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

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Background: Regulatory T cells (T_{Reg}) play an important role in controlling allergic inflammation. The transcription factor Foxp3 is a master switch gene that regulates the development and function of natural and adaptive $CD4^+CD25^+T_{Reg}$ cells whose role in the maintenance of peripheral tolerance has been demonstrated in mice and man.

Objectives: To examine the effect of grass pollen injection immunotherapy on the numbers of $Foxp3^+CD4^+$ and $Foxp3^+CD25^+$T cells and their expression of IL-10 in the nasal mucosa of hayfever sufferers.

Methods: Nasal biopsies were obtained from untreated hayfever sufferers, grass pollen-allergic participants who had received 2 years immunotherapy and normal controls. Dual immunofluorescence microscopy was used to enumerate and colocalise Foxp3 expression to $CD4^+$ and $CD25^+$ T cells in the nasal mucosa. Triple staining was performed to colocalise $Foxp3^+$ cells to $CD3^+CD25^+$ and $CD3^+$IL-10 expressing cells.

Results: Numbers of $Foxp3^+$ ($p=0.005$), $Foxp3^+CD25^+$ ($p=0.03$) and $Foxp3^+CD4^+$ ($p=0.04$) cells in the nasal mucosa were higher in immunotherapy-treated patients than in untreated hayfever sufferers, and $Foxp3^+CD25^+$ cells ($p=0.025$) were higher in immunotherapy treated patients compared to normal controls. Within the immunotherapy-treated group, 20% of $CD3^+CD25^+$cells expressed Foxp3 and 18% of $Foxp3^+CD3^+$ cells were IL-10 positive.

Conclusions: These results are consistent with the view that two subsets of T regulatory cells, $Foxp3^+CD3^+$IL-10^+ cells (inducible Tregs) and $Foxp3^-CD3^+$IL-10^+ cells (Tr-1) are recruited to the nasal mucosa following grass pollen immunotherapy.

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

Word count excluding the subtitles: 248

Keywords: Foxp3; $CD4^{+}CD25^{+}$ $T_{Reg;}$ IL-10; grass pollen immunotherapy; hayfever/allergic rhinitis

Abbreviations:

Foxp3 = forkhead winged-helix gene

T_{Reg} = regulatory T cells

IL = interleukin

Ig = immunoglobulin

TGF-β = transforming growth factor-beta

IFN-γ = interferon gamma

CTLA-4 = cytotoxic T lymphocyte antigen-4

GITR = glucocorticoid-induced TNF receptor

GP-IT = grass pollen immunotherapy

FITC = fluorescein isothiocyanate

TRITC = tetramethylrhodamine isothiocyanate

IQ = interquartile range

67 **CAPSULE SUMMARY**
68
69 Grass pollen immunotherapy is associated with increases in Fox $p3^+$ T lymphocytes in the
70 nasal mucosa. Strategies to increase local Tregs might be beneficial in the treatment of
71 hayfever.

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74 **Word counts**: Introduction – Discussion (including statements for the Online Repository
75 sections) **2180**

INTRODUCTION

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 cells in the control of immune responses, the mechanisms of which are not entirely clear. [1-3] Subsets of regulatory T cells include naturally occurring, thymic-derived $CD4^+CD25^+T_{Regs}$, inducible $CD4^+CD25^+$T cells, IL-10–producing T_{Regs} (Tr-1 cells), and TGF-β-producing Th3 type T_{Regs}.[4-6] Naturally occurring $CD4^+CD25^+T_{Regs}$ have been shown to express a variety of cell surface molecules that include CD25, $CD45RB^{low}$, CD62L, CTLA-4, GITR and most specifically the transcription factor forkhead/winged-helix gene (Foxp3). Foxp3 functions as a master switch gene in the development and function of regulatory T 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 IPEX syndrome [9], reinforcing the role of Foxp3 as the dominant transcription factor in T_{Regs}. Experimental models of inflammatory bowel disease [10] 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 Th2 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 and/or function of allergen-specific T regulatory 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 Th2 cytokine production in favour of Th1 (IFN-γ) cytokines. [21-23] GP-IT induces blunting of seasonal increases of allergen-specific IgE and substantial increases in 'blocking' allergen-specific IgG antibodies, particularly of the IgG_4 isotype. [24, 25] Elevated 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 towards allergen-specific unresponsiveness that is observed following GP-IT.[26-28] One way in which immunotherapy may be effective is by the local activation and/or recruitment from the peripheral blood/ lymph nodes of a population of T lymphocytes with a regulatory phenotype resembling $CD4^+CD25^+$T regulatory cells.[26-28]

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 $Foxp3^+CD25^+CD4^+T_{Regs}$. We also studied the influence of the pollen season on Foxp3 expression in patients who had received GP-IT compared to placebo control.

