

## ORIGINAL ARTICLE

# Sublingual grass pollen immunotherapy is associated with increases in sublingual Foxp3-expressing cells and elevated allergen-specific immunoglobulin G4, immunoglobulin A and serum inhibitory activity for immunoglobulin E-facilitated allergen binding to B cells

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## Clinical & Experimental Allergy

### Summary

**Background** The mechanisms of sublingual immunotherapy (SLIT) are less well understood than those of subcutaneous immunotherapy (SCIT).

**Objectives** To determine the effects of grass-pollen SLIT on oral mucosal immune cells, local regulatory cytokines, serum allergen-specific antibody subclasses and B cell IgE-facilitated allergen binding (IgE-FAB).

**Methods** Biopsies from the sublingual mucosa of up to 14 SLIT-treated atopics, nine placebo-treated atopics and eight normal controls were examined for myeloid dendritic cells (mDCs) (CD1c), plasmacytoid dendritic cells (CD303), mast cells (AA1), T cells (CD3) and Foxp3 using immunofluorescence microscopy. IL-10 and TGF- $\beta$  mRNA expression were identified by *in situ* hybridization. Allergen-specific IgG and IgA subclasses and serum inhibitory activity for binding of allergen-IgE complexes to B cells (IgE-FAB) were measured before, during and on the completion of SLIT.

**Results** Foxp3<sup>+</sup> cells were increased in the oral epithelium of SLIT- vs. placebo-treated atopics ( $P=0.04$ ). Greater numbers of subepithelial mDCs were present in placebo-treated, but not in SLIT-treated, atopics compared with normal controls ( $P=0.05$ ). There were fewer subepithelial mast cells and greater epithelial T cells in SLIT- compared with placebo-treated atopics ( $P=0.1$  for both). IgG<sub>1</sub> and IgG<sub>4</sub> were increased following SLIT ( $P<0.001$ ). Peak seasonal IgA<sub>1</sub> and IgA<sub>2</sub> were increased during SLIT ( $P<0.05$ ). There was a time-dependent increase in serum inhibitory activity for IgE-FAB in SLIT-treated atopics.

**Conclusions** SLIT with grass pollen extract is associated with increased Foxp3<sup>+</sup> cells in the sublingual epithelium and systemic humoral changes as observed previously for SCIT.

**Keywords** Foxp3, IgE-facilitated allergen binding, oral mucosa, specific IgG<sub>4</sub>, sublingual immunotherapy

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### Introduction

Sublingual immunotherapy (SLIT) is an effective and safe alternative to subcutaneous immunotherapy (SCIT) for the treatment of allergic rhinitis [1, 2] and other allergic

diseases. Recent attention has focused on understanding the mechanism of SLIT, particularly regarding the initial allergen – immune cell interactions [3, 4]. Murine studies have demonstrated the migration of antigen across the oral epithelium, allergen uptake by oral antigen-presenting cells (APCs) *in vitro*, and the induction of allergen-specific T cells with regulatory characteristics in local lymph nodes [5]. Allergen uptake by specialized APCs within the oral mucosa

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is presumed to be the first step in successful SLIT. Dendritic cells (DCs), including oral 'Langerhans' cells, are abundant within the human oral mucosa [6]. These cells possess high-affinity surface IgE receptors [7], are able to release IL-10 and induce T cells with a regulatory phenotype *in vitro* [8].

Studies of SCIT have identified both circulating [9] and mucosal Tregs [10, 11]. Tregs may induce B cell isotype switching to the production of protective IgG<sub>4</sub> antibodies [12] and IgA<sub>2</sub> antibodies [13]. Evidence for the induction of allergen-specific Tregs during SLIT is limited [14], but convincing increases in allergen-specific IgG<sub>4</sub> have been observed [15–17]. Post-immunotherapy sera from SCIT-treated patients have been shown to inhibit IgE-facilitated allergen binding to B cells and subsequent presentation to allergen-specific T cell clones [18–20]. The proliferative response of these T cell clones corresponds to the level of allergen-IgE binding to B cells, which can be determined by flow cytometry in a validated technique termed the IgE-facilitated allergen binding (IgE-FAB) assay [21]. The assay has been used to demonstrate a *bona fide* functional suppressive effect of induced allergen-specific antibodies in SCIT but has not as yet been extended to a study of SLIT. Induced IgG antibodies may compete with IgE for allergen binding sites and/or may bind to alternative sites on the allergen, thereby interfering with IgE-mediated co-operative binding to adjacent CD23 receptors on B cells.

The effects of SLIT at the level of the oral mucosa are incompletely understood. Given the postulated mechanisms, it is plausible that SLIT impacts on APCs and T cells within the mucosa, including the possible induction of Tregs locally. Mast cells are present in the oral mucosa at levels equivalent to the skin [6]; but their role in mediating the local side-effects of SLIT is unproven [22]. SCIT reduces nasal mast cell numbers [23] and increases IL-10 and TGF- $\beta$  expression in the nasal mucosa [13]. These cytokines might have important regulatory properties within the oral mucosa, which are exploited during SLIT.

We intended to further elucidate the local mucosal and systemic humoral responses to SLIT using sublingual biopsies and serum samples obtained in a previous double-blind, placebo-controlled trial of high-dose grass pollen SLIT [24]. The primary comparison was between SLIT- and placebo-treated atopics; secondary comparisons were made with untreated, non-atopic controls. We hypothesized that SLIT alters the immune cell constitution of the sublingual mucosa, in particular increasing the number of Tregs, and also that SLIT induces allergen-specific IgG and IgA antibodies and inhibits B cell IgE-FAB. We present novel findings including the presence of Foxp3<sup>+</sup> T cells in the oral mucosa and the induction of functional, allergen-specific antibodies following SLIT.

