After 7 days of culture with 0 and 1 $\mu\text{g/ml}$ rDer p 1, cells were analyzed for the expression of CD4, CD25 and intracellular IL-10 by flow cytometry.

etry. The CD4+ T cells were gated, and the median (and range) percentages of CD4+CD25+ T cells (**a**) and of CD4+IL-10+ T cells (**b**) among CD4+ T cells without and with stimulation by rDer p 1 are shown. O1 shows one extreme case with a value of more than 3 box lengths from the upper or lower edge of the box.

Fig. 3. A representative CFSE diluent analysis of CD4+IL-10+ T cells. CFSE-labeled PBMCs were cultured with rDer p 1 for 7 days and analyzed for the expression of CD4 and IL-10 by flow cytometry. CFSE fluorescence was analyzed on gated CD4+IL-10+ T cells using the Proliferation Wizard module of the Modfit software (Mac3.1 SP2, Verity Software House Inc.). The statistic 'precursor frequency' that the result provides is the same as the 'percent divided'. Division index = (proliferation index) (percent divided).



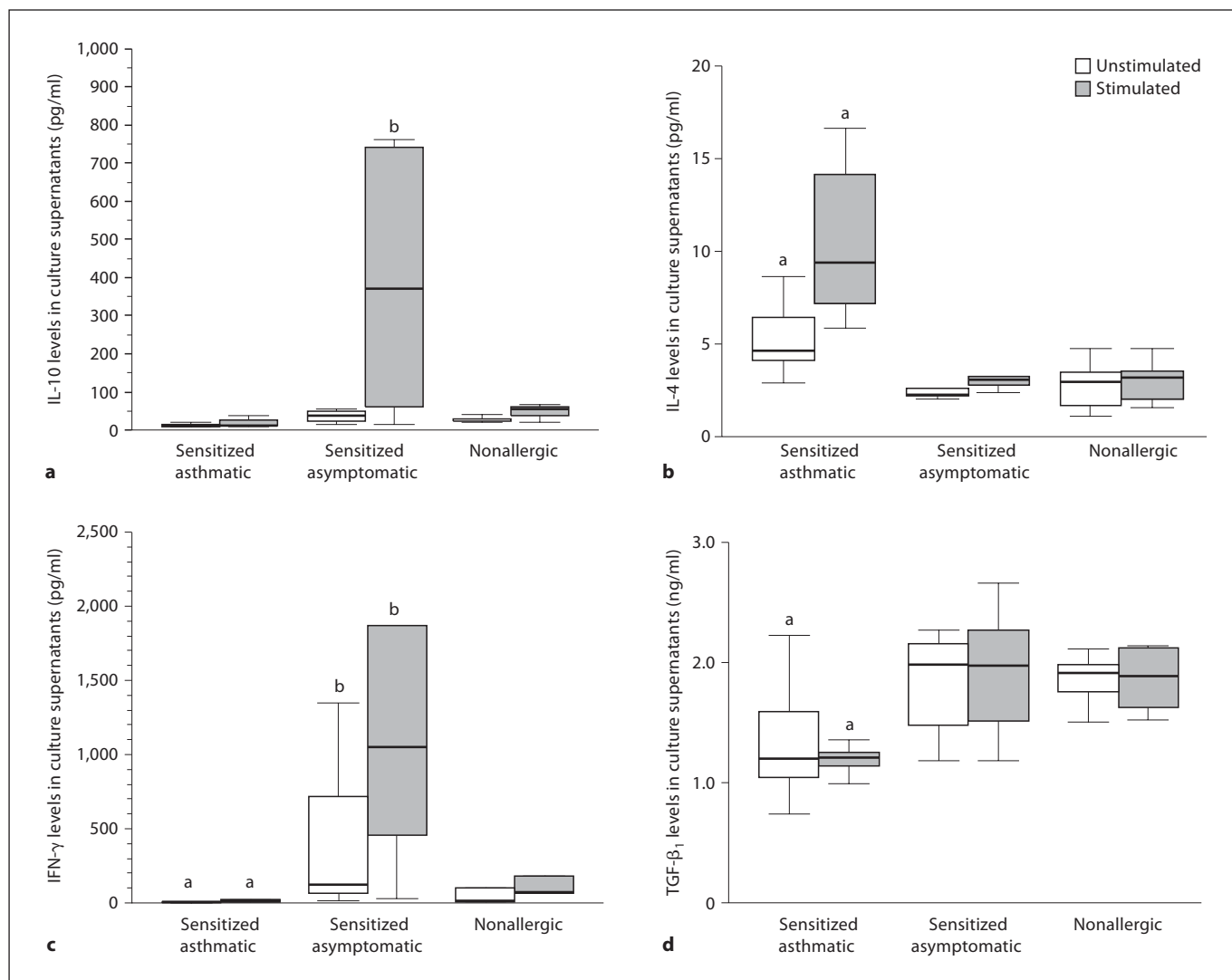


Fig. 4. Concentrations of IL-10 (**a**), IL-4 (**b**), IFN- γ (**c**) and TGF- β_1 (**d**) in culture supernatants of PBMCs derived from 20 patients sensitized to house dust mite with asthma, 24 asymptomatic subjects sensitized to house dust mite and 22 nonallergic subjects. Supernatants of unstimulated and rDer-p-1-stimulated cultures were harvested on day 7 and stored cell free in aliquots at -70°C

until analysis by ELISA. Bars represent median (and range) concentrations of these cytokines. ^a $p < 0.05$ as compared with sensitized asymptomatic subjects and nonallergic subjects; ^b $p < 0.05$ as compared with sensitized asthmatic patients and nonallergic subjects.

