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Epigenetic modifications and improved regulatory T-cell function in subjects undergoing dual sublingual immunotherapy

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Abstract

Background—Allergen-specific immunotherapy is the only mode of therapy that has been demonstrated to offer a cure in patients with IgE-mediated respiratory allergies.

Objective—We sought to demonstrate the safety and efficacy of timothy grass (TG) and dust mite (DM) dual sublingual immunotherapy (SLIT) and to begin to investigate the immune mechanisms involved in successful immunotherapy with multiple allergens.

Methods—The safety and efficacy of dual SLIT with TG and DM in children and adults with demonstrated allergies to TG and DM were investigated in a single-center, randomized, double-blind, controlled phase I study. Thirty subjects received either TG and DM dual SLIT (n = 20) or placebo (n = 10). Immune parameters were evaluated for differentiation of desensitized subjects from control subjects.

Results—Subjects treated with dual SLIT had decreased rhinoconjunctivitis scores ($P < .001$) and medication use scores ($P < .001$) and reduced responses to TG and DM allergen based on results of skin prick tests or nasal disk challenges ($P < .01$ and $P < .001$, respectively) compared with placebo-treated control subjects. An increase in TG- and DM-specific IgG₄ levels, reduced allergen-specific IgE levels, and subsequent basophil activation were observed in the active treatment group. Dual SLIT promoted allergen-specific suppressive CD4⁺CD25^{high}CD127^{low}CD45RO⁺ forkhead box protein 3 (Foxp3)⁺ memory regulatory T cells with reduced DNA methylation of CpG sites within the Foxp3 locus.

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Clinical implications: Dual SLIT with TG and DM allergen is a safe, efficacious, and promising treatment for IgE-mediated respiratory allergies. Explorative studies suggest that successful immunotherapy involves modification of Treg cells at the DNA level.

Conclusion—The results of this pilot study suggest that dual SLIT could be an effective means to treat subjects with sensitivities to a variety of allergens and that long-term tolerance might be induced by epigenetic modifications of Foxp3 in memory regulatory T cells.

Keywords

Respiratory allergies; sublingual immunotherapy; regulatory T cell; epigenetics

The only mode of therapy that has been demonstrated to offer a cure in subjects with IgE-mediated respiratory allergies by modulating immune responses and desensitizing a patient is allergen-specific immunotherapy.^{1–6} However, the underlying immune mechanism of successful immunotherapy is not completely understood. Immunotherapy consists of gradual administration of increasing quantities of an allergen extract to an allergic subject. The technique offers long-term remission by desensitizing the patient to subsequent exposure to the causative allergen. Sublingual immunotherapy (SLIT) can be self-administered regularly in a home setting, eliminating the need for burdensome and expensive office visits. Because most subjects with allergy are sensitive to many allergens, the effectiveness of simultaneous treatment to multiple allergens and the immune mechanism of tolerance are of interest.^{7–10}

During the last few decades, a basic framework in our understanding of the immunomodulatory effects of immunotherapy has emerged. There is growing evidence that regulatory T (Treg) cells play a pivotal role in immune tolerance and have been reported to mediate other effects, such as skewing T_H2/T_H1 profiles to favor noninflammatory responses and induction of CD4⁺ effector T (Teff) cell anergy or inactivation.^{11–13} Teff and Treg cells are now believed to play central roles in maintaining immune homeostasis along with other key cellular components (T_H1, T_H2, eosinophils, basophils, mast cells, and antigen-presenting cells), cytokines (IL-4, IL-13, TGF- β , and IL-10), and antibodies (IgE, IgG, IgG₄, and IgA). The sequence of events and pathways that connect these and other mediators of immune response are still incompletely understood and under investigation.

Treg cells are not homogenous, and numerous subtypes varying in gene expression and suppressive function exist. The majority of Treg cells with suppressive activity are CD4⁺, CD25⁺, transcription factor forkhead box protein 3 (Foxp3)⁺ cells. However, these markers are not unique to Treg cells. CD4 is common to effector, regulatory, and natural killer cells; CD25 is induced at low levels in nonregulatory naive CD4⁺ T cells on activation; and Foxp3 is expressed transiently in naive CD4⁺ cells on activation by TGF- β and other factors. In addition to CD4, CD25, and Foxp3, several other markers, such as CD45R0⁺, cytotoxic T-lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR), and the lack of measurable CD127 have been described to further identify Treg cells, although the functional significance of these markers is not well understood. Until a unique molecular marker is identified, identification of Treg cells necessitates the need to measure several biomarkers to positively identify them. Although a single protein biomarker has not been identified, DNA analysis of Treg cells demonstrates a unique, highly unmethylated CpG-rich Foxp3. This is in contrast to heavily methylated CpG islands of the Foxp3 locus from other T cells, such as naive CD4⁺ T cells.^{14–17}

The aim of the current pilot study was to assess both the clinical efficacy and safety of dual SLIT, investigate the immune modulations required for successful immunotherapy, and provide support and direction for larger clinical studies. We have conducted a single-center, randomized, double-blind, controlled phase I study of dual SLIT with timothy grass (TG) and dust mite (DM; *Dermatophagoides farinae*) in children and adults with demonstrated allergies to TG, DM, and oak (to assess allergen-specific responses). Subjects were examined at baseline and at 3-month intervals for 3 years (12 months of treatment followed by up to 2 years for a follow-up period). The efficacy and duration of desensitization of dual SLIT to DM and TG was evaluated along with desensitization to the additional allergen oak. Immune parameters, including the characterization of Treg cell function, basophil activation, and allergen-specific IgE and IgG₄ levels were evaluated for differentiation of desensitized subjects from control subjects.