## Methods

### Patients

Fifty-six patients were recruited to participate in a double-blind placebo-controlled parallel group study of sublingual grass pollen immunotherapy, as described previously [24]. Treatment involved a 6-week up-dosing phase followed by daily sublingual application of a standardized extract of *Phleum pratense* (Timothy grass) with a maintenance dose of 25  $\mu$ g/day or placebo that was continued for 12–18 months. Venous blood was taken from participants at baseline, after up-dosing (6 weeks), during peak pollen season (3 months) and at completion (12–18 months) of the study. At baseline and again at completion of the trial, intradermal skin testing with *P. pratense* extract (ALK-Abelló, Horsholm, Denmark, 10 SQ units) was performed in all subjects. A subgroup of 23 patients (14 on active treatment, nine on placebo) consented to undergo a sublingual biopsy. Eight non-atopic volunteers (no history of respiratory allergies, negative skin prick testing to a panel of common aeroallergens) also consented to undergo a sublingual biopsy. Biopsies were all taken following the completion of treatment after the 1999 pollen season. The study was performed with the approval of the local ethics committee and with the written informed consent of all participants.

### Sublingual biopsies

Biopsies were taken using cup and ring forceps following infiltration of the postero-lateral sublingual region with lidocaine 1%+epinephrine 1:200 000 as described previously [24]. Biopsies were divided into two halves; one half was immediately mounted in an optimal cutting temperature (OCT) compound, snap-frozen and stored at  $-80^{\circ}\text{C}$ . The other half was fixed in 4% paraformaldehyde for 2 h and dehydrated in 15% sucrose-PBS for 1 h and again overnight before being mounted in OCT and snap-frozen. Six micron acetone/methanol (60:40) cryostat sections were used throughout the study. Tonsillectomy specimens were obtained, with consent, from patients undergoing routine tonsillectomy, provided by the Ear Nose and Throat Department, Charing Cross Hospital NHS Trust, London. Tonsil tissue was used as a positive control for immunostaining.

### Immunohistochemistry

Sections were permeabilized in 0.1% saponin. Endogenous biotin was blocked using an avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Endogenous Fc-receptors were blocked by incubation in appropriate serum (20%) for 20 min. Sections were then incubated with primary antibodies. Mouse monoclonal antibodies to T

lymphocytes (CD3), mast cell tryptase (AA1) (DakoCytomation, Cambridgeshire, UK), myeloid dendritic cells (mDCs, CD1c) and plasmacytoid dendritic cells (pDCs, CD303) (MACS, Miltenyi Biotec, Surrey, UK) and goat polyclonal antibodies to Foxp3 (Abcam, Cambridgeshire, UK) were used. Primary antibody binding to CD3 and AA1 was detected using fluorescein isothiocyanate-labelled rabbit anti-mouse antibody (FITC, DakoCytomation); binding to CD1c and CD303 was detected with biotinylated horse anti-mouse antibody (Vector Laboratories), followed by streptavidin-conjugated Alexa Fluor 594 (Invitrogen, Paisley, UK); binding to Foxp3 was detected with biotinylated anti-goat antibody (Vector Laboratories) followed by streptavidin-conjugated Alexa Fluor 594. Normal goat IgG (Santa Cruz Biotech, Santa Cruz, USA) was used as an isotype control for Foxp3 antibody. Normal mouse IgG<sub>1</sub> (Vector Laboratories) was used as an isotype control for CD3, CD1c, CD303 and AA1 antibodies. Slides were mounted using fluorescence mountant, ProLong (Invitrogen). Snap-frozen, acetone-fixed slides were used for immunostaining of CD3, mDC, pDC and AA1. Foxp3 staining was carried out on a smaller, unselected subset of paraformaldehyde-fixed specimens owing to limited availability.

Sections were examined by immunofluorescence microscopy at  $\times 400$  magnification with a Nikon Eclipse (E400) microscope (Tokyo, Japan). Sections were coded with the examiner blinded to their status. Images covering the entire section were captured using a Nikon Digital Still Camera DXM1200 with Lucia 4.8 software (Prague, Czech Republic). Positively stained cells were counted and the results were reported as median numbers of cells/mm<sup>2</sup> within the epithelium and subepithelium.

#### *Foxp3 blocking peptide*

Specific Foxp3 synthetic peptide corresponding to amino acids 418–431 of human Foxp3 (Advanced Biotechnology Centre, Imperial College, UK) was used to confirm the specificity of Foxp3 staining. Peptide and Foxp3 antibody were mixed at a ratio of 30:1, incubated at room temperature for 2 h and subsequently at 4 °C overnight. The mixture was then centrifuged at 10 000 g for 15 min and the supernatant used for immunostaining as for anti-Foxp3 antibody [11].

#### *In situ hybridization*

Antisense and sense riboprobes for IL-10 and antisense riboprobe for TGF- $\beta$  were prepared from cDNA inserted in viral vectors and linearized with restriction enzymes. Transcription was performed in the presence of <sup>35</sup>S-uridine triphosphate (UTP) using an appropriate T7 or SP6 RNA polymerase. Hybridization was performed on paraformaldehyde-fixed sections permeabilized with Tri-

ton X-100 followed by proteinase K digestion. Following autoradiography, slides were incubated for 4 weeks then developed. Slides were examined under a light microscope at  $\times 200$  magnification. Specific hybridization was recognized as clear, dense deposits of silver grains in photographic emulsion overlaying tissue sections. Positive cells were counted and expressed per millimetre square of epithelium and subepithelium.

