

Induction of T 'regulatory' cells by standardized house dust mite immunotherapy: an increase in CD4⁺CD25⁺ interleukin-10⁺ T cells expressing peripheral tissue trafficking markers

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Summary

Background Clinically effective subcutaneous allergen-specific immunotherapy (SIT) is associated with altered circulating T cell cytokine production and altered local cytokine responses with increased IL-10 following allergen challenge in target organs.

Objective This study aimed to elucidate mechanisms for these T cell changes, by examining surface expression of markers for peripheral tissue trafficking on circulating cytokine-positive T cells following standardized house dust mite- (HDM-) SIT.

Methods A randomized conventional HDM immunotherapy study was performed on a panel of 12 HDM-allergic subjects. Nine subjects received treatment with conventional HDM immunotherapy using a standardized extract and three subjects were treated by standard pharmacotherapy alone. Symptom and medication scores and allergen-induced cutaneous late-phase responses were assessed before and 9 months after institution of therapy. Before and at 3 and 9 months of SIT, peripheral blood mononuclear cells were cultured for 14 days with HDM extract and CD4⁺ and CD8⁺ T cell expression of CD62L, CD49d and CCR5 and production of IL-10, IFN- γ and IL-4 were analysed by flow cytometry. Allergen-specific T cell proliferation was assessed by ³H-thymidine incorporation.

Results At 9 months, all SIT-treated patients showed reduced symptom scores and late-phase cutaneous responses to HDM compared with baseline levels. The proportions of CD4⁺ T cells which were IL-10⁺ were increased ($P < 0.01$), and the proportions of CD4⁺ and CD8⁺ T cells which were IL-4⁺ decreased ($P < 0.05$) compared with baseline. CD4⁺ and CD8⁺ T cell IFN- γ production, expression of surface markers for peripheral tissue trafficking and allergen-specific proliferation remained unchanged during SIT treatment. However, increased proportions of CD4⁺CD62L⁺, CD4⁺CD49d^{hi}, CD4⁺CCR5⁺ T cells expressing IL-10 were detected at 9 months of SIT compared with baseline ($P < 0.05$). IL-10 staining co-localized with CD4⁺CD25⁺ T cells.

Conclusion Clinically effective subcutaneous immunotherapy with a standardized HDM *Dermatophagoides pteronyssinus* preparation results in decreased numbers of IL-4⁺ T cells and expansion of CD4⁺IL-10⁺ T cells expressing a peripheral tissue trafficking phenotype. The co-localization of IL-10⁺ staining to CD4⁺CD25⁺ T cells is consistent with the induction of a T regulatory cell population by SIT.

Keywords adhesion molecules, allergy, CD4⁺CD25⁺ T cell, chemokine receptor, house dust mite, IFN- γ , IL-4, IL-10, specific immunotherapy, T regulatory cell

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Introduction

Allergic rhinitis (AR) is a common problem, affecting 10–25% of the population worldwide [1, 2]. In Melbourne, Australia, 42% of young adults report symptoms of nasal allergies [3]. Two patterns of AR are recognized – persistent and intermittent, with persistent rhinitis predominantly due

to domestic allergens such as house dust mite (HDM). With respect to dust mite-sensitive persistent AR, the initial approach to management includes dust mite avoidance and standard pharmacotherapy such as antihistamines and intranasal steroids. In patients who have symptoms despite the above measures, allergen-specific immunotherapy (SIT) is an adjunct to management, with efficacy documented in double-blind controlled trials [4–8].

Conventional aeroallergen immunotherapy involves the incremental administration of allergen extract subcutaneously to induce clinical tolerance; however, efficacy varies and the risk of side-effects is appreciable [9]. In previous studies of

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clinically successful SIT, T cell responses were shown to be modified, with a shift in cytokine production by circulating T cells from IL-4 and IL-5 to IFN- γ predominant [10–12], and increased IFN- γ^+ and IL-10 $^+$ cell numbers at peripheral sites of allergen challenge [13–16]. There is evidence that mechanisms for cytokine changes during SIT include T cell anergy [10–12], enhanced allergen-specific T cell apoptosis [17], immune deviation [10–12, 18] and induction of regulatory T cells [19, 20]. The role of IL-10 in down-regulating allergen-specific T cell cytokine production and proliferation during immunotherapy is well documented [20–22]. Recently, T cells producing IL-10 following SIT have been identified as a subset of CD4 $^+$ CD25 $^+$ regulatory T cells [19, 20]. However, it is not clear whether SIT can promote trafficking of IL-10-producing T cells to down-regulate allergen-specific T cell responses in peripheral target tissues upon allergen re-exposure.

Trafficking of T cells throughout the body, from blood vessels to tissues, is dependent on interactions between T cell expressed adhesion molecules and ligands on endothelial cells [23]. Naïve T cells express L-selectin (CD62L) which binds sulphated carbohydrates of CD34 expressed on high endothelial venules, allowing entry into lymphoid tissue. Upon T cell activation, CD62L is shed and expression of very late activation antigen-4 (VLA-4, CD49d) is up-regulated [24, 25]. CD49d binds vascular cell adhesion molecule-1 on activated peripheral vascular endothelium. Accompanying the altered expression of adhesion molecules are changes to T cell trafficking, with decreased movement through lymphoid tissue and increased migration to tissues in the periphery [25]. T cell movement into inflamed tissues is also mediated by T cell chemokine receptor expression and chemokine production in the tissues. With differential expression of chemokine receptors by T cells, such as CCR5 by Th1-type cells, it is possible that Th cell subsets traffic to different sites in the periphery [26–30]. It is possible that SIT might alter the expression of adhesion molecules and chemokine receptors by different T cell subsets.

The aim of the current study was to determine whether HDM-SIT could differentially alter expression of surface molecules that influence trafficking to peripheral tissues by allergen-specific T cells producing IL-10, IFN- γ or IL-4, and whether effective SIT results in the induction of T regulatory cells. We focussed on expression of the adhesion molecules CD62L and CD49d, as allergen-activated T cells expressing CD62L $^-$ or CD49d ^{hi} represent T cells with peripheral tissue trafficking potential and CD49d has been implicated in T cell recruitment to lung tissue after allergen challenge [31]. Several chemokine receptors are of importance in allergy [32–34]; however, in this study T cell expression of CCR5 was analysed as this receptor has been detected on T cells in allergic donor lungs and regulated on activation, normal T cells expressed and secreted, monocyte chemoattractant protein and macrophage inflammatory protein-1 α , the ligands for this receptor, have been reported to be increased in the bronchoalveolar lavage fluid from atopic asthmatics after allergen challenge [33–35]. Furthermore, we have shown increased numbers of IFN- γ^+ T cells expressing CD62L $^-$, CD49d ^{hi} and CCR5 $^+$ in cultures of HDM-allergic donor peripheral blood mononuclear cell (PBMC) stimulated with high concentrations of HDM allergen extract [36]. In the

present study, these parameters were assessed in patients receiving a new standardized HDM extract (Alustal $^{\text{®}}$ *Dermatophagoides pteronyssinus* extract, Stallergenes, Paris, France), which has had positive clinical experience in Europe. As there are no published controlled clinical trials with this product, the current study also assessed the clinical efficacy of SIT using this extract compared with an observational control group.

