

Rapid publication

Induction of IL-10⁺CD4⁺CD25⁺ T cells by grass pollen immunotherapy

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Background: Immunotherapy involves the modulation of allergen-specific T-cell responses, either T_H2-to-T_H1 immune deviation or, in bee venom-treated patients, induction of IL-10 production by CD4⁺CD25⁺ T cells. IL-10-producing CD4⁺CD25⁺ regulatory T cells have emerged as potential mediators of immune tolerance in numerous murine models of immunopathology.

Objective: The aim of this study was to evaluate the role of IL-10 production and CD4⁺CD25⁺ T cells in the response to grass pollen immunotherapy.

Methods: PBMCs were isolated from patients after 1 year of grass pollen immunotherapy and from matched untreated atopic and healthy control subjects. After 6 days of in vitro stimulation with *Phleum pratense*, production of IL-10, IL-5, IL-4, and IFN- γ and proliferation and numbers of CD4⁺CD25⁺ T cells were measured. T cells were then stimulated for a further 5 hours with phorbol 12-myristate 13-acetate and ionomycin and assessed for intracellular IL-10 by means of flow cytometry.

Results: Patients undergoing immunotherapy produced significantly more IL-10 than atopic control subjects (patients undergoing immunotherapy, 116 ± 21 pg/mL [$n = 11$]; atopic patients, 30 ± 5 pg/mL [$n = 11$]; $P < .001$), and the number of CD4⁺CD25⁺ cells identified after allergen stimulation was also greater in the immunotherapy group. The numbers of CD4⁺CD25⁺ T cells correlated positively with activation as measured by proliferation in both of the control groups but not in the immunotherapy group. Moreover, only T cells from patients undergoing immunotherapy were positive for intracellular IL-10, and these were almost exclusively CD4⁺CD25⁺ cells.

Conclusion: Grass pollen immunotherapy results in a population of circulating T cells that express the IL-10⁺CD4⁺CD25⁺ phenotype in response to allergen restimulation. (J Allergy Clin Immunol 2003;111:1255-61.)

Key words: IL-10, CD4⁺CD25⁺, T cells, regulatory, grass pollen, immunotherapy

Abbreviations used

PMA: Phorbol 12-myristate 13-acetate
rhIL-10: Recombinant human IL-10
SPT: Skin prick test
TGF: Transforming growth factor

Allergic disease is associated with T_H2 cytokine production by T cells in response to aeroallergens. Production of allergen-specific IgE and eosinophil recruitment and activation occur under the influence of IL-4 and IL-5, respectively. T_H2 cells can be detected in the peripheral blood of atopic patients^{1,2} and in the nasal and bronchial mucosae in response to allergen exposure.³⁻⁵ Specific allergen immunotherapy is an effective treatment for atopic disease, particularly for severe seasonal allergic rhinitis⁶ and for patients with severe hypersensitivity to bee or wasp stings. A variety of immunologic changes have been reported after immunotherapy, including moderate reductions in IgE, increases in allergen-specific IgG (especially of the IgG₄ isotype),⁷ and reductions in mucosa recruitment of eosinophils and basophils.⁸

Given the central importance of T cells in allergic responses, interest has inevitably focused on the role of modulation of the T-cell response in immunotherapy. Using peripheral blood T cells, some,⁹⁻¹³ but not all,¹⁴⁻¹⁷ studies have described decreased T-cell responsiveness to allergen, T_H2-to-T_H1 deviation, or both after treatment. Other studies have analyzed skin or nasal mucosa biopsy specimens taken from patients undergoing immunotherapy after an allergen challenge or during the pollen season and have demonstrated enhanced T_H1 responses, suggesting that treatment modifies the target organ response to allergen re-exposure.¹⁷⁻¹⁹

In addition to these findings, a number of studies have also identified induction of IL-10 production by T cells in patients receiving immunotherapy with insect venoms.^{20,21} IL-10 is an 18.7-kd protein expressed by a variety of human immune cells, including both T_H1 and T_H2 cells, B cells, monocytes-macrophages, dendritic cells, mast cells, and eosinophils. In mice IL-10 has been associated with suppression of colitis,²² delayed-type hypersensitivity,²³ graft rejection,²⁴ arthritis,²⁵ experimental autoimmune encephalomyelitis,²⁶ and allergic inflammation.²⁷⁻²⁹ IL-10 has a number of documented antiallergic

