
Induction of interleukin 10 by sublingual immunotherapy for house dust mites: a preliminary report

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Background: Subcutaneous specific immunotherapy has been demonstrated to be capable of inducing T-cell regulatory response. Interleukin 10 (IL-10) plays a crucial role in inducing allergen-specific tolerance; however, no previous studies have examined IL-10 production after sublingual immunotherapy (SLIT).

Objective: To evaluate T-cell proliferation and IL-10 production in patients successfully treated with SLIT for house dust mites (HDMs).

Methods: Peripheral blood mononuclear cells were isolated from patients after at least 3 years of successful HDM SLIT and from matched untreated allergic patients and healthy control subjects. After 3 and 6 days of in vitro stimulation with phytohemagglutinin (PHA), *Candida albicans*, and *Dermatophagoides farinae*, proliferation and production of IL-10 were measured.

Results: Patients treated with SLIT showed a significant reduction of proliferation induced by *C. albicans* compared with untreated atopic patients ($P < .001$), but a significant reduction was also demonstrated in healthy controls compared with untreated atopic patients ($P < .001$). Patients treated with SLIT also showed a significant increase of IL-10 production after *Candida* and PHA stimuli compared with patients with untreated rhinitis ($P < .001$ for both). Patients with untreated rhinitis did not produce IL-10.

Conclusion: This preliminary study confirms reduced T-cell proliferation and preliminarily provides the first evidence, to our knowledge, of peripheral IL-10 production in allergic patients successfully treated with HDM SLIT.

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INTRODUCTION

Specific immunotherapy (SIT) is traditionally based on the subcutaneous administration of allergen extracts to obtain clinical tolerance of the causal allergens in patients with allergic disorders.¹ No evidence exists that SIT is effective in patients with allergic rhinitis and mild forms of asthma as stated in a recent World Health Organization position paper.²

T_H2 cells play a critical role in the pathogenesis of allergic rhinitis and asthma.³ However, the immunologic mechanisms that down-modulate and protect against their development are poorly understood. A spectrum of CD4⁺ T cells, including T_H3 cells, T_R1 cells, CD4⁺CD25⁺ cells, and NK T cells, might exert a role in regulating allergic reaction.^{4,5} A better understanding of the role of regulatory cells in allergic disorders may lead to the identification of pathways involved in mechanisms of action of SIT. Recently, a role for regulatory cells has been evidenced in patients treated with SIT for grasses and house dust mites (HDMs).^{6–9} Furthermore, the

role of interleukin 10 (IL-10) as an early marker of successful immunotherapy has been more recently evidenced.¹⁰ On the basis of these findings, SIT has been recognized to generate regulatory T cells that secrete both IL-10 and transforming growth factor β and specifically to suppress allergen-induced responses.¹¹ This issue appears crucial, because a defect of T-regulatory cells has been recently identified by an experimental study of allergic patients.¹²

Because the traditional subcutaneous route of administration shows the risk of serious adverse reactions, alternative routes of immunotherapy, including oral, sublingual, and nasal, have been investigated and developed.¹³ The rationale of sublingual immunotherapy (SLIT) is that oral administration of high-dose allergen may reduce and prevent IgE responses. Methodologically, the extract is kept under the tongue for a few minutes and then swallowed: the so-called sublingual swallow.

SLIT efficacy has been evaluated in a World Health Organization position paper,² in a European Academy of Allergies and Clinical Immunology and the European Society of Paediatric Allergy and Clinical Immunology document,¹⁴ and in a Allergic Rhinitis and Its Impact on Asthma document.¹⁵ All the published studies except for 3 evidenced the clinical efficacy of SLIT on both rhinitis and asthma symptoms.^{13,16}

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Table 1. Patient Characteristics

