

ORIGINAL ARTICLE

Seasonal changes in suppressive capacity of CD4⁺ CD25⁺ T cells from patients with hayfever are allergen-specific and may result in part from expansion of effector T cells among the CD25⁺ population

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Summary

Background Suppression of allergen-stimulated peripheral blood CD4⁺ CD25[−] effector T cells by CD4⁺ CD25⁺ regulatory T cells obtained from subjects with allergic rhinoconjunctivitis is reduced during the pollen season when compared with out of season.

Objective We examined possible explanations for this effect of seasonal pollen exposure on suppression of allergen responses.

Methods CD4⁺ CD25[−] and CD4⁺ CD25⁺ T cells were isolated from blood obtained from 44 volunteers with allergic rhinoconjunctivitis during and out of the UK grass pollen season. Co-cultures were performed with grass pollen extract and house dust mite (HDM) to examine allergen specificity. The frequency of IL-5 and IL-10 producing cells was determined by ELISPOT and the expression of T cell activation markers and the CD25⁺ regulatory T cell-associated transcription factor Foxp3 were examined. Lactic acid stripping of IgE was used to determine IgE dependence of T cell responses.

Results The seasonal reduction in suppression by CD4⁺ CD25⁺ T cells was confirmed and was shown to be allergen specific because suppression of HDM-stimulated cultures was not affected significantly. The CD4⁺ CD25⁺ population contained IL-5 and IL-10 producing cells but increases in their frequencies with seasonal pollen exposure were not significant. Both activation marker and Foxp3 expression increased during the pollen season. IgE stripping reduced CD4⁺ and CD4⁺ CD25[−] T cell responses to allergen, but had no effect on suppression by CD4⁺ CD25⁺ T cells.

Conclusion The seasonal reduction in suppression of grass pollen-stimulated effector T cells by CD4⁺ CD25⁺ T cells is allergen specific and cannot be explained by increased IgE-facilitated allergen presentation. We suggest that changes in the proportion of effector to regulatory T cells among the CD25⁺ population isolated may partially explain these findings, and that trafficking to the site of allergic disease may reduce allergen-specific regulatory T cell numbers in peripheral blood.

Keywords allergen, CD4⁺ CD25⁺ regulatory T cell, elispot, grass pollen, house dust mite, pollen season, T cell

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Introduction

Atopic allergic diseases such as asthma, rhinitis and atopic dermatitis are characterized by the presence of allergen-specific IgE. Allergen-induced activation of T-helper type

2 (Th2) lymphocytes is thought to be important in allergic inflammation [1]. Such inappropriate immune activation is thought to be controlled at least in part by active suppression by regulatory T cells [2].

We have previously demonstrated a reduced suppressive capacity of CD4⁺ CD25⁺ regulatory T cells from atopic allergic individuals in allergen-stimulated cultures

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when compared with non-atopic control subjects [3]. Furthermore the degree of suppression by CD4⁺ CD25⁺ regulatory T cells of grass pollen-stimulated cultures was further reduced during the pollen season, and similar findings are reported in birch pollen allergic subjects [4]. The reason for the observed reduction in suppression by CD4⁺ CD25⁺ regulatory T cells during the pollen season could be due to 'dilution' of the regulatory cell population within isolated CD25⁺ T cells because of expansion of activated allergen-specific effector T cells (which are also CD25⁺). Other potential explanations would be recruitment of allergen specific regulatory T cells to the target organs of allergic rhinoconjunctivitis, leaving a reduced allergen-specific suppressive population in peripheral blood, or seasonal changes in IgE-mediated antigen presentation overcoming suppression by CD4⁺ CD25⁺ regulatory T cells. Here we have investigated these possible explanations for reduced suppression by peripheral blood CD4⁺ CD25⁺ regulatory T cells during the pollen season.