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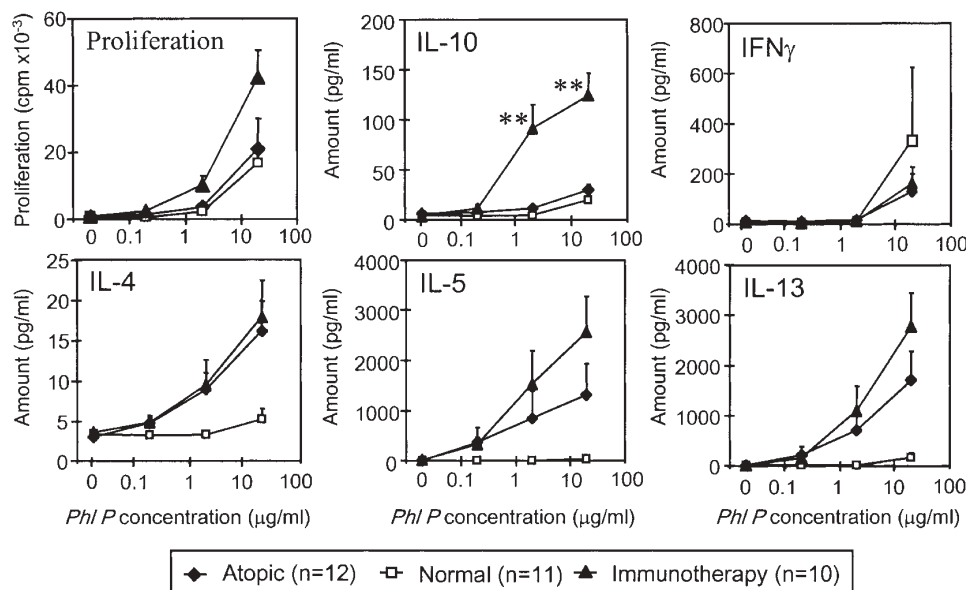


FIG 1. Proliferative responses and cytokine profiles of PBMCs from atopic donors, healthy donors, and donors undergoing immunotherapy after allergen stimulation. PBMCs were stimulated with increasing doses of grass pollen allergen for 6 days in culture, and proliferative responses (in counts per minute $\pm$ SE) and cytokine production (in picograms per milliliter $\pm$ SE) were measured. ** $P < .01$ for atopic subjects versus subjects undergoing immunotherapy.

TABLE I. Subject characterization

	Atopic subjects	Patients undergoing immunotherapy	Healthy control subjects
No. of subjects	12	10	11
Sex (F/M)	7/5	4/6	7/4
Age (y)*	30 (28,34)	38 (33,39)	35 (28,42)
SPT to grass (mm)*	8 (5,8)	8 (8,10)	0 (0,0)
RAST to grass (IU/mL)*	67 (23,88)	58 (41,100)	<0.34 (0,0)
Total IgE* (IU/mL)	234 (90,526)	178 (76,568)	15 (10,91)
Clinical efficacy (OAS)	-1 (-0.25,0.25)	2.25 (2,3) [†]	0 (0,0)

OAS, Overall assessment score.

*Median (upper quartile, lower quartile).

[†] $P < .05$.

properties that might be important to immunotherapy, including modulation of IL-4-induced B-cell IgE production in favor of IgG₄,³⁰ inhibition of IgE-dependent mast cell activation,³¹ and inhibition of human eosinophil cytokine production and survival.³² In human T cells IL-10 suppresses production of proallergic cytokines, such as IL-5,³³ and can induce a state of antigen-specific hyporesponsiveness.³⁴

