The T Cell Response to Major Grass Allergens Is Regulated and Includes IL-10 Production in Atopic but Not in Non-Atopic Subjects

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Key Words

Allergens • CD4⁺ T-lymphocytes • Flow cytometry • Hypersensitivity • Immune tolerance • Immunophenotyping • In vitro • Interleukin-10 • Phleum • Receptors • Suppression

Abstract

Background: The incidence of allergic diseases is increasing in industrialized countries and the immunological mechanisms leading to tolerance or allergy are poorly understood. Cytokines with suppressive abilities and CD4⁺CD25⁺ regulatory T cells have been suggested to play a central role in allergen-specific responses. The aim was to determine whether major grass allergens induce production of suppressive cytokines in allergic and healthy subjects and to examine the inhibitory effect of these cytokines on allergic responses. **Methods:** Peripheral blood mononuclear cells (PBMCs) were isolated from healthy and grass-allergic donors and stimulated with the major grass allergens Phl p 1 or Phl p 5. The effects of endogenous IL-10 and/or TGF- on proliferation and cytokine production were determined by use of blocking antibodies. In addition, the number of CD4⁺CD25⁺ T cells and their expression of chemokine receptors were investigated by flow cytometry. **Results:** Phl p 1 and Phl p 5 induced IL-10 production, which down-regulated prolifera-

tion and cytokine production, in PBMC cultures from atopic but not from non-atopic donors. Comparable frequencies of CD4⁺CD25⁺ T cells were present in PBMCs in the two groups, but fewer cells from atopic donors were CD4⁺CD25⁺CCR4⁺ and more cells were CD4⁺CD25⁺CLA⁺ compared to healthy donors. **Conclusion:** Allergen-specific responses of grass allergic patients but not in non-atopic subjects are influenced by regulatory cytokines produced in response to the important allergens. Differences in CD4⁺CD25⁺ T cell expression of chemokine receptors in allergic compared to non-atopic donors could suggest that the homing of CD4⁺CD25⁺ T cells is important for the regulation of allergen-specific responses.

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Introduction

Allergic diseases such as rhinitis, asthma and dermatitis mediated by IgE antibodies are referred to as atopic diseases. High levels of IgE antibodies and elevated levels of eosinophils involved in tissue inflammation are characteristic for these atopic diseases [1]. Synthesis of IL-4 and IL-5 by T helper (Th)-2 cells initiates IgE production and promotes eosinophil growth and development [2]. Given the importance of the Th2 cytokines in directing the allergic reaction, modification of the T cell response

has been of particular interest for treatment of allergic diseases. Production of Th1 cytokines such as IFN- γ as a result of allergen-specific immunotherapy (SIT) has been reported in several studies and the IFN- γ -induced counterbalance of the Th2 phenotype has been suggested to be crucial for the effect of the treatment [3–5]. More recent studies focused on regulatory T cells and production of suppressive cytokines such as IL-10 and TGF- β . Increased production of IL-10, possibly from peripheral blood CD4⁺CD25⁺ T cells, has been observed following in vitro stimulation with *Phleum pratense* (Phl p) [6], a major allergen from house dust mite (*Dermatophagoides pteronyssinus*, Der p 1) [7], birch pollen allergen (*Betula verrucosa*, Bet v 1) [7], and the major bee venom allergen phospholipase A2 (PLA) [8]. The inhibition of allergen-induced proliferation and production of Th2 cytokines demonstrated after SIT has been related to IL-10/TGF- β or CD4⁺CD25⁺ T cells or both [6, 7, 9, 10], and investigation of in vitro expanded IL-10 producing T cells from both allergic and non-atopic individuals seems to suggest that multiple suppressive mechanisms play a role in peripheral allergen tolerance [11].