Methods

Patients

Forty-four volunteers with seasonal allergic rhinitis to grass pollen (hayfever) were recruited from the Royal Brompton Hospital allergy clinic and by advertisement. All volunteers had a positive skin prick test to grass pollen extract (ALK-Abelló, Hørsholm, Denmark) and serum-specific IgE testing (Phadebas RAST, Pharmacia, Milton Keynes, UK). All had symptoms limited to seasonal exposure with no allergic disease outside the pollen season (despite IgE sensitization to other allergens in some cases). We studied these individuals between June and August when the pollen count was high and they had symptoms of hayfever, and again between November and April when they were not exposed to pollen. All out of season data was gathered between February and April the year after the in-season data except for one subject (who gave blood in November): the data from this volunteer was not clearly an outlier. All studies were approved by the Royal Brompton NHS Trust local ethics committee and all volunteers gave written informed consent.

Cell preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from 200 mL peripheral venous blood over a Ficoll[®] gradient (GE Healthcare, Chalfont St. Giles, UK). CD4⁺ T cells were isolated by negative immunomagnetic selection, followed by positive selection for CD25⁺ T cells (MACS; Miltenyi, Biscley, UK), as described previously [3]. The purity of CD4⁺ T cells was >95% in all experiments, and that of CD4⁺ CD25⁺ >90%.

Cell culture

Cells were cultured in 96-well flat-bottomed plates in triplicate at a density of 2×10^6 cells/mL in RPMI 1640 medium with 5% human AB serum, 100 µg/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (all chemicals from Sigma, Poole, UK unless stated otherwise) for 6 days with 100 µg/mL grass or house dust mite (HDM) allergen (ALK-Abelló). Autologous irradiated CD4⁺ cells were added to all cultures as antigen-presenting cells (APCs) at a density of 2×10^6 cells/mL. Medium control wells where no allergen was added were included as a negative control. Cultures were of CD4⁺ CD25⁺ cells alone, CD4⁺ CD25⁻ cells alone or mixed CD4⁺ CD25⁺ and CD4⁺ CD25⁻ cells at a ratio of 1:2 (conditions previously found to induce the maximum inhibition of proliferation [3]), or CD4⁺ CD25⁻ cells at 3×10^6 cells/mL to control for amplified cell density of the CD4⁺ CD25⁻ cultures. On day 6, 100 µL of supernatant was removed for cytokine analysis with Luminex beads (Luminex, Riverside, CA, USA). We then added ³H-thymidine (Amersham, Chalfont St. Giles, UK) for 6 h, after which cells were harvested and proliferation was measured by the amount of incorporated radioactivity. Data are expressed as counts per min (c.p.m.) in allergen-stimulated wells with medium control wells subtracted.

Elispot

Ninety-six-well flat-bottomed filter plates (Millipore, Watford, UK) were coated with 100 µL/well of capture antibody diluted in 0.1 M carbonate/bicarbonate buffer pH 9.6 overnight. The plate was washed six times with phosphate-buffered saline (PBS) and cells were cultured in triplicate at a density of 2×10^6 cells/mL (for IL-5) and 0.5×10^6 cells/mL (for IL-10) in RPMI 1640 medium with 5% human AB serum, 100 µg/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine for 2 days with 100 µg/mL grass allergen. Autologous-irradiated CD4⁺ cells were added to all cultures as APCs at a density of 2×10^6 cells/mL (for IL-5) and 0.5×10^6 cells/mL (for IL-10). Medium control wells where no allergen and recombinant IL-5 and IL-10 (R&D Systems, Abingdon, UK) wells were included as negative and positive controls, respectively. Cultures were of CD4⁺ CD25⁺ cells and CD4⁺ CD25⁻ cells. Following culture, the wells were washed six times in PBS, once in distilled water and 100 µL of biotin-labelled detector antibody diluted in reagent diluent [1% bovine serum albumin (BSA) in PBS] was added per well. The plate was incubated at 4 °C overnight and the wells washed six times in PBS. Hundred microlitres of streptavidin alkaline phosphatase (R&D Systems) diluted in reagent diluent was added per well for 2 h. The plate was washed three times in PBS, once in distilled water and 100 µL of BCIL/NBT Solution (R&D Systems) was added

per well for up to 2 h. The plate was washed in distilled water and allowed to dry. The number of spots was counted using the Bioreader 3000 (BIOSYS, Karben, Germany). Results are reported as the mean spot numbers from triplicate wells per 2×10^5 (for IL-5) and 5×10^4 (for IL-10) cells.