Materials and methods

Patients and immunotherapy protocol

Patients (18–60 years old) were recruited from the Asthma and Allergy Clinic, Alfred Hospital, Melbourne, Australia. The Alfred Hospital Research and Ethics Committee approved the study and written informed consent was obtained from each patient. Patients were selected on the basis of a history of rhinitis with symptoms consistent with dust mite sensitivity present for at least the 2 previous years, together with positive HDM-specific IgE as determined by skin prick test (weal > 5 mm above negative control) or Kallestad Allercoat EAST (Sanofi-Pasteur Diagnostics, Chaska, MN, USA; score > 1/4).

The design was a randomized open-label parallel group study in which patients were randomized in a 2:1 ratio (1) to receive SIT with Alustal $^{\text{®}}$ *D. pteronyssinus* extract (Stallergenes, Paris, France), or (2) to observe with standard pharmacotherapy and clinical monitoring (non-SIT group). For SIT, patients were given injections of the Alustal preparation according to a standard protocol, beginning at a dose of 0.1 mL of 0.1 IC/mL and increasing at weekly intervals for 13 weeks until a maintenance dose of 0.4 mL of 10 IC/mL was reached, and then continued monthly. For patients randomized to observation, persistent rhinitis was managed with pharmacotherapy including intranasal steroids and oral antihistamines. Intranasal steroids were kept at the same dose during the study and antihistamines were used as required. Topical antihistamines were not allowed.

Cutaneous late-phase response

The cutaneous late-phase response to HDM allergen was assessed before and at 9 months of treatment by the intradermal injection of 0.03 mL of 100 bioequivalent allergy units (BAU)/mL of aqueous HDM extract (Hollister-Stier $^{\text{®}}$, Richard Thomson, Sydney, Australia) into the flexor surface of the non-dominant forearm. The weal diameter was measured at 15 min. Patients returned after 24 h for measurement of the late-phase response. The outline of induration was traced onto transparency paper, digitally photographed and imported into a computer for analysis of the surface area of the response by the Alfred/Medseed Wound Imaging System (AMWIS $^{\text{®}}$) software (Bayside Health and Medseed Pty Ltd, Melbourne, Australia).

Clinical efficacy

Rhinoconjunctivitis symptom score and medication requirements A diary symptom and medication chart were maintained daily 2 weeks prior to treatment and prior to

the 9-month visit by recording chest, nose, eyes and throat symptoms, as well as medication use. Each symptom was scored from 0 (the symptom is absent) to 3 (the symptom is disabling and causes discomfort). Scores for each symptom were then added together to reach a 'total symptom score'. Rhinitis medications were also recorded, and daily dose requirements of antihistamines for symptom relief were added to give a 'total relief medication score'.

Rhinoconjunctivitis quality of life (QOL) questionnaire A disease-specific Juniper Rhinoconjunctivitis QOL Questionnaire [37] was administered before treatment and at the 9-month visit. Patients were asked to recall their experiences during the previous week and to give their responses on a 7-point scale. The rhinoconjunctivitis QOL scores from these visits were also totalled.

Antigen used for in vitro immunological studies

Freeze-dried HDM, *D. pteronyssinus*, kindly provided by Allergy Therapeutics Limited (Worthing, West Sussex, UK) was dissolved in PBS and sterilized. Endotoxin levels in the HDM extract were undetectable (<3 U/mL) as determined by the QCL-1000TM endotoxin detection kit (BioWhittaker, Oslo, Norway). Mitogenicity of the HDM extract was excluded by culturing with oligoclonal latex-reactive T cells and irradiated autologous PBMC and assessing proliferative response by ^3H -thymidine incorporation. Toxicity was excluded by culture with latex-reactive T cells and IL-2 (data not shown).

HDM extract-stimulated 14-day cultures

Peripheral blood was taken from subjects in the SIT group before and at 3 and 9 months of treatment. Analysis of immunological parameters was performed on short-term cultures of PBMC to permit detection of the low-frequency proportions of allergen-specific T cells. For this PBMC were separated from heparinized peripheral blood by density gradient centrifugation on Ficoll Paque (Amersham Pharmacia, Uppsala, Sweden). PBMC were cultured in 24-well plates (2.5×10^6 /well; Greiner, Frickenhausen, Germany) in complete medium (RPMI 1640 medium with 2 mmol/L L-glutamine, 100 IU/mL penicillin/streptomycin, Gibco Invitrogen, Auckland, NZ, and 5% screened, heat-inactivated human AB⁺ serum, Sigma, St Louis, USA) with 25 µg/mL HDM extract (final volume of 2 mL/well) for 7 days at 37 °C in 5% CO₂. On day 7 of culture, cells (1×10^6 cells/mL) were restimulated with 25 µg/mL HDM extract and irradiated (3000 rad) autologous PBMC (1×10^6 /mL) as a source of antigen presenting cells (APCs) (final volume of 2 mL/well). Recombinant human IL-2 (rIL-2; 25 U/mL; Cetus Corporation, Emeryville, CA, USA) was added on day 8 of culture, and on day 11 of culture 1 mL of culture medium was removed and replaced with fresh medium and rIL-2 (25 U/mL).

Monoclonal antibodies

Antibodies required for flow cytometry were purchased from Pharmingen (San Diego, CA, USA). T cell subsets were labelled with anti-CD4-allophycocyanin and anti-CD8-fluorescein isothiocyanate (FITC). IgG1,κ-allophycocyanin and

IgG1,κ-FITC served as isotype-specific controls. The expression of 'peripheral tissue trafficking' surface markers and chemokine receptors by T cells was analysed using anti-CD62L-FITC, anti-CD49d-Cy-chrome and anti-CCR5-FITC. IgG1,κ-FITC, IgG1,κ-Cy-chrome and IgG2a,κ-FITC served as isotype-specific controls. Intracellular cytokines were analysed using anti-IL-4-phycoerythrin (PE), anti-IL-10-PE and anti-IFN-γ-allophycocyanin with IgG1,κ-PE and IgG1,κ-allophycocyanin as isotype-specific controls.

Cell surface marker analysis

On day 14, cultured cells were labelled with anti-CD4 or anti-CD8 antibodies or isotype-specific controls, washed and then labelled with anti-CD62L, anti-CD49d or anti-CCR5 antibodies or isotype-specific controls. Percentages of T cells expressing CD62L⁺, CD49d^{hi} or CCR5⁺ were determined using a FACScaliber flow cytometer (BD Biosciences, San Jose, CA, USA) and Cellquest software. The threshold for positive staining was taken as the point at which the test and isotype histograms crossed. Background values from the isotype control histograms were then subtracted.