In murine models of immune tolerance, immunosuppressive properties have been linked to induction of CD4⁺CD25⁺ regulatory T cells producing IL-10, transforming growth factor (TGF) β , or both.^{35,36} Regulatory T cells might downregulate T-cell responses through cell-cell contact,^{35,37} as well as the direct effects of IL-10. The original immunohistochemical analysis of biopsy specimens taken from allergen-challenged skin described an increase in cutaneous CD25⁺ cells in patients undergoing grass pollen immunotherapy but not control subjects,¹⁹ and IL-10-producing CD4⁺CD25⁺ T cells have been reported after bee venom immunotherapy.²¹ In the

present study we hypothesized that IL-10-producing peripheral blood T cells can be identified after conventional grass pollen immunotherapy and that this is associated with induction of a circulating population of CD4⁺CD25⁺ putative regulatory T cells. Peripheral blood was collected from a cohort of patients undergoing grass pollen immunotherapy, untreated matched atopic control subjects, and nonatopic healthy control subjects. IL-10 production and CD4⁺CD25⁺ T cells were then examined in these samples in response to stimulation in vitro with grass pollen allergen.

METHODS

Patient characterization

The study was approved by the Ethics Committee of the Royal Brompton and Harefield Hospitals NHS Trust and performed with the written consent of all participants. The donor panel consisted of 12 atopic subjects, 11 healthy subjects, and 10 subjects receiving grass pollen immunotherapy (Table I). Atopy was defined by a pos-

itive skin prick test (SPT) response to *Phleum pratense* at least 4 mm greater than that elicited by the negative diluent control (Soluprick; ALK Abelló, Horsholm, Denmark). Atopic donors were all symptomatic, with a clear history of seasonal rhinitis symptoms. Subjects undergoing immunotherapy had received grass pollen immunotherapy for at least 1.5 years and were well matched to atopic donors for age, sex, SPT response to grass pollen extract, RAST results to grass pollen, and total IgE levels (Table I). SPTs, RASTs, and total IgE measurements were performed before immunotherapy and out of the United Kingdom pollen season. Patients were assessed for a change in hay fever symptoms by using an overall assessment score in which +3 was classified as "a lot better," +2 as "better," +1 as "a little better," 0 as "no change," -1 as "a little worse," -2 as "worse," and -3 as "a lot worse" compared with previous years. Healthy nonatopic subjects were all asymptomatic, had negative SPT responses to the same allergen panel, and had negative RAST results to grass pollen of less than 0.35 IU/mL.

Culture of T cells from peripheral blood

PBMCs were isolated from heparinized blood by means of centrifugation over Histopaque (Sigma, Poole, United Kingdom), washed twice with RPMI-1640 (Gibco, Paisley, United Kingdom), and resuspended at 1×10^6 cells/mL in RPMI-1640 supplemented with 5% human sera (Sigma), 100 U/mL penicillin-streptomycin (Gibco), and 2 mmol/L L-glutamine (Gibco). Cells were incubated with either 20, 2, 0.2, or 0 μ g/mL *P pratense* for 6 days. Cellular proliferation was measured by adding 0.5 μ Ci of tritiated methylthymidine (Amersham, Aylesbury, United Kingdom) per well for the final 8 hours of culture. For analysis of cell-surface expression of CD4 and CD25, cells were washed in FACS buffer and stained with CD25-FITC and CD4-Cy5 (Dako Cytomation, Ely, United Kingdom) for 20 minutes on ice. Cells were subsequently washed and analyzed as described below. For experiments investigating the effects of recombinant human IL-10 (rhIL-10) on PBMC cultures, cells were stimulated with 20 μ g/mL *P pratense* with various concentrations of rhIL-10 (PeproTech, London, United Kingdom). In some wells, an antibody against the IL-10 receptor was added (a kind gift from C. Akdis, Switzerland).

Intracellular cytokine analysis

Allergen-stimulated cells were resuspended at 2×10^6 cells/mL and were activated with phorbol 12-myristate 13-acetate (PMA; 25 ng/mL) and ionomycin (1 μ g/mL) for 5 hours. Brefeldin A (10 μ g/mL) was added for the final 4 hours of stimulation. Cells were subsequently washed, resuspended at 0.5×10^6 cells/mL in FACS staining buffer (PBS plus 0.1% BSA plus 0.09% sodium azide), and stained extracellularly for CD4-Cy5 and CD25-FITC (Dako Cytomation). Cells were fixed with CelFix (BD Pharmingen, Cowley, United Kingdom), permeabilized with a saponin solution (0.1% saponin plus 1% FCS in staining buffer), and incubated with IL-10-phycoerythrin or a control antibody (both BD Pharmingen) for 30 minutes at room temperature. The cells were finally washed and analyzed on a FACScalibur flow cytometer (Becton Dickinson).