METHODS

Participants and study design

A randomized, double-blind, placebo-controlled, single-center phase I study was performed to test the safety of dual SLIT with DM and TG to desensitize subjects with DM- and TG-induced allergic rhinitis. The study assessed 20 allergic subjects receiving dual SLIT versus 10 allergic subjects receiving placebo. All 30 subjects had documented clinical reactions, positive skin test results, and allergen-specific IgE levels to DM and TG. Sensitivity to oak was included to allow assessment of TG and DM dual SLIT on allergic immune responses to an allergen not included in SLIT. The 2 treatment groups did not differ significantly in terms of age, sex, race, or baseline clinical characteristics (see Table E1 in this article's Online Repository at www.jacionline.org). The main inclusion criteria were as follows: male or female subjects aged 5 years or greater; positive skin prick test responses to *Phleum pratense* (for TG), and *D farinae* (for DM), defined as a wheal diameter of 5 mm or larger than that elicited by the saline control. Subjects with chronic urticaria, significant organ system disease or chronic immune disease, moderate persistent asthma (FEV₁ < 80%), unexplained anaphylaxis within the last 2 years, use of an experimental drug in the last 30 days, or immunotherapy in the last 3 years were excluded. Healthy control subjects used for comparison in immunoassays were age matched and could not have allergies, asthma, autoimmune disease, cancer, or any chronic or acute disease; no healthy control subject took medications, and no healthy control subject had a history of smoking or currently smoked.

The subjects were randomized in a 2:1 method, dosing began 4 months before the grass pollen season (GPS) in 2009, and 20 subjects were actively treated with dual SLIT for TG and DM for 12 months. GPS was confirmed based on grass pollen counts performed by Dr Theodore Chu (San Jose, Calif). The preliminary dosing visit consisted of incremental escalating doses administered 15 minutes apart, starting with placebo (Greer Laboratories, Lenoir, NC) and ending with each Glycerinated Allergenic Extract (GAE; Greer Laboratories) of TG (Phl p 1) and *D farinae* (Der f 1 and Der f 2), as detailed in Table E2 in this article's Online Repository at www.jacionline.org. The maintenance treatment course consisted of daily self-administered doses of GAEs (15 µg of TG daily and 20 µg of DM daily) by using separate actuator bottles for TG and DM extracts that administered a metered

dose of GAE. Placebo was identical in composition and physical properties to active treatment but with no allergen extract included. The cumulative dose of allergen after the 12 months was 5475 and 7300 μ g for TG and DM, respectively. After 12 months of maintenance treatment, subjects withdrew from therapy for 6 months, and at 18 months, 16 subjects were rechallenged with TG allergen by means of nasal disk challenges (NDCs) during the non-GPS time period to test for tolerance induction or desensitization. Subjects consented under a separate Institutional Review Board protocol for the NDC. Four subjects did not consent to the NDC. Immune tolerance was defined as a lack of allergic reaction after rechallenge with allergen after treatment discontinuation. DM NDCs were not performed because of reconstruction of clinical research space at the time. Subjects and investigators were blind to treatment. Clinical symptoms were evaluated at baseline and every 3 months after treatment or placebo for the first 12-month period and then, after this time point, as available from subjects. This was primarily a phase I study to test the safety of dual SLIT. The efficacy end points included rhinoconjunctivitis symptom scores and medication use scores as published procedures,¹⁸ mucus production, and skin prick test responses. Mucus production was measured by using NDC, as in the study by Baroody et al¹⁹; details are presented in the Methods section in this article's Online Repository at www.jacionline.org. Skin prick test responses were performed with the Quintest skin device (Hollister-Stier, Spokane, Wash), and wheal-and-flare results were assessed. The study was approved by the Stanford Administrative panel on Human Subjects in Medical Research. Written informed consent was obtained from all participants or their parents (if <18 years of age) before entering the trial.

Immunophenotyping and flow cytometry

Total and allergen-specific blood IgE and IgG levels were measured in all subjects by the Clinical Laboratories at Stanford Hospital and Clinics using a standard ImmunoCAP assay (Phadia, Uppsala, Sweden). Basophils were stimulated and identified based on previously published methods²⁰; details are presented in the Methods section in this article's Online Repository.

Basic cell staining and flow cytometric methods for phenotyping are presented in the Methods section in this article's Online Repository. For T-cell assays, CD4⁺ T cells were enriched from whole blood by means of incubation with the negative selection CD4⁺ isolation kit (StemCell Technologies, Vancouver, British Columbia, Canada), followed by centrifugation at 450g for 40 minutes over Ficoll-Hypaque density gradients. The CD4⁺ T cell-containing PBMC layer was collected and washed twice with 10 mL of PBS at 4°C and resuspended in PBS. Cells were subsequently stained with Live/Dead (Invitrogen, Carlsbad, Calif) and CD4, CD25, CD127, and CD45RO cells. Memory Treg (CD4⁺CD25^{hi}CD127^{low}CD45RO⁺) and naive Treg (CD4⁺CD25^{hi}CD127^{low}) cells were stained and isolated through FACS sorting (FACSAria; BD, San Jose, Calif) before transcriptional analysis or addition to the suppressor assay. Live non-Treg cells (CD4⁺ T eff cells) were flow sorted simultaneously. Postsort analysis demonstrated that each sorted population was greater than 95% pure and that the sorted Treg cell populations were Foxp3⁺. Sorted T-cell populations were incubated in RPMI-1640 and 10% FBS after purification for 2 hours before use in further experiments.