Intracellular cytokine staining

For intracellular staining of IL-4 and IFN-γ, day-14 cultured cells were stimulated (2.5×10^5 cells/well) in 96-well U-bottom plates with immobilized anti-CD3 (OKT3; 10 µg/mL), with rIL-2 (100 U/mL) and 10 µg/mL Brefeldin A (Sigma) for 6 h at 37 °C, 5% CO₂. For intracellular staining of IL-10, cells were stimulated in the same manner but for a total of 20 h at 37 °C, 5% CO₂, adding Brefeldin A (10 µg/mL) for the last 4 h of stimulation. After stimulation, cells were surface stained with anti-CD4 and anti-CD8 antibodies. When analysing cytokine production by T cells expressing a 'peripheral tissue trafficking phenotype', cells were also labelled with anti-CD62L, anti-CD49d and anti-CCR5 antibodies or isotype-specific controls at this step. After surface staining, cells were fixed using 4% paraformaldehyde/PBS and permeabilized using FACS permeabilizing solution (BD Biosciences). After washing, cells were stained with anti-IL-4 and anti-IFN-γ antibodies or anti-IL-10 antibody or isotype-specific controls. Percentages of cytokine-positive CD4⁺ and CD8⁺ T cells were then determined by flow cytometry. In addition, the percentages of CD62L⁺, CD49d^{hi} or CCR5⁺ T cells expressing IL-4, IFN-γ or IL-10 were calculated. Absolute numbers of T cell subsets were also determined from subset percentages and the total number of cells present in the HDM extract-stimulated 14-day cultures.

T cell proliferation assays

T cells (5×10^4 per well) from the 14-day cultures were incubated in the presence of an equal number of irradiated PBMCs as feeder cells and HDM extract (10, 25 or 100 µg/mL) in round-bottom 96-well plates (final volume of 200 µL/well). Assays were performed in triplicate. Positive control cultures were T cells with IL-2 (100 U/mL) and negative control cultures were T cells alone, APC alone and T cells with APC without antigen. After 48 h, cultures were pulsed with 1 µCi ^3H -thymidine (Amersham Biosciences, Bucks, UK) and harvested 16 h later. Proliferation as correlated with

^3H -thymidine incorporation was measured by liquid scintillation spectroscopy and results expressed as Δcpm (the mean of the triplicates with antigen minus the mean of the triplicates without antigen). The maximal proliferative response detected over the allergen concentration range tested was determined for each patient.

Statistical analysis

Data were analysed with the aid of the Instat 2.0 statistical package. For clinical and immunological analyses, statistical significance of differences between results obtained at various time-points of SIT was assessed using a Wilcoxon Signed Rank test. Statistical significance between groups was analysed using a Mann-Whitney test. The Spearman Rank test was used to assess the correlations between clinical observations and immunological results. A P -value of 0.05 was considered statistically significant.

Results

Patient demographics

Sixteen subjects were recruited to the study, with 11 randomized to SIT and five to non-SIT. Of the 16 subjects, two subjects (one male, one female) randomized to SIT and two non-SIT subjects (two female) did not return for follow-up visits. The characteristics of the SIT and control non-SIT patients (12 in total) who completed the study are shown in Table 1. Due to the resultant low numbers of subjects in the non-SIT group, immunological studies were only performed on the SIT group, and statistical comparisons were made between pre- and post-SIT samples.

Clinical efficacy of SIT

At 9 months of treatment, all SIT-treated patients showed reduced symptom scores and late-phase cutaneous responses to HDM compared with baseline levels. The mean ($\pm$ SD) total symptom score in SIT patients at baseline was 103.7 ± 69.2 , and this decreased to 63.7 ± 57.8 ($P = 0.012$) at 9 months, although relief medication requirement scores did not significantly change (7.4 ± 6.2 at baseline compared with 5.9 ± 7.5 at 9 months; $P = 0.41$). The rhinitis QOL scores showed significant improvement going from 75.9 ± 23.1 at baseline to 47.6 ± 35.3 at 9 months ($P = 0.015$). None of these scores in the non-SIT group showed any significant change although the subject numbers were small ($n = 3$). The mean surface area ($\pm$ SD) of the late-phase response to HDM in the SIT group at baseline was $60 \pm 37 \text{ cm}^2$ and was reduced significantly at 9 months to $28 \pm 19 \text{ cm}^2$ ($P = 0.004$; Fig. 1a). There was no significant change in late-phase response surface area in the non-SIT group (Fig. 1b).

IL-10 production by CD4^+ T cells is increased at 9 months of SIT

The production of IL-10 by CD4^+ and CD8^+ T cells in 14-day HDM extract-stimulated cultures generated from PBMC of patients before and during SIT was analysed by intracellular cytokine staining. Representative IL-10 staining

Table 1. Clinical characteristics of subjects

	SIT	Non-SIT
Number	9	3
Age (years $\pm$ SD)	38.7 ± 9.3	29.3 ± 9.7
Male sex no. (%)	5 (55)	0 (0)
Rhinitis no. (%)	9 (100)	3 (100)
Asthma no. (%)	3 (33)	3 (100)

SIT, specific immunotherapy.

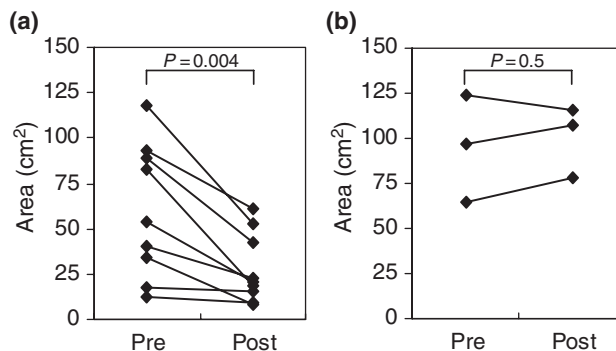


Fig. 1. Surface area of the cutaneous late-phase response to house dust mite (HDM) allergen before and at 9 months of treatment in the specific immunotherapy (SIT) group (a) and the non-SIT group (b). The late-phase response to HDM allergen was assessed by intradermal injection of 0.03 mL of 100 BAU/mL of aqueous HDM extract into the flexor surface of the non-dominant forearm and measurement of the surface area of induration at 24 h. Each symbol in the graphs represents one subject.

of CD4^+ and CD8^+ T cells for one patient receiving SIT is shown in Fig. 2a. Proportions of $\text{IL-10}^+\text{CD4}^+$ T cells and numbers of $\text{CD4}^+\text{IL-10}^+$ T cells were greater at 9 months of SIT in comparison with pre-SIT (% $P = 0.008$; number $P = 0.039$) and at 3 months of SIT (% and number $P = 0.008$; Fig. 2b). There was an inverse correlation between the change in allergen-induced late-phase responses and the change in $\text{CD4}^+\text{IL-10}^+$ T cell numbers following SIT ($r = 0.75$; $P = 0.026$). SIT had no effect on the proportions of $\text{IL-10}^+\text{CD8}^+$ T cells and $\text{CD8}^+\text{IL-10}^+$ T cell numbers (Fig. 2b).