Patient No./sex/age, y	Skin prick test result (wheal diameter)	Disease
Control group		
1/M/22	Negative	No
2/F/19	Negative	No
3/M/23	Negative	No
4/M/31	Negative	No
5/M/35	Negative	No
6/M/21	Negative	No
7/M/38	Negative	No
8/F/21	Negative	No
9/M/24	Negative	No
Untreated allergic patients		
1/M/31	HDM (6 mm)	R
2/M/22	HDM (5 mm)	R
3/F/33	HDM, grass (5–4 mm)	R, C
4/M/19	HDM, grass, <i>Parietaria</i> (7, 4, 4 mm)	R
5/M/25	HDM, cat (7, 4 mm)	R
6/F/20	HDM (6 mm)	R, C
7/M/36	HDM, grass, <i>Parietaria</i> (7, 4, 4 mm)	R, C
8/M/40	HDM, cat, grass, <i>Parietaria</i> (8, 4, 4, 4 mm)	R
9/M/21	HDM (6 mm)	R
10/M/18	HDM, grass, <i>Parietaria</i> (5, 4 mm)	R, C
Treated allergic patients		
1/M/35	HDM (7 mm)	R, C
2/M/28	HDM, <i>Parietaria</i> (6, mm)	R, C
3/F/19	HDM (8 mm)	R
4/M/37	HDM, grass (5, 4 mm)	R
5/F/24	HDM, <i>Parietaria</i> (6, 4 mm)	R, C
6/M/27	HDM, grass, <i>Parietaria</i> (7, 4, 4 mm)	R
7/M/19	HDM (6 mm)	R, C
8/M/22	HDM, <i>Parietaria</i> (5, 4 mm)	R, C
9/M/32	HDM (5 mm)	R

Abbreviations: C, conjunctivitis; HDM, house dust mite; R, rhinitis.

The SLIT efficacy for rhinitis was recently confirmed by a meta-analytic Cochrane review.¹⁷

Although the precise mechanism by which this oral tolerance is induced is not well understood, the route of allergen processing and presentation remains a critical determinant of the subsequent T-cell modulation.¹⁶ Nasal immunotherapy, a variant of local immunotherapy, has been demonstrated to be capable of decreasing allergen-specific T-cell response.¹⁸ Recently, oral SIT provided evidence of inducing a form of immunologic tolerance to allergen.¹⁹ However, the immunologic changes induced by SLIT remain unknown. Therefore, the aim of this study was to evaluate T-cell proliferation and IL-10 production in patients successfully treated with SLIT for HDMs.

METHODS

Patients

The study was approved by the Review Board of the La Spezia Navy Hospital and performed with the informed con-

sent of all participants. The donor panel consisted of 9 healthy subjects (as the control group), 10 allergic patients, and 9 patients who received SLIT for HDM allergen extract (Table 1). Sensitization was defined by a positive skin prick test response to *Dermatophagoides pteronyssinus*, cat, grass, and/or *Parietaria* of at least 4 mm greater than that elicited by the negative diluent control (Stallergenes, Milan, Italy). Allergic donors were all symptomatic, with a clear history of persistent allergic rhinitis alone.

Patients who underwent SLIT had received HDM allergen extract (Staloral 100 IR, Stallergenes) for at least 3 years and were well matched to allergic donors for age, sex, and skin prick test response (Table 1). All allergic patients followed generic environmental control measures, such as cleaning and airing only. Six patients in the untreated group and 5 patients in the treated group were polysensitized, but the sensitization for other allergens was not the cause of symptom occurrence as indicated by both their history and negative allergen-specific conjunctival challenge. The study was performed during the winter to avoid the pollen season.

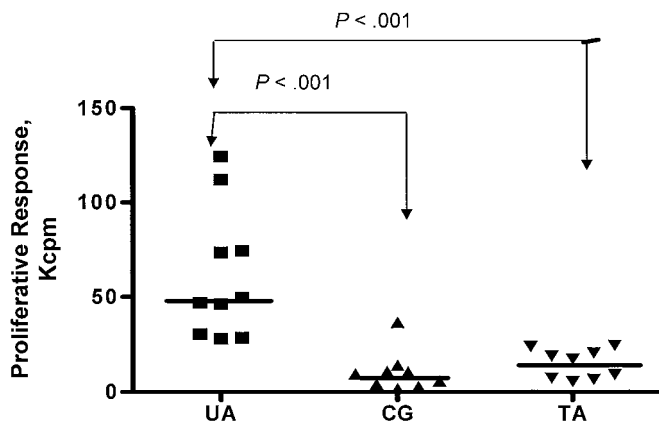


Figure 1. Proliferative response to *Candida albicans* of peripheral blood mononuclear cells from untreated (UA), healthy (CG), and sublingual immunotherapy-treated (TA) patients (in kilocounts per minute [Kcpm]).

