Immunological Effects of Sublingual Immunotherapy: Clinical Efficacy Is Associated with Modulation of Programmed Cell Death Ligand 1, IL-10, and IgG4

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Immunologic Effects of Sublingual Immunotherapy: Clinical Efficacy Is Associated with Modulation of Programmed Cell Death Ligand 1, IL-10, and IgG4

Stefania Piconi,*1 Daria Trabattoni,†,1 Veronica Rainone,† Linda Borgonovo,* Simone Passerini,* Giuliano Rizzardini,* Franco Frati,‡ Enrico Iemoli,* and Mario Clerici§,*

Sublingual immunotherapy (SLIT) is an alternate route of administration of allergen-specific immunotherapy with an improved safety profile; to clarify the immune mechanisms elicited by this therapy, we analyzed the clinical and immunologic effects of SLIT in patients with a clinical history of ragweed sensitization. To analyze possible difference among immunotherapeutic protocols, we also compared patients receiving preseasonal, seasonal, or prolonged sublingual therapy (≥3 y); patients receiving symptomatic therapy alone were enrolled as well in the study. Clinical and immunological parameters were measured twice in and out of the pollination period. Clinical benefits, as measured by the visual analog scale for symptoms and for use of drugs, were evident in all three groups of individuals receiving immunotherapy, but were significantly better in patients undergoing prolonged SLIT. Immunologically, SLIT resulted in increased IL-10 production, programmed cell death ligand 1 expression, and concentration of allergen-specific IgG4, as well as in the reduction of CD80 and CD86 expression and IL-4 production. SLIT, thus, is associated with modulation of programmed cell death ligand 1 expression and IL-10 synthesis and favors the production of allergen-specific IgG4. These effects are evident from the first pollen season, independently from therapeutic regimen (preseasonal or seasonal) even if a prolonged treatment is necessary to obtain full clinical efficacy. A more detailed understanding of the interaction of allergen and APCs within the oral mucosa will allow improved targeting of allergy vaccine. The Journal of Immunology, 2010, 185: 000–000.

Allergen-specific immunotherapy (IT) modifies the natural history of disease in atopic individuals. Such immunotherapy induces an allergen-specific immune tolerance that results in the control of clinical symptom onset upon natural exposure to the allergen (1, 2). The clinical efficacy of standard s.c. immunotherapy (SCIT) is clearly demonstrated (3–10). The effects of SCIT on immune responses have also been described in depth and include the reduction of IgE/IgG4 ratio (11–14), the augmentation in allergen-specific IgA (15), an increased IL-10 production by allergen-specific T cells (16), and possibly an effect on regulatory T cells (Tregs) (14, 17) and on nasal and bronchial mucosal effector cells (mast cells, basophils, and eosinophils) (1, 2, 18, 19).

An alternative route to perform Ag-specific immunotherapy is sublingual. Sublingual immunotherapy (SLIT) is characterized by an improved safety profile compared with SCIT (20); the mechanisms of action of this novel mode of immunization are actually under investigation. Analogously to what was observed for SCIT, a Cochrane review of 22 studies of 979 subjects confirmed the efficacy and safety of SLIT for patients with allergic rhinitis, demonstrating a mean reduction in symptoms of 42% and a mean reduction in medication use of 43% (21). SLIT was also shown to have long-lasting effects (22, 23), to prevent sensitization toward new allergens, and to reduce the risk of asthma in children affected by rhinitis (24, 25). The dose-dependent effects of SLIT were clearly reported by several studies, confirming the need of a high-dose therapy to optimize the response (26–28).

Similarly to what is observed in SCIT, SLIT affects both humoral and cell-mediated immune responses. Thus, SLIT results in a dose-dependent increase of allergen-specific IgG4 (24, 29), even if this effect is less intensive than that induced by SCIT (26, 27, 30–32) and mostly evident for seasonal aeroallergen. SLIT also reduces mucosa infiltration by effector cells (neutrophils and eosinophils) (33), increases allergen-specific IL-10 production (34–36), and modulates Th1 and Treg activity in the oral mucosa (37–43). The peculiar characteristics of APCs in the oral mucosa were suggested to be responsible for the modulation of IL-10– and TGF–β–secreting Tregs observed following sublingual immunotherapy (11, 44).