ELISA

Supernatants from allergen-stimulated cultures were assayed for the presence of IL-4, IL-5, IL-10, IL-13, IFN- γ , and TGF- β by means of ELISA. All assays used BD Pharmingen-matched antibody pairs and human recombinant cytokines as standards (PeproTech). The limits of assay detection were 4 to 10 pg/mL.

Statistics

Groups of data were analyzed by using the Wilcoxon matched-pairs signed-rank test for paired data or the Mann-Whitney *U* test

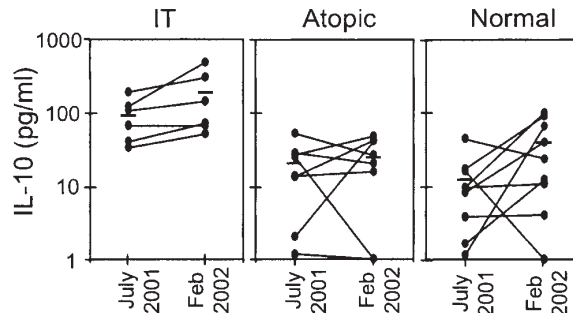


FIG 2. IL-10 production in and out of the grass pollen season. PBMCs obtained from the same subjects during and after the 2001 grass pollen season were stimulated with grass pollen allergen for 6 days in culture. The results show IL-10 production (in picograms per milliliter) as measured by means of ELISA.

for unpaired data with the GraphPad Instat program. Groups of 3 data sets were compared by using a Kruskal-Wallis test with a Dunn multiple comparison post test. *P* values of less than .05 were considered significant.

RESULTS

Effects of grass pollen immunotherapy on seasonal PBMC responses to allergen

Patients undergoing grass pollen immunotherapy reported a significant improvement of overall symptoms (median [upper quartile, lower quartile] overall assessment score, 2.5 [2,3]; *P* < .001). In contrast, hay fever symptoms were not statistically different over the same time period for atopic (-1 [-2.25, 0.25]) and healthy (0 [0,0]) donors.

P pratense-induced T-cell proliferation and production of IL-4, IL-5, IL-13, IFN- γ , and IL-10 were examined after 6 days of culture in samples collected during the pollen season of 2001 (Fig 1). In cultures stimulated with 2 or 20 μ g/mL *P pratense*, significantly higher amounts of the T_H2 cytokines IL-4, IL-5, and IL-13 were produced in both the immunotherapy and atopic groups compared with that produced in the nonatopic control group (*P* < .05). In contrast, no significant differences were recorded between the immunotherapy and untreated atopic groups in expression of any of the T_H2 cytokines. No differences in proliferation were observed between the immunotherapy, atopic, and normal groups. Thus although all groups of subjects exhibited allergen-driven proliferative responses, major differences between these groups were only revealed at the cytokine level. The most markedly different patterns of responses were seen for IL-10: production was higher in the immunotherapy-treated patients than in both the untreated atopic and healthy nonatopic groups in response to stimulation with either 2 or 20 μ g/mL *P pratense* (for both the healthy and atopic groups vs the immunotherapy group, *P* < .01). No allergen-induced TGF- β production was detected in any of the groups (data not shown).