IL-10 inhibits both Th1 and Th2 cell proliferation and cytokine production [12]. Moreover, synthesis of IgE by B lymphoblasts [13] and production of pro-inflammatory cytokines by monocytes [14, 15], macrophages [16] and eosinophils [17] is also inhibited by IL-10. TGF- β inhibits T cell differentiation and proliferation of both Th1 and Th2 cells and attracts macrophages, dendritic cells and other inflammatory cells to sites of antigen exposure. Here, TGF- β inhibits the function of these cells once they are activated [18]. Recently, it has been shown that TGF- β is able to induce differentiation of CD4⁺CD25⁺ regulatory T cells which are able to suppress via a cytokine-independent but cell contact-dependent mechanism [19, 20]. Collectively, this supports the idea that TGF- β , in addition to IL-10, may be involved in mediating tolerance toward allergens. In the present study, we analyzed the production of different cytokines, including IL-10, upon single allergen stimulation and investigated the effect of IL-10 and/or TGF- β in both atopic and non-atopic donors.

Allergens enter the immune system by penetrating the mucosal, skin and intestinal barriers and are transported to secondary lymph nodes by antigen-presenting cells. Subsequently, activated T cells recirculate, and local mucosal inflammation is characterized by these invading allergen-specific cells. Thus, in order to inhibit allergen-induced responses, CD4⁺CD25⁺ may need to migrate to sites of inflammation. CCR4 is a chemokine receptor in-

involved in guiding T cells to antigen-presenting dendritic cells at sites of inflammation and to secondary lymphoid tissues [21]. Cutaneous lymphocyte-associated antigen (CLA) is a surface molecule expressed on memory/effector T cells and plays an important role in lymphocyte migration to skin during allergen-induced inflammation [22]. CCR4 and CLA are both expressed by CD4⁺CD25⁺ T cells and are involved in the trafficking of lymphocytes to secondary lymphoid organs and sites of inflammation [23]. CCR4 expression is up-regulated upon allergen challenge of human CD4⁺ T cells [24] and CCR4 was originally connected with Th2 responses [25]. However, CCR4 is also expressed on cutaneous memory T cells and CD4⁺CD25⁺ T cells, which has generated conflicting views of the physiological role of this surface molecule [23]. In general, little is known about how the CD4⁺CD25⁺ phenotypes differ in atopic and non-atopic donors, but differences in the expression of these molecules may influence the function of CD4⁺CD25⁺ T cells in atopic and non-atopic individuals. We compared the expression of CCR4 and CLA on Tregs from these two donor groups in order to analyze whether these cells would differ in their receptor expression and thereby could differ in their ability to migrate to sites of allergen exposure.

Materials and Methods

Patients

The study was performed with 47 atopic and 13 non-atopic donors. Included subjects were males and females aged 18–65 years. Atopic donors had moderate to severe seasonal rhino conjunctivitis induced by *Phleum pratense* (Phl p). This was verified by positive skin prick test (Soluprick; ALK Abelló, Hørsholm, Denmark) and presence of specific IgE to Phl p in serum (median 22.42 kU/l, range 0.60–128.18 kU/l). Specific IgE was measured by the ADVIA-Centaur assay as earlier described by Petersen et al. [26]. The patients with grass allergy had not been previously treated with SIT. Non-atopic donors were included in the study on the basis of absence of specific IgE to a panel of 10 airborne allergens, including Phl p. The analyses were conducted in January 2002, i.e. during pollen off-season. The samples were collected before the start of a phase I trial on specific immunotherapy.

Ethics

The Danish ethical committee approved the study.

Cell Culture

PBMCs were isolated from 70 ml whole blood on a lymphoprep gradient (Axis-Shield, Oslo, Norway). Four replicates of 3×10^5 cells/well were stimulated with naturally purified major allergens Phl p 1 (5 μ g/ml) and Phl p 5 (5 μ g/ml), purified protein derivative (PPD; 25 μ g/ml; SSI, Copenhagen, Denmark) or medium control with or without blocking antibodies specific to the IL-10