#### *Allergen-specific immunoglobulin measurements*

*P. pratense*-specific IgG<sub>1</sub>, IgG<sub>4</sub>, IgA<sub>1</sub> and IgA<sub>2</sub> antibodies were measured by enzyme-linked immuno-sorbent assay. Serum samples were diluted 100-fold in PBS for IgG<sub>1</sub> and IgG<sub>4</sub>, and 10-fold for IgA<sub>1</sub> and IgA<sub>2</sub>. Microplates (Nunc-Maxisorb) were coated with 5  $\mu$ g/mL of *P. pratense* extract (ALK-Abelló) for capture. For IgG antibodies, biotinylated monoclonal antibody to IgG<sub>1</sub> (1:1000) and IgG<sub>4</sub> (1:150 000) (Sigma-Aldrich, St Louis, MO, USA) were used for detection. For IgA antibodies mouse mAbs to IgA<sub>1</sub> (clone B3506) or IgA<sub>2</sub> (clone A9603) were used (1  $\mu$ g/mL) and revealed by HRP-conjugated anti-mouse IgG (Sigma-Aldrich; 1/10 000) followed by incubation with hydrogen peroxide and tetramethylbenzidine in phosphate-citrate buffer. Optical density (OD<sub>450</sub>) was measured using an ELISA microplate reader (Emax, Molecular Probes, Eugene, OR, USA).

#### *B cell immunoglobulin E-facilitated allergen binding inhibition assay*

The B cell IgE-facilitated allergen binding inhibition assay has been described and validated previously [21]. Briefly, stock serum indicator containing a high concentration of *P. pratense*-specific IgE (> 100 IU/mL) was incubated with 1  $\mu$ m/mL allergen for 1 h at 37 °C to form allergen-IgE complexes. Patient serum was then added to the indicator serum-allergen mix. EBV-transformed B cells were then added and incubated for 1 h at 4 °C. Cells were washed and surface-bound allergen-IgE complexes detected using a polyclonal human anti-IgE FITC-labelled antibody by incubation at 4 °C for 45 min followed by flow cytometry (FACS Calibur, BD Biosciences, Rockville, MD, USA). Allergen-IgE surface binding to B cells is presented as the percentage binding in the presence of patient serum compared with binding with indicator serum alone.

#### *Statistical analysis*

Immunohistochemistry and *in situ* hybridization data are presented as median numbers of positive cells per mm<sup>2</sup>. Between-group comparisons were made by the Mann-Whitney *U*-test (Minitab Software Package, Minitab Inc., PA, USA). For antibody levels and facilitated allergen binding, within-group comparisons were performed using

repeated-measures one-way ANOVA, followed by the Friedman correction test for repeated non-parametric measurements and between-group comparisons by Mann-Whitney *U*-test (Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA). All tests were two-tailed; *P*-values  $\leq 0.05$  were considered statistically significant.

## Results

### Participant demographics and clinical response

Treatment groups were matched for age, gender and level of allergen-specific IgE to grass pollen (Table 1). As reported previously [24], significantly more SLIT-treated individuals rated their overall symptoms at the peak season as better than previous years compared with placebo-treated patients ( $P = 0.003$ ). Median symptom scores were 28% lower and rescue medication use 45% lower in SLIT- compared with placebo-treated patients ( $P = 0.48$ ,  $0.19$ , respectively). The allergen-induced late-phase skin response measured at 6 h was significantly lower following treatment in SLIT- compared with placebo-treated patients ( $P < 0.02$ ) (Table 1).

### Immunohistochemistry and in situ hybridization

There was a trend towards greater numbers of CD3<sup>+</sup> cells in the epithelium of SLIT- compared with placebo-treated atopics (Fig. 1a, Table 2). Foxp3<sup>+</sup> cells were significantly more abundant in the epithelium of SLIT-treated than placebo-treated atopics ( $P = 0.04$ ) (Fig. 1b, Table 2). In two SLIT-treated individuals, dual Foxp3<sup>+</sup> CD3<sup>+</sup> cells were identified in the epithelium. There were no differences in the number of Foxp3<sup>+</sup> or Foxp3<sup>+</sup> CD3<sup>+</sup> cells in the subepithelium between groups (Table 2). Within-tonsil sections,  $< 5\%$  of CD3<sup>+</sup> cells co-stained for Foxp3 (Fig.

1c). Foxp3 staining in tonsil and sublingual mucosa was abrogated by use of the blocking peptide (Figs 1d and f).

There were no differences in the number of mDC in the epithelium between groups (Table 2). More subepithelial mDC were present in placebo-treated atopics than in non-atopics ( $P = 0.05$ ) (Fig. 2a, Table 2). There was no difference between SLIT-treated atopics and non-atopics. pDC numbers were low in each group (Table 2).

Median number of epithelial mast cells did not differ significantly between groups. There was a trend towards a greater number of mast cells in the subepithelium of placebo- than SLIT-treated atopics ( $P = 0.1$ ) (Fig. 2b, Table 2).

Both IL-10 mRNA- and TGF- $\beta$  mRNA-positive cells were identified, particularly at the epithelial-subepithelial border (Figs 2e and f). No significant differences were evident between groups (Table 2).

### Inhibition of facilitated allergen binding

Percentage facilitated allergen binding to B cells was matched at baseline between groups (Fig. 3a, supporting information Table S1). Sera from SLIT-treated patients significantly inhibited B cell IgE-FAB at peak season and on completion of treatment compared with baseline and vs. placebo. A trend to increased inhibition was seen as early as 6 weeks.

