ORIGINAL ARTICLE

Immunotherapy Reduces CD40L Expression and Modifies Cytokine Production in the CD4 Cells of Pollen Allergy Patients

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Abstract

Background: Allergen-specific immunotherapy (SIT) is the only intervention for IgE-mediated respiratory disorders.
Objective: The aim of the study was to investigate the immunological modifications induced by SIT in patients allergic to olive and/or grass pollen by attempting to establish an association between these modifications and clinical improvements.
Methods: We studied 29 patients who were allergic to olive and/or grass pollen. Patients were randomized to 2 groups: an active treatment group, comprising 19 allergic patients who received SIT, and a control group, formed by 10 allergic patients who received pharmacological treatment for their allergic symptoms but not immunotherapy. We used flow cytometry to analyze intracellular expression of the cytokines IL-4, IFN-γ, IL-10, and TGF-β1 in $CD4^+$ T cells, as well as expression of Foxp3, the costimulatory CTLA-4 molecule, and the non–costimulatory CD40L molecule. To assess clinical changes, patients recorded their medication consumption, symptoms, and the limitation of daily activities using diary cards and quality of life questionnaires.
Results: Six months after initiation of SIT, we recorded a reduction in cell surface CD40L expression in the CD4+ T-cell population and a shift in the cytokine production profile (decrease in IL-4–producing CD4+ T cells and increase in IFN-γ, IL-10, and TGF-β1). These changes persisted after 12 months. Simultaneously, a clinical improvement was observed.
Conclusions: SIT-induced clinical improvement is the result of immunological modifications such as a reduction in CD40L expression on CD4 cells and alteration in the cytokine production profile.

Key words: Pollen allergy. Immunotherapy. Cytokines. CD40-L. Foxp3.

Resumen

Objetivo: El objetivo del presente estudio es investigar las modificaciones inmunológicas inducidas por la inmunoterapia en pacientes alérgicos al polen del olivo y/o gramíneas en relación con las mejoras clínicas inducidas por el tratamiento.
Métodos: Se estudiaron 29 pacientes alérgicos a polen del olivo y/o gramíneas. Los pacientes fueron divididos aleatoriamente en dos grupos: 19 pacientes que recibieron tratamiento con SIT subcutánea, y 10 pacientes control sin intervención con inmunoterapia. En ambos grupos se analizaron en las células T CD4 + la expresión intracelular de las citoquinas IL-4, IFN-γ, IL-10 y TGF-β1, la expresión del factor de transcripción Foxp3, y las moléculas co-estimuladoras CTLA-4 y CD40L por citometría de flujo. Para evaluar los cambios clínicos, los pacientes registraron su consumo de medicación, los síntomas y la limitación de las actividades cotidianas en tarjetas de registro diarias y en cuestionarios de calidad de vida.
Resultados: A los seis meses del establecimiento de SIT se observó en linfocitos T CD4+ una reducción en la expresión de CD40L, y un cambio en el perfil de producción de citoquinas: disminución de la producción de IL-4 y aumento para IFN-γ, IL-10, y TGF-β1. Todos estos cambios persistieron tras de 12 meses de tratamiento con inmunoterapia. Paralelamente a los cambios inmunológicos, se observó una mejoría clínica.
Conclusión: La mejoría clínica inducida por la SIT está relacionada con modificaciones inmunológicas inducidas sobre células CD4, reducción en la expresión de la molécula co-estimuladora CD40L y la modificación de su perfil de producción de citoquinas.

Palabras clave: Alergia al polen. Inmunoterapia. Citoquinas. CD40-L. Foxp3.