METHODS

Patients:

The study participants comprised 13 grass pollen allergic subjects, nine atopic hayfever sufferers who had completed two years of GP-IT (*Phleum pratense* Alutard SQ, ALK Denmark)[29] and 9 nonatopic healthy controls. We also studied the effects of seasonal pollen exposure in 37 participants in a randomised controlled trial of GP-IT.[29] For patient characteristics, details of the immunotherapy protocol, methods for skin prick tests, intradermal skin tests and recording of global hayfever assessment scores, *see Patients and Methods in the Online Repository at www.jacionline.org.*

Biopsy Collection:

Nasal biopsies (2.5 mm) were taken from the under surface of the inferior turbinate using Gerritsma forceps and 10% cocaine as local anaesthetic as previously described.[30] All samples were examined blind and independent of the clinical protocol. *For further information, see Biopsy collection in 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.

Immunohistochemistry

Foxp3 was colocalized to CD4 and CD25 T cells, and visualised by double immunofluorescence using a biotin-streptavidin system.[31] *For information, see Immunohistochemistry in Methods section in the Online Repository at www.jacionline.org.*

Synthetic peptide corresponding to aa418- 431of human Foxp3 was used in an absorption study to selectively block the binding of Foxp3 antibody (Advanced Biotechnology Centre, Imperial College London, London, UK). *For peptide absorption procedure, see Blocking Peptide in Methods in the Online Repository at www.jacionline.org.*

Colocalisation of Foxp3 to $CD3^+CD25^+$ cells and IL-10 to $CD3^+$Foxp3 expressing cells was performed with triple immunofluorescence and a combination of FITC (for CD3), TRITC (CD25 or IL-10) and Alexa Fluor 350 (Foxp3) respectively [27,31] on nasal biopsy sections from three GP-IT treated patients. *For Quantification, see Methods in the Online Repository at www.jacionline.org.*

Statistical analysis:

Between-group comparisons were performed using the Mann-Whitney *U*-test with Bonferoni correction for multiple tests. Within-group comparisons were performed using the Wilcoxon matched-pairs signed-ranks test. All analyses were performed using a statistics software package (Minitab Inc. PA, USA). All tests were two-tailed and p-values <0.05 were considered statistically significant.

RESULTS

Clinical response: Patients were asked to make an overall assessment of their hayfever severity compared with previous years (prior to 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 (interquartile range (IQ)) 3 (0,2) when compared with untreated hayfever patients 1 (2,3) (p= 0.01). GP-IT was associated with a markedly reduced late phase skin response 24 hours after intradermal grass pollen allergen, median (IQ range) 184 (175-541) mm^2 compared to untreated hayfever controls 2007 (1242, 2535) (p=0.0007). Similarly after GP-IT the early skin weal size at 15 min, 253 (179,296) mm^2 was reduced compared to untreated controls 338 (276,473) (p=0.04).

$CD4^+Foxp3^+$ and $CD25^+Foxp3^+$expressing T cells in tonsillar tissue:

Within tonsillar sections the vast majority of $Foxp3^+$ cells (more than 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 using goat anti-Foxp3 antibody in the presence/absence of a specific Foxp3 peptide that corresponded to human Foxp3 (aa 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. 1A).

$Foxp3^+$, $CD4^+Foxp3^+$ 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 16.1/mm^2 (6.5, 21.7) than

185 untreated hayfever sufferers 2.75/mm^2 (1.5, 5.3) (p=0.005), but did not differ from non-atopic
186 normal controls 4.5/mm^2 (2.8, 10.1) (p=0.13) (Fig.2A).
187
188 Likewise CD25$^+$ cells were found in the lamina propria and were present in highest
189 numbers in GP-IT patients 38.5 mm^2 (15.7, 58.1) although the difference with untreated
190 hayfever sufferers was non-significant 16.0/mm^2 (9.4, 21.55)(p=0.08) and a significant
191 difference was observed between GP-IT patients and non-atopic controls 8.0/mm^2 (4.0,17.4)
192 (p=0.01). In contrast the total number of CD4$^+$ cells was not significantly different between
193 any of the groups (data not shown).

