

Allergen-specific T cells from birch-pollen-allergic patients and healthy controls differ in T helper 2 cytokine and in interleukin-10 production

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Summary

Background T helper (Th)2 cells play an important role in the development of IgE-mediated diseases, with local overproduction of Th2 cytokines (IL-4, IL-5 and IL-13) at the site of allergic inflammation. Furthermore, IL-10 has been suggested to play a modulatory role in the induction and maintenance of allergen-specific tolerance in human atopic diseases.

Aim We studied whether circulating allergen-specific Th2 cells persist outside the season of exposure in patients mono-sensitized to birch pollen and whether healthy control individuals also have allergen-specific Th2 cells. We also studied whether IL-10-producing allergen-specific T cells can be found in circulation either in healthy controls or in allergic patients.

Methods Blood was drawn outside the birch-pollen season from 15 birch-pollen-allergic patients, with seasonal respiratory symptoms and with ($n = 12$) or without ($n = 3$) oral allergy syndrome, and from 10 matched healthy controls. Peripheral blood mononuclear cells (PBMCs) were stimulated *in vitro* with recombinant Bet v 1 allergen, control antigen tetanus toxoid (TT) and anti-CD3/CD80. In part of the cultures, rIL-4 was added in order to reinforce the allergen-specific Th2 cell responses.

Results In the presence of rBet v 1, T cells from allergic patients, but not from healthy controls, produced IL-4, IL-5 and IL-13. IL-5 production by patients' T cells was further enhanced by adding more IL-4. In contrast, rBet v 1 together with IL-4-induced significant IL-10 production in control subjects but not in patients. Both Th1 and Th2 cytokines were equally induced by polyclonal stimulation in allergic patients and controls, but in the presence of IL-4, polyclonally induced IL-10 production was lower in the patient group.

Conclusion rBet v 1-specific Th2 cells circulate outside the season of exposure in the blood of birch-pollen-allergic subjects but not in healthy controls. Allergen-specific T cells were also demonstrated in controls but these cells produce IL-10 when stimulated with rBet v 1 in the presence of IL-4. Our data reveal a different allergen-induced cytokine profile in birch-pollen-allergic patients vs. controls, and suggest that a regulatory mechanism involving IL-4-induced allergen-specific IL-10 production might be defective in allergic subjects.

Keywords allergen, Bet v 1, IL-10, immune regulation, Th2 cytokines

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Introduction

Recent insights into the development of allergic diseases such as allergic rhinitis, asthma and atopic eczema are based on the functional diversity of T helper (Th)1 and Th2 lymphocytes [1]. Th2 cells (secreting IL-4, IL-5, IL-9 and IL-13) are now considered to be responsible for the induction and for many of the manifestations of atopic diseases [1, 2]. In these diseases, IL-4 plays a key role in driving the differentiation of CD4⁺ Th precursor into Th2 and in inducing B cells to switch to IgE production [3]. IL-5 stimulates eosinophil generation, it is chemotactic for eosinophils; it activates mature eosinophils and prolongs their survival. IL-13 also is a central

mediator of allergic asthma, being required for airway hyper-responsiveness [4]. Besides Th2 cytokines, there is rising interest in the so-called regulatory cytokines that modulate Th1 and Th2 responses. IL-10 has been suggested to play a modulatory role in the induction and maintenance of allergen-specific tolerance in human atopic diseases [5] and has been shown to be an essential cytokine for the induction and the function of at least some types of regulatory T cells [6]. IL-10 also inhibits eosinophil survival and IL-4-induced IgE synthesis [7]. Regulatory T cells and IL-10 might thus play a role in the pathogenesis of atopic diseases. Whether allergic patients have altered allergen-induced IL-10 production and/or allergen-specific regulatory T cells is thus far not clear.

Local overproduction of Th2 cytokines (IL-4, IL-5 and IL-13) at the site of allergic inflammation [8, 9], and an intrinsic defect in the production of IFN- γ by T cells in atopic individuals, have now been reported by several authors [10, 11]. Predominance of Th2 clones among allergen-specific

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T cell clones from allergic individuals has nicely been demonstrated [12, 13]. Furthermore, allergic patients possess circulating allergen-specific T cells. Indeed, Till et al. have demonstrated enhanced IL-5 and IL-13 production by peripheral blood mononuclear cells (PBMCs) from allergic individuals, stimulated with grass-pollen (Phl p) or house dust mite (HDM) extract (Der p) [14, 15]. Also, in children and infants with atopic dermatitis, higher IL-4 and IL-5 production by HDM-stimulated PBMC compared with healthy controls has been demonstrated [16–18]. Those differences, however, seem to be age dependent and lost after the age of 8 years [18]. PBMC from children with bronchial asthma also have increased IL-4 and IL-13 levels after HDM stimulation, when compared with PBMC from healthy control children [19]. PBMC from individuals sensitized to grass pollen and suffering from allergic rhinitis produced IL-4 when stimulated with grass-pollen extract, if blood was drawn immediately after the peak of the grass-pollen season, whereby T cells from controls mostly failed to produce detectable IL-4 [20]. An allergen-specific Th2 predominance has thus been shown in atopics. However, whether healthy control individuals possess allergen-specific T cells is still debated.

