

Grass pollen immunotherapy induces Foxp3-expressing CD4⁺CD25⁺ cells in the nasal mucosa

Suzana Radulovic, MD, Mikila R. Jacobson, PhD, Stephen R. Durham, MD, and Kayhan T. Nouri-Aria, PhD *London, United Kingdom*

Background: Regulatory T (Treg) cells play an important role in controlling allergic inflammation. The transcription factor Foxp3 regulates the development and function of natural and adaptive CD4⁺CD25⁺ Treg cells.

Objectives: We sought to examine the effect of grass pollen injection immunotherapy on the numbers of Foxp3⁺CD4⁺ and Foxp3⁺CD25⁺ T cells in and out of season and their expression of IL-10 in the nasal mucosa of patients with hay fever.

Methods: Nasal biopsy specimens were obtained from untreated patients with hay fever, participants with grass pollen allergy who had received 2 years of immunotherapy, and healthy control subjects. Dual-immunofluorescence microscopy was used to enumerate and colocalize Foxp3 expression to CD4⁺ and CD25⁺ T cells in the nasal mucosa. Triple staining was performed to colocalize Foxp3⁺ cells to CD3⁺CD25⁺ and CD3⁺IL-10-expressing cells.

Results: At peak season, numbers of Foxp3⁺CD25⁺ ($P = .02$) and Foxp3⁺CD4⁺ ($P = .03$) cells were significantly increased in the nasal mucosa of immunotherapy-treated patients compared with numbers before treatment. Foxp3⁺CD25⁺ ($P = .03$) and Foxp3⁺CD4⁺ ($P = .04$) cells were also greater in immunotherapy-treated patients out of season compared with those in untreated patients with hay fever. Within the immunotherapy-treated group, 20% of CD3⁺CD25⁺ cells expressed Foxp3, and 18% of Foxp3⁺CD3⁺ cells were IL-10 positive.

Conclusion: The presence of local Foxp3⁺CD25⁺CD3⁺ cells in the nasal mucosa, their increased numbers after immunotherapy, and their association with clinical efficacy and suppression of seasonal allergic inflammation support a putative role for Treg cells in the induction of allergen-specific tolerance in human subjects. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Foxp3, CD4⁺CD25⁺ Treg cells, IL-10, grass pollen immunotherapy, hay fever/allergic rhinitis

The observation that neonatal thymectomy in mice leads to organ-specific autoimmune pathology and that autoimmune responses can be reversed by adoptive transfer of CD4⁺CD25⁺ T cells from healthy animals provides compelling evidence for the putative role of regulatory T (Treg) cells in the control of immune responses, the mechanisms of which are not entirely clear.¹⁻³ Subsets of Treg cells include naturally occurring, thymic-derived CD4⁺CD25⁺ Treg cells; inducible CD4⁺CD25⁺ T cells; IL-10-producing Treg cells (T_H1 cells); and TGF- β -producing T_H3-type Treg cells.⁴⁻⁶ Naturally occurring CD4⁺CD25⁺ Treg cells have been shown to express a variety of cell-surface molecules that include CD25, CD45RB^{low}, CD62L, cytotoxic T lymphocyte-associated antigen 4, glucocorticoid-induced TNF receptor, and most specifically the transcription factor Foxp3. Foxp3 functions as a master switch gene in the development and function of Treg cells.^{7,8} A mutation in Foxp3 has been reported to result in the spontaneous development of allergic airways inflammation, hyper-IgE, and eosinophilia, symptoms reminiscent of those described in immunodysregulation polyendocrinopathy enteropathy X-linked syndrome,⁹ reinforcing the role of Foxp3 as the dominant transcription factor in Treg cells. Experimental models of inflammatory bowel disease¹⁰ and clinical conditions, such as atopic dermatitis,¹¹ asthma,¹² and a number of autoimmune conditions,¹³⁻¹⁵ have been associated with impaired expression of Foxp3⁺CD4⁺CD25⁺ T cells.

Seasonal allergic rhinitis is characterized by increased production of allergen-specific IgE and tissue eosinophilia, events that are under the regulation of T_H2 T lymphocytes.^{16,17} The development of an allergic response to common inhaled allergens has been postulated to occur as a consequence of impairment in the numbers, function, or both of allergen-specific Treg cells.^{18,19}

Grass pollen injection immunotherapy (GP-IT) has been shown to be a highly effective prophylactic treatment for IgE-mediated seasonal allergic rhinitis.²⁰ GP-IT has been shown to reduce nasal mucosal recruitment of inflammatory cells and effector cells and to decrease local allergen-specific T_H2 cytokine production in favor of T_H1 (IFN- γ) cytokines.²⁰ GP-IT induces blunting of seasonal increases of allergen-specific IgE and substantial increases in "blocking" allergen-specific IgG antibodies, particularly of the IgG4 isotype.^{21,22} Increased proportions of IL-10-producing peripheral CD4⁺CD25⁺ cells and IL-10- and TGF- β -expressing cells in the nasal mucosa are believed to contribute to the allergen-specific unresponsiveness that is observed after GP-IT.^{20,23,24} One way in which immunotherapy might be effective is by the local activation, recruitment, or both from the peripheral

From the Department of Upper Respiratory Medicine, Section of Allergy and Clinical Immunology, National Heart and Lung Institute, Imperial College and Royal Brompton Hospital London.