### Grass pollen-specific immunoglobulins

Levels of both *P. pratense*-specific IgG<sub>1</sub> and IgG<sub>4</sub> antibodies were significantly greater in the SLIT-treated group when compared with placebo at peak season and the final visit, with a trend towards an increase at 6 weeks (Figs 3b and c, supporting information Table S1). Grass pollen-specific IgA<sub>1</sub> and IgA<sub>2</sub> antibody levels were significantly elevated during the season in the SLIT-treated group when compared with baseline ( $P < 0.01$  and  $0.05$ , respectively) (Fig. 3d, supporting information Table S1). There was no change in the placebo-treated group. Between group analyses revealed a trend towards greater IgA<sub>2</sub> in season ( $P = 0.065$ ) and significantly greater IgA<sub>2</sub> post-season ( $P = 0.049$ ) in SLIT- compared with placebo-treated atopics.

At the end of the study, there was a trend for a relationship between the size of the late-phase skin response and IgE-facilitated allergen binding ( $r = 0.36$ ,  $P = 0.08$ ) i.e. the higher the degree of serum inhibitory activity for IgE-FAB, the greater the inhibition of the late skin response. There was no correlation between the late skin response and allergen-specific IgG<sub>4</sub> ( $r = 0.17$ ,  $P = 0.20$ ).

## Discussion

Foxp3<sup>+</sup> cells were increased in the sublingual epithelium following grass pollen SLIT. SLIT-treated patients also showed trends for fewer subepithelial mast cells and

Table 1. Baseline characteristics, symptom improvement and effect of sublingual immunotherapy on the late-phase skin response to intradermal allergen (*Phleum pratense* 10 BU)

|                                                           | Immunotherapy      | Placebo           |
|-----------------------------------------------------------|--------------------|-------------------|
| Number of participants                                    | 28                 | 28                |
| Age; mean (range)                                         | 34 (21–53)         | 34 (21–55)        |
| Gender; m:f                                               | 13:15              | 19:9              |
| Allergen-specific IgE; mean (SD)                          | 250 (257)          | 189 (251)         |
| Skin LPR to allergen, mm <sup>2</sup> ; median (IQ range) | 2754 (1640, 3961)* | 4138 (2926, 5163) |
| Improvement in symptoms compared with the previous years  | 77% (20 of 26)**   | 39% (9 of 23)     |

Overall changes in symptoms are presented as percentages (absolute numbers) of participants reporting improvement compared with previous years.

\* $P < 0.02$ ,  $\chi^2$  test; \*\* $P = 0.003$ , Mann-Whitney *U*-test.

LPR, late-phase response.

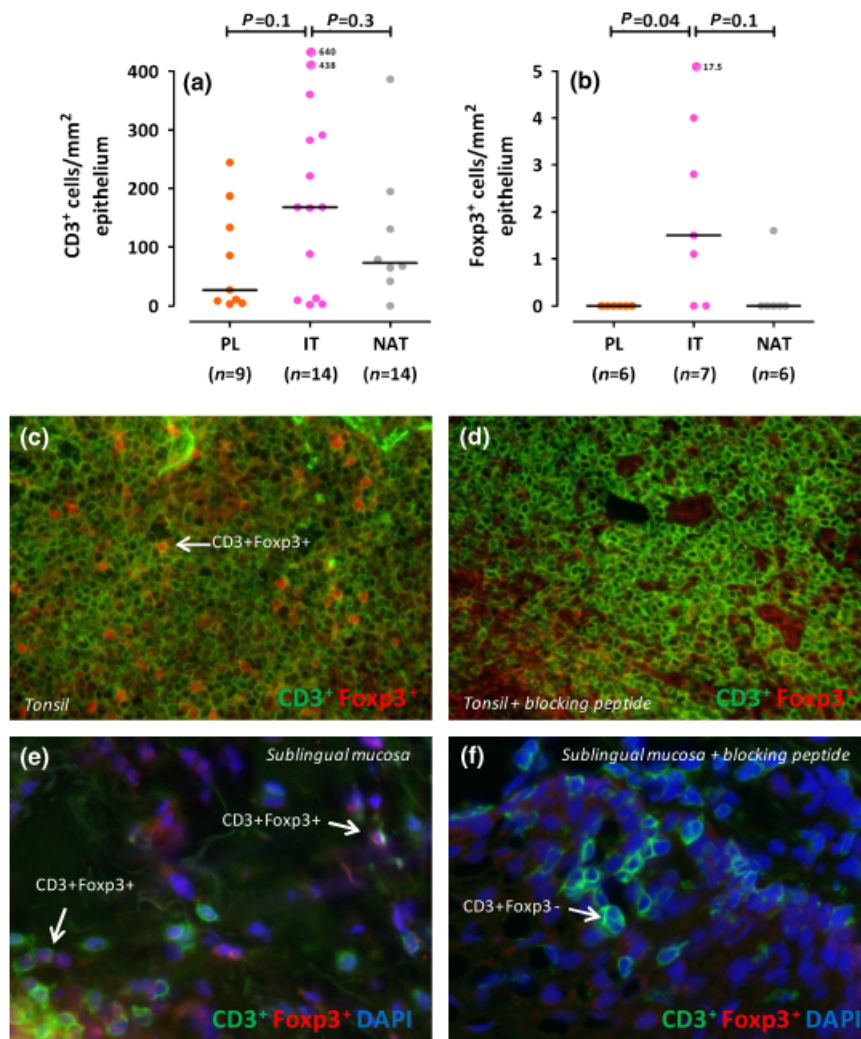


Fig. 1. CD3 and Foxp3 expression in the sublingual mucosa and tonsil. (a) Number of positive cells per mm<sup>2</sup> in the sublingual epithelium for CD3 (T cells) from placebo-treated (PL), sublingual-immunotherapy-treated (IT) and non-atopic individuals (NAT). (b) Foxp3<sup>+</sup> cells per mm<sup>2</sup> within the sublingual epithelium. (c) CD3<sup>+</sup> Foxp3<sup>+</sup> and CD3<sup>+</sup> Foxp3<sup>-</sup> cells in human tonsil ( $\times 400$  magnification). (d) Absence of CD3<sup>+</sup> Foxp3<sup>+</sup> cells in the tonsil following preincubation of anti-Foxp3 antibody with synthetic Foxp3 peptide. (e) CD3<sup>+</sup> Foxp3<sup>+</sup>, CD3<sup>+</sup> Foxp3<sup>-</sup> and CD3-Foxp3<sup>+</sup> cells in sublingual mucosa with DAPI counterstaining. (f) Foxp3-stained sublingual mucosa after preincubation with synthetic Foxp3 peptide. Circles represent individual values, horizontal bars represent medians. *P* values refer to between group comparisons by Mann-Whitney *U*-test.