The immunologic effects of SLIT are nevertheless still partially unclear, and the question of which therapeutic scheme should be used in this type of immunotherapy is hotly debated (45). We previously showed that the beneficial effects of IT in patients allergic to
Betulaceae are associated with an increase of both programmed cell death ligand 1 (PD-L1)–expressing APCs and IL-10 production (46). In this study, we confirm those results and we extend them by analyzing and comparing the clinical and immunological effects of different SLIT immunization procedures.

Materials and Methods

Study population

Sixty-two Italian patients were enrolled between February 2009 and September 2009 by the Allergy and Clinical Immunology Clinic of the Luig Sacco Hospital in Milano. Inclusion criteria were a clinical history of ragweed sensitization and skin prick test positive to ragweed pollen, clinical report of asthma and/or rhinoconjunctivitis, and age between 18 and 60 y. Exclusion criteria were as follows: perennial allergic rhinitis and/or perennial allergic asthma, a total serum IgE level >2000 kU/l, chronic nonallergic rhinitis or sinusitis, atopic dermatitis, severe asthma (mild asthma grade 1–2 was accepted), immunotherapy in the previous 5 y, and the usual contraindications to immunotherapy. House dust mite sensitivity was not an exclusion criterion. In vivo diagnosis was done by prick test using ragweed extract (Stallergen SA, Milano, Italy). The prick tests scored positive if the wheal obtained was similar or larger than the wheal elicited by the positive control (histamine hydrochloride, 10 mg/ml). Stauroral (Stallergen SA) was used at a concentration of 300 index of biological reactivity/ml (equivalent to 30 μg/ml allergen A1 variant from western ragweed pollen, Ambrosia psilostachya) (Amb a1). Patients were divided into four groups, as follows: group A = patients receiving preseasonal SLIT (May 2009–end September 2009) (n = 18); group B = patients receiving seasonal SLIT (Mid July 2009–end September 2009) (n = 17); group C = patients who received SLIT with preseasonal regimen for at least 3 y (May–end September) (n = 15); and control patients = patients who never underwent immunotherapy and received symptomatic therapy alone (antihistaminic, nasal corticosteroid, β-agonist short acting) (n = 12) (Fig. 1). Patients kept a diary during the pollen season for the daily evaluation of symptoms according to a 0–10 grading system (0 = no symptoms; 10 = very severe symptoms) (visual analog scale [VAS] symptoms) (47) and for the daily evaluation of symptomatic medication use on the base of a subjective judgment of the use of drugs by a 0–10 VAS according to validated criteria (0 = no treatment; 10 = use of all disposable symptomatic drugs) (VAS drugs) (47). Immunological evaluations were performed out of pollen season, before starting immunotherapy (April 2009) and at the peak of pollination (August/September 2009) (Fig. 1). The Institutional Review Board approved the study protocol; all patients were informed about the aim of the trial and gave their consent.

Pollens counts

Ragweed pollen count for the 2009 season was obtained from Azienda Sanitaria Locale Milano 1, Dipartimento di Prevenzione Medica, Unità Operativa Complessa Igiene e Sanità Pubblica Monitoraggio Aerobiologico, Rho Station (Fig. 2).

Immunophenotypic analyses

Lymphocyte subsets were evaluated by flow cytometric analysis, using 50 μl EDTA peripheral blood incubated for 30 min at 4˚C with fluorochrome-labeled mAbs (CD4, CD19, CD14, CD86, CD80). After incubation, erythrocyte lysis and fixation of marked cells were performed using the Immuno-Prep EPICS kit and Q-prep Work Station (Coulter Electronics, Hialeah, FL).