Funding for this project was from the Medical Research Council UK. K.T.N.-A. was supported by the Advanced Drug Discovery Initiative, a collaborative project between Imperial College Trust and GlaxoSmithKline. ALK-Abelló, Hørsholm, Denmark, supported the clinical trial of grass pollen subcutaneous immunotherapy.

Disclosure of potential conflict of interest: S. R. Durham has consulting arrangements with ALK-Abelló; has received research support from ALK-Abelló; is partially funded by the Immune Tolerance Network of the National Institutes of Health; and is on the speakers' bureau for ALK-Abelló, Allergy Therapeutics, and Stallergenes. The rest of the authors have declared that they have no conflict of interest.

Received for publication June 27, 2007; revised March 10, 2008; accepted for publication March 18, 2008.

Reprint requests: Kayhan T. Nouri-Aria, PhD, Allergy and Clinical Immunology, National Heart and Lung Institute at Imperial College London, Sir Alexander Fleming Building, London SW7 2AZ, United Kingdom. E-mail: k.nouri-aria@imperial.ac.uk. 0091-6749/\$34.00

© 2008 American Academy of Allergy, Asthma & Immunology
doi:10.1016/j.jaci.2008.03.013

Abbreviations used

GP-IT: Grass pollen injection immunotherapy

IQR: Interquartile range

Treg: Regulatory T

blood/lymph nodes of a population of T lymphocytes with a regulatory phenotype resembling CD4⁺CD25⁺ Treg cells.²⁰

In this report, in patients with allergic rhinitis, we examine the influence of GP-IT on the nasal mucosal expression of Foxp3 by CD4⁺ and CD25⁺ T lymphocytes and on the proportion of Foxp3⁺CD3⁺ cells expressing IL-10. We compared the findings in the nose with those in the tonsil, a tissue with naturally occurring thymic-derived Foxp3⁺CD25⁺CD4⁺ Treg cells. We also studied the influence of the pollen season on Foxp3 expression in a controlled trial of patients who had received GP-IT compared with placebo treatment.

METHODS**Patients**

The study participants comprised 13 patients with grass pollen allergy, 9 atopic patients with hay fever who had completed 2 years of GP-IT (*Phleum pratense*, Alutard SQ, ALK-Abelló, Hørsholm, Denmark), and 9 nonatopic healthy control subjects.²⁵ We also studied the effects of seasonal pollen exposure in 37 participants in a randomized controlled trial of GP-IT.²⁵ For patient characteristics, details of the immunotherapy protocol, methods for skin prick tests and intradermal skin tests, and recording of global hay fever assessment scores, see the **Methods** section in the Online Repository at www.jacionline.org.

Biopsy specimen collection

Nasal biopsy specimens (2.5 mm) were taken from the undersurface of the inferior turbinate by using Gerritsma forceps and 10% cocaine as a local anesthetic, as previously described.²³ All samples were examined blind and independent of the clinical protocol. For further information, see the **Methods** section in the Online Repository at www.jacionline.org. Tonsillectomy specimens were obtained from patients undergoing routine tonsillectomy and were provided by the Ear, Nose, and Throat Department, Charing Cross Hospital NHS Trust, London, United Kingdom.

Immunohistochemistry

Foxp3 was colocalized to CD4 and CD25 T cells and visualized by means of double immunofluorescence with a biotin-streptavidin system.²⁶ For information, see the **Methods** section in the Online Repository at www.jacionline.org.

Synthetic peptide corresponding to amino acids 418 to 431 of human Foxp3 was used in an absorption study to selectively block the binding of Foxp3 antibody (Advanced Biotechnology Centre, Imperial College London, London, United Kingdom). For the peptide absorption procedure, see the **Methods** section in the Online Repository at www.jacionline.org.

Colocalization of Foxp3 to CD3⁺CD25⁺ cells and IL-10 to CD3⁺ Foxp3-expressing cells was performed with triple immunofluorescence and a combination of fluorescein isothiocyanate (for CD3), tetramethylrhodamine isothiocyanate (CD25 or IL-10), and Alexa Fluor 350 (Foxp3), respectively,²⁶ on nasal biopsy sections from 3 GP-IT-treated patients. For quantification information, see the **Methods** section in the Online Repository at www.jacionline.org.

Statistical analysis

Between-group comparisons were performed by using the Mann-Whitney U test with the Bonferroni correction for multiple tests. Within-group comparisons were performed by using the Wilcoxon matched-pairs signed-rank test.

All analyses were performed with a statistical software package (Minitab, Inc, State College, Pa). All tests were 2-tailed, and *P* values of less than .05 were considered statistically significant.

RESULTS**Clinical response**

Patients were asked to make an overall assessment of their hay fever severity compared with that of previous years (before immunotherapy treatment) on a scale from +3 ("a lot better") to −3 ("a lot worse"). GP-IT was highly effective in improving patients' overall assessment of seasonal symptom severity (median, 3; interquartile range [IQR], 0-2) when compared with assessment among patients with untreated hay fever (median, 1; IQR, 2-3; *P* = .01). GP-IT was associated with a markedly reduced late-phase skin response 24 hours after intradermal grass pollen allergen (median, 184 mm²; IQR, 175-541 mm²) compared with that seen in untreated control subjects with hay fever (median, 2007 mm²; IQR, 1242-2535 mm²; *P* = .0007). Similarly, after GP-IT, the early skin wheal size at 15 minutes (median, 253 mm²; IQR, 179-296 mm²) was reduced compared with that seen in untreated control subjects (median, 338 mm²; IQR, 276-473 mm²; *P* = .04).