Introduction

Allergic diseases have a remarkable clinical impact. Their prevalence has increased dramatically during recent decades to the extent that they affect up to 30% of the population in industrialized countries [1]. Allergy is defined as an exaggerated response of the immune system to innocuous environmental substances known as allergens [1]. Although recent advances have raised the possibility that other mechanisms could be implicated [2], the prevailing consensus is that the immunological basis of allergic disease results from inappropriate type 2 helper T cell (T_H2) responses to allergens, since T_H2 cytokines contribute to eosinophilic inflammation (IL-5), play a key role in bronchial hyperresponsiveness (IL-9 and IL-13), and participate in the production of IgE (IL-4 and IL-13), which characterizes most allergic responses [1]. In addition to cytokines produced by T_H2 lymphocytes, the interaction between CD40 on B cells and CD40 ligand (CD40L) on the surface of $CD4^+$ T cells is essential for B-cell activation and generation of T-cell–dependent IgE antibodies [3,4].

Nevertheless, it remains unclear why some individuals have allergic disease and others with the same exposure to allergens do not. Studies suggest that the balance between T_H2, type 1 helper T cells (T_H1), and a third T-cell subtype with immunoregulatory properties known as T regulatory cells (Treg) is essential for determining the development of a healthy or allergic immune response [5]. Through a variety of suppressor factors, such as IL-10, TGF-β, and CTLA-4, Treg have the ability to directly inhibit activation of allergen-specific T_H2 cells, thus minimizing production of IL-4, IL-5, IL-13, and IL-9 and suppressing allergic inflammation through direct action on mast cells, basophils, and eosinophils [6,7]. Low Treg counts and high T_H2 counts are observed in allergic individuals, whereas in nonallergic individuals, a mixed T_H1/T_H2 response is associated with a high Treg count [8].

Today, allergen-specific immunotherapy (SIT), which involves administration of increasing doses of allergen extracts, remains the only cure for allergic diseases. SIT is the only clinically validated approach that induces antigen-specific Treg and peripheral tolerance with the capacity to restore homeostasis in humans [9-10].

Knowledge of the mechanisms underlying SIT is crucial if we are to understand the pathogenesis of the disease and select optimal therapy. No biomarkers enabling simple evaluation of the effect of immunotherapy on the immune system have been identified.

The aim of the present study was to investigate the immunological alterations induced by treatment with SIT in patients who are allergic to olive and/or grass pollen and to establish an association between these alterations and clinical manifestations of disease.

Material and Methods

Patients

The study population comprised patients screened at the Allergy Outpatient Clinic of Hospital General Universitario de Ciudad Real, Ciudad Real, Spain. The inclusion criteria were age 12-50 years, allergic rhinitis or asthma due to sensitization to olive and/or grass pollen, positive skin prick test (SPT) results to *Olea europaea* and/or *Lolium perenne*, and no positive prick test results with other aeroallergens. The exclusion criteria were rhinitis or chronic asthma, previous treatment with SIT, pregnancy, and autoimmune or severe organic diseases.

We recruited 29 allergic patients, who were randomized to 2 groups: an active treatment group, comprising 19 allergic patients who received SIT, and a control group, formed by 10 allergic patients who received pharmacological treatment for their allergic symptoms but not immunotherapy.

Treatment

All patients received medication with antihistamines, $ß_2$-adrenergic agonists, or corticosteroids during the pollen season in order to control their allergic symptoms. The SIT group received immunotherapy subcutaneously using an extract of *O europaea* and/or a mixture of 5 grasses (Pangramin Plus, ALK-Abelló, SA). Extracts were biologically standardized with the major allergens (Ole e 1 and Phl p 5, respectively) quantified in mass units. The SIT protocol comprised 8 increasing weekly doses followed by maintained monthly doses. The samples for the analysis of immunological parameters were taken just before the monthly dose. The criteria for the administration of grasses, olive, or both types of SIT were defined according to the result of the SPT and the season when symptoms were recorded.

Immunological Parameters

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood from patients who were allergic to olive pollen and/or grasses. The cells (5×10^6 cells/mL) were stimulated with pollen extracts containing 10 µg/mL of Phl p 5 and 10 µg/mL of Ole e 1 at 37ºC in 5% CO_2 for 72 hours. Our previous assays demonstrated that the maximum IL-4 production was obtained under these conditions at a range of 5-10 µg/mL of Phl p 5 (data not shown).