PBMC separation and stimulation

PBMCs were separated on lymphocyte separation medium (Organon Teknika, Durham, NC) from whole blood samples collected by venipuncture in Vacutainer tubes containing EDTA (BD Biosciences, Rutherford, NJ). PBMCs were then incubated for 18 h without any Ag or in the presence of Amb or CMV grade II Ag (10 mg/ml; Microbix Biosystem, Mississauga, Ontario, Canada) (control Ag). Anti-CD28 Ab (R&D Systems, Minneapolis, MN) was added during incubation (1 μg/well) to facilitate costimulation. For cytokine analyses, 10 μg/ml brefeldin A (Sigma-Aldrich, St. Louis, MO) was added to the cultures during the last 6 h of stimulation to block protein secretion.

Identification of Treg lymphocytes

Unstimulated PBMCs were incubated with anti-CD4 and anti-CD25 for 15 min at room temperature. The intracellular detection of Foxp3 was performed following the manufacturer’s staining protocol (eBioscience, San Diego, CA). Intracellular Foxp3 was performed by flow cytometry on CD4+CD25bright gated T CD4+ cells.

Immunofluorescent staining

The following mAbs were used: anti-human CD4 (mouse IgG1 isotype) PE-Cy7, anti-human CD4 (mouse IgG1 isotype), anti-human CD14 (mouse IgG2a isotype), anti-human CD19 (mouse IgG1 isotype) R-PE–cytotoxicine 5 tandem (PE-Cy5), and anti-CD25 (mouse IgG2a PE-Texas red (energy-coupled dye) (Beckman Coulter, Fullerton, CA); anti-human CD66 (mouse IgG1 isotype) FITC (Serotec, Oxford, U.K.); anti-human PD-L1 (mouse IgG1 isotype) PE-Cy7 (R&D Systems); anti-Foxp3 (rat IgG2a isotype) PE-Cy5, anti-human IL-4 (mouse IgG1 isotype) R-PE, and anti-human IL-10 (mouse IgG2b isotype) FITC (R&D Systems).

The cytometric analyses of phenotype and cytokine-secreting lymphocytes were performed using a FACsFlow flow cytometer (Beckman Coulter) equipped with a double 15–mW argon ion laser operating at 456 and 488 interfaced with Intercorp computer. For each analysis, 200,000 events were acquired and gated on CD4, CD14, and CD19 expression and side scatter properties. Samples were first run using isotype control or single fluorochrome-stained preparations for color compensation.

Allergen-specific IgG4

Amb a1 Ag was used to coat ELISA microplates overnight at 37˚C, 7% CO2. After washing and blocking procedures, plasma samples were added and incubated for 1 h at 37˚C, 7% CO2. A HRP-conjugated mouse anti-human IgG4 mAb (isotype IgG1; Lifespan Biosciences, Seattle, WA) (1:25,600) was dispensed in the plates and incubated for 1 h at 37˚C, 7% CO2. Finally, 100 μl/well o-phenylenediamine (0.4 mg/ml; Sigma-

Table I. Epidemiologic, clinical, and immunotherapeutic characterization of the patients enrolled in the study

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (range)</td>
<td>40.5 (19–58)</td>
<td>42 (26–51)</td>
<td>44.5 (21–57)</td>
<td>43.5 (31–60)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Gender (M/F)</th>
<th>8/18</th>
<th>7/10</th>
<th>7/8</th>
<th>5/7</th>
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<tbody>
<tr>
<td>RC + A</td>
<td>5/18</td>
<td>5/17</td>
<td>7/15</td>
<td>6/12</td>
</tr>
<tr>
<td>RC</td>
<td>13/18</td>
<td>12/17</td>
<td>8/15</td>
<td>4/12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amb a1 (ng/dl)</th>
<th>120 (60–120)</th>
<th>120 (60–120)</th>
<th>120 (120)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Months of SLIT</td>
<td>5</td>
<td>3</td>
<td>5 for 3 y</td>
</tr>
</tbody>
</table>

*Mean values and range.