IL-10 responses to *P pratense* were re-evaluated in 6 patients undergoing immunotherapy outside the grass

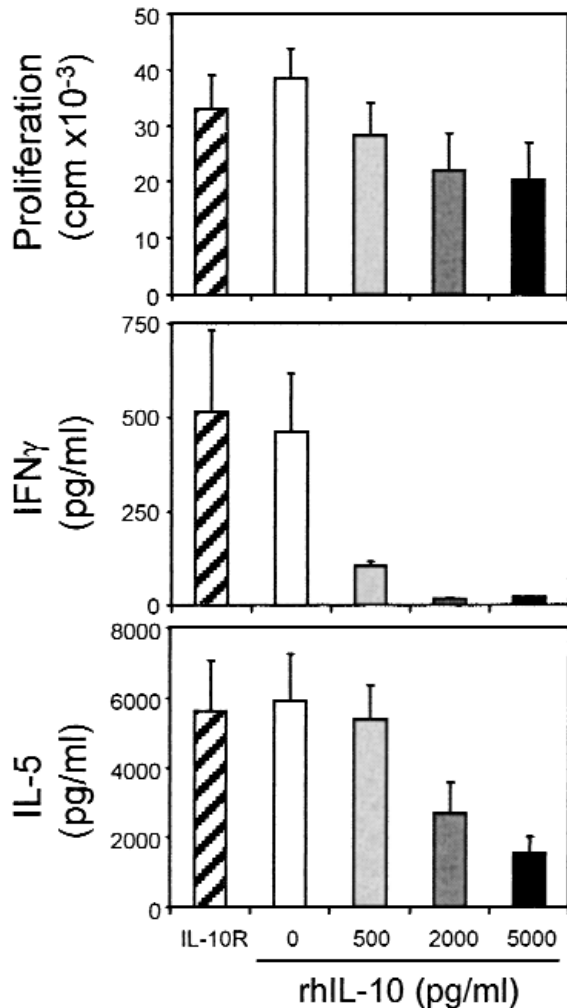


FIG 3. Effects of rhIL-10 on proliferative responses and cytokine profiles. Cells derived from donors undergoing immunotherapy ($n = 4$) were stimulated with 20 μ g/mL *P pratense* in the presence or absence of various concentrations of rhIL-10 or IL-10 receptor, as shown. Similar effects are also observed with atopic donors ($n = 3$).

pollen season in February 2002 to determine the effect of seasonal allergen exposure. IL-10 was still produced in cultures of patients undergoing immunotherapy (Fig 2). Indeed, in 5 of the 6 patients tested, IL-10 responses were marginally increased, although this might have reflected the fact that these patients had received immunotherapy for longer by February 2002.

Exogenous IL-10 inhibits T-cell responses to *P pratense*

Because we did not detect inhibition of *P pratense*-induced proliferation and cytokine production in conjunction with the observed increases in IL-10 production after immunotherapy, we questioned whether this was because the concentrations of IL-10 produced were too low to have had an effect on T cells under the experimental conditions used. We showed that exogenous IL-10 added to *P pratense*-stimulated PBMCs could indeed

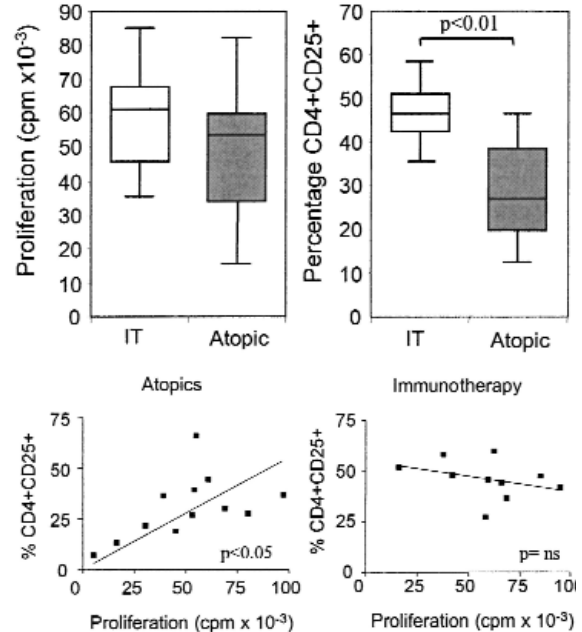


FIG 4. Expression of CD4 and CD25 by allergen-stimulated cells. After 6 days of culture with 20 μ g/mL *P pratense*, cells were analyzed for the expression of the cell-surface markers CD4 and CD25 by means of flow cytometry. The results show the percentage of CD4+CD25+ cells from the total population of lymphocytes. In addition, proliferative responses (in counts per minute) from the same donors are shown.

inhibit proliferation, IFN- γ production, and IL-5 production, although only at high concentrations (Fig 3). The medium effective dose for this response was between 500 and 2000 pg/mL rhIL-10, which exceeded the maximal IL-10 production seen in the patients undergoing immunotherapy. Moreover, the addition of an antibody against the IL-10 receptor did not influence the proliferative response or cytokine profile induced by the allergen.