194 The median number of Foxp3$^+$CD25$^+$ cells/mm^2 in the IT- treated patients 9.3/mm^2,
195 (3.9, 18.5) was significantly greater than in untreated hayfever sufferers 2.0/mm^2 (1.5, 2.9)
196 (p=0.03) and in nonatopic controls 1.7/mm^2 (0, 4.9) (p=0.025) (Fig.2B). Similarly, the
197 numbers of Foxp3$^+$CD4$^+$ cells/mm^2 in the IT- treated patients 8.6/mm^2 (0.8, 16.3) were
198 significantly higher than in the hayfever sufferers 0/mm^2 (0, 2.9) (p=0.04), whereas the
199 difference between the IT-treated patients and the nonatopic controls was not significant
200 2.0/mm^2 (0.7, 10.3) (p=0.7) (Fig.1B & Fig. 2C). For the effects of seasonal exposure on
201 Foxp3 expression before/after immunotherapy *see Table 1 Results in the online respository at*
202 *www.jacionline.org.*

203

204 **CD3$^+$CD25$^+$Foxp3$^+$ cells and CD3$^+$Foxp3$^+$IL-10$^+$ in tonsillar tissue and nasal mucosa:**

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

207 In tonsillar tissue IL-10 expression was completely absent in $Foxp3^{+}CD3^{+}$ cells. In
208 contrast, within the nasal mucosa 18% of $CD3^{+}$IL-10 expressing cells were Foxp3 positive.
209 (Fig 1E, 1F & 1G).

210

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 to untreated hayfever sufferers. 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 co-expression of Foxp3 provides additional evidence in favour of an increase in T regs 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 elevations 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-positive and Foxp3-negative $IL\text{-}10^+CD25^+$ T cells co-exist in the nasal mucosa following GP-IT support the emergence of phenotypically distinct populations of regulatory cells[3-5], i.e. Foxp3 expressing adaptive Tregs and IL-10 producing Tr-1 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, the consensus being T cells are capable of synthesizing but not storing cytokines. Here we have, convincingly demonstrated $IL\text{-}10^+$ T cells in the nasal mucosa using paraformaldehyde fixed tissue [31] (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 may imply that immunotherapy was responsible for the induction and/or recruitment of Tregs (adaptive/inducible) in allergic rhinitis.

Our findings are in agreement with those reported in murine models of colitis and in patients with ulcerative colitis and/or Crohn's disease, where 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] In studies of cells derived from peripheral blood of birch allergic subjects, a recombinant Bet v 1 allergen-S-layer fusion proteinprimed the development of naïve T cells into IL-10 producing $CD25^+Foxp3^+CLTA4^+$ cells capable of active suppression.[33] Functionally active, antigen specific $Foxp3^+CD4^+CD25^+$ T cells have also been identified in peripheral blood of atopic and nonatopic individuals.[34]

The mechanisms of suppression by T regulatory subsets remain controversial; both cell-cell contact and down regulation through the immunosuppressive cytokines IL-10 and TGF-β have been described.[6, 35] It was not possible to perform functional studies on local $Foxp3^+$ cells in view of the inevitable small size of nasal biopsies and the relatively low numbers of cells present. In the absence of functional data the identification of local T cells with a so-called regulatory phenotype can only be made by inference. In man, in contrast to mice, a proportion of activated T cells have also been shown to express Foxp3 mRNA.[36,37] Therefore the expression of this transcription factor on its own may 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 elevations in Foxp3+CD25+ and Foxp3+CD4+ cells in the nasal mucosa after immunotherapy were accompanied by reductions in local IL-5+ cells and eosinophils.[23] Our previous findings of elevated proportions of IL-10-producing peripheral $CD4^+CD25^+$ cells [26] and increased numbers of IL-10 [27] and TGF-β [28] expressing T cells ~~in parallel with a reduction in inflammatory cells including eosinophils~~[23] within the nasal mucosa following immunotherapy are also consistent with the view that T regulatory cells play a role in allergen-specific tolerance during successful immunotherapy. In patients with bronchial asthma, corticosteroids have also been shown to modify peripheral T_{Reg} function with increased levels of IL-10 production and Foxp3 expression which accompanied the alleviation of asthma symptoms.[12]

In summary, following grass pollen immunotherapy, $Foxp3^+CD25^+$ and $Foxp3^+CD4^+$ cells were elevated 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 may contribute to the prevention of Th2 cell recruitment and activation. Future studies should focus on the underlying mechanisms controlling the phenotype and function of T_{regs} following immunotherapy. Our data suggest that strategies to increase local Tregs might be beneficial in the treatment of hayfever.