A, asthma; F, female; M, male; R, rhinitis; RC, rhinoconjunctivitis.
Aldrich) (0.05 M) was added and the color reaction was stopped by adding 1 N H₂SO₄. The absorbance was measured at 450 nm, and the concentrations of allergen-specific IgG₄ were calculated from the standard curve.

**Statistical analysis**

As data were normally distributed, procedures were based on parametric analyses. Comparisons between the different groups were made using a two-tailed t test. Possible relationships were evaluated using Pearson’s or Spearman’s correlation tests. Statistical analysis was performed using the SPSS statistical package (SPSS, Chicago, IL).

**Results**

**Study population and clinical efficacy**

Epidemiological, clinical, and therapeutic features of all the patients enrolled in the study are presented in Table I. Four group B patients reported adverse events (two sublingual edema and asthma; two sublingual edema and abdominal and gastric pain), interrupted SLIT treatment after 3 wk, and were excluded from final immunological analyses (Figs. 1, 2).

The efficacy of SLIT was evaluated by a significant improvement of a 10-point VAS applied to rhinitis, asthma, and conjunctivitis, and need for therapy, according to the guidelines of symptom severity assessment of allergic rhinitis (48). Clinical efficacy data showed a significant improvement in overall SLIT compared with control patients. This improvement was significantly better when prolonged SLIT patients (group C) were compared with control patients. The differences were significant also when prolonged SLIT were compared with either preseasonal or seasonal patients (Fig. 3).

**Phenotypic analyses: CD80 and CD86 expression on APCs**

CD80⁺/CD14⁺ cells were significantly reduced during pollination in all SLIT-treated compared with control patients (A, \( p = 0.015 \); B, \( p = 0.009 \); C, \( p = 0.014 \)); a similar trend that, nevertheless, did not reach statistical significance was also seen for CD86⁺/CD14⁺ cells (data not shown). Both CD80⁺/CD14⁺ and CD86⁺/CD14⁺ were significantly lower during pollination compared with out-season data in patients receiving either preseasonal (\( p = 0.001 \)) or prolonged SLIT (\( p = 0.05 \)). Finally, CD80⁺/CD19⁺ and CD86⁺/CD19⁺...
CD19⁺ B cell lymphocytes were also significantly reduced during pollination in individuals undergoing seasonal SLIT compared with controls (p = 0.009 in both cases) (data not shown) and compared with patients receiving seasonal SLIT (p = 0.043).

**Allergen-induced modulation of PD-L1 expression on APCs**

During pollination, Amb a1-stimulated PD-L1⁺ expressing CD14⁺ and CD19⁺ cells were significantly increased in all SLIT-treated compared with control patients (Fig. 4). Comparison of in- and out-season data showed that Amb a1-stimulated PD-L1⁺/CD14⁺ cells were reduced in control patients during pollination (p < 0.001).

**Allergen-induced cytokine production by T cells and APCs**

Amb a1-specific IL-4- and IL-10-producing cells during pollination behaved in a diverging way. Thus, whereas IL-4-producing cells were reduced in all SLIT-receiving patients compared with controls, IL-10-producing cells were significantly increased by SLIT during pollination. This behavior was confirmed in all three populations of Amb a1-stimulated and cytokine-producing cells (CD4⁺ T lymphocytes, CD19⁺ B lymphocytes, and CD14⁺ monocytes) (Fig. 5).

Comparison of Amb a1-stimulated IL-4 and IL-10 production in or out of the pollination season showed that IL-4-secreting cells were reduced and IL-10-producing cells were increased during pollination in SLIT-treated patients. Control patients behaved in an opposite way, as follows: IL-4 production augmented and IL-10 generation diminished during pollination (Fig. 5).

**IL-10-producing and PD-L1-expressing immune cells**

During pollination, IL-10-secreting and PD-L1-expressing CD14⁺ and CD19⁺ cells were significantly increased in SLIT patients. Comparison of data obtained out and in season showed that both IL-10-secreting and PD-L1-expressing CD14⁺ and CD19⁺ cells were increased in SLIT patients, but decreased in control individuals (Fig. 6).