It is also controversial whether enhanced Th2 cytokine production in atopic individuals is allergen specific or is a general characteristic of T cells. Indeed, enhanced IL-4 production by PBMC in grass-pollen-allergic patients compared with healthy controls was found after allergen-specific T cell stimulation, but not if T cells were polyclonally stimulated [21]. Others found, however, increased IL-4 and IL-5 production by PBMC from atopics when compared with healthy controls if cells were stimulated with superantigen (SEB) as well as with allergens (HDM and ryegrass) [22]. Moreover, poly-sensitized patients produced higher amounts of IL-4 upon polyclonal stimulation as compared with mono-sensitized allergics [23].

Here, we wanted to study whether circulating allergen-specific Th2 cells are persisting outside the birch-pollen season in patients mono-sensitized to birch pollen and whether healthy control individuals also possess allergen-specific T cells. IL-4 is known to increase IL-5 and IL-13 production [24, 25]. In order to increase the sensitivity of allergen-specific Th2 cytokine detection, we also studied allergen-specific T cell responses in the presence of IL-4. Furthermore, we addressed the question whether a Th2 predominance is a general characteristic of T cells from allergic individuals. Finally, we studied whether IL-10-producing allergen-specific T cells are circulating either in healthy controls or in allergic patients.

Materials and methods

Subjects

The study included 15 adult patients (seven males and eight females), mono-sensitized to tree pollen, who attended the outpatient clinic of the Allergy Division, Department of Internal Medicine, University Hospital Gasthuisberg. All patients had early spring seasonal symptoms and positive skin prick test (SPT) to birch-pollen extract (HAL, Haarlem, the Netherlands). Twelve out of 15 patients presented with oral allergy syndrome. Four out of 15 patients also had another

positive SPT without any symptoms related to that sensitization at present or in the past: dog or cat dander (2), grass pollen (2) or HDM (2). Ten matched healthy adult volunteers (four males and six females), without a history of allergic symptoms, and negative SPT were used as controls. SPT were performed on the volar aspects of the forearms with a panel of standardized extracts (from HAL), including grass-pollen mixture, tree-pollen (birch, alder and hazel), weed-pollen mixture, moulds, HDM and pets in symptomatic patients, and with standardized extracts to birch-, alder- and hazel-pollen in healthy controls. Aas' criteria were used to score the SPT; a reaction was regarded as positive when the weal was identical to or larger than that of the histamine control [26, 27]. The blood was drawn outside the birch-pollen season, i.e. between September and December 2001.

The study was approved by the local ethical committee of the Faculty of Medicine, Leuven.

Isolation of peripheral blood cells

PBMC were obtained by venous puncture on Sodium-Heparin vacuum tubes.

Blood was centrifuged on Ficoll-Hypaque density gradients. Interphase cells were washed three times with Dulbecco's phosphate-buffered saline (PBS; Boehringer Ingelheim Bioproducts, Heidelberg, Germany). They were resuspended at a concentration of 10×10^6 cells/mL in complete medium, consisting of RPMI 1640 (Boehringer Ingelheim Bioproducts) supplemented with 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% bovine calf serum (Hyclone, Logan, UT, USA) (for cytokine measurements). Optimal doses of stimulatory agents and of the cytokines used, together with kinetics of cytokine secretion, were determined in an independent pilot study on four allergic and four healthy adult volunteers.

Monoclonal antibodies (mAbs) and cytokines

Monoclonal mouse anti-human IL-4R antibody (clone 25463.11) (azide-free) was bought from R&D (Abingdon, UK). The mAb was raised by immunizing mice with recombinant soluble IL-4R α . The mAb blocks the bioactivity of both IL-4 and IL-13 and 3–6 ng of mAb neutralizes 50% of the bioactivity of 200 pg of IL-4 on 10^5 TF-1 cells in a 1-mL volume. One milligram contains <10 ng endotoxin and the mAb is sold azide-free. We have demonstrated that this mAb has an efficient blocking effect on the *in vitro* IL-4 consumption and on the IL-4 activity at the level of both B cells and T cells [28]. Moreover, the mAb by itself has no stimulating effect on B or T cell proliferation. In all experiments the mAb was used at a concentration of 2.5 µg/mL [28].

MAb UCHT-1 (mouse anti-human CD3) was a gift from Dr P. Beverley, E. Jenner Institute for vaccine Research (Berkshire, UK). rIL-4 was kindly provided by Innogenetics (Ghent, Belgium). Recombinant human IFN- γ and rIL-2 were purchased from Boehringer Mannheim (Mannheim, Germany), whereas rIL-10 was bought from PeproTech Inc. (Rocky Hill, NJ, USA).