Suppression assay

Standard thymidine-based suppression assays were performed to analyze Treg cell function, as previously published.²¹ Details are presented in the Methods section in this article's Online Repository.

DNA methylation and quantitative PCR analysis

RNA was isolated with RNAeasy kits (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Similar cell numbers were used for each subject. cDNA was synthesized with 500 ng of total RNA transcribed with cDNA transcription reagents (Applied Biosystems, Foster City, Calif) by using random hexamers, according to the manufacturer's protocol. Gene expression was measured in real time with primers and reagents purchased from Applied Biosystems and SuperArray. All PCR assays were performed in triplicates. Data were presented as relative fold expression of Foxp3 to the expression of the housekeeping gene β_2 -microglobulin. For quantification of CpG methylation islands, genomic DNA (Qiagen) purified from memory Treg cells before and after SLIT was bisulfite treated (Chemicon-Millipore, Temecula, Calif) and sequenced for CpG methylation sites in the promoter (n = 8 sites) and intronic (n = 13 sites) regions of the Foxp3 locus.

Statistics

Power calculations were conducted with PROC POWER in SAS 9.2 TS2M3 (SAS Institute, Inc, Cary, NC) at the .025 level and were based on the effects and variances we observed in previous studies using single SLIT for DM or TG.^{6,22–24} We estimated mean effect sizes and variance by using the pessimistic tails from 80% CIs generated from these data and estimated a greater than 0.80 power at an α level of .025 to detect a difference in Treg cell function between active treatment and placebo with the proposed sample size of 20 for active treatment versus 10 for placebo.

Where appropriate, data are represented as means $\pm$ SEMs. All statistical procedures were performed with Prism software (GraphPad Software, Inc, La Jolla, Calif). Between- and within-group comparisons were used the non-parametric Wilcoxon rank sum and signed-rank tests, respectively. Pairwise correlations used the Spearman test. Differences with a *P* value of less than or equal to .05 were considered statistically significant.

RESULTS

Efficacy of dual SLIT

This study was designed as a phase I study to determine whether dual SLIT was safely tolerated in subjects. In addition, this study was designed to test immune mechanisms of immunotherapy by using dual SLIT.

Thirty subjects were randomized in this single-center, double-blind, placebo-controlled phase I study of DM and TG SLIT (dual SLIT) to receive either active treatment (20 participants) or placebo (10 participants). All 30 subjects had documented clinical reactions, positive skin test responses, and allergen-specific IgE levels to DM and TG. The 2 treatment

groups did not differ with regard to age, sex, race, or baseline clinical characteristics (see Table E1). Dosing began 4 months before the TG season (mid-April to mid-June), and 20 subjects were actively treated with dual TG and DM SLIT for 12 months (see Fig E1 in this article's Online Repository at www.jacionline.org). At the preliminary dosing visit, subjects received up to 5 incrementally escalating doses of separate DM and TG extracts administered sublingually every 15 minutes up to a maximum tolerable dose (see Table E2), which was self-administered daily for 12 months. Subjects were evaluated every 3 months during the treatment period and every 6 months during the 1-year follow-up period. After 12 months of maintenance therapy, subjects withdrew from therapy and were rechallenged at 18 months to test for tolerance (ie, no allergic reaction on rechallenge) or desensitization (ie, an allergic reaction on rechallenge).

Clinical symptoms were evaluated at baseline and every 3 months during the treatment phase of the protocol. Adverse effects were well tolerated and mild to moderate and consisted of local reactions, such as itching (mouth, throat, ear, and eyes), headaches, edema (lip, tongue, or throat), urticaria, rhinitis, sneezing, lethargy, or gastrointestinal complaints (see Tables E3 and E4 in this article's Online Repository at www.jacionline.org). Total baseline clinical symptom scores did not differ between subjects receiving placebo and those receiving dual SLIT; however, symptoms in subjects receiving active treatment during the GPS were improved compared with those in placebo-treated subjects after 18 months of treatment (see Table E5 in this article's Online Repository at www.jacionline.org). Finally, NDCs with TG allergen demonstrated that mucus production was significantly decreased in dual SLIT-treated subjects compared with that seen in the placebo group ($P < .0001$; Fig 1, D). Sixteen of the total 20 subjects receiving active therapy withdrew from treatment for 6 months, and on rechallenge, 7 subjects did not react to NDCs to TG and might have achieved tolerance (no further allergic reaction after treatment discontinuation). The remaining 9 subjects did not achieve tolerance. Collectively, assessment of the clinical features (clinical symptom scores and adverse effects for both TG and DM and NDC results for TG) from the active and placebo groups suggests that dual SLIT could be clinically effective for TG and DM allergies.