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**FIGURE 3.** VAS symptom and VAS drug score. VAS symptom score (A) and VAS drug score (B) in preseasonal, seasonal, prolonged SLIT individuals, and in controls. Mean values, SE, and p values are shown.

**FIGURE 4.** PD-L1 expression. A, Percentage of PD-L1-expressing CD14⁺ cells in preseasonal, seasonal, prolonged SLIT individuals, and in controls upon Amb a1 stimulation. B, Percentage of PD-L1-expressing CD19⁺ cells in preseasonal, seasonal, prolonged SLIT individuals, and in controls upon Amb a1 stimulation. Data obtained out (shaded area) and in (white area) season, mean values, SE, and p values are shown.
Tregs

Tregs (CD4+/CD25bright/Foxp3+ cells) were increased in SLIT compared with control patients both during pollination and out season; nevertheless, these differences did not reach statistical significance (data not shown).

Allergen-specific IgG4

Amb a1-specific IgG4 were significantly increased during pollination in all SLIT-treated compared with control patients. When in and out of season data were compared, significant differences were seen in patients undergoing prolonged SLIT alone (Fig. 7).

Correlations

Significant positive correlations were observed during pollination between Amb a1-specific IgG4 concentration and Amb a1-stimulated, IL-10–secreting, PD-L1–expressing CD14+ (Spearman correlation: \( p = 0.01 \)) and CD19+ cells (Spearman correlation: \( p = 0.01 \)), as well as between Amb a1-specific IgG4- and IL-10–secreting CD4+ T cells (Pearson correlation: \( p = 0.01 \)). Moreover, in-season data showed a negative correlation between allergen-specific IgG4 and V AS symptom score (Spearman correlation: \( p = 0.01 \)) and V AS drug scores (Spearman correlation: \( p = 0.01 \)) (Fig. 8).

Discussion

We evaluated the clinical and immunological effects of sublingual immunotherapy by analyzing V AS scores and allergen-specific humoral and cellular immune parameters and compared the results in patients undergoing different SLIT regimes. Thus, we analyzed, in and out of the pollination period, the expression of costimulatory molecules on APCs, cytokine production, Treg function, and IgG4 synthesis in patients with allergy to Ragweed that had undergone specific prolonged SLIT or that received SLIT for the first time during the observation period. The aim of our study was to compare preseasonal and seasonal SLIT with prolonged SLIT to identify the clinical and immunological effects of these different regimens.

Results in this study demonstrate that, whereas all three protocols were associated with clinical improvement, prolonged SLIT is needed to optimize such improvements. Thus, even if VAS symptom data showed that SLIT was able to improve clinical rhinoconjunctivitis and asthma also after the first treatment cycle, VAS symptoms and VAS drugs were significantly different in prolonged compared with seasonal SLIT. Notably, the effects of SLIT on immune parameters were similar in the three treated groups. In particular, in SLIT-treated patients compared with patients who received symptomatic therapy alone: 1) CD80 and

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**FIGURE 5.** Cytokine synthesis. Percentage of IL-4- and IL-10–producing CD4+ T cells (A, B), CD14+ cells (C, D), and CD19+ cells (E, F) in preseasonal, seasonal, prolonged SLIT individuals, and in controls upon Amb a1 stimulation. Data obtained out (shaded area) and in (white area) season, mean values, SE, and \( p \) values are shown.
CD86 expression on APCs was reduced; 2) IL-10 production was increased and IL-4 synthesis was reduced; 3) PD-L1 expression and PD-L1–associated IL-10 synthesis were augmented; and 4) allergen-specific IgG4 concentration was increased. Interestingly, no significant effects of SLIT were noticed on systemic Tregs. Finally, allergen-specific IgG4 showed a positive correlation with IL-10–secreting APCs and a negative one with VAS symptom and VAS drug scores.