Cytokine measurements

The concentrations of IL-4, IL-5, IL-10, IL-13 and IFN- γ in culture supernatants were measured with a sandwich ELISA technique using combinations of unlabelled and biotin-coupled mAb to different epitopes of each cytokine. Coating mAb for these five assays, respectively, were 860A4B3 (Medgenix Diagnostics, Fleurus, Belgium), TRFK5 (Pharmingen, San Diego, CA, USA), JES3-9D7 (Pharmingen), JES10-5A2 (Pharmingen) and 350B10G6 (Medgenix Diagnostics). Biotinylated detection mAb were 860F10H12 (Medgenix Diagnostics), JES1-SA10 (Pharmingen), JES3-12G8 (Pharmingen), B69-2 (Pharmingen) and 67F12A8 (Medgenix Diagnostics). Human recombinant IL-4, IL-5, IL-10, IL-13 and IFN- γ were used as standards. The lower detection limit for all cytokines was <20 pg/mL.

Activation of T cells for cytokine production

PBMC (1×10^6) were cultured in 24-well culture plates in a 1 mL volume at 37 °C in 5% CO₂/95% air and supernatants were collected after 3 or 7 days of stimulation as indicated in the results. For allergen-induced T cell stimulation, rBet v 1 (Biomay, Linz, Austria) was used at a concentration of 1 μ g/mL. Recombinant Bet v 1 has been compared with natural Bet v 1 and had comparable functional effects [29]. Human albumin was used as a control protein at a concentration of 1 μ g/mL (Behring, Marburg, Germany). For recall Ag-induced T cell stimulation, PBMC were cultured with soluble tetanus toxoid (TT) (RIVM, Bilthoven, the Netherlands) (40 lab U/mL). T cells were also stimulated with soluble anti-CD3 mAb (UCHT-1) captured by mouse P815/CD80 mastocytoma cells, at a T/P815 ratio of 1/1. The P815 cell line is an NK-resistant DBA/2-derived mouse mastocytoma cell line that expresses mouse Fc γ RII and Fc γ RIII. P815 cells transfected with human CD80 were a gift from L. L. Lanier (then at DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA, USA). P815 cells were mitomycin-C treated and washed five times before use. rIL-4, if added, was used at a concentration of 5 ng/mL. rIL-10, where added, was used at a concentration of 20 ng/mL.

Intracellular staining

PBMC (1×10^6) were cultured in a 1 mL volume at 37 °C in 5% CO₂/95% air for 6 days in the absence or presence of either rBet v 1 (Biomay), rIL-4 (Innogenetics) or both. During the last 24 h of culture, 3 mM monensin (Sigma Diagnostics, St Louis, MO, USA) was added to the cultures. Phorbol myristate acetate (Sigma Chemical, St Louis, MO, USA) (1 ng/mL) and Ionomycin (Sigma Chemical) (0.5 μ g/mL) were added during the last 4 h of culture to express the imprinted cytokine pattern [30]. For membrane staining either 10 μ L fluorescein isothiocyanate (FITC)-conjugated IgG1 antibody (Becton Dickinson, San Diego, CA, USA), FITC-labelled anti-CD3 mAb (Pharmingen) or FITC-labelled anti-CD14 mAb (Becton Dickinson) was added for 30 min at 4 °C. Cells were washed with PBS containing 0.5% bovine serum albumin (BSA; Sigma Diagnostics), collected and fixed with 500 μ L paraformaldehyde (Janssen Pharmaceutica, Beerse, Belgium) 2% in PBS at 4 °C for 10 min. For permeabilization, cells were washed with PBS containing 0.5% BSA (Sigma

Diagnostics) and 0.2% saponin (Sigma Diagnostics). Cells were then resuspended in 100 μ L of this permeabilization buffer and 10 μ L normal mouse serum was added for 5 min at room temperature. Ten microlitres of either PE-conjugated control IgG1 mAb (Pharmingen) or PE-labelled anti-human IL-10 mAb (Pharmingen) was added. Tubes were gently mixed for 30 min in the dark at room temperature. Cells were resuspended and washed twice with permeabilization buffer, resuspended in PBS and analysed with a FACSort flowcytometer (Becton Dickinson). Calibration was carried out with CaliBRITE 3, unlabelled, FITC, PE, PerCP (Becton Dickinson).

Plots were analysed with the Cell Quest software.

Statistical analysis

Results were compared with either non-parametric Mann-Whitney *U*-test, two- or one-tailed where appropriate, or non-parametric Wilcoxon's paired test, where possible. A *P*-value of 0.05 was considered as statistically significant. Calculations were performed with Prism Graph 3 (GraphPad Software Inc., San Diego, CA, USA) for Macintosh.

Results

Th1 and Th2 cytokine production by allergen-stimulated T cells

We studied the *in vitro* allergen-induced T cell cytokine profile in PBMC cultures of 15 birch-pollen-allergic patients and 10 matched healthy controls. PBMC (1×10^6 /mL) were stimulated with rBet v 1, the major allergen from birch pollen, and cytokines were measured in the supernatants collected on day 7. To block *in vitro* IL-4 consumption, anti-IL-4R mAb was added in those conditions used to measure IL-4 [28], as described in Materials and methods. As shown in Fig. 1a, rBet v 1-induced significant IL-5, IL-13 and IL-4 production in cultures from allergic subjects but not in those from healthy controls. rBet v 1 did not induce significant IFN- γ production in controls or in allergic subjects (data not shown). Cytokine profiles in allergic patients did not significantly differ from the profiles of healthy controls if PBMC were cultured either in medium alone (Fig. 1a) or in the presence of human albumin used as a negative control (data not shown).