IL-4 production by T cells is reduced at 9 months of SIT

IL-4 and IFN- γ production by T cells in 14-day HDM extract-stimulated cultures generated from PBMC from patients receiving SIT was also analysed before and after treatment. Intracellular IL-4 and IFN- γ profiles of CD4^+ and CD8^+ T cells for one patient are shown in Fig. 3. The proportions of $\text{IL-4}^+\text{CD4}^+$ and CD8^+ T cells were significantly decreased at 9 months of SIT in comparison with pre-SIT ($P < 0.05$; Table 2). SIT had no significant effect on $\text{CD4}^+\text{IL-4}^+$ and $\text{CD8}^+\text{IL-4}^+$ T cell numbers (data not shown). There was a trend for decreased proportions of IFN- $\gamma^+\text{CD4}^+$ T cells at 9 months of SIT in comparison with pre-SIT levels; however, statistical significance was not reached ($P = 0.07$; Table 2). Proportions of IFN- $\gamma^+\text{CD8}^+$ T cells were high at 9 months of SIT for seven of the nine patients, but overall there was no significant difference between pre-SIT and post-SIT (Table 2). At the time-points studied, SIT had no significant effect on $\text{CD4}^+\text{IFN-}\gamma^+$ and $\text{CD8}^+\text{IFN-}\gamma^+$ T cell numbers (data not shown).

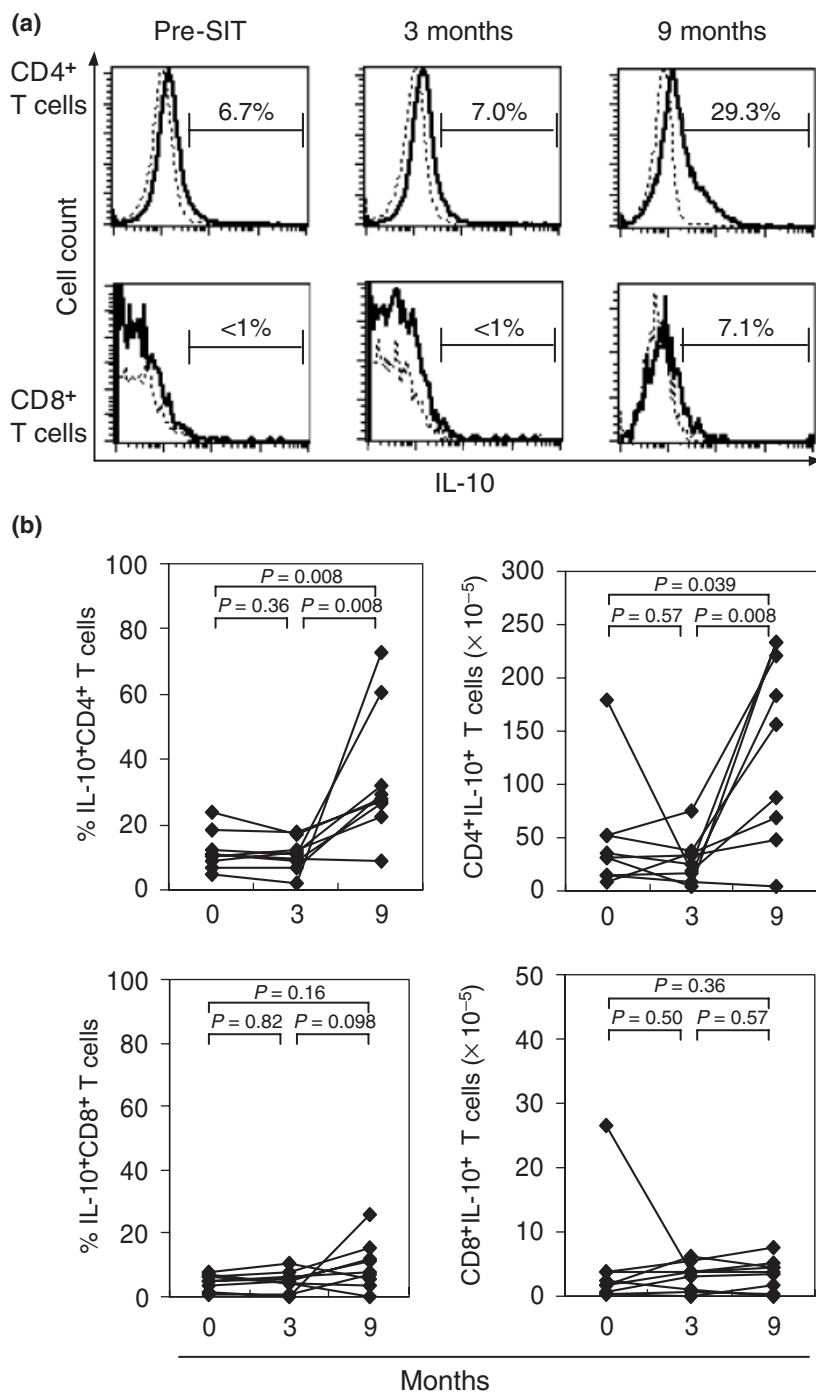


Fig. 2. IL-10 production by $CD4^+$ and $CD8^+$ T cells before and at 3 and 9 months of specific immunotherapy (SIT). Peripheral blood mononuclear cells (PBMCs), from the nine subjects receiving SIT, were cultured with house dust mite (HDM) extract for 14 days and stimulated with anti-CD3/IL-2 for 20 h with Brefeldin A added for the last 4 h. Cells were labelled with anti-CD4, anti-CD8 and anti-IL-10 antibodies and analysed by flow cytometry. (a) Representative IL-10 profiles for $CD4^+$ and $CD8^+$ T cells pre-SIT and at 3 and 9 months of SIT are shown. Positive staining of the test histogram (solid line) was determined at the level of intensity where the isotype control histogram (dotted line) intensity was less than 1% positive. (b) Percentages of $IL-10^+CD4^+$ T cells and the absolute numbers of $CD4^+IL-10^+$ T cells were significantly greater at 9 months of SIT in comparison with pre-SIT (% $P = 0.008$; number $P = 0.039$) and at 3 months of SIT (% and number $P = 0.008$). No significant differences in the percentages of $IL-10^+CD8^+$ T cells and the absolute numbers of $CD8^+IL-10^+$ T cells were observed between pre-SIT and at 3 or 9 months of SIT. Each symbol in the graphs represents one subject receiving SIT.

SIT does not alter overall expression of surface markers involved in peripheral T cell trafficking

The effect of SIT on the expression of the chemokine receptors CCR5 and adhesion molecules, CD62L and CD49d, by $CD4^+$ and $CD8^+$ T cells, was analysed by staining 14-day HDM extract-stimulated cultures and flow cytometry. No significant differences in the proportions of $CD4^+$ or $CD8^+$ T cells expressing CCR5 were observed between pre-SIT and 9 month post-SIT (Table 3; $CD8^+$ T cell data not shown). Analysis of $CD62L^-$ and $CD49d^{hi}$ expression by $CD4^+$ or $CD8^+$ T cells revealed no differences

between pre-SIT and 9 month post-SIT (Table 3; $CD8^+$ T cell data not shown). SIT had no effect on $CD4^+CD62L^-$, $CD8^+CD62L^-$, $CD4^+CD49d^{hi}$, $CD8^+CD49d^{hi}$, $CD4^+CCR5^+$ and $CD8^+CCR5^+$ T cell numbers in 14-day HDM extract-stimulated cultures (data not shown).