After in vitro stimulation with olive and grass pollen extracts, we applied flow cytometry to study $CD4^+$ T cells for intracellular expression of IL-4, IFN-γ, IL-10, and TGF-β1. We also used flow cytometry to investigate Foxp3 (a transcription factor that is essential for the regulatory function of Treg cells [11,12]), CTLA-4 (a costimulatory molecule that inhibits T-cell responses and regulates peripheral T-cell tolerance [13]), CD40L (a non–costimulatory molecule that contributes to isotype class switching towards IgE [4]), and CD63 expression on basophils (as an indicator of degranulation). Brefeldin A (50 ng/mL) and phorbol 12-myristate 13-acetate (50 ng/mL) plus ionomycin (500 ng/mL) were added during the last 6 hours of culture in order to accumulate synthesized proteins inside the endoplasmic reticulum and to express the imprinted cytokine pattern. $CD4^+$ T cells were gated in an SSC/FL1 dot plot and analyzed as the percentage of $CD4^+$ T cells for the expression of each molecule in a 2-color analysis (Figure 1). The cut off was defined in each assay by staining with only the FL1 anti-CD4 antibody. For the cytometry study, we used the following monoclonal antibodies: anti-CD4-FITC

(clone RPA-T4), anti-IL4-PE (clone 8D4-8), anti-IFNγ-PE (clone 4SB3), anti-IL10-PE (clone JESE-19F1), anti-Foxp3-PE (clone 259D/C7), anti-CTLA-4-PE (clone BNI3), and anti CD40L-PE (clone TRAPI), all of which were purchased from BD Biosciences; we also used anti-TGFβ1-PE (clone 9016) (R&D Systems). Foxp3 staining was performed using fixation and permeabilization buffers provided with the FOXP3 kit (BD Biosciences). In addition, CD63 expression was investigated using a pollen-specific basophil activation test (BASOTEST, Glycotope Biotechnology). Optimum results were obtained with a concentration of 1 µg/mL of allergen.

In the active group, samples were collected before initiating SIT (baseline) and at 6 months and 1 year after starting treatment. In the control group, samples were collected at baseline and after 6 months. All samples were studied outside the pollen season.

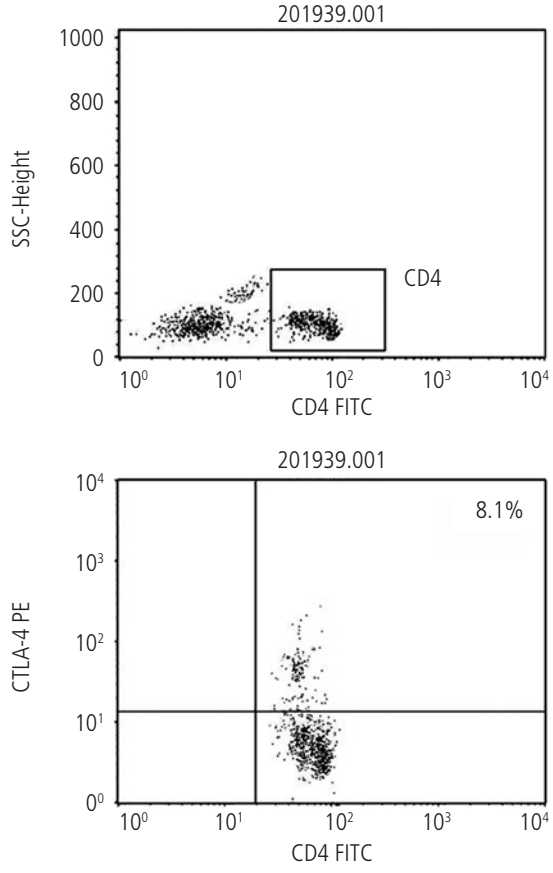

Figure 1. Dot plot used to select CD4+ T cells from peripheral blood mononuclear cells and dual fluorescence dot plot used for analysis of immunological parameters (CD4-FITC/CTLA-4-PE).