receptor (aIL-10R: 5 µg/ml, clone 3F9; BD Biosciences, San Jose, Calif., USA), and TGF-β (aTGF-β 1, 2, and 3: 10 µg/ml, clone 1D11; R&D, Minneapolis, Calif., USA) or to the IL-4 receptor (IL-4R: 2.5 µg/ml, clone 25463.111; R&D). Allergens were purified from grass pollen extracts as described elsewhere [27] and concentrations of allergens were selected on the basis of previous studies on grass allergen-specific T cell responses [28, 29]. Endotoxin levels were below 2.5 EU/ml in cultures with Phl p 5 and below 0.5 EU/ml in cultures with Phl p 1. The medium consisted of RPMI 1640 (Bio Whittaker, Vallengbaek Strand, Denmark) with 5% human AB serum (Bio Whittaker) and 1% penicillin/streptomycin (Gibco BPR, Roskilde, Denmark). The cells were cultured in 150 µl medium in 96-well round-bottomed plates at 37°C and 5% CO₂ for 6 days before supernatants were harvested for detection of cytokine levels. Subsequently, 0.5 µCi/well ³H-thymidine (New England Nuclear, Boston, Mass., USA) was added and proliferation was measured by counts per minute (cpm) in a β-scintillation counter (Wallax, Pharmacia, Turku, Finland) after an additional 16–20 h of incubation. In previous studies [28, 29] and in pilot experiments, these experimental conditions were found to be generally applicable for detection of grass allergen-specific T cell proliferation and detection of Th1 and Th2 cytokines as well as IL-10. The stimulation index (SI) was calculated by the mean cpm of allergen or PPD stimulated cells divided by the mean cpm of cells in medium (median 906 cpm, range 293–4,845 cpm).

Cytokine Measurement

After 6 days, 50 µl supernatant were harvested and pooled from replicate stimulations and stored at –20°C until IL-2, IL-4, IL-5, IL-10 and IFN-γ levels were analyzed using human Th1/Th2 cytometric bead array (CBA; BD Bioscience, Pharmingen, San Diego, Calif., USA). The CBA was measured using a FACSCalibur flow cytometer equipped with BD Bioscience CBA software version 1.1. Due to the detection range of the CBA, values below 10 pg/ml were assigned the value 10 pg/ml and values above 6,000 pg/ml were assigned the value 6,000 pg/ml.

FACS Analysis of Tregs

5 × 10⁵ freshly isolated PBMCs were used per sample for FACS analysis of the Treg phenotype of atopic and non-atopic donors. The cells were stained with CD4-RPE-Cy5 and either CD25-RPE or CD25-FITC in combination with either CLA-FITC or CCR4-RPE. For each donor, some cells were stained with the following isotype controls: mouse-IgG1-RPE-Cy5 (control for CD4 staining), mouse IgG1-FITC (control for CD25 staining), mouse IgG1-RPE (control for CD25 staining), rat-IgM FITC (control for CLA staining), and mouse IgG1 R-PE (control for CCR4 staining). All antibodies were from BD Pharmingen except CCR4, which was from RD Systems. Cells incubated for 30 min on ice with conjugated antibodies were subsequently washed twice in PBS plus 2% FCS. Cells were fixed using 1% paraformaldehyde and analyzed on the FACSCalibur flow cytometer (BD). The fluorescence intensity of the cytometer was adjusted to the same level daily using Flow-Set FluoroSphere (Beckman Coulter, Fullerton, Calif., USA). In the following data analysis, gates were positioned to gate 1% of isotype control cells stained positive. CD4⁺ T cells were divided into different subpopulations depending on the expression frequency of CD25 molecules. To be able to compare the number and phenotype of cells with the highest frequency of CD25 molecules

(CD4⁺CD25^{+(high)} T cells) for different donors, the following gating strategy was used (fig. 1). The mean fluorescence intensity (MFI) for CD25 staining was determined on the basis of the core of the main CD4⁺CD25⁺ population. The MFI was multiplied by 2.5 in order to determine the position of the left-hand site of the gate on CD4⁺CD25^{+(high)} T cells. A fixed factor was chosen to avoid bias in the data analysis and the value 2.5 was the average multiplication factor of 20 analyses gated by hand. Each of the subpopulations CD4⁺CD25^{+(high)}, CD4⁺CD25^{+(all)}, and CD4⁺CD25^(negative) were analyzed for expression of CLA or CCR4 in separate histograms, as illustrated in figure 1.