Fluorescence-activated cell sorter staining

Labelled antibodies and isotype controls (BD Pharmingen, Oxford, UK) were added to 0.2×10^6 cells in staining buffer (SB, 0.2% BSA in PBS) for 30 min. The cells were washed in SB and resuspended in ice-cold fixative (Cellfix[®], Becton Dickinson, Oxford, UK) until analysis by flow cytometry using a Becton Dickinson FACSCalibur (San Jose, CA, USA). A minimum of 10 000 events were acquired. Intracellular Foxp3 staining was performed using a FITC-conjugated antibody from e-Biosciences (San Diego, CA, USA) with appropriate isotype control.

Immunoglobulin E stripping

Autologous CD4⁺ cells were resuspended in 1 mL lactic acid buffer (0.14 M NaCl, 0.005 M KCL, 0.01 M lactic acid, pH 3.9) on ice for 5 min. Stripped cells were washed three times and irradiated as APCs.

Statistics and data analysis

All analyses were performed using GraphPad Prism[®] Version 3.02. Experimental data are presented as the median $\pm$ interquartile range. For paired data (in and out of season) the statistical significance (*P*) was determined using the Wilcoxon test.

Results

Seasonal reduction in suppression by CD4⁺ CD25⁺ regulatory T cells is restricted to grass pollen allergen

A total of 44 grass pollen allergic volunteers were studied (Table 1). Firstly we confirmed a seasonal reduction in suppression of CD4⁺ CD25⁺ T cell proliferation and cytokine production by CD4⁺ CD25⁺ T cells when compared with cultures performed out of season in the same individuals (Fig. 1a). For whole CD4⁺ T cell cultures there was a significantly increased proliferative response to

grass pollen in season compared with out of season, but this was not the case for cultures of CD4⁺ CD25⁺ T cells (Fig. 1b). To determine whether the CD4⁺ CD25⁺ T cell population isolated might have a generally reduced regulatory T cell component, we examined allergen specificity of the seasonal change in suppression. In volunteers sensitized to both grass pollen and HDM allergens there was no significant change in the ability of the CD4⁺ CD25⁺ T cell population to suppress HDM-stimulated cultures of CD4⁺ CD25⁺ T cells, despite a reduction in ability to suppress grass pollen-stimulated cultures in the same subjects (Fig. 1c).

CD25⁺ T cell population contains both regulatory and effector cells but this proportion does not change significantly with allergen season

The grass pollen season had no effect on the numbers of IL-5 and IL-10-producing CD4⁺ CD25⁺ cells or IL-10-producing CD4⁺ CD25⁺ cells (Figs 2a and b). There was a non-significant trend for an increase in the frequency of IL-5 producing cells among the CD4⁺ CD25⁺ population (Fig. 2a).

There was a significant increase in activation markers such as CD25, CD69 and HLA-DR on PBMCs in the grass pollen season compared with out of season (Fig. 2c). However, the regulatory T cell marker, transcription factor Foxp3, was also significantly increased in PBMCs in the grass pollen season (Fig. 2c).

In order to examine the potential effect of IgE-facilitated antigen presentation of grass pollen allergen on the response of CD4⁺ T cells in culture, including suppression by CD4⁺ CD25⁺ T cells, we used a published protocol of lactic acid stripping to remove surface IgE from APCs [5]. This protocol did not affect proliferative responses to phytohaemagglutinin (data not shown). The stripping of IgE from the surface of APCs with lactic acid did not affect the CD4⁺ CD25⁺ – induced suppression of proliferation by CD4⁺ CD25⁺ cells [median 92.8% (range 84.1–97.5%) unstripped vs. 93.2% (66.9–94.0) *P* = 0.44]. However, the proliferation of CD4⁺ and CD4⁺ CD25⁺ cells was significantly reduced with stripping (Fig. 3). The median serum concentration of grass-specific IgE for these patients was 14.6 IU/L (range 3.9–55.2).

