

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

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Repository sections: Suzana Radulovic et al.**Methods:****Patients:**

Participants were recruited from the Royal Brompton Allergy Clinic or following 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 grass pollen allergic subjects (7 male, mean age 36 yrs (range 24-59 yrs), skin prick test $47.3 \pm 8.0 \text{ mm}^2$ (mean $\pm$ SEM) and nine atopic hayfever sufferers who had completed two years of grass pollen injection immunotherapy (6 male, mean age 31 yrs (range 22-38 yrs), skin prick test $55.5 \pm 6.6 \text{ mm}^2$).^{E1} The nonatopic healthy controls comprised 5 males and 4 females, mean age 29 yrs (range 24-41 yrs), skin prick tests negative to a panel of 11 common aeroallergens {Grass pollen, birch pollen, mixed tree pollen, *Dermatophagoides Pteronyssinus*, cat, dog, horse, and moulds (*Alternaria*, *Cladosporium* and *Aspergillus Fumigatus*) and negative (diluent) and positive (histamine 10 mg/ml) controls} Soluprick ALK Denmark, 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 randomised controlled trial^{E2}. Twenty subjects [10 male, median age 32yrs (range 26-36yrs)] received GP-IT and 17 subjects received placebo injections [9 male, median age 34yrs (range 29-34yrs)]. GP-IT Injections (Alutard SQ Phleum Pratense, ALK Abello) or placebo injections were given during a cluster 4 week up dosing phase followed by monthly maintenance injections (containing 20 mcg major allergen Phleum p5) for 2 years.

Nasal biopsies were taken at baseline and during the pollen season following 2 years treatment, as described.^{E2}

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

Skin prick tests and intradermal skin tests were performed using grass pollen extract (*Phleum Pratense*, Soluprick, ALK Abello, Denmark). Skin prick tests were performed on the flexor aspect of the forearm. Intradermal tests were performed using 0.02 ml of diluent containing 10 Biological units (BU)/ml. on the extensor aspect of the forearm and the early skin response (weal size) at 15 minutes and late swelling after 24 hours were recorded as previously described^{E1}.

Biopsy Collection:

Nasal biopsies were collected outside the UK pollen season from **October - March** from GP-IT treated patients and untreated hayfever sufferers and between **June - August** from nonatopic healthy controls. None of the patients were symptomatic or taking medication at the time of biopsy as their biopsies were taken remote from the pollen season. Immunotherapy-treated patients had received treatment for at least 2 years and remained on treatment at the time of biopsy, with biopsies being taken 2-4 weeks after the most recent monthly maintenance injection. The effect of seasonal exposure was examined on biopsies taken at baseline and during the peak pollen season after treatment.^{E1}

Immunohistochemistry:

Six micron, 4% paraformaldehyde fixed cryostat sections were used in the study. Foxp3 colocalized to CD4 and CD25 T cells, was visualised by double immunofluorescence using a biotin- streptavidin system. Monoclonal antibodies to CD4 and CD25 (Dako Cytomation, UK), polyclonal goat anti Foxp3 (Abcam, Cambridge, UK) for the detection of nuclear Foxp3 and monoclonal antibody to human IL-10 (Santa Cruz Biotechnology, Santa Cruz, CA) 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 two or three step staining. FITC- labelled rabbit anti-mouse (2 steps), or rabbit anti-mouse secondary antibodies (3 steps) (Dako Cytomation) were used for lymphocyte subsets, and biotinylated anti-goat antibody (Jackson ImmunoResearch) for Foxp3 detection. These were followed by TRITC-swine anti- rabbit Ig (Dako Cytomation) for T cell subsets (3 steps), and streptavidin-Alexa Fluor 488 (for Foxp3 detection) (Invitrogen, Paisley, Scotland, UK).^{E3} Normal goat IgG, mouse IgG1 and IgG_{2b} (Dako Cytomation) 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 two grids depth along the whole length of biopsy, and results expressed as the number of positive cells per square millimetre. On average this involved counting cells in 8 fields which is equivalent to 0.8mm^2 ($0.2 - 1.8\text{mm}^2$) of sub-epithelial tissue per biopsy. The sections stained for Foxp3⁺, CD4⁺ and CD25⁺ cells and dual stained sections for Foxp3⁺, CD4⁺ and CD25⁺ cells and colocalization of Foxp3 to CD4 and CD25 cells were examined and counted at 400× magnification with a Nikon Eclipse (E400) fluorescent microscope (Tokyo, Japan) under appropriate absorption/emission wavelengths (590/617nm for CD4⁺ & CD25⁺ cells, TRITC, and 488/519 nm for Foxp3, Alexa Fluor 488). The images were captured using Nikon Digital Still Camera DXM1200 with Lucia 4.8 software (system for Image processing and analysis; Prague, Czech Republic).

Results:

The effect of seasonal grass pollen exposure on Foxp3 expression in the nasal mucosa is shown in Table 1. Seasonal increases in Foxp3⁺ cells compared to baseline 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 cells^{E4} are also included along with numbers of Foxp3 expressing cells in Table 1. Seasonal increases were observed for both eosinophils and IL-5 mRNA expressing cells in placebo treated patients which were inhibited after immunotherapy.

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121 **References:**

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

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