Statistics

Statistical analysis was performed using GraphPad Prism Version 4.02. Non-parametric tests either paired (Wilcoxon test) or non-paired (Mann-Whitney) with 95% confidence intervals were used, depending on the type of comparison.

Results

Proliferation and Cytokine Production upon Single Allergen Stimulation

PBMCs from atopic donors proliferated significantly more than PBMCs from non-atopic donors when stimulated with the allergens Phl p 1 ($p < 0.0002$) and Phl p 5 ($p < 0.0001$; fig. 2). In contrast, stimulation with the control antigen, PPD of mycobacterium tuberculosis, induced comparable proliferation levels ($p < 0.9429$) of atopic and non-atopic donors.

Stimulation with Phl p 1 and Phl p 5 induced significantly more IL-4 and IL-5 production in cultures from atopic compared to non-atopic donors ($p < 0.0001$; fig. 3). However, IL-4 secretion was only detectable in the presence of blocking antibodies to the IL-4 receptor. Cultures from non-atopic donors produce more IL-10 than cultures from atopic donors when stimulated with PPD ($p < 0.0057$) but not when stimulated with Phl p 5 ($p = 0.1769$) or Phl p 1 ($p = 1.000$). IFN-γ was not detectable in most of these allergen-stimulated cultures from non-atopic donors and INF-γ expression was not possible to differentiate between atopic and non-atopic donors (Phl p 5: $p < 0.0860$; Phl p 1: $p < 0.3575$). Cultures stimulated with the control antigen PPD produced IL-5 and IFN-γ but no IL-4, and there was no detectable difference between atopic and non-atopic donors (IL-5: $p < 0.7060$; IL-4: $p < 0.1970$; IFN-γ: $p < 0.3575$). IL-2 concentrations were generally below the detection limit for all stimulations (data not shown) possibly because the supernatants were harvested at day 6 where IL-2 has been consumed by the proliferating T cells.

Fig. 1. Illustration of gating strategy for FACS analysis of Tregs. **a** Gates were positioned to gate 1% of isotype control cells stained positive, in order to position the quadrants separating CD4⁺ from CD4-negative T cells as well as CD25-positive and -negative cells. The mean fluorescence intensity (MFI) for CD25 staining was determined of the core of the main CD4⁺CD25⁺ pop-

ulation. This number was multiplied by 2.5 in order to determine the position of the left-hand side of the gate on CD4⁺CD25^{+(high)} T cells. These cells were analyzed for expression of CLA or CCR4 as illustrated in **b**, **c** and **d**, where gates were positioned to gate 1% of isotype control cells stained positive.

Fig. 2. Proliferation of PBMCs from atopic and non-atopic donors. There was significantly stronger proliferation of PBMC cultures of atopic compared to non-atopic donors when stimulated with

Phl p 1 or with Phl p 5. No difference was detected between the groups when using the control antigen PPD for stimulation. The black bars indicate mean values.

Fig. 3. Cytokine production upon Phl p 5, Phl p 1, and PPD stimulations of PBMCs. Significantly higher IL-5 and IL-4 but not IL-10 and IFN- γ cytokine levels were observed in PBMC cultures of atopic compared to non-atopic donors when stimulated with Phl p 5 or Phl p 1 for 7 days. Cells stimulated with the control antigen

PPD produced IL-5, IL-10, and INF- γ but not IL-4. There was no detectable difference between the two groups of donors except for the IL-10 production. IL-4 secretion was only detectable in the presence of blocking antibodies to the IL-4 receptor. The black bars indicate mean values.

IL-10 Production Is Induced upon Stimulation with Purified Major Allergens

Further results from measurements of IL-10 levels in cultures from both atopic and nonatopic donors are shown in figure 4. Interestingly, addition of a combination of blocking antibodies, which were specific for IL-10R and TGF- β , resulted in significantly higher IL-10 levels in Phl p 5 ($p = 0.0039$), and Phl p 1 ($p = 0.0035$)-

stimulated cultures from atopic donors, whereas cultures from non-atopic donors were unaffected ($p = 0.5589$ and $p = 0.6250$, respectively). In contrast, IL-10 levels increased significantly by addition of blocking antibodies to PPD-stimulated cultures from both groups. In PPD-stimulated cultures, the IL-10 production was significantly higher for non-atopic donors (123 ± 23 pg/ml) compared to atopic donors (78 ± 8 pg/ml; $p <$