Dual SLIT favors IgG₄ production and decreased basophil activation

Allergen-specific IgE mediates the type I hypersensitivity response observed in patients with allergic rhinitis and allergic conjunctivitis. Previous reports have indicated that successful allergen-specific immunotherapy induces an allergen-specific immune tolerance through reduction of the IgE/IgG₄ ratio.²⁵ Peripheral blood samples from subjects undergoing dual SLIT or placebo-treated control subjects were collected longitudinally and analyzed for immunoglobulin levels and basophil activation to assess immune tolerance and to determine whether the development of tolerance is allergen specific. Plasma levels of specific IgE to TG and DM were 25% (12.5% to 50%) and 11% lower in the active group compared with increases within the placebo group observed over time from 12 to 24 months after treatment (Fig 2, A, and data not shown; placebo vs active treatment for both DM and TG-specific responses, $P < .05$). In contrast, levels of IgG₄ specific to TG and DM, which has been indicated in blocking allergen-specific IgE,^{26,27} increased in the active group by 50% and 18%, respectively, compared with those seen in the placebo group (Fig 2, B, and data not

shown; placebo vs active treatment, $P < .05$ for time points 9–24 months after treatment for both DM- and TG-specific responses). In general, allergen-specific IgE and IgG₄ levels did not significantly differ in the GPS (Fig 2). Levels of oak-specific IgG₄ or IgE did not change over time in either placebo- or dual SLIT-treated subjects. These data indicate that dual SLIT is effective in modulating the humoral response toward TG and DM allergens.

IgE binds to the FcεRI present on basophils and, on cross-linking with allergen, can mediate basophil activation. On activation, basophils release histamine and express the surface activation markers CD11b, CD63, CD123, and CD203c. Several studies have suggested that basophils are a useful tool for diagnosis and monitoring of allergies.^{28,29} In particular, the expression of CD203c on the surface of basophils stimulated *ex vivo* with allergen is indicative of allergen-specific immune responses.³⁰ Surface expression of CD203c was measured on basophils collected from peripheral blood and stimulated *ex vivo* with allergens to determine the effect of dual SLIT on basophil activation. Data in Fig 3 show that basophil activation after *ex vivo* stimulation with either TG (Fig 3, A) or DM (Fig 3, B) is decreased in dual SLIT-treated subjects 24 months after treatment compared with that seen before treatment ($P < .001$), whereas no significant changes in basophil activation to TG or DM within the placebo group were observed (Fig 3, D and E). The decreased basophil activation was specific for TG and DM allergens because *ex vivo* stimulation with oak allergen did not demonstrate significant differences in basophil activation over time from either the active or placebo treatment groups (Fig 3, C and F). These *ex vivo* analyses of basophil function suggest that decreased basophil activation is one factor contributing to the clinical efficacy of dual SLIT.

Dual SLIT promotes suppressive memory Treg cells

Treg cells are important for modulating the allergic immune response and are considered responsible for successful immunotherapy, driving immune responses, such as the production of IgG₄.^{11,12} The effect of dual SLIT on Treg cell proliferation and suppressive function, as well as allergen-specific Teff cell proliferation, was examined. The suppressive function of Treg cells on allergen-specific Teff cells from active and placebo group subjects before treatment and 12 months after baseline was assessed by measuring Teff cell proliferation in the presence of Treg cells. Total mixed CD4⁺CD25^{high}CD127^{low}Foxp3⁺ Treg cells (including memory/induced and naive Treg cells) after treatment were found to suppress Teff cell proliferation to DM- and TG-specific allergens, as denoted by increased percentages of Treg cell function compared with those seen in Treg cells from before treatment ($P < .05$; Fig 4, A). Suppressive allergen-specific Treg cells did not appear to be induced in the placebo arm because this effect was not observed (Fig 4, C). Treg cells were further purified by memory phenotype and used as effector cells in the suppression assay to directly assess the role for CD4⁺CD25^{hi}CD127^{lo}CD45RO⁺Foxp3⁺ memory Treg cells in suppressing allergen-specific responses. Data in Fig 4, B, demonstrate that memory Treg cells are the predominant subset of Treg cells that mediate suppressive function. In the absence of Treg cells, Teff cells from before and after dual SLIT treatment responded to allergens and the negative control antigen tetanus comparably, with the exception of a slight decrease in Teff cell proliferation in response to TG (Fig 4, D). Total Treg cells from dual SLIT-treated subjects also had significant suppressive function on oak-specific Teff cell

proliferation ($P < .05$; Fig 4, A), but this effect was not observed when memory Treg cells were used as effector cells (Fig 4, B). In addition, Treg cells induced during dual SLIT did not modulate Teff cell responses against tetanus antigen (Fig 4, A). These data indicate that the memory Treg cells induced during TG and DM dual SLIT are antigen specific and suppress TG and DM Teff cell responses.