CD4⁺Foxp3⁺- and CD25⁺Foxp3⁺-expressing T cells in tonsillar tissue

Within tonsillar sections, the vast majority of Foxp3⁺ cells (>90%) were either CD4⁺ or CD25⁺ cells, whereas only 7% of CD4⁺ cells and 44% of CD25⁺ cells were Foxp3⁺. The specificity of the Foxp3 antibody used was confirmed by absorption studies with goat anti-Foxp3 antibody in the presence/absence of a specific Foxp3 peptide that corresponded to human Foxp3 (amino acids 418-431). Addition of the "blocking" Foxp3 peptide resulted in complete abrogation of the binding of goat anti-Foxp3 within both tonsillar and nasal tissue sections known to express Foxp3 (Fig 1, A).

Foxp3⁺-, CD4⁺Foxp3⁺-, and CD25⁺Foxp3⁺-expressing T cells in the nasal mucosa

Foxp3⁺ cells were predominantly distributed within the lamina propria of the nasal mucosa. GP-IT-treated patients had significantly more Foxp3⁺ cells (median, 16.1/mm²; IQR, 6.5-21.7/mm²) than untreated patients with hay fever (median, 2.75/mm²; IQR, 1.5-5.3/mm²; *P* = .005), but these levels did not differ from those in nonatopic healthy control subjects (median, 4.5/mm²; IQR, 2.8-10.1/mm²; *P* = .13; Fig 2, A).

Likewise, CD25⁺ cells were found in the lamina propria and were present in the highest numbers in patients receiving GP-IT (median, 38.5/mm²; IQR, 15.7-58.1/mm²), although the difference with numbers in untreated patients with hay fever was non-significant (median, 16.0/mm²; IQR, 9.4-21.55/mm²; *P* = .08), and a significant difference was observed between patients receiving GP-IT and nonatopic control subjects (median, 8.0/mm²; IQR, 4.0-17.4/mm²; *P* = .01). In contrast, the total number of CD4⁺ cells was not significantly different between any of the groups (data not shown).

The median number of Foxp3⁺CD25⁺ cells per square millimeter in the immunotherapy-treated patients (median, 9.3/mm²; IQR, 3.9-18.5/mm²) was significantly greater than in

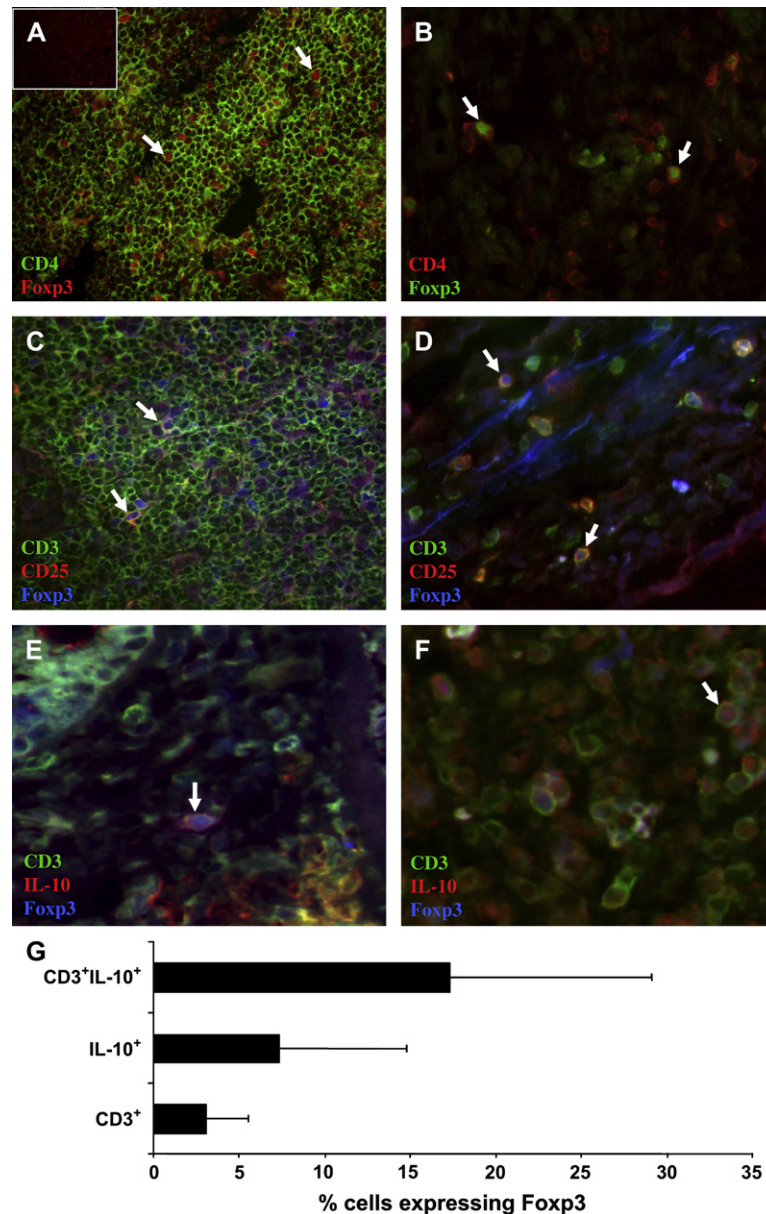


FIG 1. Immunofluorescence staining of tonsillar sections and nasal sections from GP-IT-treated patients. **A**, Foxp3⁺CD4⁺ cells in the tonsil (*inset* shows control after preadsorption with human Foxp3 peptide, magnification ×200). **B**, Foxp3⁺CD4⁺ cells in the nose (magnification ×400). **C**, CD3⁺CD25⁺Foxp3⁺ cells in the tonsil (magnification ×200). **D**, Foxp3⁺CD3⁺CD25⁺ cells in the nose (magnification ×400). **E and F**, Foxp3⁺CD3⁺IL-10⁺ cells in the nose (magnification ×1000). **G**, Percentage of Foxp3⁺ cells that were CD3⁺, IL-10⁺, or CD3⁺IL-10⁺.