Fig. 4. IL-10 levels of PBMC cultures with or without blocking antibodies. IL-10 levels of PBMC cultures from atopic and non-atopic donors upon 7 days of stimulation with Phl p 1, Phl p 5 or PPD with (shaded symbols) or without (open symbols) blocking antibodies to the IL-10 receptor (IL-10R) and TGF- β (1, 2, 3). The presence of blocking antibodies reveals that the actual IL-10 lev-

els, in cultures stimulated with Phl p5 and Phl p 1, were higher in cultures from atopic but not non-atopic donors. For both groups stimulation with control antigen (PPD) resulted in higher IL-10 levels in presence of blocking antibodies. Unstimulated cultures (medium) show that IL-10 levels were unchanged by addition of blocking antibodies. The black bars indicate mean values.

0.0133). In all control cultures, medium IL-10 values were below the detection limit; therefore, the data were not adjusted for background IL-10 production. Blocking antibodies did not affect IL-10 production in these cultures.

Suppressive Cytokines Are Involved in Controlling Immune Response toward Allergens and Antigens

We investigated whether these relatively low amounts of IL-10, produced upon allergen stimulation, had any effect on proliferation and cytokine production in the cul-

Fig. 5. Effect of suppressive cytokines on allergic response in vitro. Addition of anti-IL-10R and anti-TGF- β (1, 2, 3) (indicated by $\blacktriangle$) to cultures of PBMCs from atopic donors ($\triangle$), which were stimulated with Phl p 5 for 7 days, resulted in a significant increase in proliferation, significant increase in IFN- γ levels, and a non-significant increase of IL-5 levels. Addition of the blocking antibody-

ies to analogue cultures, stimulated with Phl p 1, resulted in unchanged proliferation levels, significant increase IFN- γ levels, and significant increase in IL-5 levels. In contrast to this, addition of blocking antibodies ($\bullet$) to Phl p 1-stimulated PBMCs from non-atopic donors ($\circ$) did not induce proliferation or cytokine production.

tures by blocking the effects of both IL-10 and TGF- β (see results in fig. 5). Indeed, proliferation of Phl p 5-stimulated PBMCs from atopic donors was significantly higher in the presence of blocking antibodies ($p < 0.0038$). Phl p 1 induced lower proliferation levels than Phl p 5, and in these cultures a similar tendency but no significant increase was detected ($p < 0.6639$). Thus, suppressive cyto-

kines induced by single allergens inhibit proliferation in this culture system. Blocking of suppressive cytokines resulted in an increased cytokine production in cultures from atopic donors. As illustrated in figure 5, IFN- γ increased in almost all Phl p 5 ($p < 0.0001$), and Phl p 1 ($p < 0.0024$)-stimulated cultures by addition of blocking antibodies. An increase of IL-5 was detected for 30 of the

47 atopic donors stimulated with Phl p 5, even though no significant difference was observed ($p < 0.3835$). For Phl p 1-stimulated cultures, IL-5 increased significantly ($p < 0.0115$) by addition of the blocking antibodies. Thus, IL-10 and/or TGF- β were shown to influence both T cell proliferation and cytokine production and seem primarily to down-regulate IFN- γ production.

Addition of anti IL-10R and anti-TGF- β antibodies to Phl p 1-stimulated cultures from non-atopic donors did not result in increased proliferation ($p < 0.5418$), IL-5 (undetectable) or IFN- γ ($p < 0.5000$; see fig. 5). Similar results were obtained for Phl p 5 stimulation of cultures from non-atopic donors (data not shown). Thus, the effects of IL-10 and/or TGF- β do not seem to be involved in the unresponsiveness toward allergen stimulation in PBMC cultures from non-atopic donors.