Clinical Parameters

All patients were given diary cards to record their daily consumption of antiallergic medication and scores for their conjunctival, nasal, and bronchial symptoms during the pollen season according to the following scale [14]: 0, no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms. Graded symptoms were summarized with a weighted score for the drugs used as follows: 0, no drugs; 1, oral antihistamines, ß$_2$-adrenergic agonists, or both; 2, nasal or bronchial corticosteroids; and 3, systemic corticosteroids.

SPT was performed to assess outcome after treatment with immunotherapy, since specific IgE levels require long periods of treatment to be significantly modified.

In order to determine the activities of daily life where patients were most limited, patients also completed 2 quality of life questionnaires (QLQ): the miniAQLQ (patients with allergic asthma) and the miniRQLQ (patients with allergic rhinitis). The questions posed in these instruments are divided into 4 domains (symptoms, activity limitation, emotional function, and environmental stimuli). Patients had to respond on a 7-point scale (7, no impairment; 1, severe impairment). The overall AQLQ/RQLQ score was the mean of all responses; the individual domain scores are the means of the items in those domains.

The present study was approved by the Ethics Committee of Hospital General Universitario de Ciudad Real. All patients gave their signed informed consent to participate.

Statistical Analysis

The statistical analysis was performed using SPSS, version 19.0. Given the small size of the samples, nonparametric tests were used to compare the difference in statistical significance between the groups (Mann-Whitney test) and differences within the groups (Wilcoxon test).

Results

Study Population

The characteristics of the study population are shown in Table 1. Patients of both groups were similar in age and gender. Most had rhinoconjunctivitis and asthma and were sensitized to both grass pollen and olive pollen. Only 6 patients were monosensitized, 4 to grass pollen and 2 to olive pollen.

Table 1. Clinical Characteristics of All Patients at Baseline[a]

		Control Group	Active Group
No.		10	19
Sex, No. (%)	Female	4 (40)	10 (52.6)
	Male	6 (60)	9 (47.4)
Age	Minimum-Maximum	13.5-47.1	12.6-45.6
	Mean (SD)	29.5 (11.1)	26.1 (10.0
Diagnosis, No. (%)	Rhinoconjunctivitis	1 (10)	5 (26.3)
	Rhinoconjunctivitis + asthma	9 (90)	14 (73.7)
SPT, mean (SD) diameter, mm	Grass	6.60 (2.5)	7.23 (3.0)
	Olive	5.88 (1.3)	6.20 (2.3)
SIT	Grass	0	12
	Olive	0	6
	Grass + olive	0	1

[a]There were no significant differences for any of the analyzed parameters. SPT indicates skin prick test; SIT, allergen-specific immunotherapy.

Immunological Parameters

At baseline, both groups showed similar values for all immunological parameters, with no significant differences (Table 2). Six months after implementation of SIT, differences

Table 2. Immunological Parameters Analyzed at Baseline[a]

	Control Group	Active Group	*P* Value
No.	10	19	
IL-4	22.7 (9.5)	27.0 (14.5)	.33
IFN-γ	9.7 (6.0)	6.7 (5.0)	.20
IL-10	3.8 (2.3)	3.0 (1.8)	.80
TGF-β1	10.6 (6.3)	8.0 (3.5)	.54
Foxp3	12.5 (6.1)	15.3 (5.1)	.19

[a]The results are shown as the mean (SD) of the percentage of cytokine-producing CD4+ T cells and for intracellular Foxp3 expression.