untreated patients with hay fever (median, 2.0/mm²; IQR, 1.5–2.9/mm²; $P = .03$) and in nonatopic control subjects (median, 1.7/mm²; IQR, 0–4.9/mm²; $P = .025$; Fig 2, B). Similarly, the numbers of Foxp3⁺CD4⁺ cells per square millimeter in the immunotherapy-treated patients (median, 8.6/mm²; IQR, 0.8–16.3/mm²) were significantly higher than in the patients with hay fever (median, 0/mm²; IQR, 0–2.9/mm²; $P = .04$), whereas the difference between the immunotherapy-treated patients and the nonatopic control subjects was not significant (median, 2.0/mm²; IQR, 0.7–10.3/mm²; $P = .7$; Figs 1, B, and 2, C).

Effects of seasonal exposure on Foxp3 expression after immunotherapy

The effect of seasonal grass pollen exposure on Foxp3 expression in the nasal mucosa is shown in Table I. Seasonal increases in Foxp3⁺ cells compared with baseline values were observed in both placebo- and immunotherapy-treated subjects, whereas significant increases in dual-positive Foxp3⁺CD4⁺ cells and Foxp3⁺CD25⁺ cells were observed only in the immunotherapy-treated group. For comparison with the effects of immunotherapy on seasonal allergic inflammation, previously reported counts for eosinophils and IL-5–expressing cells²⁷ are also included, along

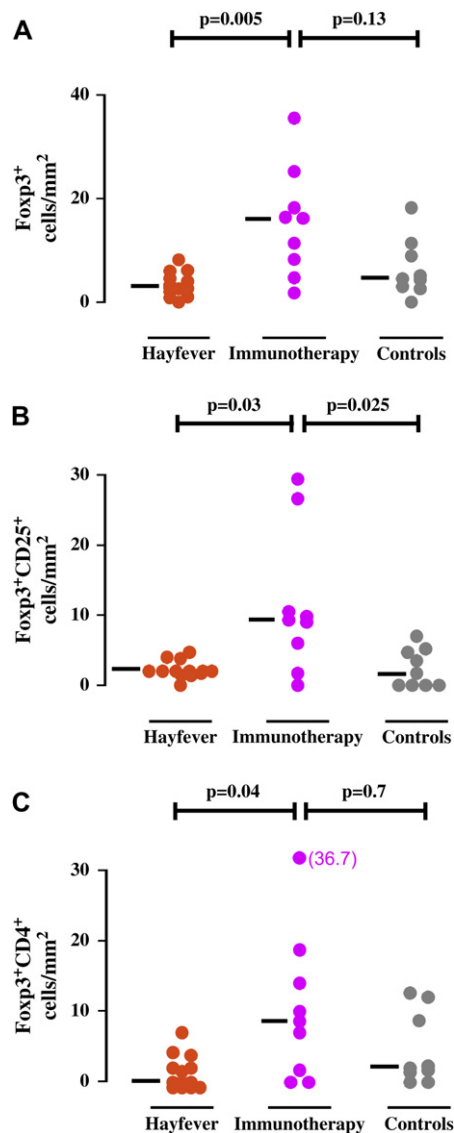


FIG 2. Foxp3⁺ (A), Foxp3⁺CD25⁺ (B), and Foxp3⁺CD4⁺ (C) cells in patients with hay fever, GP-IT-treated patients, and nonatopic control subjects. Statistical analysis was performed by using the Mann-Whitney *U* test.

with numbers of Foxp3-expressing cells in Table I. Seasonal increases were observed for both eosinophils and IL-5 mRNA-expressing cells in placebo-treated patients, which were inhibited after immunotherapy.

CD3⁺CD25⁺Foxp3⁺ cells and CD3⁺Foxp3⁺IL-10⁺ cells in tonsillar tissue and nasal mucosa

In tonsillar sections 47% of CD3⁺CD25⁺ cells expressed Foxp3 (Fig 1, C). In the nasal mucosa after immunotherapy, 20% of CD3⁺CD25⁺ cells also expressed Foxp3 (Fig 1, D).

In tonsillar tissue IL-10 expression was completely absent in Foxp3⁺CD3⁺ cells. In contrast, within the nasal mucosa 18% of CD3⁺ IL-10-expressing cells were Foxp3 positive (Fig 1, E-G).