Increased numbers of CD4+CD25+ cells in *P pratense*-stimulated cultures of patients undergoing immunotherapy

Numbers of CD4+CD25+ T cells were assessed in patients undergoing grass pollen immunotherapy and control subjects. In *P pratense*-stimulated cultures, CD4+CD25+ T cells (as a proportion of all lymphocytes) were significantly increased compared with the numbers seen in untreated atopic subjects and healthy control subjects (Fig 4). In contrast, numbers of CD4+CD25+ T cells were not increased in the unstimulated peripheral blood of patients undergoing immunotherapy (data not shown). Because CD25 is expressed by activated T cells, we also examined the relationship between CD25 expression and T-cell activation, as measured on the basis of proliferation. Although in both the atopic and healthy groups the numbers of CD4+CD25+ T cells correlated closely with proliferation, no correlation was observed in the immunothera-

py group (atopic group, $P < .05$; healthy control group, $P < .01$). Thus in the patients undergoing immunotherapy, the increased CD4⁺CD25⁺ T cells appeared to proliferate disproportionately poorly to *P pratense*.

P pratense-stimulated CD4⁺CD25⁺ cells of patients undergoing immunotherapy produce IL-10

Allergen-stimulated cells were activated for a further 5 hours with PMA and ionomycin, followed by intracellular staining for IL-10, to further characterize the CD4⁺CD25⁺ T cells observed in the patients undergoing immunotherapy. By using this technique, a clear population of IL-10⁺CD4⁺ T cells could be identified in the patients undergoing immunotherapy (Fig 5, A). When CD4⁺CD25⁻ and CD4⁺CD25⁺ subsets were analyzed, IL-10 staining almost exclusively colocalized to CD4⁺CD25⁺ cells (Fig 5). Moreover, expression of IL-10 by the CD4⁺CD25⁺ subset was allergen dependent because IL-10 was not expressed by T cells that had not been stimulated with *P pratense* before stimulation with PMA and ionomycin (Fig 5, B). Finally, IL-10⁺CD4⁺CD25⁺ T cells could only be detected in patients who had undergone immunotherapy (Fig 5, B): the mean percentages of CD4⁺CD25⁺ T cells that were IL-10⁺ in patients undergoing immunotherapy, atopic control subjects, and healthy control subjects were $20.5\% \pm 1.5\%$, $2.6\% \pm 1.0\%$, and $1.3\% \pm 0.3\%$, respectively.

DISCUSSION

After immunotherapy, T cells acquire the capacity to produce IL-10, regardless of whether cells are analyzed during natural allergen exposure (ie, in or out of the pollen season). The production of IL-10 did not appear to have any modulatory effect on allergen-induced proliferation and T_H2 cytokine expression, although the addition of exogenous IL-10 to cultures at higher concentrations did downregulate allergen-driven responses. Concomitant with the IL-10 response, we observed allergen-induced increases in the percentage of CD4⁺CD25⁺ T cells. Finally, by using intracellular cytokine staining, we demonstrate that in the patients undergoing immunotherapy, allergen-induced IL-10 colocalizes almost exclusively with this CD4⁺CD25⁺ subset.