Table 3. Immunological Parameters Analyzed at 6 and 12 Months of Treatment in Patients Undergoing Allergen-Specific Immunotherapy (n=19)[a]

	6 Months	12 Months	*P*
IL-4	14.3 (10.3)	16.3 (6.4)	.83
IFN-γ	16.5 (6.9)	19.4 (12.6)	.86
IL-10	13.4 (6.3)	19.8 (12.2)	.20
TGF-β	29.8 (12.9)	31.5 (12.6)	.31
Foxp3	16.1 (6.4)	18.3 (9.9)	.24
CTLA-4	16.9 (13.1)	14.3 (11.2)	.38
CD40L	1.8 (0.9)	1.1 (0.7)	.32
CD63 (BAT)	29.4 (12.2)	36.7 (15.5)	.47

Abbreviation: BAT, basophil activation test.
[a]The results are shown as the mean (SD) of the percentage of cytokine-producing CD4+ T cells and of CD4+ T cells with expression of Foxp3, CTLA-4, or CD40L. The value for the BAT is shown as the mean (SD) percentage of CD63 expression on the basophil surface. *P* values are based on the Wilcoxon test.

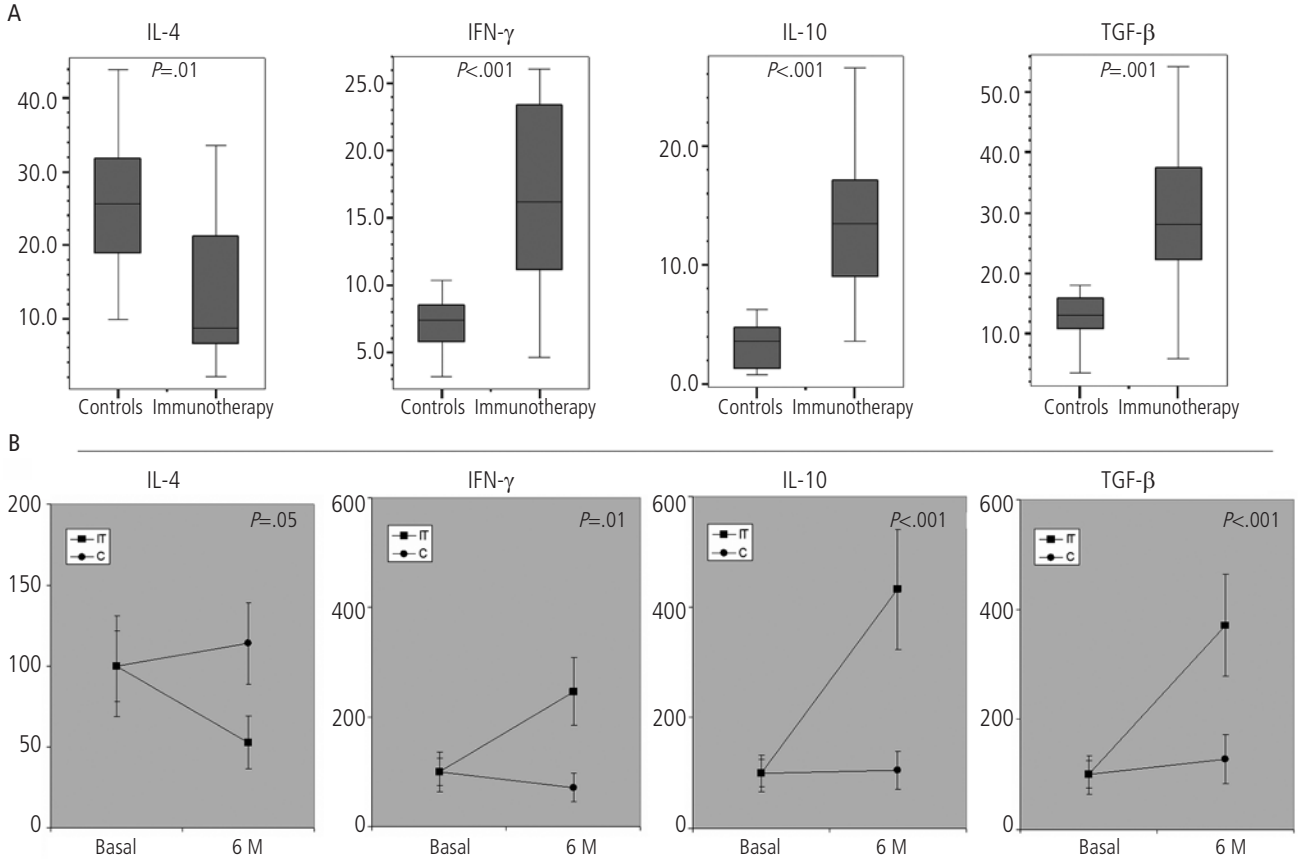

Figure 2. Changes in the cytokine production profile in CD4+ T cells induced by immunotherapy in pollen-allergic patients after 6 months of treatment. A, Differences between untreated patients (controls) and patients undergoing immunotherapy. The vertical bar represents the 95% confidence interval, the box is the interquartile range (50% of the outcomes), and the horizontal line is the median. The P values were calculated using the Mann-Whitney test. B, Relative change at 6 months in the percentage of cytokine-producing CD4+ T cells in controls and patients undergoing immunotherapy. The P values were calculated using the Wilcoxon test in absolute values from patients undergoing immunotherapy. No significant differences were detected in the controls for any of the parameters analyzed. IT indicates immunotherapy; C, controls.