SIT Schedule

Patients were treated only with HDM allergen extract. The sublingual treatment was self-administered at home. Drops were held under the tongue for 2 minutes before swallowing. A 15-day sublingual induction phase was followed by a maintenance phase of 36 months. The initial dose was 0.015 μg and the top dose was 51 μg administered every second day. The treatment schedule was adjusted according to the individual patient's tolerance.

Clinical Evaluation

Patients were evaluated for a change in symptoms. The following symptoms were evaluated by the patient, answering the questions made by the investigator: nasal obstruction, sneezing, rhinorrhea, and itchy nose. Each symptom was evaluated on the following scale: 0 indicates absent; 1, mild (symptom was present but was not annoying or troublesome); 2, moderate (symptom was frequently troublesome but did not interfere with either normal daily activity or sleep); and 3, severe (symptom was sufficiently troublesome to have interfered with normal daily activity or sleep). Total symptom score (TSS) was the sum of each individual symptom.

SLIT-treated patients were evaluated at baseline (ie, before SLIT) and after 3 years during clinical examination. Untreated allergic patients were evaluated at the same times. Allergic patients were evaluated after appropriate withdrawal of pharmacologic treatment.

Allergen-Specific Proliferative Response

Patients were instructed to discontinue use of medications before blood sampling and clinical evaluation: 1 week for antihistamines and 2 weeks for intranasal corticosteroids. No patient consumed corticosteroids or antibiotics in the previous 4 weeks.

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on a Ficoll-Hypaque gradient for 30 minutes at 1,800 rpm. The PBMCs were suspended at $2 \times$

$10^6/\text{mL}$ in RPMI 1640 (Sigma, Milan, Italy) supplemented with 10% autologous plasma and 2 mm of L-glutamine (Sigma) and cultured in duplicate on 96 flat-bottomed plates (Nunc, Kamstrup, Denmark) for 6 days in the presence of the allergen extract (20 $\mu\text{g}/\text{mL}$) *Dermatophagoides farinae* (donated by Stallergenes). In all groups, we included duplicate medium controls and a negative control in the presence of $2 \times 10^6/\text{mL}$ of autocleaved bodies of *Candida albicans*, as the recall antigen, or phytohemagglutinin (PHA) (1 $\mu\text{g}/\text{mL}$). After a 6-hour pulse with 0.5 μCi of (3H)-thymidine per well (3H-TdR; Amersham International, Bucks, UK), cultures were harvested and incorporated radioactivity was counted as the index of proliferation.

Quantification of IL-10 Production

To evaluate cytokine production at day 3, we harvested 100 μL of supernatant of the cultures and the samples were frozen at -70°C until used. The quantitative determination of IL-10 was performed by a commercial enzyme-linked immunosorbent assay (Pantec s.r.l., Turin, Italy).

Statistical Analysis

We compared proliferation data and cytokine concentrations between groups with the nonparametric Mann-Whitney *U* test with Prism software (version 3; Graph Pad, San Diego, CA).

RESULTS

Clinical Efficacy of SLIT

Allergic patients undergoing HDM SLIT showed a significant improvement of nasal symptoms (mean $\pm$ SD TSS, 2.7 ± 0.8) compared with untreated patients (mean $\pm$ SD TSS, 4.4 ± 1.3) ($P = .009$).