DISCUSSION

Successful grass pollen immunotherapy was associated with markedly higher numbers of CD25⁺, Foxp3⁺, Foxp3⁺CD25⁺,

and Foxp3⁺CD4⁺ cells in the nasal mucosa compared with numbers seen in untreated subjects with hay fever. These differences could not be explained by atopic status alone and were not a feature of the nasal mucosa of normal subjects. Whereas CD25 expression alone is a feature of both “activated” and “regulatory” T cells,¹⁸ the coexpression of Foxp3 provides additional evidence in favor of an increase in Tregs that accompanies GP-IT. Increases in Foxp3-expressing cells in the nasal mucosa during the pollen season were observed in both placebo- and GP-IT-treated patients. However, seasonal increases in dual-positive Foxp3⁺CD4⁺ and Foxp3⁺CD25⁺ cells, more indicative of a regulatory phenotype, were observed only in immunotherapy-treated patients, which implies that local accumulation of T cells with a regulatory phenotype are a feature of immunotherapy rather than a “natural” regulatory mechanism in patients with allergic rhinitis during seasonal allergen exposure. The observation that Foxp3⁺ and Foxp3⁺IL-10⁺CD25⁺ T cells coexist in the nasal mucosa after GP-IT supports the emergence of phenotypically distinct populations of regulatory cells³⁻⁵: Foxp3-expressing adaptive Tregs and IL-10-producing T_H1 cells.

The dual- and triple-immunofluorescence methods used in this study provided highly sensitive and accurate methods of identifying double/triple-positive cells in the same, rather than consecutive, sections. Immune reactive cytokine detection in the tissue had not been easy in the past, with the consensus being that T cells are capable of synthesizing but not storing cytokines. Here we have convincingly demonstrated the presence of IL-10⁺ T cells in the nasal mucosa by using paraformaldehyde-fixed tissue,²³ which prevents the release of newly synthesized cytokines from T cells, rather than snap-frozen sections. This allowed us to colocalize IL-10 and Foxp3 within individual cell types.

Tonsillar sections were primarily used in this study as a positive control for the nasal sections. However, the observation that Foxp3⁺CD3⁺ Tregs in the tonsil lack IL-10 expression raises the possibility that these cells, although phenotypically consistent with “naturally occurring” Tregs, do not express IL-10 *de novo* in the absence of stimulation.⁸ In contrast, the expression of IL-10, which is known to promote tolerance, by Foxp3⁺CD3⁺ cells in the nasal mucosa of immunotherapy-treated patients might imply that immunotherapy was responsible for the induction, recruitment, or both of Tregs (adaptive/inducible) in patients with allergic rhinitis.

Our findings are in agreement with those reported in murine models of colitis and in patients with ulcerative colitis, Crohn’s disease, or both, in whom IL-10-producing Foxp3⁺CD4⁺CD25⁺ cells were present at increased density in the colon and the presence of IL-10 was associated with amelioration of colitis.^{13,28} In studies of cells derived from peripheral blood of subjects with birch allergy, a recombinant Bet v 1 allergen-S-layer fusion protein primed the development of naive T cells into IL-10-producing CD25⁺Foxp3⁺CLTA4⁺ cells capable of active suppression.²⁹ Functionally active, antigen-specific Foxp3⁺CD4⁺CD25⁺ T cells have also been identified in peripheral blood of atopic and nonatopic individuals.³⁰

The mechanisms of suppression by Treg subsets remain controversial; both cell-cell contact and downregulation through the immunosuppressive cytokines IL-10 and TGF- β have been described.^{6,31} It was not possible to perform functional studies on local Foxp3⁺ cells in view of the inevitably small size of nasal biopsy specimens and the relatively low numbers of cells present. In the absence of functional data, the identification of local T cells

TABLE I. Effects of grass pollen immunotherapy on Foxp3⁺CD4⁺CD25⁺ T cells in the nasal mucosa

	Placebo (n = 17)		Immunotherapy (n = 20)	
	Before treatment (out of season)	After treatment (in season)	Before treatment (out of season)	After treatment (in season)
CD4 ⁺ /mm ²	54.5 (33-78)	53.6 (18-138)	80.4 (30-120)	74.9 (35-154)
CD25 ⁺ /mm ²	14.0 (8-21)	18.2 (8-25)	13.7 (8-24)	20.9 (12-36)
Foxp3 ⁺ /mm ²	2.8 (1-4)	5.7* (1-8)	3.8 (2-5)	6.0‡ (4-10)
Foxp3 ⁺ CD4 ⁺ /mm ²	0 (0-3)	3.1 (0-81)	1.5 (0.3-3)	4.6* (3-7)
Foxp3 ⁺ CD25 ⁺ /mm ²	2.0 (1-3)	3.1 (0.6-6)	1.9 (0-3)	4.4‡ (3-7)
Eosinophil granules-2 ⁺ §/mm ²	7.5 (2.8-28.8)	39.2‡# (16.4-76)	12 (3.2-21)	14.2* (9.4-27)
IL-5 mRNA§/mm ²	0 (0-1.4)	2.2* (0-17)	0 (0-0)	0.7 (0-4.6)

**P* < .05, †*P* < .01, and ‡*P* < .001, after treatment (in season) compared with before treatment (out of season).

§Data obtained from Nouri-Aria et al^{E3} (available in the [Online Repository](http://www.jacionline.org) at www.jacionline.org).

||*P* = .16 and #*P* = .04, immunotherapy-treated group compared with placebo-treated group.