were observed in the cytokine profile of $CD4^+$ T cells from patients undergoing treatment, in contrast to control patients (Figure 2). A significant decline in the percentage of IL-4–producing $CD4^+$ T cells (P=.01) and an increase in the IFN-γ–producing $CD4^+$ T cells (P<.001), IL-10 (P<.001), and TGF-β1 (P=.001) were observed in patients undergoing treatment compared with control patients (Figure 2A) and baseline (Figure 2B). These changes reached statistical significance in patients undergoing SIT but not in control patients. Similarly, SIT had an impact on surface expression of the non–costimulatory CD40L molecule in $CD4^+$ T cells. A significant difference was recorded in the number of $CD4^+$ T cells with CD40L surface expression (P<.001) between the active treatment group at 6 months after starting SIT and the controls (Figure 3A). In contrast, membrane expression of the negative costimulatory CTLA-4 molecule was not altered by SIT (Figure 3B). Similarly, after 6 months of treatment we found that SIT did not significantly alter expression of transcription factor Foxp3 in $CD4^+$ T cells (13.8% [6.3%] in controls vs 16.1% [6.4%] in active patients; P=.12).

Furthermore, patients undergoing SIT had a significant reduction in the basophil activation test results with pollen extracts (69.3% [16.2%] vs. 29.4% [12.2%]; P<.001).

These results persisted at 12 months of treatment. No significant differences were observed in any of the parameters studied after 6 months of SIT (Table 3).

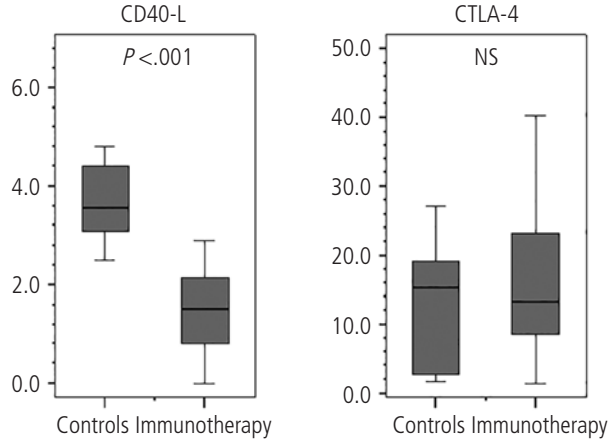

Figure 3. Expression of CD40L (A) and CTLA-4 (B) molecules on $CD4^+$ T cells from allergic patients at 6 months after starting allergen-specific immunotherapy. Results are expressed as the percentage of $CD4^+$ T cells from untreated patients (controls) and patients undergoing immunotherapy. The vertical bar represents the 95% confidence interval, the box the interquartile range (50% of the outcomes), and the horizontal line the median. The P value was calculated using the Mann-Whitney test. NS indicates nonsignificant.