Discussion

In this study we have confirmed that suppression of grass allergen-stimulated peripheral blood CD4⁺ CD25⁺ T cells by CD4⁺ CD25⁺ T cells is reduced during the pollen season compared with out of season. This effect was allergen-specific because no significant change was seen in suppression of HDM-stimulated cultures. The CD4⁺ CD25⁺ population contained both IL-5 producing and IL-10 producing T cells, with a trend to increase in the

Table 1. Characteristics of participants (*n* = 44)

Age	33 (1)
M/F	10/34
Total IgE (IU/L)	494 (157)
Grass pollen-specific IgE (IU/L)	28 (4)

Values are mean (SEM). M/F, male/female.

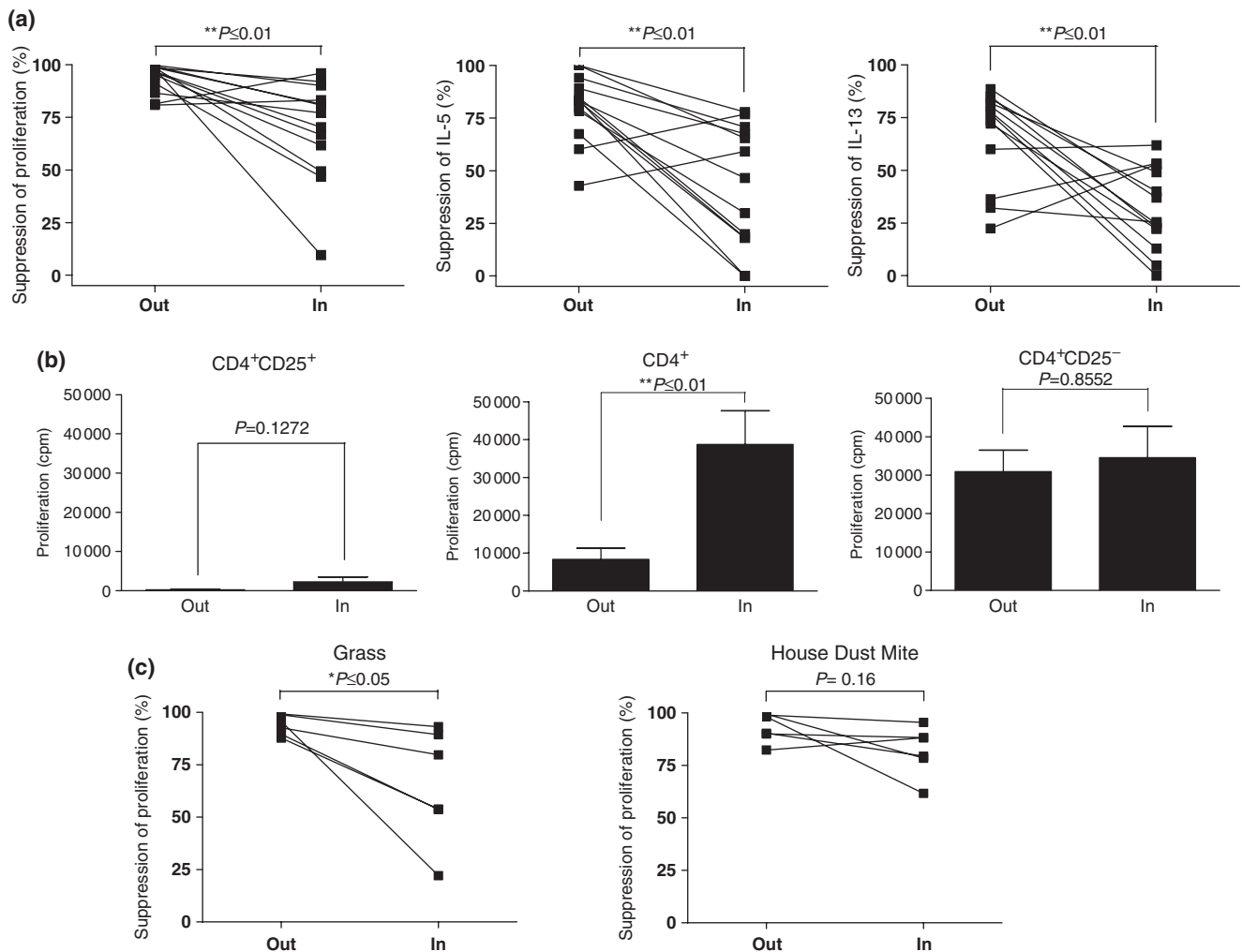


Fig. 1. (a) Percentage suppression by CD4⁺ CD25⁺ T cells for proliferation, IL-5 and IL-13 production by grass pollen allergen-stimulated CD4⁺ CD25⁻ T cells in and out of the grass pollen season ($n = 12$). (b) Grass pollen allergen-induced proliferation of CD4⁺ and CD4⁺ CD25⁻ cells in and out of the grass pollen season ($n = 12$). (c) Percentage suppression of proliferation by CD4⁺ CD25⁺ cells in response to grass pollen allergen stimulation and house dust mite allergen stimulus in and out of the grass pollen season ($n = 6$).

frequency producing IL-5 during the pollen season. In addition, both markers for T cell activation (CD69 and HLA-DR) and regulatory T cells (Foxp3) increased during the season. Stripping of IgE from APCs did not affect suppression during the season suggesting that increased allergen-specific IgE was not overcoming suppression by allergen focussing.