SIT enhances IL-10 production by $CD4^+$ T cells expressing a peripheral tissue trafficking phenotype

Production of IL-10 by $CCR5^+$, $CD62L^-$ or $CD49d^{hi}$ T cells in 14-day HDM extract-stimulated cultures generated from

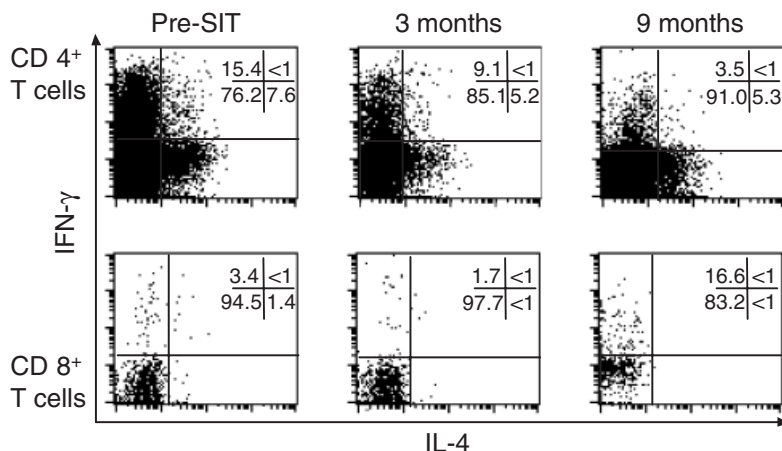


Fig. 3. Representative intracellular IL-4 and IFN- γ staining of CD4⁺ and CD8⁺ T cells pre-specific immunotherapy (SIT) and at 3 and 9 months of SIT. Peripheral blood mononuclear cells (PBMC) from a subject receiving SIT was cultured with house dust mite (HDM) extract (25 μ g/mL) for 14 days, stimulated with anti-CD3/IL-2 for 6 h in the presence of Brefeldin A. Following stimulation, cells were labelled with anti-CD4, anti-CD8, anti-IL-4 and anti-IFN- γ antibodies and analysed by flow cytometry. Isotype controls were used to set quadrant markers and quadrant percentages are shown.

Table 2. Proportions of CD4⁺ and CD8⁺ T cells expressing IL-4 and IFN- γ before and during SIT ($n = 9$)

Subject	CD4 ⁺ T cells						CD8 ⁺ T cells					
	% IL-4 ⁺			% IFN- γ ⁺			% IL-4 ⁺			% IFN- γ ⁺		
	Pre-SIT	3 months	9 months	Pre-SIT	3 months	9 months	Pre-SIT	3 months	9 months	Pre-SIT	3 months	9 months
1	7.58	5.21	5.26	15.36	9.11	3.52	1.44	0.30	0.20	3.39	1.73	16.64
2	25.55	25.04	25.27	14.39	8.56	3.20	2.21	2.88	2.27	2.01	2.53	0.09
3	26.38	30.73	10.12	4.88	3.71	12.50	3.87	2.86	0.10	1.22	1.35	49.76
4	8.24	11.18	10.99	13.03	9.91	12.56	0.70	0.80	0.90	11.48	16.44	44.37
5	19.27	16.24	15.62	3.03	4.38	1.74	3.90	2.60	0.80	1.06	0.50	35.41
6	15.00	20.00	7.60	17.20	26.61	8.27	0.50	0.10	1.00	78.99	83.70	13.60
7	9.89	1.75	9.43	52.50	28.10	15.10	3.20	0.50	0.90	8.66	13.14	28.85
8	2.30	4.30	1.20	14.70	12.50	5.25	1.50	2.00	0.10	4.10	5.90	32.00
9	9.70	12.70	2.08	7.40	12.30	10.00	1.90	3.50	0.70	1.30	2.70	56.30
Mean	13.77	14.13	9.73*	15.83	12.80	8.02	2.14	1.73	0.77*	12.47	14.22	30.78

Peripheral blood mononuclear cells were cultured for 14 days with 25 μ g/mL house dust mite extract. Cells were stimulated with anti-CD3/IL-2 for 6 h, labelled with anti-CD4 and anti-CD8 antibodies and stained for intracellular IL-4 and IFN- γ . Percentages of CD4⁺ and CD8⁺ T cells expressing IL-4 or IFN- γ were determined by flow cytometry.

SIT, specific immunotherapy.

* $P < 0.05$ between pre-SIT and 9 months of SIT.

Table 3. Expression of CCR5, CD62L[−] and CD49d^{hi} by CD4⁺ T cells before and during SIT ($n = 9$)

Surface marker	Mean % CD4 ⁺ T cells ($\pm$ SD)		
	Pre-SIT	3 months	9 months
CCR5 ⁺	26.89 ($\pm$ 21.93)	22.93 ($\pm$ 21.36)	24.39 ($\pm$ 17.89)
CD62L [−]	29.24 ($\pm$ 12.38)	26.13 ($\pm$ 15.19)	23.67 ($\pm$ 12.95)
CD49d ^{hi}	77.00 ($\pm$ 10.43)	69.16 ($\pm$ 26.51)	82.93 ($\pm$ 11.19)

Peripheral blood mononuclear cells were cultured for 14 days with 25 μ g/mL house dust mite extract. Cells were then labelled with anti-CD4, anti-CCR5, anti-CD62L and anti-CD49d antibodies and analysed by flow cytometry.

SIT, specific immunotherapy.

PBMC from patients before and during SIT was determined by surface marker and intracellular cytokine staining. Histograms of intracellular IL-10 staining of CD4⁺CCR5⁺, CD4⁺CD62L[−] and CD4⁺CD49d^{hi} T cells are shown for one patient receiving SIT in Fig. 4a. Comparing results for the nine patients receiving SIT revealed significant increases in the proportions of CD4⁺CCR5⁺ T cells expressing IL-10 at 9 months of SIT in comparison with pre-SIT ($P = 0.027$) and to

3 months of SIT ($P = 0.004$; Fig. 5a). The CD4⁺CCR5⁺IL-10⁺ T cell numbers were increased at 9 months of SIT in comparison with 3 months ($P = 0.004$; Fig. 5a). Significant increases in proportions of CD4⁺CD62L[−] T cells expressing IL-10 at 9 months of SIT in comparison with pre-SIT ($P = 0.019$) and to 3 months of SIT ($P = 0.004$; Fig. 5b) were seen. CD4⁺CD62L[−]IL-10⁺ T cell numbers were increased at 9 months of SIT in comparison with 3 months ($P = 0.019$; Fig. 5b). A trend for increased CD4⁺CD62L[−]IL-10⁺ T cell numbers at 9 months of SIT was observed in comparison with pre-SIT; however, statistical significance was not reached ($P = 0.074$). Proportions of CD4⁺CD49d^{hi} T cells expressing IL-10 and numbers of CD4⁺CD49d^{hi}IL-10⁺ T cells were significantly greater at 9 months of SIT in comparison with pre-SIT (% and number $P = 0.039$) and 3 months of SIT (% and number $P = 0.004$; Fig. 5c).