of IL-10 increased in the sensitized asymptomatic group ($p = 0.016$) and the nonallergic group ($p = 0.028$), but not in the sensitized asthmatic group, ($p = 0.474$). Moreover, the mean concentration of IL-10 dramatically increased to 6.8 times higher than in unstimulated cultures in the sensitized asymptomatic group, while it increased to only 3.2 times higher than that in unstimulated cultures in the nonallergic group. Therefore, cultured PBMCs from the sensitized asymptomatic group displayed the highest level of IL-10 among the 3 groups in rDer-p-1-stimulated

conditions ($p = 0.002$ as compared with the sensitized asthmatic group, $p = 0.01$ as compared with the nonallergic group).

The baseline level of IL-4 in supernatants from the sensitized asthmatic group was higher than those from the sensitized asymptomatic ($p = 0.017$) and nonallergic groups ($p = 0.013$). This difference was even more striking in rDer-p-1-stimulated cultures. The mean level of IL-4 in the sensitized asthmatic group doubled after stimulation, while it was not affected in the sensitized

Table 3. Concentrations of IL-10, IL-4, IFN- γ and TGF- β_1 in supernatants of cultured PBMCs derived from sensitized asthmatic patients, sensitized asymptomatic subjects and nonallergic subjects

Group	IL-10, pg/ml		IL-4, pg/ml		IFN- γ , pg/ml		TGF- β_1 , ng/ml	
	unstimulated	stimulated	unstimulated	stimulated	unstimulated	stimulated	unstimulated	stimulated
Asthmatic	13.67 (7.95–27.62)	44.76 (8.04–244.63)	5.33 (2.89–8.64) ^a	10.65 (5.84–16.62) ^a	3.65 (1.77–4.63) ^a	13.90 (4.71–24.37) ^a	1.13 (0.74–2.23) ^a	1.21 (0.85–2.14) ^a
Sensitized asymptomatic	57.66 (13.54–226.35)	390.74 (15.43–761.86) ^b	3.34 (2.20–5.05)	3.43 (2.00–5.68)	454.36 (17.48–1,351.43) ^b	1,056.20 (31.85–1,871.36) ^b	1.99 (1.18–2.27)	1.98 (1.18–2.66)
Nonallergic	26.21 (9.45–40.46)	84.05 (21.28–317.49)	2.76 (1.59–3.56)	3.35 (1.07–4.78)	40.11 (5.47–100.33)	105.59 (62.77–181.99)	1.79 (1.30–2.11)	1.89 (1.52–3.03)

Data are median (range in parentheses) concentrations of these cytokines. ^a $p < 0.05$ versus sensitized asymptomatic and nonallergic subjects; ^b $p < 0.05$ versus sensitized asthmatic patients and nonallergic subjects.