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288

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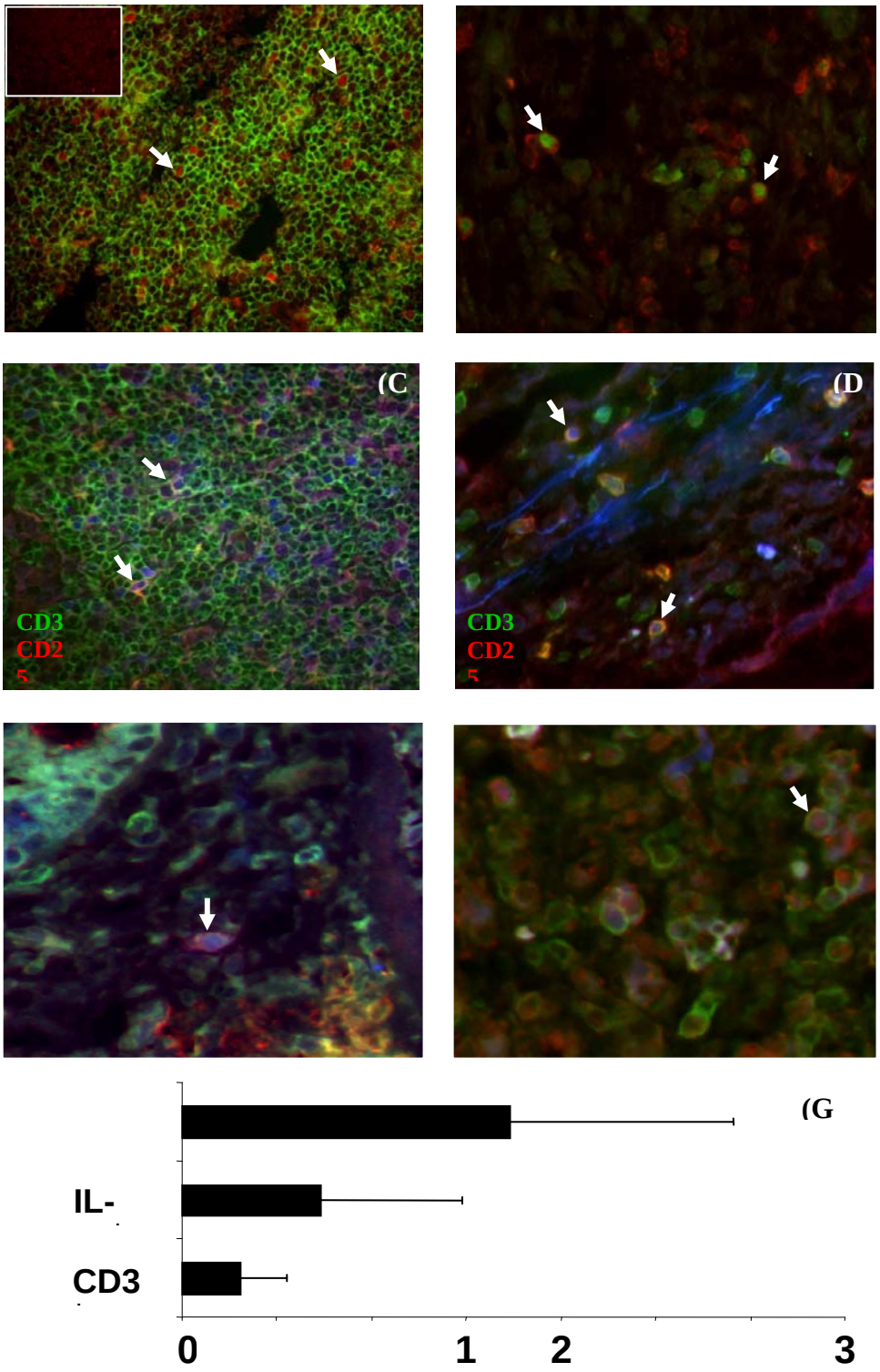
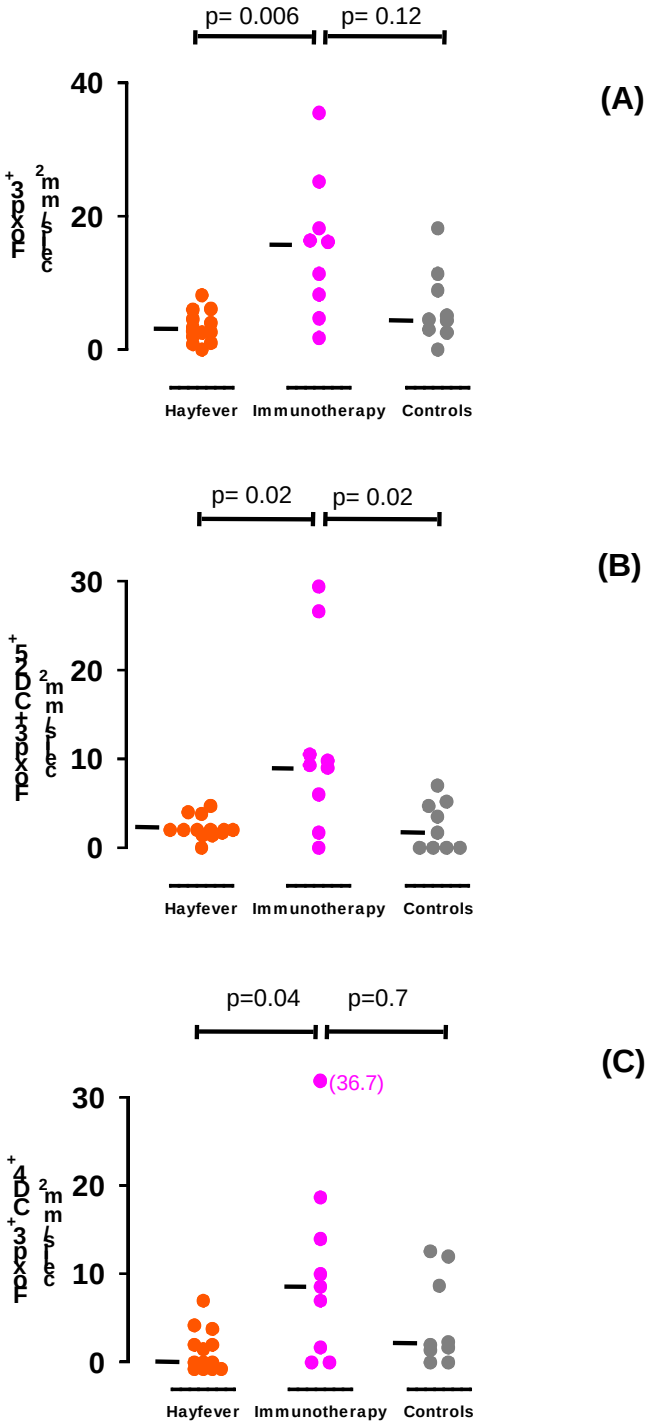
403 **LEGENDS TO FIGURES**
404
405
406 Figure 1:



407

Immunofluorescence staining of tonsillar sections and nasal sections from GP-IT treated patients. (A) $Foxp3^{+}CD4^{+}$ cells in tonsil (inset shows control following pre-adsorption with human Foxp3 peptide)(x200) (B) $Foxp3^{+}CD4^{+}$cells in nose (x400) (C) $CD3^{+}CD25^{+}Foxp3^{+}$ cells in tonsil (x200) , (D) $Foxp3^{+}CD3^{+}CD25^{+}$ cells in nose (x400), (E&F) $Foxp3^{+}CD3^{+}IL\text{-}10^{+}$ cells in nose (x1000) (G) % $Foxp3^{+}$ cells that were $CD3^{+}$, $IL\text{-}10^{+}$ or $CD3^{+}IL\text{-}10^{+}$.

415 Figure 2:



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417

418 (A) Foxp3$^+$, (B) Foxp3$^+$CD25$^+$, and (C) Foxp3$^+$CD4$^+$ cells in hayfever sufferers, GP-IT

419 treated patients and nonatopic controls. Statistical analysis was performed using Mann-

420 Whitney *U* test.