Within the CD8⁺ T cell population, the proportions of CD8⁺CCR5⁺ T cells expressing IL-10 and numbers of CD8⁺CCR5⁺IL-10⁺ T cells were unaffected by HDM-SIT (data not shown). Significant increases in proportions of CD8⁺CD62L[−] T cells expressing IL-10 were observed at 9 months of SIT in comparison with pre-SIT ($P = 0.018$) and at

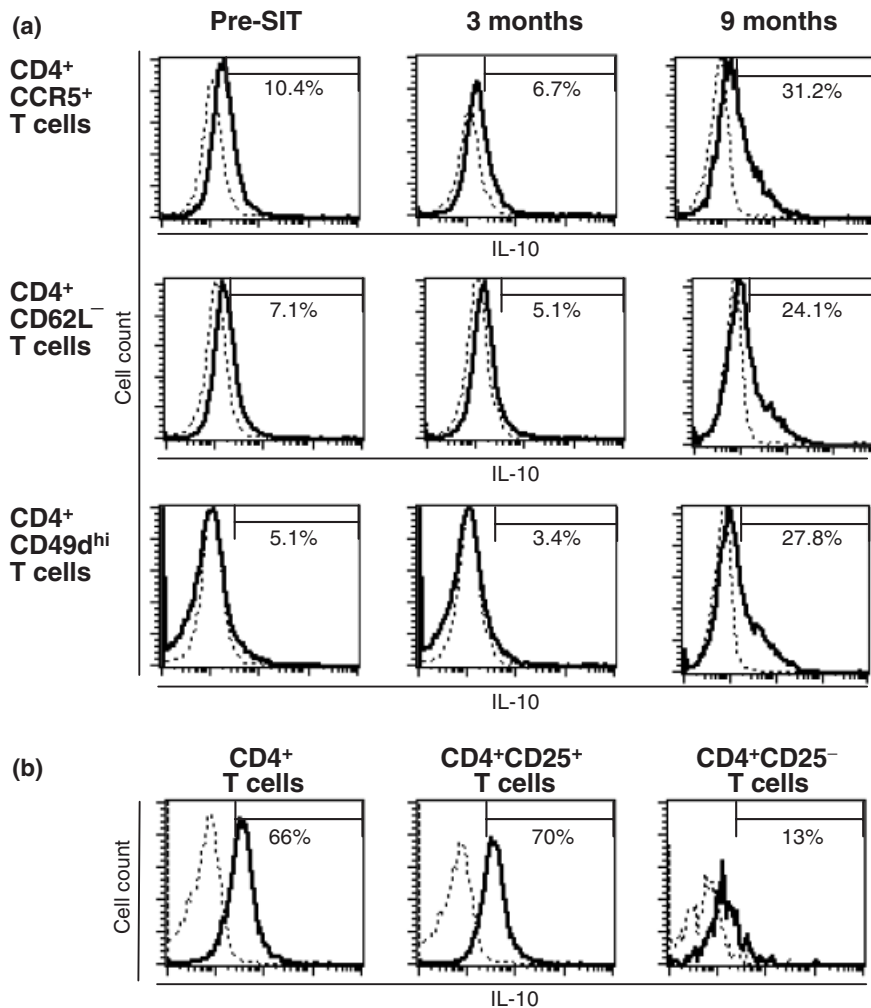


Fig. 4. Representative histograms of intracellular IL-10 staining of $CD4^+CCR5^+$ T cells, $CD4^+CD62L^-$ T cells, $CD4^+CD49d^{hi}$ T cells and $CD4^+CD25^+$ T cells at pre-SIT and at 3 and 9 months of SIT. (a) Peripheral blood mononuclear cells (PBMCs) from a subject receiving specific immunotherapy (SIT) were cultured with house dust mite (HDM) extract (25 μ g/mL) for 14 days, stimulated with anti-CD3/IL-2 for 20 h with Brefeldin A added for the last 4 h. Following stimulation, cells were labelled with anti-CD4, anti-CCR5, anti-CD62L, anti-CD49d and anti-IL-10 antibodies and analysed by flow cytometry. Positive staining of the test histogram (solid line) was determined at the level of intensity where the isotype control histogram (dotted line) intensity was less than 1% positive. (b) Representative histograms of intracellular IL-10 staining of $CD4^+$ T cells, $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells at 9 months of SIT. PBMC from a subject receiving SIT were cultured with HDM extract (25 μ g/mL) for 14 days, stimulated with anti-CD3/IL-2 for 20 h with Brefeldin A added for the last 4 h. Following stimulation, cells were labelled with anti-CD4, anti-CD25 and anti-IL-10 antibodies and analysed by flow cytometry.

3 months of SIT ($P = 0.039$; data not shown). $CD8^+CD62L^-IL-10^+$ T cell numbers were unaffected during treatment. There were no differences in the proportions of $CD8^+CD49d^{hi}$ T cells expressing IL-10 and the numbers of $CD8^+CD49d^{hi}IL-10^+$ T cells at the time-points tested (data not shown).

Intracellular IL-10 staining co-localizes to $CD4^+CD25^+$ T cells at 9 months of SIT

T cells producing IL-10 following SIT have been identified as a subset of $CD4^+CD25^+$ regulatory T cells [19, 20]. Hence, IL-10-producing $CD4^+$ T cells were analysed for CD25 expression in 14-day HDM extract-stimulated cultures from four patients at 9 months of dust mite SIT (data for one patient is shown in Fig. 4b). The proportions of $CD4^+CD25^+$ T cells expressing IL-10 (mean, 47%; range, 27–70) were significantly greater ($P = 0.025$) than the proportions of

$CD4^+CD25^-$ T cells expressing IL-10 (mean, 16%; range, 13–23).

IL-4 and IFN- γ production by $CD4^+$ T cells expressing a peripheral tissue trafficking phenotype remains unchanged during SIT

The IL-4 and IFN- γ phenotype of $CCR5^+$, $CD62L^-$ or $CD49d^{hi}CD4^+$ and $CD8^+$ T cells in 14-day HDM extract-simulated cultures generated from PBMC from patients receiving SIT was examined. When comparing the results from the nine patients receiving SIT, there were no significant differences in the percentages of $CD4^+CCR5^+$, $CD4^+CD62L^-$ or $CD4^+CD49d^{hi}$ T cells expressing either IL-4 or IFN- γ when comparing pre-SIT to 3 or 9 months of SIT (Table 4). Correspondingly, SIT had no effect on the numbers of $CD4^+CCR5^+$, $CD4^+CD62L^-$ or $CD4^+CD49d^{hi}$ T cells expressing IL-4 or IFN- γ (data not shown). In addition, there were no