The observation of reduced suppression of allergen-stimulated CD4⁺ CD25⁻ T cells we reported initially [3] has been confirmed [4]. Here we reconfirm our previous findings for seasonal reduction in suppression of proliferation and cytokine production. Furthermore we found an increase in proliferation of total CD4⁺ T cells to grass pollen allergen during the season in these patients with hayfever, but did not detect any differences in allergen responses of either CD4⁺ CD25⁻ or CD4⁺ CD25⁺ individually. This finding is compatible with a reduced suppres-

sive capacity of CD4⁺ CD25⁺ T cells during the pollen season. There was no reduction in the number of regulatory T cells among the CD25⁺ population isolated in season. Furthermore, the seasonal effect was allergen specific, with no significant change in suppression of HDM-stimulated cultures from patients sensitized to both grass pollen and HDM. The relative allergen specificity of the reduction in suppressive activity of CD4⁺ CD25⁺ T cells would also argue against an effect of allergen exposure in inducing pro-inflammatory cytokines such as IL-6 and TNF- α which can overcome suppression by CD4⁺ CD25⁺ T cells [6, 7], induced by allergen exposure, because such an effect would not be allergen specific.

Another potential explanation for the seasonal difference in suppression is that the CD4⁺ CD25⁺ T cell population isolated during the season contained an increased frequency of activated allergen-specific effector