To study whether the observed difference in IL-5 production was allergen specific, cells were stimulated with a non-allergenic recall protein: TT. As shown in Fig. 1b, IL-5 as well as IL-13 and IL-4 were induced by TT in allergic patients and in control subjects. The amount of those cytokines produced by T cells from the allergic subjects in response to TT was in fact lower than the amount produced by T cells from healthy controls.

In the same line, T cells were polyclonally stimulated with anti-CD3 mAb presented on a P815 cell-line transfected with human CD80. As can be seen in Fig. 1b, no differences in polyclonally induced IL-5, IL-13 or IL-4 production could be observed between birch-pollen-allergic patients and healthy controls. IFN- γ production (median 29 ng/mL in the control group and 16 ng/mL in the patient group, respectively) and

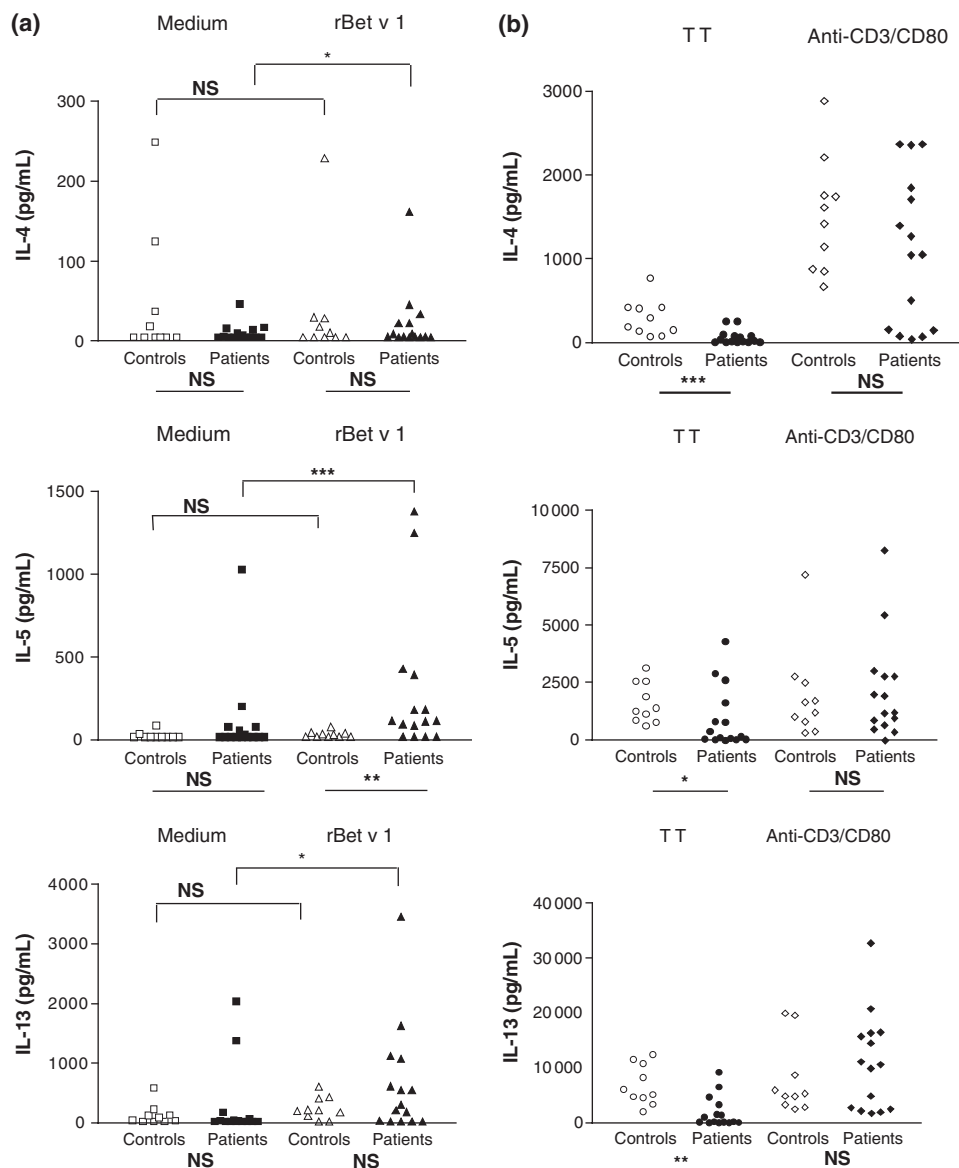


Fig. 1. T helper 2 cytokine production by stimulated T cells. Peripheral blood mononuclear cells (1×10^6 in 1 mL) were cultured in medium alone (a) or stimulated with either rBet v 1 (a) ($1 \mu\text{g/mL}$) or tetanus toxoid (TT) (b) or anti-CD3 monoclonal antibody (mAb) ($2 \mu\text{g/mL}$) presented on 1×10^6 P815/CD80-transfected cells (b). In cultures for IL-4 measurement, anti-IL-4R mAb ($2.5 \mu\text{g/mL}$) was added. Supernatants were collected after 3 (anti-CD3/CD80) or 7 days (medium, rBet v 1 and TT), and the concentrations of IL-4, IL-5 and IL-13 in the supernatants were detected by ELISA. The results of the experiments from 10 controls and 15 birch-pollen-allergic patients are shown. Wilcoxon's paired test was used to test the differences in cytokine concentration between medium and Bet v 1 conditions. Mann-Whitney *U*-test was used to test differences between healthy controls and birch-pollen-allergic patients. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and NS, not significant.

the Th2/Th1 ratios were also comparable between both groups (data not shown).

Thus, an rBet v 1-specific response was detected only in birch-pollen-allergic patients and was characterized by rBet v 1-induced production of IL-5, IL-13 and IL-4. Enhanced Th2 cytokine secretion, however, was not a general characteristic in this group of birch-pollen-allergic patients.

Response of allergen-stimulated T cells to IL-4

IL-4 increases the production of both IL-5 and IL-13 [24, 25]. In order to reinforce the allergen-specific Th2 cytokine production, we stimulated PBMC from allergic patients and healthy controls with rBet v 1 in the absence or presence of

rIL-4. 'rBet v 1-induced' IL-5 produced by T cells from allergic subjects (but not from controls) was further increased in the presence of rIL-4 (Fig. 2a). 'rBet v 1-induced' IL-13 production in patient samples on the contrary remained unchanged with IL-4 addition (Fig. 2b). It can be concluded that IL-4 further enhances IL-5 production by allergen-specific T cells in allergic individuals but does not enhance allergen-specific IL-5 production in healthy individuals.

Differences in T cell IL-10 production between healthy individuals and healthy controls

IL-10 has been suggested to play a modulatory role in the induction and maintenance of allergen-specific tolerance in