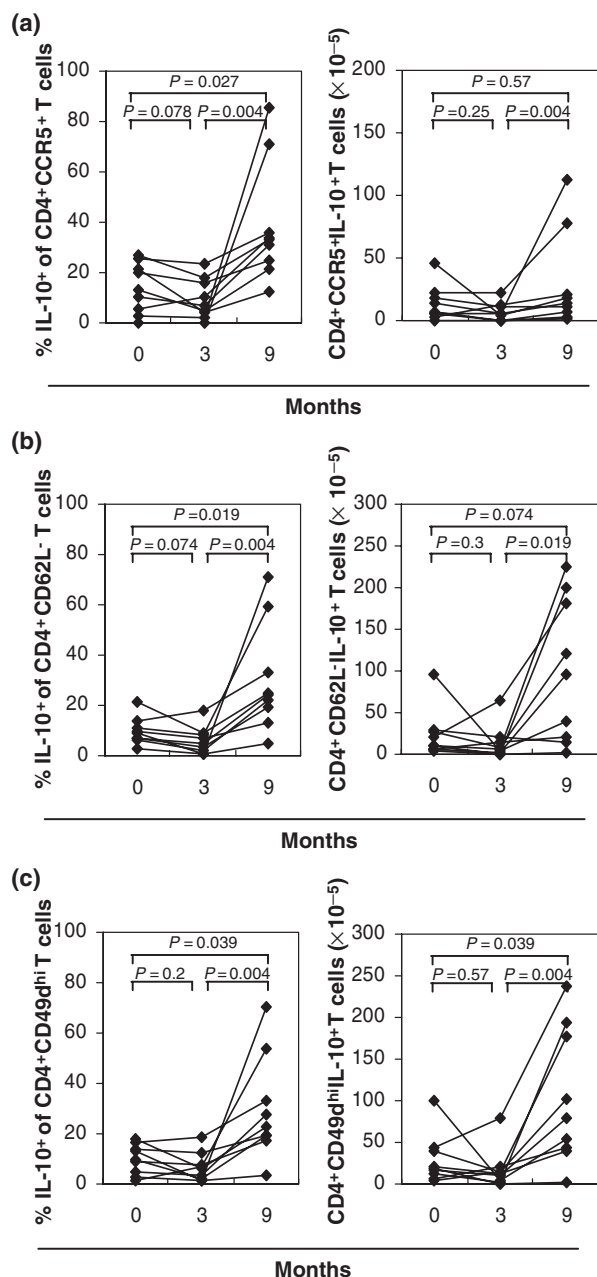


Fig. 5. IL-10 production by CD4⁺CCR5⁺ T cells, CD4⁺CD62L⁻ T cells and CD4⁺CD49d^{hi} T cells at pre-specific immunotherapy (SIT) and at 3 and 9 months of SIT. Peripheral blood mononuclear cells (PBMCs) from the nine subjects receiving SIT were cultured with house dust mite (HDM) extract (25 µg/mL) for 14 days and stimulated with anti-CD3/IL-2 for 20 h with Brefeldin A added for the last 4 h. Cells were labelled with anti-CD4, anti-CCR5, anti-CD62L, anti-CD49d and anti-IL-10 antibodies and analysed by flow cytometry. (a) Significant increases in the proportions of CD4⁺CCR5⁺ T cells expressing IL-10 at 9 months of SIT in comparison with before ($P = 0.027$) and to 3 months of SIT ($P = 0.004$) were observed. CD4⁺CCR5⁺IL-10⁺ T cell numbers were increased at 9 months of SIT in comparison with 3 months ($P = 0.004$). (b) Significant increases in the proportions of CD4⁺CD62L⁻ T cells expressing IL-10 at 9 months of SIT in comparison with pre-SIT ($P = 0.019$) and to 3 months of SIT ($P = 0.004$) were also seen. CD4⁺CD62L⁻IL-10⁺ T cell numbers were increased at 9 months of SIT in comparison with 3 months ($P = 0.019$). There was a trend for increased CD4⁺CD62L⁻IL-10⁺ T cell numbers at 9 months of SIT in comparison with pre-SIT ($P = 0.074$). (c) The percentage of CD4⁺CD49d^{hi} T cells expressing IL-10 and CD4⁺CD49d^{hi}IL-10⁺ T cell numbers were significantly greater at 9 months of SIT in comparison with pre-SIT (% and number $P = 0.039$) and to 3 months of SIT (% and number $P = 0.004$). Each symbol in the graphs represents one subject receiving SIT.

differences in the percentages or numbers of CD8⁺CD62L⁻, CD8⁺CD49d^{hi} or CD8⁺CCR5⁺ T cells expressing IL-4 or IFN-γ during SIT (data not shown).

Allergen-specific proliferation following SIT

The 14-day HDM extract-stimulated cultures generated from PBMC obtained from SIT-treated patients were tested for T cell reactivity to HDM extract by proliferation assay (Table 5). Before treatment, all patients showed strong T cell proliferative responses to HDM extract. At 9 months of SIT in comparison with pre-SIT, the proliferative response to HDM extract was decreased in five of nine patients. Of these five patients, four also showed decreased proliferation at 3 months in comparison with pre-SIT with only one showing increased proliferation. However, as the other four patients showed increased proliferative responses to HDM extract at 9 months of SIT and had variable responses at 3 months of SIT, overall there were no significant changes in allergen-induced proliferation following immunotherapy.

For one patient (subject 7) who displayed reduced allergen-specific T cell proliferation at 9 months of SIT, we assessed whether addition of IL-2 could restore the T cell proliferative response thus indicating reversal of anergy or inhibition of T regulatory cell-mediated suppression [38, 39]. Stimulating patient 7's 14-day HDM extract-stimulated culture with HDM extract (100 µg/mL) in the presence of IL-2 resulted in increased proliferation from 24 193 Δcpm (without IL-2) to 68 577 Δcpm.

Discussion

In this study of allergen immunotherapy using a standardized HDM preparation, the immunological mechanisms involved in effective desensitization of allergic individuals were shown to include decreased T cell production of IL-4 with increased CD4⁺ T cell production of the immunoregulatory cytokine IL-10. The IL-10⁺CD4⁺ T cells also expressed CD25 consistent with induction of T regulatory cells. This study also demonstrated for the first time that the allergen SIT-induced IL-10-producing T cells express a peripheral tissue trafficking phenotype.

Consistent with standardized SIT for other allergens [6, 40–42], the HDM-SIT was efficacious with significant improvement in symptom scores in all patients. Although relief medication usage remained unchanged, all patients showed significant reductions in cutaneous late-phase responses to HDM allergen. The possibility that these decreases may have been due to the degradation of the allergen skin test reagent was excluded by an unchanged response in non-SIT patients over the same time period.