In contrast, the blocking of suppressive cytokines in PPD-stimulated cultures resulted in increased proliferation for both atopic ($p < 0.0001$) and non-atopic ($p < 0.0391$) donors (data not shown). In control cultures with medium, the mean background levels of cytokines were lower than 18 pg/ml with or without blocking antibodies (data not shown).

Comparison between Treg Phenotype in Atopic and Non-Atopic Donors

It is possible that differences in phenotype and/or the number of Tregs between atopic and non-atopic donors may explain the level of reactivity toward allergens observed in these two groups. Therefore, we compared the frequency of the total population of CD4⁺CD25^{+(all)} T cells as well as the frequency of CD4⁺CD25^{+(high)} T cells in PBMC from atopic and non-atopic donors. The frequency of both these CD25 populations was within a very narrow range in all donors investigated and did not differ between atopic and non-atopic donors (see table 1). Subsequently, we compared the phenotypes of these cells with respect to expression of CCR4 and CLA. For this analysis, we gated the cells into the following subpopulations: CD4⁺CD25^{+(high)} T cells, CD4⁺CD25^{+(all)} T cells, and CD4⁺CD25^(negative) T cells. The percentage of cells expressing a CCR4 or CLA was analyzed for each of these cell populations. These subpopulations were differentiable in regard to expression of CCR4 and CLA. This is illustrated in figure 6, in which the percentage of each subpopulation expressing CCR4 and CLA of the total CD4⁺ T cell population is depicted. For each result a comparison was made between the atopic and non-atopic group. We found the frequencies of CCR4-expressing cells to be significantly lower in all subpopulations in atopic donors

Table 1. Frequency of Tregs in PBMC from atopic and non-atopic subjects

Percent (%) of cells in the PBMC population	Atopic (n = 44)	Non-atopic (n = 12)	p value
CD4 ⁺ CD25 ^{+(high)}	0.48 $\pm$ 0.03	0.57 $\pm$ 0.08	<0.2593
CD4 ⁺ CD25 ^{+(all)}	3.44 $\pm$ 0.14	3.59 $\pm$ 0.39	<0.7645

Comparison of the frequency of the total population of CD4⁺CD25^{+(all)} T cells as well as the frequency of CD4⁺CD25^{+(high)} T cells in freshly isolated PBMC from atopic and non-atopic donors did not show any statistical differences between the two groups.

compared to the respective subpopulations from non-atopic donors. The frequency of CLA-expressing CD4⁺CD25^{+(high)} T cells was higher in atopic donors than in non-atopic donors. Thus, the phenotype of Tregs with respect to these surface receptors is differentiable for atopic and non-atopic donors.

Discussion

The importance of T cell regulation in allergic sensitization and in active disease has been a matter of debate during the last decade. As mentioned above, the involvement of CD4⁺CD25⁺ T cells and the effect of regulatory cytokines (IL-10 and/or TGF- β) have been investigated for various allergens in an attempt to describe the effect of SIT [6, 9, 30, 31]. Experiments with grass allergen extracts (Phl p) have shown modest induction of IL-10 from PBMC in vitro after grass pollen immunotherapy [6, 10, 32]. However, allergen extracts include a variety of allergenic and non-allergenic proteins, and it has not previously been determined whether the IL-10 production observed was initiated by major grass allergens. We also found that atopic donors before SIT treatment produce IL-10 in response to Phl p 5 and Phl p 1 (fig. 3 and 4) which around 90% of patients with grass allergy are sensitized towards [33, 34]. Similarly, Bet v 1-specific IL-10 production has also been demonstrated in birch pollen-allergic patients prior to SIT treatment [35].

Stimulation with the recall antigen PPD resulted in IL-10 production in PBMC cultures from both atopic and non-atopic donors. However, a slightly impaired IL-10 production in atopic donors compared to non-atopic donors was observed (fig. 3), suggesting that the level of T

Fig. 6. Comparisons of Tregs from atopic and non-atopic donors by flow cytometry. PBMCs were analyzed for co-expression of CD4, CD25 and CCR4 or CLA. The cells were divided into the following subpopulations: CD4⁺CD25^{+(high)} T cells, CD4⁺CD25^{+(all)} T cells, and CD4⁺CD25^(negative) T cells. The per-

centage of cells expressing CCR4 or CLA was determined for each subpopulation. For each result a comparison was made between the atopic and non-atopic group. NS = Not significant; *** $p \leq 0.001$.

cell regulation in atopic individuals in general is reduced. This is supported by studies in infants (6–18 months old) with atopic dermatitis stimulated with various antigens and allergens [36] as well as studies in birch pollen-allergic patients [35, 37].