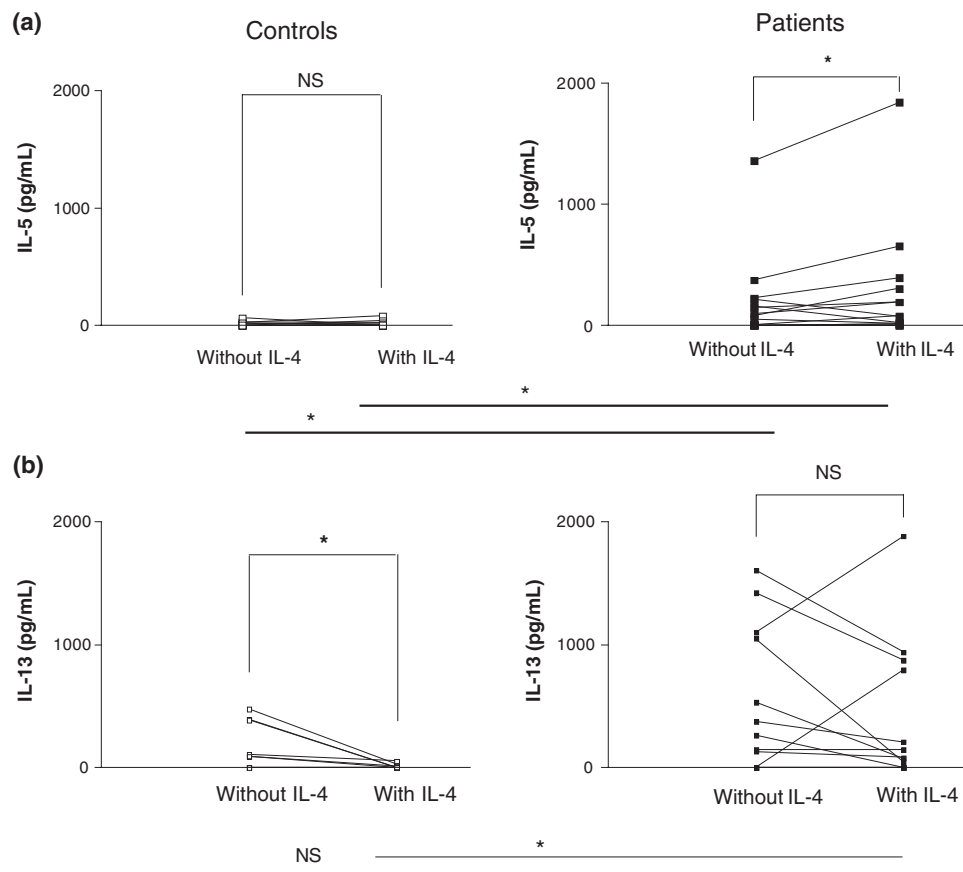


Fig. 2. Effect of IL-4 on rBet v 1-stimulated T cell cytokine production. Peripheral blood mononuclear cells (1×10^6 in 1 mL) were stimulated with rBet v 1 (1 μ g/mL) in the absence or presence of rIL-4 (5 ng/mL). Supernatants were collected after 7 days, and the concentrations of IL-5 (a) and IL-13 (b) in the supernatants were detected by ELISA. rBet v 1-induced production of IL-5 and IL-13 was calculated by subtracting the value obtained in medium conditions from its value in medium+rBet v 1. If the value obtained in medium conditions was higher than in medium+rBet v 1, the calculated production was zero. The paired results without and with the addition of rIL-4 (tested by Wilcoxon's paired test) of the experiments from 10 controls and 15 birch-pollen-allergic patients are shown. The differences between healthy controls and birch-pollen-allergic patients were tested by the use of the Mann-Whitney *U*-test. * $P \leq 0.05$ and NS, not significant.

human atopic diseases [5]. It is an essential cytokine for the induction and the function of at least some types of regulatory T cells [6]. We therefore wanted to study whether allergic patients have altered allergen-induced IL-10 production. rBet v 1 did not induce significant IL-10 production in neither patients or in controls. Since IL-10 is at least partly IL-4 dependent [28, 31], we also tested IL-10 production in the presence of IL-4. Adding rIL-4 to PBMC cultured in medium significantly enhanced IL-10 production both in cultures from control subjects and from allergic patients (Fig. 3a). Importantly, in the presence of IL-4, rBet v 1 induced significant IL-10 production in PBMC cultures from controls (Fig. 3a), but not from allergic individuals.

In order to study the source of the allergen-induced IL-10 production, 1×10^6 PBMC from four healthy donors were stimulated in the absence or presence of rBet, rIL-4 or both. In the gated anti-CD14-positive population (monocytes), no significant IL-10 production was detectable (data not shown). However, in a significant percentage of CD3-positive cells, intracellular IL-10 production could be detected after stimulation with rBet v 1 alone or rBet v 1+rIL-4 (Table 1). Comparison of these results with those of protein analysis in the supernatants indicates that IL-4 is important not for

the induction but for the secretion of IL-10 by allergen-specific T cells.

To study whether the defect in allergen-induced IL-10 production was a general phenomenon in allergic individuals, IL-10 production by polyclonally activated T cells was studied by stimulating PBMC for 3 days with anti-CD3 mAb (2 μ g/mL) captured by P815/CD80-transfected cells, in the absence or presence of rIL-4. Controls and patients produced similar amounts of IL-10 in the absence of rIL-4 (median 3131 and 1349 pg/mL, respectively). Adding rIL-4 to these polyclonally activated cultures significantly enhanced the IL-10 production by T cells from healthy controls (median 3773 pg/mL, $P = 0.03$) but did not influence the productions of this cytokine in allergic individuals (median 1468 pg/mL, $P = 0.34$) (Fig. 3b). Upon stimulation with anti-CD3- and CD80-transfected cells in the presence of rIL-4, IL-10 production was significantly higher in healthy controls compared with allergic patients ($P = 0.012$) (see Fig. 3b).

We can conclude that significant IL-10 production in response to rBet v 1 and IL-4 can be induced in cultures from control subjects but not from patients. Moreover, in the presence of IL-4, polyclonally activated T cells from birch-pollen-allergic patients produce lower amounts of IL-10 when