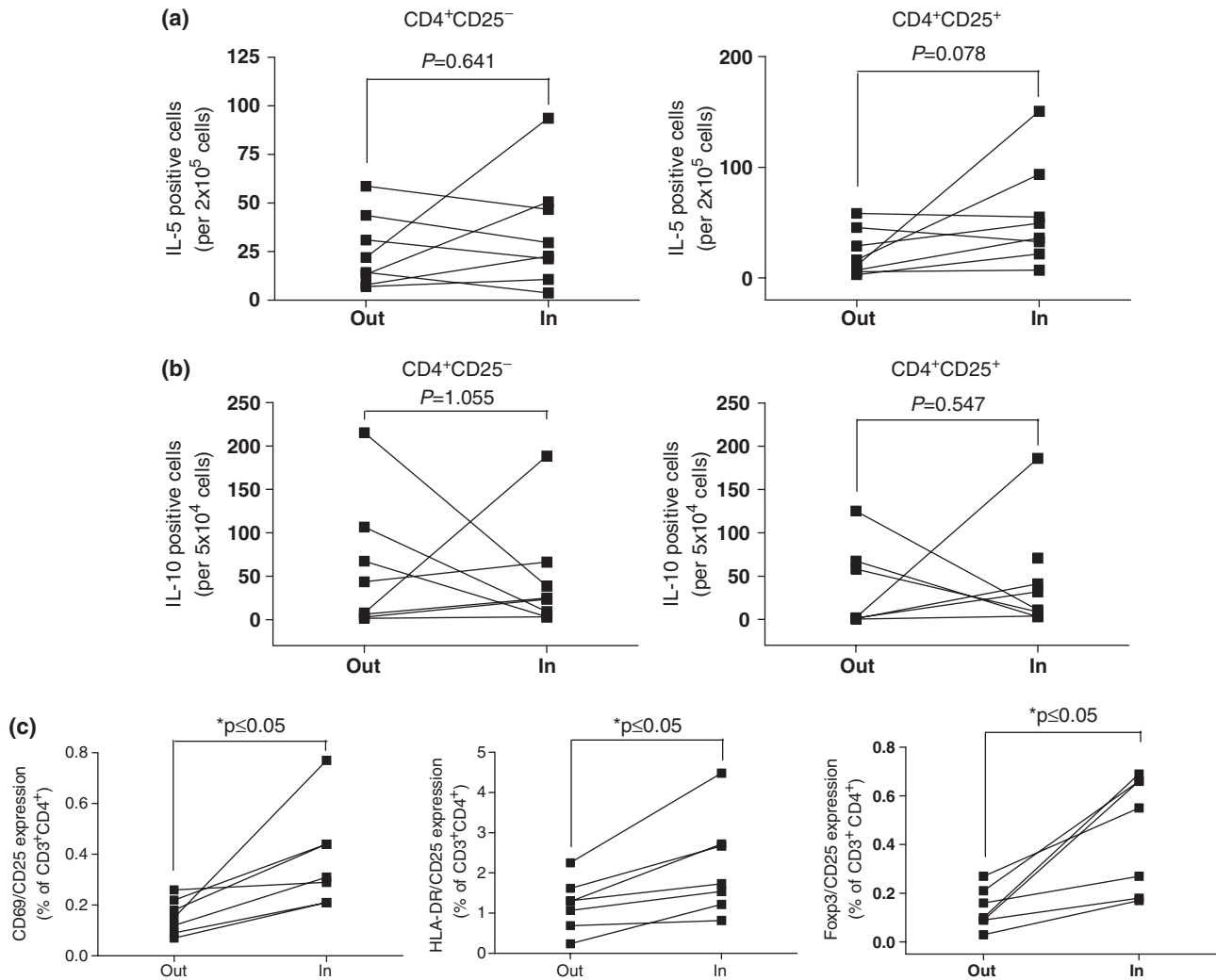


Fig. 2. (a) Grass pollen allergen-induced frequency of IL-5 producing cells in CD4⁺ CD25⁻ and CD4⁺ CD25⁺ cells in and out of the grass pollen season measured by ELISPOT assay ($n = 8$). (b) Grass pollen allergen-induced frequency of IL-10 producing cells in CD4⁺ CD25⁻ and CD4⁺ CD25⁺ cells in and out of the grass pollen season measured by ELISPOT assay ($n = 8$). (c) FACS staining of activation markers and Foxp3 on PBMCs in and out of the grass pollen season ($n = 8$).