Effects of SLIT on the Proliferative Response

C. albicans-induced T-cell proliferation. SLIT-treated patients showed significantly lower proliferation than untreated allergic patients ($P < .001$) (Fig 1). Untreated allergic pa-

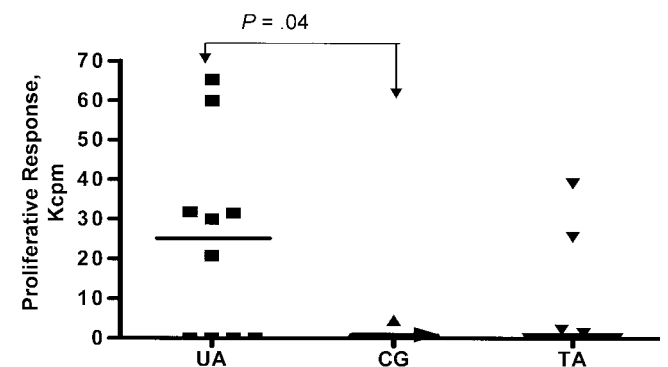


Figure 2. Proliferative response to *Dermatophagoides farinae* of peripheral blood mononuclear cells from untreated (UA), healthy (CG), and sublingual immunotherapy-treated (TA) patients (in kilocounts per minute [Kcpm]).