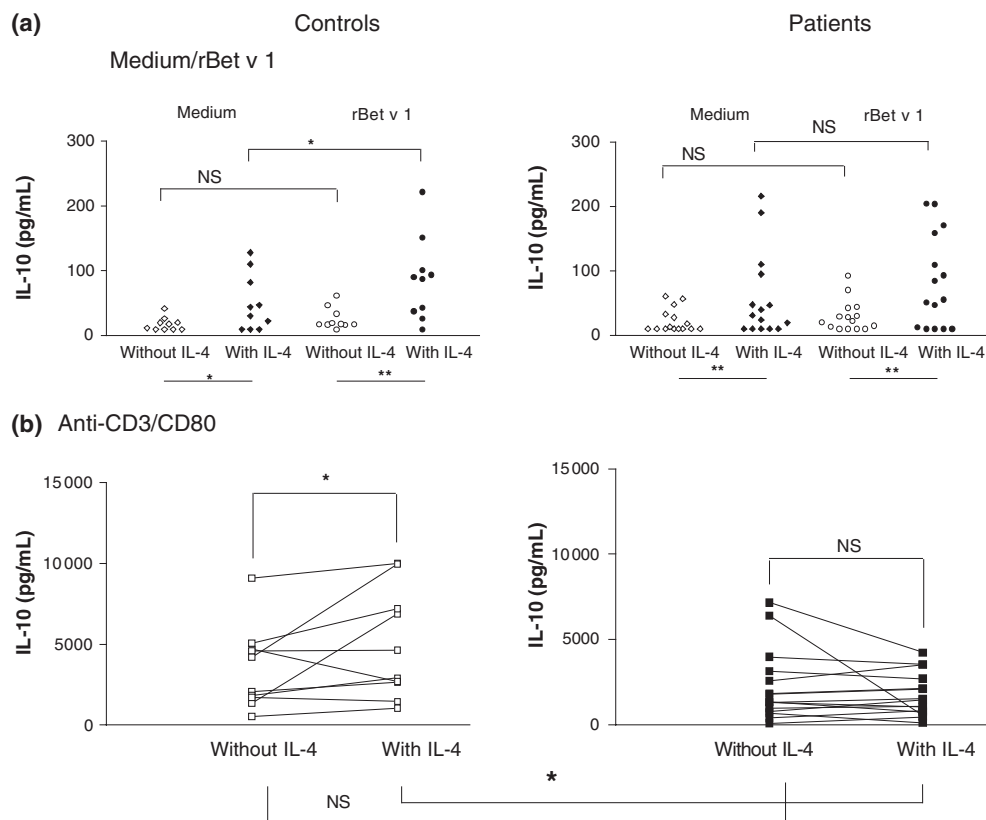


Fig. 3. Allergen-induced and polyclonally induced IL-10 production. 1×10^6 peripheral blood mononuclear cells were cultured either in medium alone (a) or in the presence of rBet v 1 ($1 \mu\text{g/mL}$) (a) or anti-CD3 monoclonal antibody presented on 1×10^5 p815/CD80 cells (b) in the absence or presence of rIL-4 (5 ng/mL). Supernatants were collected after 7 (a) or 3 (b) days, and the concentrations of IL-10 in the supernatants were detected by ELISA. The results without and with addition of rIL-4 of the experiments from 10 controls and 15 birch-pollen-allergic patients are shown. Effect of IL-4 was tested by Wilcoxon's paired test and the differences between healthy controls and birch-pollen-allergic patients were tested by the use of Mann-Whitney *U*-test. * $P \leq 0.05$, ** $P \leq 0.01$ and NS, not significant.

compared with T cells from healthy controls. These data point towards a defect in IL-10 production in birch-pollen-allergic subjects.

Discussion

We demonstrate here that, even outside the birch-pollen season, rBet v 1-specific Th2 cells producing IL-4, IL-5 and

IL-13 upon allergen contact can be detected in the blood of allergic patients sensitized to birch pollen, but not in healthy controls. Circulating allergen-specific Th2 cells therefore persist outside the birch-pollen season, at least in adults. An increased number of IL-13-producing peripheral blood T cells in birch- or grass-pollen allergics compared with controls after stimulation with the allergen and outside the pollen season has also been found by Gabrielsson et al. [32]. Our findings are in contrast to the findings of Rudin et al. [17], who suggested that grass-pollen-specific T cells from children,

Table 1. Percentage of intracellular IL-10-positive CD3⁺ T cells among PBMC from healthy control individuals

Culture condition*	% IL-10 expressing cells†			
	Donor 1	Donor 2	Donor 3	Donor 4
PBMC	1.42	1.24	2.34	1.89
PBMC+rBet v 1	7.77	5.16	8.49	4.99
PBMC+rIL-4+rBet v 1	8.16	3.46	7.79	3.75