Epigenetic modification of induced memory Treg cells during dual SLIT

Foxp3 is a major transcription factor that determines the fate and identity of Treg cells. Transient expression of Foxp3 in peripheral CD4⁺ T cells induces suppressive characteristics, but constitutive expression is required for stable suppressive function. Recently, several studies have demonstrated a link between reduced methylation of CpG islands on the Foxp3 locus and increased stable Foxp3 expression (reviewed in Lal and Bromberg³¹). The number of CpG sites methylated within the Foxp3 locus from purified memory Treg cells was quantified before and 12 months after treatment to determine whether suppressor functions in memory Treg cells induced during dual SLIT have characteristics of stable Foxp3-expressing Treg cells. As shown in Fig 5, A, DNA methylation was decreased in CD45RO⁺ memory Treg cells from subjects after 12 months of dual SLIT treatment ($P < .05$), whereas no modifications of DNA methylation occurred over time in memory Treg cells from placebo-treated or healthy control subjects. Decreased CpG methylation within the Foxp3 locus was specific for memory Treg cells from dual SLIT-treated subjects because sorted naive Treg cells (CD45RA⁺) showed no significant changes in CpG methylation levels over time (Fig 5, A). Consistent with decreased methylated CpG sites, Foxp3 transcript levels were increased in memory Treg cells after dual SLIT treatment ($P < .05$; Fig 5, B). An increase in Foxp3 transcript levels was not observed in placebo-treated or healthy control subjects over time. Of note, placebo-treated subjects with allergic responses to TG and DM had increased methylation of CpG islands within the Foxp3 locus, as well as reduced Foxp3 transcript levels, regardless of time point, compared with that seen in healthy subjects (Fig 5).

Absolute CD4⁺CD25^{hi}CD127^{lo}CD45RO⁺Foxp3⁺ memory Treg cell numbers were measured to determine whether subjects who had achieved tolerance could be distinguished by immune indicators. Preliminary examination demonstrates a significant trend for increased memory or induced Treg cell numbers in the subjects who did not react to NDCs at 18 months after stopping SLIT for 6 months ($n = 7$) compared with subjects who had positive reactions to NDCs ($n = 9$, as shown in Fig 1) between 9 and 24 months after baseline (tolerant vs desensitized, $P < .05$; Fig 6). Collectively, these data (Figs 4 and 6) suggest that memory Treg cells (1) suppress allergen-activated Teff cells and (2) modulate the immune tolerance necessary for successful immunotherapy.

Programmed death 1 and Foxp3 levels in memory Treg cells are predictive markers of subjects with tolerance

To better define the Treg cell population induced during dual SLIT, we further characterized Treg cells from subjects undergoing active treatment or placebo by means of surface phenotyping and intracellular staining using flow cytometry. We assessed known indicators of Treg cells, including CTLA-4, Helios, programmed death 1 (PD-1), GITR, granzyme B,

CD25, Foxp3, and IL-10. The data in Fig 7, A, demonstrate that memory Treg cells (CD4⁺CD25^{hi}CD45RO⁺) from subjects receiving dual SLIT increased expression of PD-1, Foxp3, and IL-10 over time (baseline vs 12 months after treatment, $P < .01$). No changes in phenotype were observed over time from CD45RA⁺ naive Treg cells (Fig 7, A) or from memory Treg cells from the placebo group (Fig 7, B). Of note, baseline expression levels of all Treg cell indicators assessed were similar between the memory Treg cells from dual SLIT-treated and placebo-treated subjects.

We then determined whether PD-1, Foxp3, and IL-10 memory Treg cell markers were associated with tolerance. Baseline (before treatment) levels of PD-1, Foxp3, and IL-10 were assessed in memory Treg cells from both groups, as in Fig 7, A and B. Memory Treg cells from subjects who eventually became tolerized after dual SLIT treatment tended to have increased levels of all 3 markers assessed compared with Treg cells from subjects who were desensitized, although Foxp3 was the only marker that showed a significant increase in this small sample size ($P < .05$; Fig 7, C). These preliminary data suggest that the expression levels of memory Treg cell markers, in particular Foxp3 and PD-1, might be predictive of clinical tolerance.

DISCUSSION

The present phase I study assesses the safety and efficacy of TG and DM dual SLIT in subjects with demonstrated allergies to TG and DM. Consistent with previous single SLIT studies, our study demonstrated clinical efficacy, as observed by significant decreases in rhinoconjunctivitis symptom scores, medical use scores, and mucus production in dual SLIT-treated subjects. Multiple allergen studies conducted with grass and 9 other allergens in a 10-month study did not show benefit.⁷ This could have been due to the large volume of SLIT, the low GPS geographically, and the short time period to determine response.^{7–10} By contrast, when challenged with either TG or DM antigen, the dual SLIT-treated group had reduced mucus production (TG) and responses to skin prick tests (TG or DM) compared with the placebo group. In our study immune tolerance was defined as a lack of allergic reaction after rechallenge with allergen (as determined by means of NDCs). We chose the NDC assay because NDC measurements can be performed by using objective assessments. Our NDC methods, measurements, and results for placebo and allergen versus diluent challenge are similar to those published previously.¹⁹ We used standard rhinosinusitis scoring and medication use scoring, as previous published,¹⁸ and our results showed a trend toward clinical significance. However, we realize that additional studies are needed with a larger sample size to fully define immune tolerance and to determine statistically significant clinical efficacy measures for dual SLIT therapy. These preliminary data suggest that SLIT performed with multiple allergens could be an effective and efficient means of treating subjects with sensitivities to a variety of allergens and supports the need for a larger long-term clinical study.