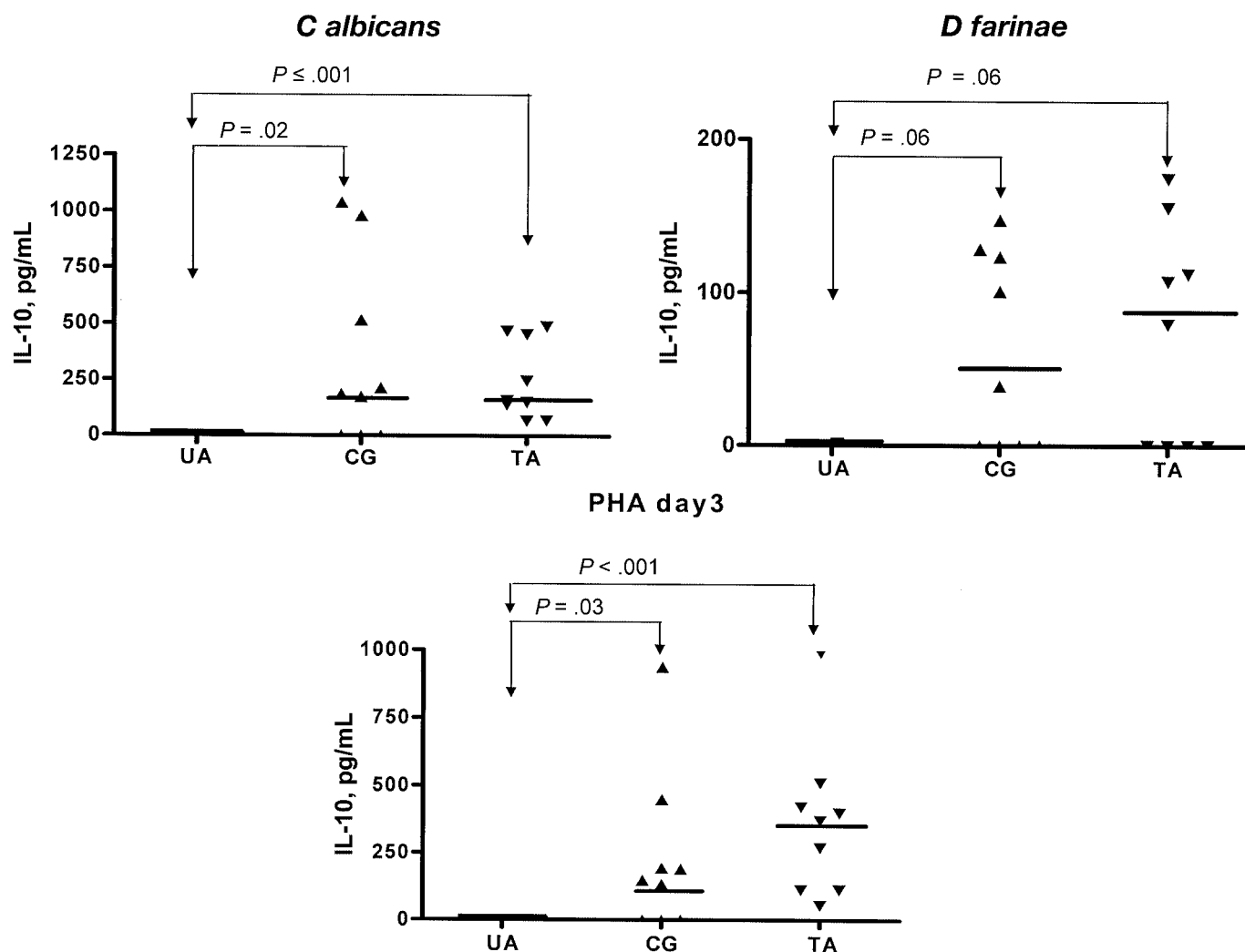


Figure 3. Interleukin 10 (IL-10) production after 3-day cultures with different stimuli (*Candida albicans*, *Dermatophagoides farinae*, and phytohemagglutinin [PHA]) of peripheral blood mononuclear cells from untreated (UA), healthy (CG), and sublingual immunotherapy-treated (TA) patients.

tients showed significantly higher proliferation than healthy subjects ($P < .001$). There was no difference between SLIT-treated patients and healthy donors.

***D. farinae*-induced T-cell proliferation.** SLIT-treated patients showed lower proliferation than untreated allergic patients, even though this difference was not statistically significant (Fig 2). There was no statistically significant difference between patients with treated rhinitis and healthy subjects, whereas there was a statistically significant difference between untreated allergic patients and healthy subjects ($P = .04$).

Effects of SLIT on IL-10 Production

IL-10 production was evaluated after 3 days of culture with different stimuli (Fig 3). PHA-induced IL-10 production was

present only in SLIT-treated patients and healthy controls compared with untreated atopic patients ($P < .001$ and $P = .03$, respectively). For *C. albicans*-induced IL-10 production, only SLIT-treated patients and healthy subjects experienced synthesis compared with untreated patients ($P < .001$ and $P = .02$, respectively). *D. farinae*-induced IL-10 production was detectable only in SLIT-treated patients with rhinitis and healthy donors compared with untreated allergic patients who did not produce IL-10 ($P = .06$).

DISCUSSION

Allergic rhinitis is characterized by a T_H2 -polarized inflammation. T_H2 -derived cytokines have been previously investigated in patients with allergic rhinitis, particularly concerning

the role of IL-4 and IL-13.²⁰ IL-4 is a pivotal cytokine that orchestrates allergic inflammation, because it is the most important signal to induce T_H2 polarization in allergic patients.²¹ IL-4 and IL-13 promote IgE synthesis, up-regulate adhesion molecules selective for eosinophil recruitment, and cause increased mucous production and airway hyperreactivity.²¹ Thus, there is agreement about the role exerted by both IL-4 and IL-13 as main pathogenic factors in inducing, maintaining, and amplifying inflammatory allergic inflammation.