greater numbers of epithelial T cells. Placebo-treated atopics had higher subepithelial mDC numbers than non-atopics, whereas there were no differences observed after SLIT. IL-10 and TGF- $\beta$  mRNA<sup>+</sup> cells were detectable in the sublingual mucosa of both allergic and non-atopic individuals with no detectable changes after SLIT. SLIT induced time-dependent increases in allergen-specific IgG<sub>1</sub> and IgG<sub>4</sub> levels that were accompanied by serum inhibitory activity for IgE-FAB. Increases in specific IgA<sub>1</sub> and IgA<sub>2</sub> were also observed after SLIT. These data suggest a similar mechanism of action of SLIT to that of SCIT, with the likelihood of additional regulatory changes locally within the oral mucosa.

SLIT- and placebo-treated patients in this study were well-matched; serum and tissue samples were coded with

investigators blinded to their status. Foxp3 expression was detected using techniques used previously for nasal mucosa [11]; the specificity of the antibody was proven using a recombinant blocking peptide. Conversely, the power of the data is limited due to the relatively low number of biopsies available. Although there were significant improvements in global symptom scores, the primary clinical outcomes – symptom and medication scores – failed to achieve statistical significance [24]. In retrospect, we believe this is likely to be explained by a lack of power as the clinical effect sizes observed (28% lower symptom scores and 45% lower medication scores compared with placebo) were very similar to those observed using similar doses (15 mcg Phl p 5 daily) in the form of grass allergen tablets (Grazax<sup>R</sup>, ALK-Abelló) in adequately powered

**Table 2.** Positively stained cells per mm<sup>2</sup> within the epithelium and subepithelium in immunotherapy-treated patients, placebo-treated patients and non-atopic controls

|                                             | Sample number<br>(IT, PL, NAT) | Immunotherapy (IT)<br>(median, IQR) | Placebo (PL)<br>(median, IQR) | Non-atopics (NAT)<br>(median, IQR) |
|---------------------------------------------|--------------------------------|-------------------------------------|-------------------------------|------------------------------------|
| <b>Epithelium (cells/mm<sup>2</sup>)</b>    |                                |                                     |                               |                                    |
| CD3                                         | 14, 9, 8                       | 168* (31.7–289.0)                   | 27.3* (8.4–187.0)             | 73.2 (59.0–146.6)                  |
| mDC                                         | 14, 9, 8                       | 128.4 (87.1–180.1)                  | 113.9 (108.9–154.0)           | 121.2 (98.3–168.6)                 |
| pDC                                         | 14, 9, 8                       | 0 (0–2.0)                           | 0 (0–1.4)                     | 0 (0–0)                            |
| Mast cell                                   | 14, 9, 8                       | 9.5 (3.1–20.6)                      | 8.4 (4.0–18.7)                | 8.6 (1.0–18.7)                     |
| Foxp3 <sup>+</sup>                          | 7, 6, 6                        | 1.5** (0.6–3.4)                     | 0** (0–0)                     | 0 (0–0)                            |
| Foxp3 <sup>+</sup> CD3 <sup>+</sup>         | 7, 6, 6                        | 0 (0–1.4)                           | 0 (0–0)                       | 0 (0–0)                            |
| IL-10 mRNA <sup>+</sup>                     | 6, 6, 5                        | 0 (0–3.1)                           | 0 (0–8.3)                     | 1.4 (0–6.0)                        |
| TGF- $\beta$ mRNA <sup>+</sup>              | 6, 6, 5                        | 0 (0–2.7)                           | 0 (0–7.1)                     | 0 (0–4.4)                          |
| <b>Subepithelium (cells/mm<sup>2</sup>)</b> |                                |                                     |                               |                                    |
| CD3                                         | 14, 9, 8                       | 35.7 (2.4–56.9)                     | 16.7 (1.9–93.9)               | 7.9 (4.7–21.8)                     |
| mDC                                         | 14, 9, 8                       | 30.0 (9.6–53.2)                     | 40.6** (34.8–44.7)            | 22.6** (12.1–35.3)                 |
| pDC                                         | 14, 9, 8                       | 3.6 (0.4–9.6)                       | 2.4 (0–8.7)                   | 1.6 (0–5.9)                        |
| Mast cell                                   | 14, 9, 8                       | 28.0* (10.4–51.0)                   | 68.4* (26.3–77.1)             | 30.7 (26.2–79.6)                   |
| Foxp3 <sup>+</sup>                          | 7, 6, 6                        | 4.3 (2.3–7.9)                       | 4.9 (2.1–8.2)                 | 4.5 (1.8–6.9)                      |
| Foxp3 <sup>+</sup> CD3 <sup>+</sup>         | 7, 6, 6                        | 0.8 (0–2.0)                         | 0 (0–1.2)                     | 0 (0–0)                            |
| IL-10 mRNA <sup>+</sup>                     | 6, 6, 5                        | 1.0 (0–1.9)                         | 2.0 (0–3.2)                   | 0 (0–4.3)                          |
| TGF- $\beta$ mRNA <sup>+</sup>              | 6, 6, 5                        | 0.5 (0–2.3)                         | 3.3 (0–8.8)                   | 0 (0–1.7)                          |

Sections were immunostained for myeloid dendritic cells (mDC), plasmacytoid dendritic cells (pDC), T cells (CD3), Mast cells, Foxp3, Foxp3 and CD3, and examined for IL-10 and TGF- $\beta$  mRNA expression by *in situ* hybridization. Between group comparisons by Mann–Whitney *U*-test.