* 1×10^6 PBMC were incubated with either rBet v 1 ($1 \mu\text{g/mL}$), rIL-4 (5 ng/mL) or both. After 5 days, monensin (3 mM/mL) was added for 24 h. PMA (1 ng/mL) and Ionomycin ($0.5 \mu\text{g/mL}$) were added during the last 4 h.

†Cells were stained with $10 \mu\text{L}$ of either FITC-conjugated IgG1 control mAb or FITC-labelled anti-CD3 mAb, permeabilized and stained with either PE-conjugated IgG1 control mAb or PE-conjugated anti-IL-10 mAb. 100 000 PBMC per condition were analysed on the FACS. Gates were defined on the basis of anti-CD3-FITC mAb alone. The percentage of CD3⁺ cells expressing IL-10 is shown. Results of four independent experiments on different donors are shown. PBMC, peripheral blood mononuclear cell; PMA, phorbol myristate acetate; FITC, fluorescein isothiocyanate.

mono-allergic to grass pollen, stop circulating without seasonal activation or 'bystander' activation by a Th2 microenvironment in the regional lymph nodes. Perhaps several pollen seasons are necessary to induce a substantial number of circulating allergen-specific cells, which could explain the differences found between our adult patients and the allergic children in the study of Rudi et al. We, however, cannot rule out that circulating birch-pollen-specific Th2 cells in our patient group, of whom the majority also suffers from oral allergy syndrome, could also persist due to regular contact of these patients with cross-reactive allergens in fruit or vegetables, outside the birch-pollen season.

We furthermore show that enhanced production of Th2 cytokines is not a general characteristic of our patient group, as no differences with controls were found after polyclonal stimulation. After recall-antigen (TT) stimulation, allergic individuals even produced less Th2 cytokines as compared with healthy controls, and Th2/Th1 ratios (data not shown) were comparable. Comparable Th1/Th2 ratios between allergic patients and healthy controls after TT stimulation have also been found by others [15, 33]. On the basis of these results, we conclude that allergen-specific Th2 cells could only be demonstrated in allergic subjects, and that enhanced Th2 cytokine production is not a general characteristic of these birch-pollen-allergic individuals.