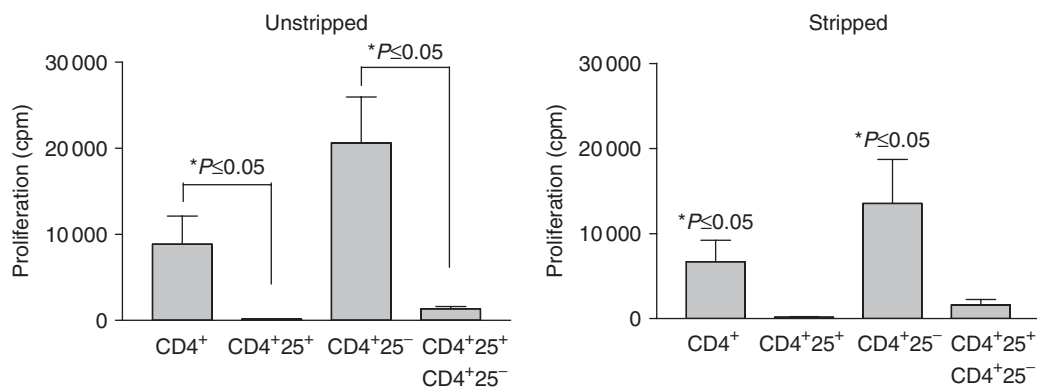


Fig. 3. Grass pollen allergen-induced proliferation of CD4⁺ T cells, CD4⁺ CD25⁺, CD4⁺ CD25⁻ and mixed CD25⁺ and CD25⁻ cells in response to antigen-presenting cells that have been untreated or stripped of IgE with lactic acid. The significance denoted after IgE stripping are in comparison with unstripped data ($n = 6$).

cells which overcame the suppressive effect of regulatory CD25^{hi} cells. Increased expression of CD69 and HLA-DR on CD4⁺ T cells during the season argues for *in vivo* T cell activation. To examine the frequency of allergen reactive T cells among both CD25⁺ and CD25⁻ CD4⁺ T cells we used ELISPOT analysis of IL-5 production. This technique has been used previously to estimate frequencies of antigen-reactive T cells [8] and has the advantage that cells are isolated in a matrix preventing cell-contact-dependent suppression of allergen-reactive effector cells by regulatory T cells in the CD25⁺ population. ELISPOT analysis for IL-5 did detect a significant proportion of allergen-reactive T cells among the CD25⁺ T cells isolated, and there was a non-significant trend for an increased frequency of IL-5 producing cells among CD4⁺ CD25⁺ T cells during the pollen season. Thus, part of the explanation for the seasonal effect on suppression may be due to an increase in activated allergen-specific effector cells among the CD4⁺ CD25⁺ T cell population isolated.

It has been suggested that IL-10 may play a role in suppression by CD4⁺ CD25⁺ T cells [9], although other data suggest IL-10 producing regulatory T cells are a distinct population [10–12]. In our previous report blockade of IL-10 receptor did not alter the suppressive capacity of peripheral blood CD4⁺ CD25⁺ T cells [3]. Here we detected IL-10 producing T cells after allergen stimulation among both the CD25⁻ and CD25⁺ populations and the frequencies did not change with pollen season. Because IL-10 is also produced by activated Th2 cells and non-T cells further studies will be required to determine the cell source for this cytokine.

APCs express IgE receptors and binding of allergen–IgE complexes to these receptors can facilitate antigen presentation to T cells [13]. Because allergen-specific IgE increases during the pollen season one explanation for reduced suppression during the season might be that IgE-mediated allergen presentation is increased and hence abrogates suppression. Stripping IgE from the surface of APCs did indeed reduce allergen-stimulated proliferation of both whole CD4⁺ and CD4⁺ CD25⁻ T cells. However, this treatment did not increase suppression by CD4⁺ CD25⁺ T cells during the season. Another possible explanation for the seasonal effect on suppression of grass pollen-stimulated cultures is that the effect of exposure to increased grass pollen allergen could ‘sensitize’ APC for the production of factors with the potential to inhibit regulatory T cell function by a mechanism that does not involve surface IgE (such as via allergen-specific IgG). This would presumably not be the case for the effect of HDM.

Recently our group has reported increased numbers of Foxp3⁺ T cells in the nasal mucosa of patients with rhinitis during the pollen season [14]. It seems likely that allergen-specific regulatory T cells are recruited to the nasal mucosa during the pollen season, and thus may be underrepresented in the peripheral blood. Use of allergen-

MHC tetramers may allow exploration of this possibility in the future [15]. It is of note that immunotherapy was associated with a further increase in seasonal Foxp3⁺ cells in the nose [14], and treatment that expands and recruits regulatory T cells to sites of allergic inflammation would be of great interest.

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Conflict of interest statement: D. S. R. is currently an employee of Laboratorios Leti SL, Madrid, Spain.

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