with a so-called regulatory phenotype can only be made by inference. In human subjects, in contrast to mice, a proportion of activated T cells has also been shown to express Foxp3 mRNA.^{32,33} Therefore the expression of this transcription factor on its own might not represent regulatory function of these cells. Nonetheless, we have shown that distinct subsets of Foxp3⁺ T cells within the local target organ were associated with allergen-specific immunotherapy along with suppression of allergen-induced late-phase cutaneous responses,²⁰ which are known, at least in part, to be T-cell dependent. Furthermore, seasonal increases in Foxp3⁺CD25⁺ and Foxp3⁺CD4⁺ cells in the nasal mucosa after immunotherapy were accompanied by reductions in local IL-5⁺ cells and eosinophils.²⁰ Our previous findings of increased proportions of IL-10-producing peripheral CD4⁺CD25⁺ cells²⁰ and increased numbers of IL-10-expressing²³ and TGF-β-expressing²⁴ T cells within the nasal mucosa after immunotherapy are also consistent with the view that Treg cells play a role in allergen-specific tolerance during successful immunotherapy. In patients with bronchial asthma, corticosteroids have also been shown to modify peripheral Treg function with increased levels of IL-10 production and Foxp3 expression, which accompanied the alleviation of asthma symptoms.¹²

In summary, after grass pollen immunotherapy, Foxp3⁺CD25⁺ and Foxp3⁺CD4⁺ cell numbers were increased in the nasal mucosa. Seasonal increases in these cells were accompanied by suppression of local allergic inflammation. Immunotherapy also resulted in the recruitment of IL-10-producing Foxp3⁺CD3⁺ and IL-10-producing Foxp3⁺CD3⁺ cells, both of which can contribute to the prevention of T_H2 cell recruitment and activation. Future studies should focus on the underlying mechanisms controlling the phenotype and function of Tregs after immunotherapy. Our data suggest that strategies to increase local Tregs might be beneficial in the treatment of hay fever.

Clinical implications: These data support the local involvement of Foxp3⁺ T cells, presumed Treg cells, in the mechanisms of allergen immunotherapy and identify a potential target for immunomodulation.

REFERENCES

- Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, et al. Immunologic tolerance maintained by CD25⁺ CD4⁺ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* 2001;182:18-32.
- Maloy KJ, Powrie F. Regulatory T cells in the control of immune pathology. *Nat Immunol* 2001;2:816-22.
- O'Garra A, Vieira P. Regulatory T cells and mechanisms of immune system control. *Nat Med* 2004;10:801-5.
- Le NT, Chao N. Regulating regulatory T cells. *Bone Marrow Transplant* 2007;39:1-9.
- Vieira PL, Christensen JR, Minaae S, O'Neill EJ, Barrat FJ, Boonstra A, et al. IL-10 secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4⁺CD25⁺ regulatory T cells. *J Immunol* 2004;172:5986-93.
- Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4⁺CD25⁺ regulatory T cells is mediated by cell surface-bound transforming growth factor-β. *J Exp Med* 2001;194:629-44.
- Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25⁺CD4⁺ regulatory cells that control intestinal inflammation. *J Exp Med* 2000;192:295-302.
- Lim HW, Broxmeyer HE, Kim CH. Regulation of trafficking receptor expression in human forkhead box p3⁺ regulatory T cells. *J Immunol* 2006;177:840-51.
- Freitas A, Wildin RS. IPEX and FOXP3; clinical and research perspectives. *J Autoimmun* 2005;25:56-62.
- Uhlig HH, Coombes J, Mottet C, Izcue A, Thompson C, Fanger A, et al. Characterisation of Foxp3⁺CD4⁺CD25⁺ and IL-10-secreting CD4⁺CD25⁺ T cells during cure of colitis. *J Immunol* 2006;177:5852-60.
- Verhagen J, Akdis M, Traidl-Hoffmann C, Schmid-Grendelmeier P, Hijnen D, Knol EF, et al. Absence of T-regulatory cell expression and function in atopic dermatitis skin. *J Allergy Clin Immunol* 2006;117:176-83.
- Karagiannidis C, Akdis M, Holopainen P, Woolley NJ, Hense G, Ruckert B, et al. Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. *J Allergy Clin Immunol* 2004;114:1425-33.
- Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 1999;190:995-1004.
- Battaglia M, Stabilini A, Migliavacca B, Horejs-Hoeck J, Kaupper T, Roncarolo M-G. Rapamycin promotes expansion of functional CD4⁺CD25⁺FOXP3⁺ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J Immunol* 2006;177:8338-47.
- Ruprecht C, Gattorno M, Ferlito F, Gregorio A, Martini A, Lanzavecchia A, et al. Co-expression of CD25 and CD27 identifies FoxP3⁺ regulatory T cells in inflamed synovia. *J Exp Med* 2005;201:1793-803.
- Bochner BS, Schleimer RP. Mast cells, basophils and eosinophils: distinct but overlapping pathways for recruitment. *Immunol Rev* 2001;179:5-15.
- Hansen I, Klimek L, Mösges R, Hörmann K. Mediators of inflammation in the early and late phase of allergic rhinitis. *Curr Opin Allergy Clin Immunol* 2004;4:159-63.
- Ling EM, Smith T, Nguyen XD, Pridgeon C, Dallman M, Arbery J, et al. Relation of CD4⁺CD25⁺ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004;363:608-15.
- Karlsson MR, Rugtveit J, Brandtzaeg P. Allergen-responsive CD4⁺CD25⁺ regulatory T cells in children who have outgrown cow's milk allergy. *J Exp Med* 2004;199:1679-88.
- Till SJ, Francis JN, Nouri-Aria K, Durham SR. Mechanisms of immunotherapy. *J Allergy Clin Immunol* 2004;113:1025-34.
- Aalberse R, van der Gaag CR, van Leeuwen J. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. *J Immunol* 1983;130:722-7.
- Gehlhar K, Schlaak M, Becker W, Bufe A. Monitoring allergen immunotherapy of pollen-allergic patients: the ratio of allergen-specific IgG4 to IgG1 correlates with clinical outcome. *Clin Exp Allergy* 1999;29:497-506.