Taken together these observations could indicate that an impaired regulation may lead to allergic sensitization and that the allergen-specific production of this regulatory cytokine may be boosted in order to enhance down-regulation of the response towards the allergens.

Jutel et al. [7] reported cooperative suppressive effects of IL-10 and TGF- β upon stimulation with the major allergens from house dust mite (Der p 1) and birch pollen (Bet v 1), suggesting that both these cytokines might be important for regulation of the allergen-specific response. In the present study, we cannot rule out that TGF- β contributes to the down-regulation observed since the effects of aIL-10R and aTGF- β were not analyzed separately because a limited experimental setup was needed to analyze the high number of cell samples. In addition, available assays for measurements of TGF- β include activation of all TGF- β present in the supernatants, which may not represent changes in the biologically active form of this cytokine. Our attempts to measure naturally activated TGF- β in culture supernatants failed, so changes in TGF- β production were not evaluated. However, Francis et al.

[6] did not find Phl p 1-induced TGF- β production in PBMC cultures from non-atopic or atopic donors. Moreover, blocking of TGF- β in PBMC from birch pollen-allergic patients did not influence Bet v 1-specific proliferation or cytokine production and had only a marginal effect on IFN- γ production in non-atopic controls [37]. This suggests that the involvement of TGF- β in the regulation of responses to allergens in non-SIT-treated allergic patients is minimal and the effect of TGF- β will not be discussed further.

A suppressive effect on Phl p 5-induced proliferations and suppression of Phl p 1-induced production of IL-5 was observed, while blocking of the regulatory cytokines resulted in increased production of IFN- γ for both allergens investigated (fig. 5). Minor differences in the sensitization patterns for individual patients may in part account for these different effects on individual allergens, while the strong effect of IL-10 on allergen-specific IFN- γ production has been found in several recent studies [7, 31, 37]. In general, the strength of the responses differed considerably suggesting that a strong T cell proliferation (Phl p 5) may be highly sensitive to the effects of IL-10, while for IL-5 production low levels (Phl p 1) may be inhibited most efficiently by IL-10. In addition, evaluation of inhibitory effect on individual parameters may depend on the experimental setup because IL-10 blockade does

not seem to influence Bet v 1-specific proliferation but strongly enhances IFN- γ production in allergic patients in a recent study [37], while IL-10-mediated inhibition of both proliferation and cytokine production has been reported previously for Bet v 1 [7].

Grass pollen extracts, 20 $\mu\text{g/ml}$ Phl p ($\sim 2\text{--}4\ \mu\text{g/ml}$ Phl p 5) [6] or 100 $\mu\text{g/ml}$ Phl p ($\sim 1,020\ \mu\text{g/ml}$ Phl p 5) [38], have been shown to induce moderate T cell proliferation in PBMC from non-atopic donors. In contrast, we found no proliferation upon stimulation of PBMCs from non-atopic donors with purified major allergens Phl p 1 or Phl p 5 (5 $\mu\text{g/ml}$) (fig. 2). Therefore, it is likely that other proteins in the high concentrations of allergen extracts are causing the T cell activation described in the previous reports, but direct comparison of the response to extracts and single allergens or even stimulations with non-allergen preparations are needed to determine this.