Otherwise, accumulating evidence shows that T_H1-related cytokines, such as interferon- γ and IL-12, may suppress and counteract this T_H2 response and vice versa, because there is functional dichotomy between T_H1 and T_H2 cells.²² However, evidence exists that T_H1 cells may be themselves proinflammatory, because it has been demonstrated that the development of a T_H1-associated inflammatory response could exacerbate asthma.²³ Furthermore, the increase in prevalence of atopic diseases during the past 20 years has been accompanied by an increase in the prevalence of autoimmune, T_H1-driven diseases, also in the same patient.²⁴ Thus, forms of immunity other than pure T_H1 responses must be effective in regulating the allergic inflammation, such as regulatory T cells.³ As recently evidenced, allergic patients shows a defect of T-regulatory cells.¹² This defect may be replaced by subcutaneous immunotherapy as indicated by several studies.^{6–10} Particularly, successful SIT has been demonstrated to be capable of inducing IL-10 production by T cells.¹¹ IL-10 exerts a number of documented activities that are relevant for understanding the mechanisms of action of SIT. IL-10 properties include the modulation of allergen-specific IgE production in favor of IgG4,²⁵ the inhibition of allergen-dependent mast cell activation,²⁶ and the suppression of activation and survival of eosinophils.²⁷ Furthermore, IL-10 reduces the production of proallergic cytokines²⁸ and induces hyporeactivity to allergens.²⁹ Recently, it has been demonstrated that peripheral T cells of healthy subjects produce IL-10 when stimulated with recombinant allergen, explaining how natural allergen-specific tolerance may be achieved.³⁰

SLIT is a safe and effective route of administration of allergen extracts in allergic patients. However, its mechanism of action is still poorly understood, because there are few data concerning the immunologic changes induced by SLIT.^{13,16} Few studies have shown a relevant effect on the IgG4 response after SLIT; thus, it could seem that SLIT does not reduce constantly the IgE production and increase the IgG4 synthesis.¹³ Furthermore, Arikan et al³¹ showed that children treated with SLIT for asthma had elevated PHA-stimulated IL-12 levels and *D pteronyssinus*-stimulated interferon- γ was released by PBMCs compared with controls. On the contrary, a decrease of serum IL-13 was demonstrated after SLIT.³² Furthermore, it has been reported that the T-cell proliferative response was reduced by SLIT.³³ The same effect was demonstrated also for other local routes of SIT, such as nasal¹⁸ and oral.³³ These findings confirm numerous

experimental studies in animal models, showing that the oral route is tolerogenic and can reverse the T_H2 polarization.^{35–37}

The present study proves that allergic patients successfully treated with SLIT for HDMs showed reduced T-cell proliferation induced by *C albicans*, which is a ubiquitous recall antigen. Furthermore, *C albicans* may induce T_H1 and T_H2 response and T-regulatory cell immunity.³⁸ A previous study did not show reduction of tetanus toxoid-induced proliferation, probably because of low allergen dose schedule.³³

Thus, these data confirm that SLIT is capable of inducing immunologic hyporeactivity directed to environmental antigens. As recently evidenced by several studies,^{6–10} the decreased T-cell proliferation induced by SLIT may be a consequence of T-regulatory cell involvement. In this regard, this study provides evidence that allergic patients treated with SLIT significantly produce IL-10 compared with untreated allergic patients who do not produce IL-10. IL-10 exerts a crucial role in regulating tolerance and modulating IgE production. Therefore, IL-10 production and decreased T-cell proliferation may be attributed to the generation of regulatory cells capable of inducing tolerance to the causal allergen. This tolerance may be involved in the clinical improvement achieved by SLIT-treated patients.

In conclusion, we report that systemic immunologic changes are induced by the administration of allergens via the sublingual mucosa. It is well known that oral-associated lymphoid tissue shows a particular capacity for inducing immune tolerance.^{39,40} According to previous studies, by analyzing the events that occur during mucosal immunotherapy, such as via the nasal,¹⁸ oral,¹⁹ and sublingual routes,³³ we may conclude that HDM SLIT induces both a decrease in T-cell reactivity and an immunologic tolerance. In fact, this pilot study shows that successful SLIT induces production of IL-10, thus providing the first evidence for this kind of mucosal route and confirming findings obtained with the subcutaneous route.⁴¹

Furthermore, new experimental evidence has demonstrated that in mice oral administration of mite allergen expressed by zucchini yellow mosaic virus in cucurbit species down-regulated allergen-induced airway inflammation and IgE synthesis.⁴² Therefore, these results may be considered clinically relevant proof of inducing a regulatory response after SLIT treatment and add new data in the field of mechanisms of action of SIT as recently reviewed.^{43–45} Finally, the present preliminary findings have to be confirmed by further studies.

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