Because the link between clinical efficacy and immunologic alteration is not clear, the secondary goal of the current study was to elucidate the mechanism of tolerance induced during allergen-specific immunotherapy. Antigen-specific immunotherapy targets the immunologic process underlying allergic diseases by altering allergen-specific T- and B-cell

responses and inducing long-term tolerance. The IgE and IgG₄ responses against TG and DM allergen over time reflect the humoral responses observed in previous SCIT and SLIT studies.³² Anti-TG and anti-DM IgG₄ levels increased by 50% and 18%, respectively, and anti-TG and anti-DM IgE levels were reduced by 25% and 11%, respectively, in the active treatment group compared with the placebo control group (Fig 2). Our results during GPS are similar to those published by other groups using SLIT for TG.¹⁸ Consequent to the reduction in allergen-specific IgE levels, basophils from dual SLIT-treated subjects showed lower expression of the surface activation marker CD203c after stimulation with allergen *ex vivo*. Finally, in agreement with other studies, Treg cells were found to be a major factor in the suppression of allergen-specific T-cell responses because Treg cells from the active treatment group suppressed TG and DM Teff cell responses, whereas Treg cells from the placebo group did not have an allergen-specific suppressor function. We observed a minimal bystander effect to oak allergen but not to tetanus antigen in the suppression assay when naive Treg cells from dual SLIT-treated subjects were used. However, our data demonstrate that immune regulation during immunotherapy, for the most part, is specific in nature and that a bystander effect, if any, is minimal; further studies are required to fully understand this effect. In addition to suppressive function, we observed an increase in Foxp3 transcript levels and reduced methylation of CpG islands within the Foxp3 locus within memory CD4⁺CD25^{hi}CD127^{low}CD45RO⁺ Treg cells isolated from subjects undergoing dual SLIT (Fig 5). This is the first study that demonstrates epigenetic regulation of Treg cells after allergen immunotherapy and suggests the promotion of stable suppressive Treg cells during the tolerance induction/immune mechanism associated with SLIT.

The mucosal immune response is required to respond appropriately to pathogens while maintaining tolerance to harmless environmental antigens. A breakdown of tolerance to particles, such as pollens and animal dander, results in the development of allergic pathology. Treg cells are critically involved in the maintenance of self-tolerance and the dampening of overactive immune responses. Foxp3 is a key transcription factor for both the development and suppressive activities of Treg cells, and it is expressed in the thymus in naturally occurring CD4⁺ Treg cells. Transient expression of Foxp3 can be induced in peripheral naive CD4⁺ T cells to generate T cells with suppressive characteristics. However, constitutive expression is required for stable suppressor function, as observed from natural Treg cells.³³ Previous studies have demonstrated that epigenetic modifications in the CpG-rich regions within the Foxp3 locus are associated with stable Foxp3 expression and thus suppressor functions of Treg cells.^{34,35} Methylation of CpG dinucleotides downmodulates transcription by preventing the binding of positive transcription factors and by recruiting repressor molecules. Accordingly, a CpG-rich island of Foxp3 is unmethylated in suppressive Treg cells but heavily methylated in naive CD4⁺ T cells, activated CD4⁺ T cells, and TGF- β -induced Treg cells. One can speculate that Treg cells, having a more stable suppressive phenotype, are more efficient at promoting long-term tolerance and would be better targets for immunotherapy. Thus immunotherapies that promote memory Treg cells with unmethylated CpG and more stable Foxp3 expression leading to enhanced suppressor functions might be indicative of treatment success. Additionally, assessing a patient's Foxp3⁺ Treg cells throughout the course of immunotherapy might become a useful tool to monitor the treatment progress of therapy. The scope of the current study only partially

addresses immune memory, and a larger long-term study would better define the promotion of long-term tolerance in patients receiving dual SLIT.

Further phenotypic analysis demonstrated that memory Treg cells promoted during dual SLIT have increased IL-10, Foxp3, and PD-1 expression, which provides some insight into the mechanism of Treg cell-mediated immune modulation. IL-10 redirects class-switching in favor of IgG₄. PD-1 regulates T-cell activity by delivering negative signals on interaction with its 2 ligands, programmed death ligand 1 and programmed death ligand 2 (reviewed in Jin et al³⁶). Further analysis of Treg cell function would include analysis of the production of TGF- β by Treg cells; increased TGF- β levels in the nasal mucosa associated with local increased IgA production and mucosal induction of protective allergen-specific IgA has been shown after successful immunotherapy.^{25,37} Finally, even though Treg cells have been indicated as major regulatory cells during SLIT, other immune cells, such as natural killer T cells or $\gamma\delta$ T cells, have regulatory function and could be involved in maintaining airway tolerance.^{38,39} Even though we have provided insight into the immune mechanism of successful immunotherapy in this study, complete understanding of the immune factors involved warrants further investigation. Assessment of these additional immune parameters would be a major goal of future clinical studies.