23. Nouri-Aria KT, Wachholz PA, Francis JN, Jacobson MR, Walker SM, Wilcock LK, et al. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol* 2004;172:3252-9.
24. Pilette C, Nouri-Aria KT, Jacobson MR, Wilcock LK, Detry B, Walker M, et al. Grass pollen immunotherapy induces an allergen-specific IgA2 antibody response associated with mucosal TGF- β expression. *J Immunol* 2007;178:4658-66.
25. Walker SM, Pajno GB, Lima MT, Wilson DR, Durham SR. Grass pollen immunotherapy for seasonal rhinitis and asthma: a randomized, controlled trial. *J Allergy Clin Immunol* 2001;107:87-93.
26. Nouri-Aria KT, Pilette C, Jacobson MR, Watanabe H, Durham SR. IL-9 and c-Kit⁺ mast cells in allergic rhinitis during seasonal allergen exposure: effect of immunotherapy. *J Allergy Clin Immunol* 2005;116:73-9.
27. Wilson DR, Nouri-Aria KT, Walker SM, Pajno GB, O'Brien F, Jacobson MR, et al. Grass pollen immunotherapy: Symptomatic improvement correlates with reductions in eosinophils and IL-5 mRNA expression in the nasal mucosa during the pollen season. *J Allergy Clin Immunol* 2001;107:971-6.
28. Liu H, Hu B, Xu D, Liew FY. CD4⁺CD25⁺ regulatory T cells cure murine colitis: the role of IL-10, TGF- β , and CTLA4. *J Immunol* 2003;171:5012-7.
29. Gerstmayr M, Ilk N, Schabussova I, Jahn-Schmid B, Egelseer EM, Sleytr UB, et al. A novel approach to specific allergy treatment: The recombinant allergen-S-Layer fusion protein rSbsC-Bet v 1 matures dendritic cells that prime Th0/Th1 and IL-10 producing regulatory T cells. *J Immunol* 2007;179:7270-5.
30. Maggi L, Santarlasci V, Liotta F, Frosali F, Angeli R, Cosmi L, et al. Demonstration of circulating allergen-specific CD4⁺CD25^{high}Foxp3⁺ T-regulatory cells in both nonatopic and atopic individuals. *J Allergy Clin Immunol* 2007;120:429-36.
31. Zheng SG, Wang J, Wang P, Gray JD, Horwitz DA. IL-2 is essential for TGF- β to convert naïve CD25⁺CD4⁺ cells to CD25⁺Foxp3⁺ regulatory T cells and for expansion of these cells. *J Immunol* 2007;178:2018-27.
32. Mantel P-Y, Ouaked N, Ruckert B, Karagiannidis C, Welz R, Blaser K, et al. Molecular mechanisms underlying Foxp3 induction in human T cells. *J Immunol* 2006;176:3593-602.
33. Morgan ME, van Bilsen JHM, Bakker AM, Heemskerk B, Schilham MW, Hartgers FC, et al. Expression of Foxp3 mRNA is not confined to CD4⁺CD25⁺ T regulatory cells in human. *Hum Immunol* 2005;66:13-20.

METHODS

Patients

Participants were recruited from the Royal Brompton Allergy Clinic or after placement of an advertisement in the local newspaper. The study was performed with approval of the ethics committee of the Royal Brompton Hospital and with the informed consent of the participants. The patients comprised 13 untreated subjects with grass pollen allergy (7 male subjects; mean age, 36 years [range, 24–59 years]; skin prick test, $47.3 \pm 8.0 \text{ mm}^2$ [mean $\pm$ SEM]) and 9 atopic patients with hay fever who had completed 2 years of GP-IT (6 male patients; mean age, 31 years [range 22–38 years], skin prick test, $55.5 \pm 6.6 \text{ mm}^2$).^{E1} The nonatopic healthy control group comprised 5 male and 4 female subjects (mean age, 29 years) (range, 24–41 years) with negative skin prick test responses to a panel of 11 common aeroallergens (grass pollen, birch pollen, mixed tree pollen, *Dermatophagoides pteronyssinus*, cat, dog, horse, and molds [*Alternaria* species, *Cladosporium* species, and *Aspergillus fumigatus*] and negative [diluent] and positive [histamine, 10 mg/mL] controls); Soluprick, ALK-Abelló) and no symptoms during the pollen season. The effects of GP-IT on Foxp3⁺ cells during the pollen season was studied in 37 patients who participated in a previously reported randomized controlled trial.^{E2} Twenty subjects (10 male subjects; median age, 32 years [range, 26–36 years]) received GP-IT, and 17 subjects (9 male subjects; median age, 34 years [range, 29–34 years]) received placebo injections. GP-IT (Alutard SQ *Phleum pratense*, ALK-Abelló) or placebo injections were administered during a clustered 4-week up dosing phase, followed by monthly maintenance injections (containing 20 μg of the major allergen Phl p 5) for 2 years. Nasal biopsy specimens were taken at baseline and during the pollen season after 2 years of treatment, as previously described.^{E2}

A global assessment of seasonal hay fever severity was performed at the end of the pollen season, in which patients recorded their hay fever severity on a scale of -3 to $+3$ compared with previous years or compared with symptoms before commencing immunotherapy.