Results obtained upon analyzing CD80 and CD86 expression on monocytes (CD14) and B lymphocytes (CD19) showed that cells expressing these costimulatory molecules were reduced in season in vaccinated individuals compared with unvaccinated patients. Such reduction was also observed, during pollination, in SLIT-treated groups compared with out-season data. SLIT, thus, down-modulates APC functions by reducing the expression of costimulatory molecules. PD-L1 is an additional costimulatory molecule that has recently been identified (49); ligation of PD-L1–expressing APCs results in the preferential production of IL-10 (49–52). Results showed that both CD14- and CD19-expressing cells were increased in vaccinated individuals compared with controls irrespective of pollination. Interestingly, these cells were further increased during pollination in individuals undergoing SLIT. The specificity of SLIT procedures was confirmed by the observation that Amb a 1-specific, but not CMV-specific cells were modulated by immunotherapy. Thus, allergen-specific and PD-L1–expressing APCs increased only in vaccinated patients, whereas these cell types decreased in unvaccinated individuals. These results support the suggestion that PD-L1 could be a major target of allergen-specific immunotherapy, and that the modulation of its expression could be used as a clinical efficacy marker.

Recent data in mice on PD-L1 showed an important role of this inhibitory costimulator in developing and maintaining Tregs. These observations confirm the crucial role of PD-L1 in conditioning the clinical response to immunotherapy in allergy. We did not observe a significant modification in Treg induction and no correlation of this parameter with PD-L1 expression. This could be due to methodological problems or to the prevalent effect of PD-L1 in tissue tolerance. Additional studies are needed to better understand the relationship between PD-L1 overexpression and tolerance induction (53, 54).

Ligation of PD-L1 results in IL-10 production (50). Results showed that Amb a 1-stimulated, IL-10–producing, PD-L1–expressing CD14+ and CD19+ cells were increased during pollination in vaccinated compared with control patients. Additionally, these cells were significantly augmented in SLIT patients, but reduced in control individuals, when in- and out-season data were compared. The effect of SLIT on IL-10 synthesis by CD4+ T cells was similar to that observed on APCs, suggesting that one of the main biological effects of specific SLIT is the up-regulation of an IL-10–mediated immunosuppressive loop. This justifies the clinical benefits of SLIT, as increased IL-10 production has repeatedly been observed in patients undergoing allergen-specific IT and has been shown to be a major player in the beneficial effects of therapy (33, 34).

Amb a 1-specific IgG4 were significantly increased as well in SLIT-treated patients. These Abs are suggested to play a protective role in SLIT-induced down-modulation of APC functions by reducing the expression of costimulatory molecules.
role (12, 55–58) and to mediate the beneficial effects of immunotherapy in allergy as they compete with IgE for the binding of allergens, even if some disagreements still surround this interpretation of the biological role of IgG4 in allergy (59). Interestingly, the isotype switch toward the γ4 chain is induced by IL-10, the production of which is up-regulated by SLIT. The observed significant correlations between Amb a 1-specific IgG4 concentrations and IL-10–secreting immune cells confirm the key role played by costimulatory molecules expressed by APCs in inducing immune tolerance during allergen-specific immunotherapy and reinforce the suggestion that the clinical efficacy of SLIT is associated with the modulation of the APC/PD-L1/IL-10/IgG4 immunoregulatory loop.

One of the most important and debated aspects of SLIT is which is the optimal regimen to administer such sublingual immunotherapy. Currently used protocols use either seasonal (start and end with the pollen season) or preseasonal (start before the pollen season and continue until the end of pollination) procedures. The overall evaluation of the available literature on SLIT suggests that preseasonal regimen would be the best choice for pollen SLIT. Our data indicate that both seasonal and preseasonal regimens result in comparable favorable clinical and immunologic effects; optimal benefits are nevertheless obtained only after repeated cycles of SLIT.

In conclusion, our data show that the major systemic effect of SLIT is the modulation of the APC/PD-L1/IL-10/IgG4 immunoregulatory loop. This results in a significant amelioration of symptoms and a reduced need for drugs. These benefits are evident from the first pollen season independently of the administration regimen. However, repeated preseasonal treatments are necessary to reach optimal clinical efficacy.

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Disclosures
The authors have no financial conflicts of interest.

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