Since the difference between both groups was most clear for IL-5, and since IL-5 production is at least partly driven by IL-4, we furthermore studied allergen-specific Th2 cytokine production in the presence of rIL-4. rIL-4 significantly enhanced the already high IL-5 production by allergen-specific T cells from birch-pollen-allergic patients, without influencing the IL-5 production by T cells from healthy controls. rBet v 1-induced IL-13 production by T cells from allergic patients on the other hand was not influenced by rIL-4, whereas the rBet v 1-induced IL-13 production found in a few healthy individuals decreased in the presence of IL-4. IL-13 in humans can also be produced by Th1 cells [34]. This could perhaps explain why IL-5 and IL-13 behave differently in response to rIL-4.

Recently, there has been a lot of interest in IL-10 as a cytokine that inhibits allergic reactions. We demonstrate here that control individuals, but not allergic patients, respond to allergen + rIL-4 with increased IL-10 production. These data suggest that healthy controls do have allergen-specific T cells but they mainly produce IL-10. Our results are in contrast with a recent publication in which the birch-pollen-induced IL-10 production by PBMC from birch-pollen-allergic patients was significantly higher as its production by PBMC from healthy controls [33]. In that study, however, blood was drawn during the birch-pollen season following the birch-pollen peak, whereas our study had been performed between September and December. This could potentially explain the differences in results and requires further investigation. Our results further suggest an intrinsic difference in T cell IL-10 production in general between birch-pollen-allergic patients and healthy controls. If PBMC were polyclonally stimulated, IL-4 induced a significant induction in IL-10 production by T cells from healthy controls but not from birch-pollen-allergic patients. This is of importance as IL-10 production by regulatory T cells (Treg) might be required to balance our immune system [6, 35]. We hypothesize that the allergen-

specific IL-10-producing cells found in control individuals in the presence of IL-4 could be Treg cells. It has been shown that Treg cells have similarities with Th2 cells, and the influence of IL-4 on these cells might in fact be comparable with its influence on Th2 cells [36]. IL-4, moreover, induces the proliferation and increases the functional capacity of Treg cells *in vitro* [37]. The expression of IL-10 may also play a role in the induction and maintenance of allergen-specific tolerance in allergic disorders [38]. It has been shown that human IL-10 can down-regulate the antigen-specific Th2 cytokine production by Th2 clones [39]. Pre-treatment of DC with IL-10 also inhibits the production of Th2 cytokines by T cells in coculture [40]. Furthermore, adding 20 ng rIL-10 to our *in vitro* culture system significantly decreased the production of IL-4, IL-5 and IL-13 by rBet v 1-stimulated PBMC from all three allergic donors tested (unpublished observations). Although control individuals did not produce detectable concentrations of IL-4 in response to allergen, low IL-4 amounts could be sufficient to induce allergen-specific IL-10 production in control individuals. We can speculate that, if 'by accident' T cells are induced to produce IL-4 upon contact with a 'harmless' allergen, the regulatory T cell population releases IL-10 and counteracts further Th2 development and/or Th2 cytokine production. Regulatory T cell activity could also depend on other mechanisms of action (e.g. cell-cell contact), which could also help to counteract inappropriate immune responses against allergens [41]. We can further speculate that this regulatory mechanism is defective in allergic individuals and that the initial IL-4 induction will lead instead to further Th2 development and enhanced IL-5 secretion.

The conclusions of our study apply to birch-pollen-allergic subjects, not necessarily to atopsics in general. We have to stress, indeed, that our population of mono-allergic birch-pollen-reactive patients, of which most had also an oral allergy syndrome, is a select population. As recently stated, 'atopy' should probably be defined as 'a familial tendency to produce IgE antibodies to low doses of ubiquitous and multiple allergens, usually starting in infancy, with the development of typical symptoms such as asthma, rhinoconjunctivitis and eczema/dermatitis' [42]. On the other hand, the numerous cases of IgE-mediated allergy occurring at any age later in life, which sometimes present as asthma or rhinoconjunctivitis and are usually caused by single isolated allergens could rather be called 'non-atopic IgE-mediated allergy' [42]. Twelve out of our 15 birch-pollen-allergic patients started their allergic symptoms in adulthood and none of them had symptoms in early infancy. We do not know whether the differences in allergen-specific T cell cytokine production as reported here can also be found in the so-called 'atopsics', and current experiments are focusing on this issue.

In conclusion, here we provide evidence that rBet v 1 induces a Th2 cytokine profile in T cells from birch-tree pollen-allergic individuals. Control subjects, on the other hand, have allergen-specific T cells that produce IL-10 in response to rBet v 1 and IL-4, and these cells could be Treg cells. Furthermore, IL-4 induces significant IL-10 production in polyclonally activated T cells from control subjects but not from allergic patients, perhaps reflecting an intrinsic difference between allergic patients and healthy controls.

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