Skin prick tests and intradermal skin tests were performed by using grass pollen extract (*Phleum pratense*, Soluprick, ALK-Abelló). Skin prick tests were performed on the flexor aspect of the forearm. Intradermal tests were performed with 0.02 mL of diluent containing 10 BU/mL on the extensor aspect of the forearm, and the early skin response (wheal size) at 15 minutes and late swelling after 24 hours were recorded as previously described.^{E1}

Biopsy collection

Nasal biopsy specimens were collected outside the United Kingdom pollen season from October through March from GP-IT-treated patients and untreated patients with hay fever and between June and August from nonatopic healthy control subjects. None of the patients were symptomatic or taking medication at the time of biopsy because their biopsy specimens were taken remote from the pollen season. Immunotherapy-treated patients had received treatment for at least 2 years and continued treatment at the time of biopsy, with biopsy specimens being taken 2 to 4 weeks after the most recent monthly maintenance injection. The effect of seasonal exposure was examined on biopsy specimens taken at baseline and during the peak pollen season after treatment.^{E1}

Immunohistochemistry

Six-micrometer, 4% paraformaldehyde-fixed cryostat sections were used in the study. Foxp3 colocalized to CD4 and CD25 T cells was visualized by means

of double immunofluorescence with a biotin-streptavidin system. Monoclonal antibodies to CD4 and CD25 (DakoCytomation, Cambridgeshire, United Kingdom), polyclonal goat anti-Foxp3 (Abcam, Cambridge, United Kingdom) for the detection of nuclear Foxp3, and mAb to human IL-10 (Santa Cruz Biotechnology, Santa Cruz, Calif) were used to colocalize Foxp3 and IL-10 in T-cell subsets. The method for the detection of IL-10 used in the present study has been previously validated.^{E2} T-cell subsets were detected by means of 2- or 3-step staining. Fluorescein isothiocyanate-labeled rabbit anti-mouse antibody (2 steps) or rabbit anti-mouse secondary antibodies (3 steps; DakoCytomation) were used for lymphocyte subsets, and biotinylated anti-goat antibody (Jackson ImmunoResearch, Suffolk, United Kingdom) was used for Foxp3 detection. These were followed by tetramethylrhodamine isothiocyanate-swine anti-rabbit immunoglobulin (DakoCytomation) for T-cell subsets (3 steps) and streptavidin-Alexa Fluor 488 (for Foxp3 detection; Invitrogen, Paisley, Scotland).^{E3,E4} Normal goat IgG and mouse IgG1 and IgG2b (DakoCytomation) were used as controls for Foxp3, CD4/CD25, and IL-10, antibodies respectively.

Blocking of Foxp3 staining

Foxp3 synthetic peptide was incubated with goat anti-Foxp3 antibody at a ratio of 30:1. The mixture was incubated at 4°C overnight. The mixture was then centrifuged for 15 minutes at 10,000g, and the supernatant was used for immunostaining of tonsil sections.

Quantification

Coded sections were counted independently from the clinical protocol and in a blinded fashion. Positively stained cells were counted immediately beneath the epithelium to 2 grids' depth along the whole length of the biopsy specimen, and results were expressed as the number of positive cells per square millimeter. On average, this involved counting cells in 8 fields, which is equivalent to 0.8 mm^2 ($0.2\text{--}1.8 \text{ mm}^2$) of subepithelial tissue per biopsy specimen. The sections stained for Foxp3⁺, CD4⁺, and CD25⁺ cells; dual-stained sections for Foxp3⁺, CD4⁺, and CD25⁺ cells; and colocalization of Foxp3 to CD4 and CD25 cells were examined and counted at $\times 400$ magnification with a Nikon Eclipse (E400) fluorescent microscope (Tokyo, Japan) under appropriate absorption/emission wavelengths (590/617 nm for CD4⁺ and CD25⁺ cells [tetramethylrhodamine isothiocyanate] and 488/519 nm for Foxp3 [Alexa Fluor 488]). The images were captured with a Nikon Digital Still Camera DXM1200 with Lucia 4.8 software (system for image processing and analysis; Prague, Czech Republic).

REFERENCES

- E1. Walker SM, Pajno GB, Lima MT, Wilson DR, Durham SR. Grass pollen immunotherapy for seasonal rhinitis and asthma: a randomized, controlled trial. *J Allergy Clin Immunol* 2001;107:87-93.
- E2. Nouri-Aria KT, Wachholz PA, Francis JN, Jacobson MR, Walker SM, Wilcock LK, et al. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol* 2004;172:3252-9.
- E3. Nouri-Aria KT, Pilette C, Jacobson MR, Watanabe H, Durham SR. IL-9 and c-Kit⁺ mast cells in allergic rhinitis during seasonal allergen exposure: effect of immunotherapy. *J Allergy Clin Immunol* 2005;116:73-9.
- E4. Wilson DR, Nouri-Aria KT, Walker SM, Pajno GB, O'Brien F, Jacobson MR, et al. Grass pollen immunotherapy: symptomatic improvement correlates with reductions in eosinophils and IL-5 mRNA expression in the nasal mucosa during the pollen season. *J Allergy Clin Immunol* 2001;107:971-6.