A pivotal role for CD4⁺ T cells in controlling the allergic immune response is well recognized, with recent data also suggesting a role for CD8⁺ T cells [18, 43]. In our study, clinically effective HDM-SIT was accompanied by modulation of T cell function as reflected by changes in T cell proliferation and cytokine production. Allergen-specific T cell proliferation was decreased in five of nine patients, but failed to reach statistical significance. Francis et al. [19] similarly failed to observe an overall decrease in allergen-specific T cell

Table 4. Proportions of IL-4⁺ and IFN- γ ⁺ CD4⁺ T cells expressing CCR5⁺, CD62L⁻ or CD49d^{hi} before and during SIT ($n = 9$)

Surface marker	Mean % IL-4 ⁺ ($\pm$ SD)			Mean % IFN- γ ⁺ ($\pm$ SD)		
	Pre-SIT	3 months	9 months	Pre-SIT	3 months	9 months
CCR5 ⁺	18.21 ($\pm$ 11.19)	17.24 ($\pm$ 11.72)	14.88 ($\pm$ 12.55)	10.92 ($\pm$ 11.71)	11.98 ($\pm$ 10.78)	10.69 ($\pm$ 9.58)
CD62L ⁻	12.46 ($\pm$ 9.82)	10.57 ($\pm$ 12.22)	8.18 ($\pm$ 10.28)	8.91 ($\pm$ 7.91)	12.03 ($\pm$ 10.59)	9.77 ($\pm$ 9.31)
CD49d ^{hi}	11.98 ($\pm$ 8.31)	11.35 ($\pm$ 10.39)	7.13 ($\pm$ 7.97)	4.24 ($\pm$ 7.09)	5.24 ($\pm$ 6.38)	4.82 ($\pm$ 6.00)

Peripheral blood mononuclear cells were cultured for 14 days in the presence of 25 μ g/mL house dust mite extract. Cells were stimulated with anti-CD3/IL-2 for 6 h, labelled with anti-CD4, anti-CCR5, anti-CD62L and anti-CD49d antibodies and stained for intracellular IL-4 and IFN- γ . Percentages of CD4⁺CCR5⁺, CD4⁺CD62L⁻ and CD4⁺CD49d^{hi} cells expressing IL-4 or IFN- γ were determined by flow cytometry.

SIT, specific immunotherapy.

Table 5. T cell proliferation to house dust mite (HDM) extract before and during specific immunotherapy (SIT) ($n = 9$)

Subject	Proliferation (Δ cpm)		
	Pre-SIT	3 months	9 months
1	31 761	5491	611
2	43 982	73 779	48 067
3	26 251	11 531	34 776
4	10 827	28 669	46 745
5	23 957	6776	4820
6	47 459	33 962	41 110
7	138 335	25 174	23 051
8	31 722	35 477	15 967
9	8007	6904	27 893
Mean	40 256	25 307	27 004

Maximal proliferative responses of 14-day HDM extract-stimulated cultures generated from peripheral blood mononuclear cells of subjects receiving SIT expressed as Δ cpm. Background cpm: median 1461; range 128 – 15 326.

proliferation following SIT, but suggested that this reflected failure to achieve adequate intensity of IL-10 T cell response. In a study of semi-rush SIT by Jutel et al. [20], a statistically significant decrease in allergen-specific T cell proliferation was observed. After SIT, levels of IL-10 produced by allergen-stimulated peripheral blood T cells in short-term cultures were considerably higher in study of Jutel et al. compared with that of Francis et al. The concentration of IL-10 induced during SIT and therefore effect on allergen-specific T cell proliferative response may vary according to the allergen preparation and treatment protocol. The cytokine transforming growth factor- β may also down-regulate allergen-specific T cell responses following SIT [20] and could be analysed in future studies.

A significant decrease in IL-4 production following SIT was observed for both CD4⁺ and CD8⁺ T cells, as has been shown previously [44, 45]. In contrast, Majori et al. [45] reported no change in CD8⁺ T cell IL-4 production following HDM-SIT, but PBMC were non-specifically stimulated with phorbol 12-myristate 13-acetate and ionomycin rather than allergen. Observed changes in CD4⁺ and CD8⁺ T cell IFN- γ production following HDM-SIT have been more variable [44–47]. Although in our study we observed a trend of decreased IFN- γ production by CD4⁺ and increased production by CD8⁺ T cells, this did not reach statistical significance. Taken together our results suggest that SIT induced T cell suppression rather than immune deviation. Our observed increase in IL-10 production by CD4⁺ T cells

during SIT is consistent with induction of T regulatory cells as a possible mechanism for this suppression. Confirmation of IL-10 as a contributory mechanism could be obtained by neutralization of inhibition following co-incubation with IL-10R blocking antibodies [20]. Previous *in vitro* studies demonstrated that neutralization of IL-10 post-bee venom SIT can reverse SIT-induced suppression of T cell cytokine production and proliferation [21, 22].

In addition to altered peripheral blood T cell cytokine production, SIT can induce changes in peripheral tissue cytokine expression with increased numbers of IFN- γ mRNA⁺ cells detected in allergen-challenged nasal mucosa and skin after grass pollen SIT [13–15]. Increased IL-10⁺ cell numbers and decreased IL-4⁺ cell numbers were observed in skin biopsies of allergen-induced late-phase responses following wasp venom SIT [16]. The observed increase in local T cell numbers after allergen provocation [48] suggests that successful SIT may modify the functional phenotype of T cells trafficking to sites of peripheral allergen encounter. Thus, we analysed surface expression of selected adhesion molecules and a chemokine receptor involved in T cell peripheral tissue trafficking and correlated this with cytokine production. However, we found no SIT-induced differences in allergen-stimulated CD4⁺ or CD8⁺ T cell expression of CD62L, CD49d and CCR5 or in IL-4 or IFN- γ production by allergen-stimulated CD4⁺ or CD8⁺ T cells expressing a peripheral tissue trafficking phenotype, i.e. CD62L⁻, CD49d^{hi}, or CCR5⁺. These findings are in contrast to our *in vitro* observations where high concentration allergen stimulation increased IFN- γ production by T cells expressing trafficking markers [36]. This discrepancy may be due to the kinetics of blood sampling with respect to allergen administration or to down-regulation of IFN- γ production by IL-10. Important roles in peripheral allergen-specific T cell trafficking have been suggested for other adhesion molecules and chemokine receptors, with increased CCR4⁺ T cells detected in allergen-challenged atopic asthmatic bronchial biopsies [32].

Our observed increase in IL-10 production by T cells expressing a CD4⁺CD25⁺ regulatory phenotype following HDM-SIT provides further evidence for induction of T regulatory cells as a mechanism for effective allergen-SIT [19, 20]. The observed co-expression of CD62L⁻, CD49d^{hi} and CCR5 is consistent with a peripheral tissue trafficking phenotype of these CD4⁺IL-10⁺ T cells. Induction of 'trafficking' CD4⁺IL-10⁺ T cells by SIT is consistent with increased IL-10 production at sites of allergen-induced late-phase responses observed following wasp venom SIT [16], and may be responsible for decreased allergen-induced

late-phase responses following HDM-SIT. IL-10 has been shown to down-regulate allergic inflammation by inhibiting T cell proliferation and production of IL-4, IL-5 and IFN- γ [49–51]. Our results suggest that enhanced IL-10-mediated immune suppression through development of standardized allergen preparations and new administration regimens may lead to improved clinical and immunological efficacy of SIT.

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