\**P* = 0.1; \*\**P* ≤ 0.05.

IQR, interquartile range.

studies in a comparable patient group [17]. Whereas IL-10 and TGF- $\beta$  mRNA<sup>+</sup> cells were detectable within the sublingual mucosa, their numbers did not alter following SLIT. Co-localization of these regulatory cytokines to Foxp3<sup>+</sup> cells would provide stronger evidence of a regulatory phenotype and discount the possibility that these cells merely represent recently activated cells. This was not possible owing to the limited availability of tissue sections. Cells were counted in epithelium and subepithelium separately to reflect the structural and possible functional differences between these regions – the epithelium having a barrier function in addition to being a site of allergen-immune cell interaction.

Current models of SLIT suggest an uptake of allergen by APCs in the oral mucosa, followed by migration to regional lymph nodes [3, 4, 25]. The distribution of Langerhan's, mDCs and pDCs within the oral mucosal epithelium and subepithelium has been described in mice [5]. Subsets of mDCs and pDCs were shown to be competent at allergen uptake *in vitro* and capable of inducing T cells secreting IFN- $\gamma$  and/or IL-10 with suppressor function. Uptake was enhanced by linking an allergen (ovalbumin) to a mucoadhesive polysaccharide core. SLIT efficacy in a mouse asthma model was also improved using the mucoadhesive formulation [5]. Together, these results provide further evidence for the importance of oral DCs in SLIT.

A candidate APC, the oral Langerhan's cell, is abundant in the human oral mucosa and capable of allergen

presentation and IL-10 release *in vitro*; the latter being enhanced by TLR-4 ligation [8]. Interestingly, significantly higher levels of these cells are present in the mucosa of the oral vestibulum compared with the sublingual tissue [6]. Circulating allergen-specific, IL-10-secreting T cells have been reported following SLIT [14] and reductions in eosinophils have been demonstrated at effector mucosae [26, 27]. To date, Foxp3<sup>+</sup> cells in oral tissues have largely been studied in the context of periodontitis [28–30] or aphthous ulceration [31]. Results have been conflicting, with the demonstration of CD25<sup>+</sup> Foxp3<sup>+</sup> cells in healthy tissue only [30], in contrast to their overwhelming presence in the disease tissue [28, 29].

In this study, increases in serum IgG<sub>1</sub> and IgG<sub>4</sub> were accompanied by an increase in serum inhibitory activity for IgE-FAB. Inhibition of IgE-FAB provides a surrogate for the suppression of B cell IgE-facilitated allergen presentation to, and activation of, T cells *in vivo*, and suggests these antibodies may impair subsequent T-helper type 2 responses following allergen exposure. The trend for a correlation between the size of the late skin response and IgE-FAB (*P* = 0.08) is consistent with this concept as allergen-induced late skin responses are known to be largely T cell driven [32]. That no such trend was seen for IgG<sub>4</sub> lends support the use of the IgE-FAB assay as a functional assay of IgG inhibitory activity. The time course of change in serum antibody responses is