Modulation of mucosal tolerance has been recognized as a promising therapeutic approach for allergic responses. Even though clinical efficacy has been well documented for antigen-specific immunotherapy, the underlying immune mechanism has yet to be fully elucidated. Further understanding of the immune mechanism could support the use of immunotherapy as a mainstay treatment for allergy. Previous reports have shown that successful immunotherapy involves the downregulation of the T_H2 response,⁴⁰ blocking antibody production⁴¹ and the generation of Treg cells.¹¹ In the current study we have shown that memory Treg cells are responsible for the modulation of allergen-specific Teff cell responses in TG and DM dual SLIT. These results not only provide further insight into the mechanism of SLIT but provide further evidence for SLIT as a possible long-term treatment for allergy symptoms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

CTLA-4	Cytotoxic T-lymphocyte antigen 4
DM	Dust mite
Foxp3	Forkhead box protein 3
GITR	Glucocorticoid-induced TNF receptor
GPS	Grass pollen season
NDC	Nasal disk challenge
PD-1	Programmed death 1
SLIT	Sublingual immunotherapy
Teff	CD4 ⁺ effector T
TG	Timothy grass
Treg	Regulatory T

References

1. Akdis CA, Akdis M. Mechanisms of allergen-specific immunotherapy. *J Allergy Clin Immunol.* 2011; 127:18–29. [PubMed: 21211639]
2. Canonica GW, Bousquet J, Casale T, Lockey RF, Baena-Cagnani CE, Pawankar R, et al. Sublingual immunotherapy: World Allergy Organization Position Paper 2009. *Allergy.* 2009; 64(suppl 91):1–59. [PubMed: 20041860]
3. Durham SR, Leung DY. One hundred years of allergen immunotherapy: time to ring the changes. *J Allergy Clin Immunol.* 2011; 127:3–7. [PubMed: 21211638]
4. Jutel M, Akdis CA. Immunological mechanisms of allergen-specific immunotherapy. *Allergy.* 2011; 66:725–32. [PubMed: 21466562]
5. Nelson HS. Subcutaneous immunotherapy. *J Allergy Clin Immunol.* 2011; 128:907, e3. [PubMed: 21962798]
6. Wilson DR, Lima MT, Durham SR. Sublingual immunotherapy for allergic rhinitis: systematic review and meta-analysis. *Allergy.* 2005; 60:4–12. [PubMed: 15575924]
7. Amar SM, Harbeck RJ, Sills M, Silveira LJ, O'Brien H, Nelson HS. Response to sublingual immunotherapy with grass pollen extract: monotherapy versus combination in a multiallergen extract. *J Allergy Clin Immunol.* 2009; 124:150–6. e1–5. [PubMed: 19523672]
8. Calderon MA, Casale TB, Togias A, Bousquet J, Durham SR, Demoly P. Allergen-specific immunotherapy for respiratory allergies: from meta-analysis to registration and beyond. *J Allergy Clin Immunol.* 2011; 127:30–8. [PubMed: 20965551]
9. Marogna M, Bruno M, Massolo A, Falagiani P. Long-lasting effects of sublingual immunotherapy for house dust mites in allergic rhinitis with bronchial hyperreactivity: a long-term (13-year) retrospective study in real life. *Int Arch Allergy Immunol.* 2007; 142:70–8. [PubMed: 17016060]
10. Nelson HS. Multiallergen immunotherapy for allergic rhinitis and asthma. *J Allergy Clin Immunol.* 2009; 123:763–9. [PubMed: 19217653]
11. Francis JN, Till SJ, Durham SR. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol.* 2003; 111:1255–61. [PubMed: 12789226]
12. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszczyk M, Blaser K, et al. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol.* 2003; 33:1205–14. [PubMed: 12731045]
13. Scadding GW, Shamji MH, Jacobson MR, Lee DI, Wilson D, Lima MT, et al. Sublingual grass pollen immunotherapy is associated with increases in sublingual Foxp3-expressing cells and