Table 4. Daily Recording of Symptoms and Medication Required During the Pollen Season and the Diameter of the Wheal at 6 Months After Initiating Immunotherapy

	Control Group	Active Group	*P* Value
Score diary cards	183.50 (239.83)	173.44 (176.38)	0.823
Wheal diameter, mm	6.38 (2.07)	4.08 (1.80)	0.27

[a]Results are expressed as mean (SD).

Clinical Parameters

An association was established between immunological data and clinical improvement as assessed using quality of life questionnaires and diary cards during the pollen season. Clinical data are shown in Tables 4 and 5. Although the frequency of symptoms and the limitations in daily activities decreased in the active group compared to the control group, the differences were not statistically significant. Nevertheless, a considerable difference was observed for SPT reactivity between patients undergoing SIT and control patients 6 months after starting treatment (Table 4).

Discussion

In our study, we recorded a SIT-induced gain in the number of $CD4^+$ cells producing the regulatory cytokines IL-10 and TGF-β. This gain appeared simultaneously with a shift in helper T-cell activity from T_H2 to T_H1. It has been reported that SIT can induce activation of Treg cells secreting IL-10, which leads to long-term hyporesponsiveness of allergen-specific $CD4^+$ T cells, decreases IgE levels and the number of mast cells, and inhibits production of eosinophils [15]. Functional studies on antigen-specific Treg cells revealed that the modulation of TH1 and T_H2 responses depends mostly on secretion of the cytokine IL-10. In fact, IL-10 inhibits the proliferative response of peripheral cells against specific allergens [16]. In vitro, IL-10 modulates eosinophil function and reduces proinflammatory mediators released by mast cells [17]. TGF-β, which was also recently identified as a key cytokine in successful SIT, downregulates IgE synthesis [18], promotes the production of other antibody isotypes, and increases the expression of mucosal and circulating TGF-β1–producing T cells [19,20]. In our study, we tested enhanced production of IL-10 and TGF-β induced by SIT. Nevertheless, expression of Foxp3 in $CD4^+$ cells was not significantly

Table 5. Results of Quality of Life Questionnaires

	Overall	Symptoms	Emotion	Environment	Activities
Control group	22.3 (12.5)	12.7 (7.9)	13.2 (7.3)	21.3 (8.2)	69.5 (32.1)
Active group	28.8 (4.1)	19.9 (1.8)	16.8 (2.4)	25.1 (3.3)	90.6 (8.7)
P value	.586	.112	.271	.615	.254

[a]Results are expressed as mean (SD).

increased in patients undergoing SIT. These findings suggest that SIT leads mainly to immunoregulation through cytokine secretion. Groux et al [21] reported an inducible Treg cell population that did not constitutively express Foxp3, although it released the regulatory cytokines IL-10 and TGF-β. Naturally occurring $CD4^+CD25^+Foxp3^+$ Treg and inducible Treg cells differ in a number of important biological features, including the ability to differentiate to cells that show a specific response to antigens and the patterns by which they function (ie, through secretion of cytokines or cell-cell contact mechanisms) [22]. Other IL-10–secreting T cells may constitute an additional mechanism that is responsible for peripheral tolerance. Type 1 Treg cells, CD46-stimulated IL-10–secreting T cells, and IL-10–secreting T cells induced by vitamin D3 and dexamethasone are induced populations with significant regulatory activities [23].

The absence of the negative costimulatory molecule CTLA-4 in SIT also suggests that SIT promotes immune regulation through secretion of IL-10 and TGF-β rather than through cell-to-cell contact [24]. Induction of peripheral T-cell tolerance in SIT is initiated by the autocrine action of IL-10 and TGF-β, which are increasingly produced by antigen-specific induced Treg cells [25].