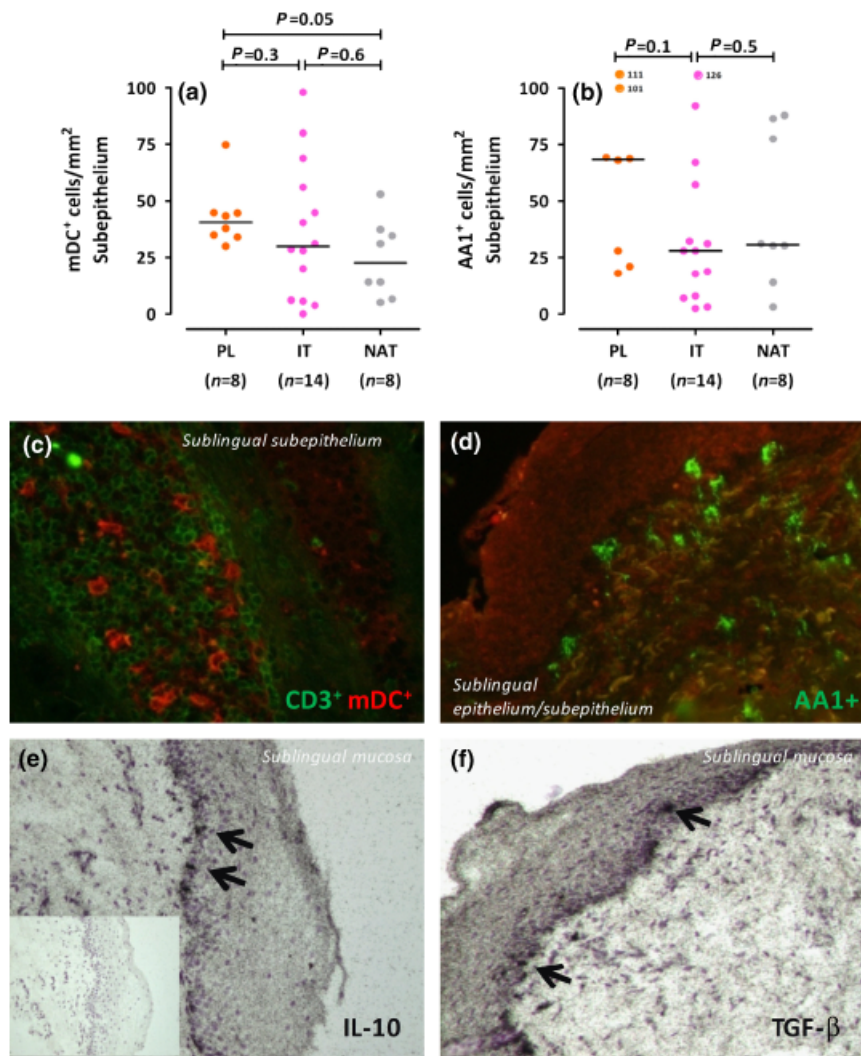


Fig. 2. Dendritic cells, mast cells and cytokine expression in the sublingual mucosa. (a) Number of positive cells per mm<sup>2</sup> in the subepithelium for CD1c (myeloid dendritic cells). (b) AA1 (tryptase) positive cells (mast cells) in the subepithelium. (c) Example of mDC staining (red cells,  $\times 400$ ). (d) Example of mast cell staining (green cells,  $\times 400$ ). (e) *In situ* hybridization for IL-10 mRNA<sup>+</sup> cells using an antisense RNA probe ( $\times 200$ ) (inset: IL-10 sense probe as negative control). (f) *In situ* hybridization for TGF- $\beta$ ; circles represent individual values, horizontal bars represent medians.

in-keeping with the known clinical response to SLIT. Trends for IgG<sub>1</sub>, IgG<sub>4</sub> and inhibition of IgE FAB at 6 weeks, with further increases at 3 months mirror the observations that at least 8, or preferably 16 weeks, pre-seasonal treatment provides optimal clinical outcomes [15, 17]. Indeed, this is also similar for SCIT where maximal changes in IgG and FAB inhibition are reached at 16 weeks [12].

The increase in specific IgA in SLIT-treated patients may be the result of enhanced TGF- $\beta$  expression within the nasal mucosa, from either APCs or possibly T regulatory cells. Although TGF- and IL-10-mRNA positive cells were present in the oral mucosa, their numbers did not increase despite increases in Foxp3<sup>+</sup> cells. Biopsies were only taken following the completion of SLIT treatment and it is possible that increased mucosal expression of

these regulatory cytokines occurs at an earlier time-point during immunotherapy. Alternatively, TGF- $\beta$ /IL-10 production may occur in regional lymph nodes. Sublingual biopsy samples taken earlier during the course of SLIT and/or within 12–24 h of an immunotherapy dose may have been more sensitive in identifying cells expressing regulatory cytokines.

Our observation that greater numbers of subepithelial mDC were found in placebo-treated, but not SLIT-treated atopics, compared with non-atopic controls, would be consistent with a possible role for these cells in SLIT, although it is not clear whether a decrease in the number of these cells would either augment or limit the efficacy of SLIT during treatment. Low numbers of pDC within the sublingual mucosa are consistent with previous findings in human oral tissue [4], but contrast with murine studies

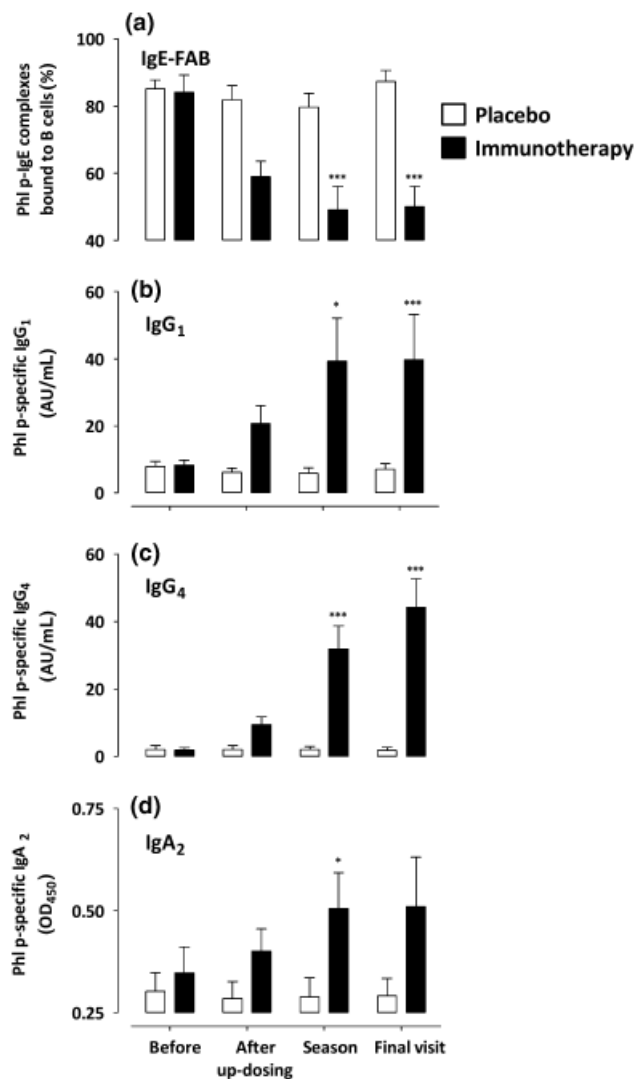


Fig. 3. Antibody responses following sublingual immunotherapy. (a) Serum inhibitory activity measured by facilitated allergen-binding assay. Phl p-specific IgG<sub>1</sub> (b), IgG<sub>4</sub> (c) and IgA<sub>2</sub> (d) antibody concentrations measured by enzyme-linked immuno-sorbent assay. Closed bars: sublingual immunotherapy -treated patients; open bars: placebo-treated patients. Data are shown as mean ( $\pm$ SE). \*\*\* $P < .001$  and \* $P < 0.05$  (one-way ANOVA, Friedman correction for repeated non-parametric measurements).

where pDC were relatively prominent [5]. As yet, there is little evidence for a pro-tolerogenic role for pDC in SLIT in human studies.