Table 4. Serum concentrations of IL-10, IL-4, IFN- γ and TGF- β_1 in sensitized asthmatic patients, sensitized asymptomatic and nonallergic subjects

Group	IL-10, pg/ml	IL-4, pg/ml	IFN- γ , pg/ml	TGF- β_1 , ng/ml
Sensitized asthmatic	6.76 (5.30–11.85)*	8.62 (4.74–15.63)*	22.80 (15.55–30.00)*	8.45 (3.07–13.39)*
Sensitized asymptomatic	8.31 (6.58–18.20)	5.20 (3.08–9.44)	38.40 (19.37–40.21)	4.42 (2.12–6.33)
Nonallergic	9.23 (6.87–14.94)	4.86 (2.27–8.69)	42.94 (21.03–45.58)	4.82 (0.69–8.59)

Data are median (range in parentheses) concentrations of these cytokines. * $p < 0.05$ versus sensitized asymptomatic subjects and nonallergic subjects.

asymptomatic and non-allergic groups. The concentration of IL-4 in supernatants from the sensitized asymptomatic group was comparable to those from the nonallergic group with and without stimulation.

The sensitized asthmatic group had the lowest baseline concentration of IFN- γ in culture supernatants, while the sensitized asymptomatic group had the highest baseline concentration among these 3 groups ($p = 0.031$). Although all increased after rDer p 1 stimulation, the concentration of IFN- γ in the sensitized asymptomatic group was still the highest among all ($p = 0.017$). When compared with supernatants from the sensitized asthmatic group, the mean level of IFN- γ in supernatants from the sensitized asymptomatic group was 124 times higher than that in unstimulated conditions and was still 75 times higher in rDer-p-1-stimulated conditions.

Concentration of TGF- β_1 in supernatants from the sensitized asthmatic group was lower than in the other 2 groups without and with stimulation (all $p < 0.05$). Stimulation with rDer p 1 did not affect the TGF- β_1 level in these 3 groups. Concentration of TGF- β_1 in supernatants

from the sensitized asymptomatic group was similar to that from the nonallergic group both in unstimulated and stimulated cultures.

To sum up, in unstimulated cultures, supernatants from the sensitized asthmatic patients displayed higher concentrations of IL-4 but lower concentrations of IFN- γ and TGF- β_1 than those from sensitized asymptomatic and nonallergic subjects. These differences were even more striking in rDer-p-1-stimulated cultures. Supernatants from sensitized asymptomatic and nonallergic subjects displayed similar levels of IL-4 and TGF- β_1 both in unstimulated and rDer-p-1-stimulated cultures. Interestingly, concentrations of IL-10 and IFN- γ dramatically increased after rDer p 1 stimulation in supernatants from sensitized asymptomatic subjects, which was not the case in supernatants from sensitized asthmatic patients and nonallergic subjects.

Serum concentrations of IL-10, IL-4, IFN- γ and TGF- β_1 were determined in each group and shown in table 4. Serum samples from the sensitized asthmatic group showed higher levels of IL-4 but lower levels of IFN- γ and

IL-10 than those from the other 2 groups, which is consistent with our findings in culture supernatants. The serum concentration of TGF- β_1 was the highest in the sensitized asthmatic group. Sera of the sensitized asymptomatic and nonallergic groups showed similar levels of IL-10, IL-4, IFN- γ and TGF- β_1 .

Discussion

Our study indicated that CD4+CD25+ T_{reg} cells in patients with allergic asthma might have insufficient reaction to specific allergen stimulation, so that they could not suppress excessive Th2 responses. In this study, both CD4+CD25+ T cells and CD4+IL-10+ T cells were present in cultured human PBMCs without allergen stimulation, and their relative percentages in sensitized asthmatic patients were not significantly different from those in nonallergic subjects. However, patients with allergic asthma had decreased percentages of CD4+CD25+ T cells, but not of CD4+IL-10+ T cells, as compared with nonallergic subjects after *in vitro* stimulation with rDer p 1. In addition, we found that IL-4 secretion in samples from allergic asthmatics dramatically increased after allergen stimulation and was much higher than that in sensitized asymptomatic subjects and nonallergic subjects, which could support this conclusion. A similar interesting observation regarding the role of CD4+CD25+ T_{reg} cells in the manifestation of allergy was studied by Karlsson et al. [22] in children with cow's milk allergy. Children who outgrew their allergy had higher numbers of CD4+CD25+ T cells in their blood than those who maintained clinically active allergies. The increased numbers coincided with decreased *in vitro* T-cell-proliferative responses to the food allergen, bovine β -lactoglobulin, but not with proliferative responses to polyclonal stimulation, also indicating the presence of allergen-specific CD4+CD25+ T_{reg} cells. Moreover, another study described defective suppression of Th2 cytokines by CD4+CD25+ T_{reg} cells in subjects allergic to birch pollen [14].