Results for the effect of SIT on the allergen-induced production of IFN-γ are conflicting. Although SIT inhibits both the T_H1 and the T_H2 response [26,27], we detected a decrease in IL-4 production and an increase in IFN-γ production in patients undergoing SIT. This same effect has been described elsewhere [28-29]. The effects of immunotherapy on T_H1 cells are not clearly established. Nevertheless, SIT clearly induces specific T-cell tolerance and diminishes IgE-mediated activation of mast cells and basophils and thus release of histamine and other mediators.

During allergen sensitization, cooperation between T and B cells through CD40–CD40L interaction is a basic signal for isotype class switching towards IgE [30]. In addition, crosstalk between T cells and bronchial fibroblasts in asthmatic patients involves an interaction between CD40L and VLA-5 and increases the production of profibrogenic cytokine IL-6 [31]. In the present study, we report that SIT reduces expression of CD40L on the CD4 cells of patients with pollen allergy treated with SIT compared to control patients not undergoing treatment. The reduction in CD40L expression may be an active mechanism through which treatment with SIT modulates the response to allergens. Suzuki et al [32] recently showed that inhibition of CD40 expression using small interfering RNA in ovalbumin-sensitized mice results in decreased production of T_H2 cytokines and increases the number of Treg cells. However, in a murine model of grass pollen allergy, blockade of costimulation inhibits allergic sensitization but does not affect established allergy [33]. We did not analyze the type of CD4 cell (T_H1, T_H2, or Treg) that reduces CD40L expression. Downregulation of IL-4–producing T_H2 cells might explain the decreased expression of CD40L. Treg cells do not require costimulation (CD40–CD40L) to trigger their activity [34]. Isotype switching is initiated by the CD40–CD40L interaction, although the specificity of the isotype is determined by cytokines [35]. SIT may work through both mechanisms, ie, decreasing expression of CD40L and changing the profile of cytokines produced by $CD4^+$ cells, to elicit reduced production of specific IgE and degranulation of effector cells. Moreover, the use of surface expression of CD40L to identify lymphocytes that specifically recognize allergens has been reported [36,37]. According to these data, CD40L is correlated with the specific immune response against allergens. The decrease in surface expression after treatment with SIT might indicate a lower immune response against the allergen that would anticipate the beneficial effect of immunotherapy. CD40L might behave as a simple surface biomarker that indicates the effectiveness of immunotherapy in treated patients.

All immune alterations induced by SIT can be observed 6 months after implementation of treatment and are maintained at 12 months. These data are consistent with those reported elsewhere [38], which show that the production of IL-10 starts quickly at the beginning of SIT, remains at 12 months, and is clearly linked to clinical improvement. We recently compared the levels of IL-4 in pollen-allergic patients and healthy controls under the same conditions as those of the present study [39]. Levels of IL-4–producing cells by patients undergoing immunotherapy for 6 months decreased to levels similar to those of healthy individuals.

Our study shows a clear trend towards clinical improvement, although our findings were not statistically significant. This inconsistency could be due to low grass and olive pollen counts compared with normal pollen seasons (2011) in central Spain [40]. In addition, both basophil degranulation and the SPT result were altered by SIT, suggesting that immunotherapy modifies the immune response. The absence of differences in symptoms and medication use did not compromise the results for T cells and basophils, because the medication required during the pollen season in both groups studied was equivalent (Table 4). Clinical differences between both groups should be more pronounced during a season with high pollen levels. Consequently, performing the studies during a season with high pollen levels could better clarify the relationship between immunological changes and clinical manifestations of allergic disease.

In conclusion, SIT considerably modifies immune activity towards allergens in patients who are allergic to grass and olive pollens. SIT diminishes the expression of CD40L on $CD4^+$ T cells by generating interactions between T cells and B cells, modifies the pattern of cytokine production from T_H2 to T_H1, and decreases production of the regulatory cytokines IL-10 and TGF-β.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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