Most studies that have addressed the effects of immunotherapy on T-cell responses have used readouts on the basis of responses of T cells isolated from peripheral blood to allergen extracts in vitro. A number of studies of patients treated with venom or pollen immunotherapy reported a reduction in the proliferation of peripheral blood T cells to allergen.^{9-12,38} Superimposed on this reduced reactivity was a shift away from T_H2 toward T_H1 responses after treatment.⁹⁻¹³ Nevertheless, in addition to the findings presented here, other studies have failed to observe similar changes after conventional grass pollen immunotherapy.¹⁴⁻¹⁷ On the other hand, modulation of T-cell cytokine responses in the skin and nasal mucosae of

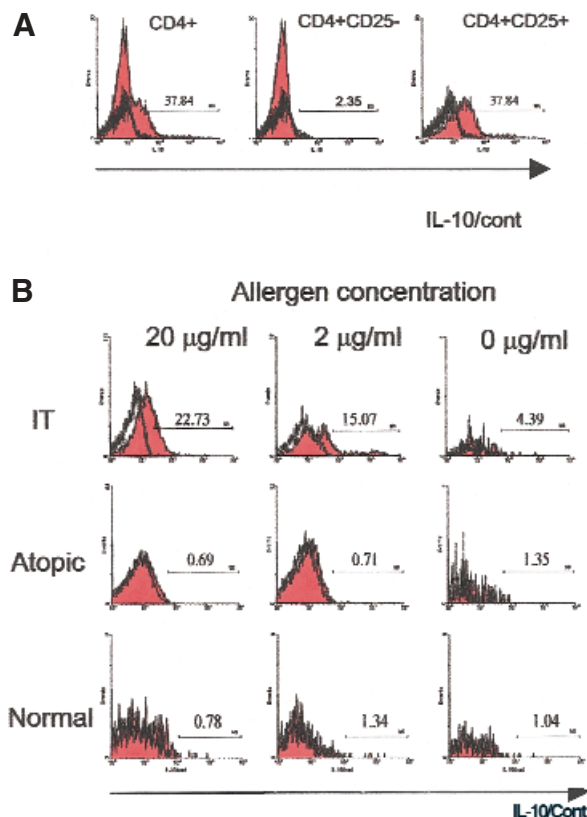


FIG 5. Intracellular expression of IL-10 by allergen-stimulated lymphocytes. **A**, PBMCs derived from donors undergoing immunotherapy ($n = 5$) were stimulated with $20 \mu\text{g/mL}$ *P pratense* for 6 days. Cells were further activated, stained for CD4 and CD25 expression, permeabilized, and further stained for intracellular IL-10. Fluorescence histograms for a representative donor gating on the lymphocyte population and either all CD4⁺, CD4⁺CD25⁻, or CD4⁺CD25⁺ cells are shown. The filled lines represent staining for IL-10, and the open lines represent staining by a control antibody. The markers represent the percentage of IL-10⁺ population, as determined by means of control staining. **B**, Cells from healthy subjects, atopic subjects, and patients undergoing immunotherapy ($n = 3-5$) were stained as above. Fluorescence histograms for representative donors gating on the lymphocyte population and CD4⁺CD25⁺ cells are shown.

patients undergoing immunotherapy is a consistent finding.^{6,15,17-19,39} Overall, these findings suggest that peripheral blood T-cell responses after immunotherapy are variable and that induction of T_H2/T_H1 immune deviation and hyporesponsiveness in peripheral blood T cells is not a necessary feature of clinically successful immunotherapy.

The presence of peripheral blood T cells that produce IL-10 in response to allergen stimulation after immunotherapy has, however, emerged as a consistent finding from studies of venom immunotherapy. Bellinghausen et al²⁰ were the first to describe this phenomenon, and Akdis et al²¹ also described an increase in IL-10 production after venom immunotherapy that was superimposed on a suppression of T-cell cytokine and proliferative responses. More recently, IL-10 mRNA-expressing cells have been identified in venom-challenged skin of patients undergoing immunotherapy.⁴¹

It might be possible that both immunodeviation of the T_H1/T_H2 response and immunoregulation through IL-10 production might occur after immunotherapy in an independent or interdependent manner. For example, although IL-10 can downregulate immune responses, allergen reactivation in the presence of IL-12 generated after high-dose grass pollen vaccination might induce preferential T_H1 deviation.⁴⁰ In contrast, during natural high-pollen exposure, mast cell-derived IL-4 might reactivate cells in favor of the T_H2 phenotype. Thus although initial allergen activation might be downregulated by exposure to IL-10, subsequent reactivation of allergen-specific cells might be influenced by the local cytokine milieu.