CD4+CD25+ T cells may represent either activated or T_{reg} cells. CD4+CD25+ T_{reg} cells are characterized by expression of the transcription factor Foxp3 [9, 25]. We determined Foxp3 expression in CD4+CD25+ T cells using antihuman Foxp3 (clone PCH101) and found that most CD4+CD25+ T cells express Foxp3 in unstimulated and rDer-p-1-stimulated cultures but not in PHA-stimulated cultures. The hypothesis was that CD25 expression in unstimulated and rDer-p-1-stimulated PBMCs was not

due to recent cell activation. Other studies investigated the surface expression of activation markers on CD4+CD25+ T cells isolated from untreated and PHA-stimulated PBMCs. CD69 expression was detectable on CD4+CD25+ T cells isolated from PHA-stimulated PBMCs, but not in the majority of CD4+CD25+ T cells isolated from untreated PBMCs [26, 27]. Moreover, the percentages of CD4+CD25+ T cells detected in our study are in agreement with Sakaguchi et al. [28], who first described CD4+CD25+ T_{reg} cells as a small subpopulation of CD4+ T cells whose ratio is about 5–10% of circulating CD4+ T cells in humans. In this study, cells from different groups were cultured under the same conditions, so we conclude that the decrease in the baseline percentage and insufficient allergen-induced increment in CD4+CD25+ T_{reg} cells in patients with allergic asthma is valid.

Our present study also indicated that asymptomatic sensitized subjects displayed a different pattern of immunological response to allergen stimulation. CD4+CD25+ T_{reg} cells and CD4+IL-10+ T_{reg} cells in sensitized asymptomatic subjects were normal, for their frequency was comparable to that in nonallergic subjects (or even higher in some samples) without and with stimulation by rDer p 1. In addition, sensitized asymptomatic subjects had higher percentages of both CD4+CD25+ T_{reg} cells and CD4+IL-10+ T_{reg} cells than sensitized asthmatic patients after allergen stimulation. Sensitized asymptomatic subjects differ from nonallergic subjects in their high levels of IL-10 and IFN- γ after specific allergen stimulation. Moreover, their cytokine pattern differed from that of sensitized asthmatics, i.e. they displayed low levels and only small increments of IL-4 but high levels and conspicuous increments of IL-10 and IFN- γ production after allergen stimulation, a pattern similar to that of allergic individuals who received immunotherapy in other researches [29, 30]. Jutel et al. [20] investigated immunotherapy with house dust mite and found allergen-induced IL-10 and TGF- β productions by T cells in parallel to a global suppression of Th2-proliferative responses and cytokine production. IL-10 and TGF- β were detectable at 7 days into a semi-rush immunotherapy protocol. Similar IL-10 responses were observed in healthy nonallergic subjects. The conclusion was that immunotherapy restored a tolerant T-cell response in allergic subjects similar to that observed in healthy individuals. Moreover, other studies have shown that increased IL-10 production after immunotherapy is not limited to T cells, other cells in PBMCs such as B cells, monocytes and macrophages also produce IL-10 after immunotherapy [19, 20, 31]. The

similar immunological changes might account for the occurrence of 'naturally' induced immunology tolerance [19, 22] and can partly explain the immunological characteristics associated with sensitized asymptomatic subjects.

In summary, cultured PBMCs from patients with allergic asthma displayed an impaired CD4+CD25+ T_{reg} cell increase reaction after specific allergen stimulation and therefore had the highest level of IL-4 secretion; sensitized asymptomatic subjects had a normal frequency of CD4+CD25+ and CD4+IL-10+ T cells but the highest levels of IL-10 and IFN- γ responses to allergen, which can partly explain the occurrence of asymptomatic sensitization to house dust mite; the rDer-p-1-induced increment in number of CD4+CD25+ T_{reg} and CD4+IL-10+ T_{reg} cells in sensitized asthmatic patients was less than that in sensitized asymptomatic subjects. All these data indicate that the frequency of CD4+CD25+ T_{reg} and CD4+IL-10+ T cells and the subsequent cytokine pattern play an important role in the manifestations of allergy and allergic diseases. Cultures of human blood mononuclear cells

might provide only a crude analysis of immune interactions and responses in lymphoid tissues, mucosal tissues or both. More research into the mechanism by which T_{reg} cells promote the development of tolerance to environmental allergens is needed. It may lead to novel strategies for the prevention and treatment of allergic diseases, including asthma.

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