Moreover, only marginal IL-10 production was observed toward these major grass allergens in non-atopic donors (fig. 3 and 4). In addition, blocking antibodies to IL-10 and TGF- β did not lead to an increased response toward the allergens (fig. 5), which indicates that regulatory cytokines are not involved in the lack of response of cells from non-atopic donors towards grass allergens. Previous reports on individuals who were tolerant to bee venom suggest that IL-10 mediates this tolerance. The fact that these donors have been exposed to high venom allergen concentrations working as bee keepers may explain this difference [9]. However, involvement of IL-10 has also been reported for responses to Der p 1 and Bet v 1 in non-atopic controls [7, 11]. Thus, tolerance mediated by IL-10 may be more relevant for some allergens than for others. In accordance with our *in vitro* results, Nouri-Aria et al. [10] found a low frequency of IL-10 mRNA⁺ T cells in nasal mucosa of non-atopic donors during the peak grass pollen season. The authors concluded that IL-10 was not involved in the mucosal tolerance in healthy donors. Other mechanisms of tolerance were found to be involved in tolerance to Bet v 1 and Der p 1 [11] and these cell-contact-dependent mechanisms [38, 39] may dominate in the responses towards grass allergens in healthy controls.

As an alternative to differences in the production of regulatory cytokines, a compromised function or insufficient amounts of CD4⁺CD25⁺ T cells might explain why allergen exposure induces allergy in some but not all individuals. In accordance with previous reports [39], we found no difference in the number of CD4⁺CD25⁺ T cells between the groups investigated (table 1).

In a recent study, depletion of CD4⁺CD25⁺ T cells or subgroups of these T cells (CD4⁺CD25⁺CD127^(high/low)) did not affect the *in vitro* response from atopic or non-atopic cells toward grass pollen [40], questioning the involvement of such regulatory T cells in allergic responses. However, increased frequency of CCR4⁺CD4⁺CD25⁺ T cells relative to other CCR4⁺CD4⁺ T cells migrating to inflamed tissue has been shown to correlate positively with tolerance induction [41]. In line with this, we found a reduced frequency of CCR4⁺CD4⁺CD25⁺ T cells in grass-allergic patients suffering from hay fever (fig. 6). However, also other CCR4⁺CD4⁺ T cell subpopulations were lower in atopic compared to non-atopic donors, which makes it difficult to interpret the physiological implications of this result.

In contrast, we found a higher frequency of cells expressing the skin-homing receptor CLA exclusively among CD4⁺CD25⁺ T cells from atopic compared to non-atopic donors. Similarly, patients suffering from atopic dermatitis (AD) had a higher frequency of CLA⁺ Tregs than both asthmatic and non-atopic donors [42], but how CLA-mediated homing influences the effect of such regulatory T cells in hay fever and AD remains to be established. A direct link between the CD4⁺CD25⁺CCR4^{+/−} or CLA^{+/−} T cells and regulation could have been obtained by further phenotypic characterization of the cells including staining for IL-10, Fox p 3 or CD127 or by functional analyses of isolated subpopulations. Such experiments were performed by Reefer et al. [43], who demonstrated that CD4⁺CD25⁺CCR4⁺ T cells were increased in AD patients, and the percentage of these cells was linked to the severity of disease. Moreover, within this population cells expressing additional markers of regulatory T cells, Fox p 3 and GITR were increased and co-expression of CCR4 and CLA was also demonstrated, suggesting that both these receptors are related to T cell regulation [43]. In line with this, Nguyen et al. [44] and Ahern et al. [45] demonstrated most recently that expression of CCR4 (as well as CCR8) is linked to CD4⁺CD25⁺ T cell with regulatory properties and that CCR4-CCL17/22 signaling is intact in allergic patients, in contrast to CCR8-CCL1 signaling, which seems to be compromised in severely diseased patients.

In conclusion, we found that the major allergens Phl p 5 and Phl p 1 are able to induce IL-10 production in cultures from atopic donors and that both allergen-induced proliferation and cytokine production were influenced by regulatory cytokines. In contrast, IL-10 and TGF- β were not involved in the lack of reactivity to allergens in non-atopic donors, suggesting that it is either due to lack

of allergen-specific T cells or due to tolerance mediated by other mechanisms than these suppressive cytokines. Equal frequencies of CD4⁺CD25⁺ T cells were found in atopic and non-atopic donors. However, the differences

observed in CCR4 and CLA expression of CD4⁺CD25⁺ T cells in atopic and healthy donors might influence the balance between effector T cells and regulatory T cells at sites of allergic inflammation.

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