The finding of elevated numbers of epithelial T cells and Foxp3<sup>+</sup> cells within the sublingual mucosa is consistent with the possibility that T cells with a regulatory phenotype either migrate from local lymphoid organs during SLIT, or alternatively may be induced within the oral mucosa through local DC-T cell interaction following repeat allergen exposure [3, 4]. One way in which regulatory T cells may act is via the release of regulatory cytokines such as IL-10 and TGF- $\beta$  [12]. Our data illustrate that both IL-10- and TGF- $\beta$ -producing cells are present

within the oral mucosa. Together with secretory IgA, the presence of these cells may contribute to the pro-tolerogenic state of the oral mucosa [4].

Local itching within the mouth is a common side-effect of SLIT, which generally disappears within the first 1–2 weeks of treatment. We found mast cells to be abundant within the sublingual mucosa, as demonstrated previously [6]. Furthermore, levels were equivalent in atopic and non-atopic individuals. It appears highly plausible that mast cells are the mediators of local side-effects during SLIT, although this remains to be proven.

Targeting of allergen to DCs may improve the efficacy of SLIT [33]. This might be achieved through the use of adjuvants; indeed, some adjuvants may increase the propensity of DCs to release IL-10 [8]. An ideal vaccine would target DCs, yet not be recognized by mast cell surface IgE.

The data presented suggest that the IgE-FAB assay may prove more useful as a biomarker of the efficacy of SLIT than allergen-specific IgG<sub>4</sub> levels. However, confirmation of this will require comparisons with clinical outcomes in larger clinical trials.

In summary, these results suggest that high-dose grass pollen SLIT works by mechanisms which parallel many of those observed in SCIT and, furthermore, that the oral mucosa plays an active role in both the mechanisms and side effects of SLIT.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Statistical analysis of relative IgE-facilitated allergen binding and *P. pratense*-specific IgG1, IgG4, IgA1, and IgA2 antibodies in subjects treated with sublingual grass pollen-immunotherapy or placebo.

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|                                                                              | Before<br>(Mean± SE) | After up-dosing<br>(Mean± SE) | Peak season<br>(Mean± SE) | Final<br>(Mean± SE)  |
|------------------------------------------------------------------------------|----------------------|-------------------------------|---------------------------|----------------------|
| Allergen-IgE complexes bound to B cells [%]                                  |                      |                               |                           |                      |
| Sublingual immunotherapy (n=13)                                              | 84.10 (5.24)         | 58.99 (4.53) ††               | 49.02 (7.04) ***†††       | 50.04 (5.99)*** †††  |
| Placebo (n=11)                                                               | 85.11 (2.64)         | 81.78 (4.29)                  | 79.59 (4.16)              | 87.27 (3.37)         |
| <i>P.pratense</i> -specific IgG <sub>1</sub> antibodies [AU/ml]              |                      |                               |                           |                      |
| Sublingual immunotherapy (n=13)                                              | 8.23 (1.52)          | 20.63 (5.40) †                | 39.35 (12.76) *††         | 39.72 (13.56) ***††† |
| Placebo (n=11)                                                               | 7.84 (1.54)          | 6.21 (1.14)                   | 5.81 (1.63)               | 7.04 (0.98)          |
| <i>P.pratense</i> -specific IgG <sub>4</sub> antibodies [AU/ml]              |                      |                               |                           |                      |
| Sublingual immunotherapy (n=13)                                              | 1.85 (0.73)          | 9.43 (2.32) †                 | 31.84 (6.78) *††          | 44.15 (8.50) ***†††  |
| Placebo (n=11)                                                               | 1.98 (1.23)          | 2.00 (1.21)                   | 1.96 (1.05)               | 1.68 (0.98)          |
| <i>P.pratense</i> -specific IgA <sub>1</sub> antibodies [OD <sub>450</sub> ] |                      |                               |                           |                      |
| Sublingual immunotherapy (n=8)                                               | 0.82 (0.12)          | 0.91( 0.10)                   | 1.11 (0.12)**             | 1.15 (0.16)          |
| Placebo (n=8)                                                                | 0.91(0.13)           | 0.82 (0.14)                   | 0.89 (0.13)               | 0.96 (0.16)          |
| <i>P.pratense</i> -specific IgA <sub>2</sub> antibodies [OD <sub>450</sub> ] |                      |                               |                           |                      |
| Sublingual immunotherapy (n=8)                                               | 0.35 (0.06)          | 0.40 (0.05)                   | 0.50 (0.08)*              | 0.51(0.12) †         |
| Placebo (n=8)                                                                | 0.30 (0.04)          | 0.28 (0.04)                   | 0.29 (0.04)               | 0.29 (0.04)          |

**Supplementary Table 1: Statistical analysis of relative IgE-facilitated allergen binding and *P.pratense*-specific IgG<sub>1</sub>, IgG<sub>4</sub>, IgA<sub>1</sub>, and IgA<sub>2</sub> antibodies in subjects treated with sublingual grass pollen-immunotherapy or placebo.** Data is expressed as mean ± standard error. Comparisons with baseline measurements were determined by using one-way ANOVA with Friedman correction for repeated non-parametric measurements where \*\*\* and \*\* and \* represent p<0.001, p<0.01 and p<0.05 respectively. Statistical differences between sublingual grass pollen-immunotherapy and placebo treated-groups within visit were determined by Mann-Whitney U-test, where †††, †† and † represent p<0.001, p<0.01 and p<0.05 respectively.