Although the IL-10 production described here was not associated with suppression of endogenous T-cell activation and cytokine production in response to allergen, exogenous recombinant IL-10 did inhibit grass pollen-stimulated T-cell responses when added in concentrations in excess of those produced after immunotherapy. Therefore although induction of IL-10-producing T cells might be a key event in immunotherapy, whether the effects of IL-10 are manifest as suppression plus or minus immune deviation within the same cultures, as described after venom immunotherapy,⁴¹ likely reflects the intensity of the IL-10 response induced by particular clinical protocols and the extent to which this is revealed by the precise in vitro culture conditions used. Importantly, this report has not established that IL-10-producing $CD4^+CD25^+$ cells can modulate ongoing immune responses, and this information is critical to establishing the functional immune regulatory functions of this population.

In human T cells IL-10 suppresses production of proallergic cytokines³³ and is able to induce a state of antigen-specific hyporesponsiveness (anergy).³⁴ This might occur as a result of IL-10 receptor-dependent blockade of CD28 T-cell costimulation.⁴² CD28 tyrosine phosphorylation and subsequent signaling in T cells in response to ligation by B7 molecules on antigen-presenting cells is inhibited by IL-10. This costimulatory pathway is required in addition to T-cell receptor-MHC class II binding for T-cell responses to allergen in both human subjects⁴³ and mice.⁴⁴ An important question concerns the relationship between a T_H2 -to- T_H1 shift and induction of IL-10 responses. In studies of bee venom immunotherapy in which IL-10 caused inhibition of T-cell responses in vitro, it was noted that addition of IL-2 restored proliferation and T_H1 , but not T_H2 , cytokine production.⁴⁵ Thus microenvironmental IL-2 produced in the nasal mucosa might act in combination with IL-10 to cause T_H2 -to- T_H1 immune deviation. Alternatively, these changes could occur through entirely independent but superimposed mechanisms. For example, immunotherapy might induce IL-10-producing regulatory T cells (see below) but also separately modify antigen-presenting cell-T-cell interactions so as to increase T_H1 cytokine expression by allergen-specific T cells.

Similar phenomena to those described here are well documented in murine models of immune tolerance, with

immunosuppressive properties being linked to induction of $CD4^+CD25^+$ regulatory T cells producing IL-10, TGF- β , or both.⁴⁶ IL-10-producing $CD4^+CD25^+$ T cells have been identified in patients undergoing bee venom immunotherapy.²¹ In the original immunohistochemical description of biopsy specimens taken from allergen-challenged skin, an increase in $CD25^+$ cells was observed in patients undergoing grass pollen immunotherapy but not in control subjects.¹⁹ Results described here represent the first description of increased numbers of $CD4^+CD25^+$ T cells in patients who have received immunotherapy of any kind. Increased percentages of $CD4^+CD25^+$ cells were only observed within PBMCs that had been stimulated with grass pollen allergen. These percentages are assumed to be representative of the absolute numbers of $CD4^+CD25^+$ cells present after allergen stimulation. The population of $CD4^+CD25^+$ cells observed in these cultures range from 2.3% to 4.2%, demonstrating that allergen-driven responses are restricted to the $CD4^+$ subset. Although $CD25^+$ is expressed by activated T cells, there are a number of reasons why we believe that the increased number of $CD4^+CD25^+$ cells does not merely represent increased T-cell reactivity to allergen in vitro after immunotherapy. First, although in untreated atopic patients and healthy control subjects the numbers of $CD4^+CD25^+$ T cells detected after allergen stimulation closely correlated with activation as measured by cell proliferation, the same was not true of patients undergoing immunotherapy. Therefore the increased numbers of $CD4^+CD25^+$ cells observed in patients undergoing immunotherapy might represent an additional regulatory population. Second, when allergen-stimulated PBMCs were examined for intracellular IL-10, the $CD4^+CD25^+$ population in the patients undergoing immunotherapy, but not in the control subjects, almost exclusively colocalized to IL-10.

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