- elevated allergen-specific immunoglobulin G4, immunoglobulin A and serum inhibitory activity for immunoglobulin E-facilitated allergen binding to B cells. *Clin Exp Allergy*. 2010; 40:598–606. [PubMed: 20184605]
14. Scadding G, Durham S. Mechanisms of sublingual immunotherapy. *J Asthma*. 2009; 46:322–34. [PubMed: 19484663]
 15. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3⁺ regulatory T cells in the human immune system. *Nat Rev Immunol*. 2010; 10:490–500. [PubMed: 20559327]
 16. Miyara M, Sakaguchi S. Human FoxP3(+)CD4(+) regulatory T cells: their knowns and unknowns. *Immunol Cell Biol*. 2011; 89:346–51. [PubMed: 21301480]
 17. Wieczorek G, Asemisen A, Model F, Turbachova I, Floess S, Liebenberg V, et al. Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. *Cancer Res*. 2009; 69:599–608. [PubMed: 19147574]
 18. Nelson HS, Nolte H, Creticos P, Maloney J, Wu J, Bernstein DI. Efficacy and safety of timothy grass allergy immunotherapy tablet treatment in North American adults. *J Allergy Clin Immunol*. 2011; 127:72–80. e1–2. [PubMed: 21211643]
 19. Baroody FM, Ford S, Proud D, Kagey-Sobotka A, Lichtenstein L, Naclerio RM. Relationship between histamine and physiological changes during the early response to nasal antigen provocation. *J Appl Physiol*. 1999; 86:659–68. [PubMed: 9931205]
 20. Gernez Y, Tirouvanziam R, Yu G, Ghosn EE, Reshamwala N, Nguyen T, et al. Basophil CD203c levels are increased at baseline and can be used to monitor omalizumab treatment in subjects with nut allergy. *Int Arch Allergy Immunol*. 2011; 154:318–27. [PubMed: 20975283]
 21. Nadeau K, McDonald-Hyman C, Noth EM, Pratt B, Hammond SK, Balmes J, et al. Ambient air pollution impairs regulatory T-cell function in asthma. *J Allergy Clin Immunol*. 2010; 126:845–52. e10. [PubMed: 20920773]
 22. Acquistapace F, Agostinis F, Castella V, Kantar A, Novembre E, Perrone MR, et al. Efficacy of sublingual specific immunotherapy in intermittent and persistent allergic rhinitis in children: an observational case-control study on 171 patients. The EFESO-children multicenter trial. *Pediatr Allergy Immunol*. 2009; 20:660–4. [PubMed: 19320852]
 23. Penagos M, Compalati E, Tarantini F, Baena-Cagnani R, Huerta J, Passalacqua G, et al. Efficacy of sublingual immunotherapy in the treatment of allergic rhinitis in pediatric patients 3 to 18 years of age: a meta-analysis of randomized, placebo-controlled, double-blind trials. *Ann Allergy Asthma Immunol*. 2006; 97:141–8. [PubMed: 16937742]
 24. TePas EC, Hoyte EG, McIntire JJ, Umetsu DT. Clinical efficacy of microencapsulated timothy grass pollen extract in grass-allergic individuals. *Ann Allergy Asthma Immunol*. 2004; 92:25–31. [PubMed: 14756461]
 25. Scadding GW, Shamji MH, Jacobson MR, Lee DI, Wilson D, Lima MT, et al. Sublingual grass pollen immunotherapy is associated with increases in sublingual Foxp3-expressing cells and elevated allergen-specific immunoglobulin G4, immunoglobulin A and serum inhibitory activity for immunoglobulin E-facilitated allergen binding to B cells. *Clin Exp Allergy*. 2010; 40:598–606. [PubMed: 20184605]
 26. Mothes N, Heinzkill M, Drachenberg KJ, Sperr WR, Krauth MT, Majlesi Y, et al. Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. *Clin Exp Allergy*. 2003; 33:1198–208. [PubMed: 12956739]
 27. Nouri-Aria KT, Wachholz PA, Francis JN, Jacobson MR, Walker SM, Wilcock LK, et al. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol*. 2004; 172:3252–9. [PubMed: 14978133]
 28. Buhring HJ, Streble A, Valent P. The basophil-specific ectoenzyme E-NPP3 (CD203c) as a marker for cell activation and allergy diagnosis. *Int Arch Allergy Immunol*. 2004; 133:317–29. [PubMed: 15031605]
 29. Kleine-Tebbe J, Erdmann S, Knol EF, MacGlashan DW Jr, Poulsen LK, Gibbs BF. Diagnostic tests based on human basophils: potentials, pitfalls and perspectives. *Int Arch Allergy Immunol*. 2006; 141:79–90. [PubMed: 16837789]

30. Gernez Y, Tirouvanziam R, Yu G, Ghosn EE, Reshamwala N, Nguyen T, et al. Basophil CD203c levels are increased at baseline and can be used to monitor omalizumab treatment in subjects with nut allergy. *Int Arch Allergy Immunol.* 2011; 154:318–27. [PubMed: 20975283]
31. Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. *Blood.* 2009; 114:3727–35. [PubMed: 19641188]
32. Dahl R, Kapp A, Colombo G, de Monchy JG, Rak S, Emminger W, et al. Efficacy and safety of sublingual immunotherapy with grass allergen tablets for seasonal allergic rhinoconjunctivitis. *J Allergy Clin Immunol.* 2006; 118:434–40. [PubMed: 16890769]
33. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol.* 2007; 8:277–84. [PubMed: 17220892]
34. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol.* 2003; 4:330–6. [PubMed: 12612578]
35. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 2003; 299:1057–61. [PubMed: 12522256]
36. Jin HT, Ahmed R, Okazaki T. Role of PD-1 in regulating T-cell immunity. *Curr Top Microbiol Immunol.* 2011; 350:17–37. [PubMed: 21061197]
37. Pilette C, Nouri-Aria KT, Jacobson MR, Wilcock LK, Detry B, Walker SM, et al. Grass pollen immunotherapy induces an allergen-specific IgA2 antibody response associated with mucosal TGF-beta expression. *J Immunol.* 2007; 178:4658–66. [PubMed: 17372025]
38. Zuany-Amorim C, Ruffie C, Haile S, Vargaftig BB, Pereira P, Pretolani M. Requirement for gammadelta T cells in allergic airway inflammation. *Science.* 1998; 280:1265–7. [PubMed: 9596580]
39. Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T, et al. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat Med.* 2003; 9:582–8. [PubMed: 12669034]
40. Ebner C, Siemann U, Bohle B, Willheim M, Wiedermann U, Schenk S, et al. Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from TH2 to TH1 in T-cell clones specific for Phl p 1, a major grass pollen allergen. *Clin Exp Allergy.* 1997; 27:1007–15. [PubMed: 9678832]
41. Wachholz PA, Durham SR. Induction of 'blocking' IgG antibodies during immunotherapy. *Clin Exp Allergy.* 2003; 33:1171–4. [PubMed: 12956735]

FIG 1. Clinical efficacy of dual SLIT. **A** and **B**, Rhinoconjunctivitis symptom scores (RCS; Fig 1, A) and medication use scores (MS; Fig 1, B). **C**, Responses to skin prick test challenges. **D**, NDCs to TG at 18 months. The orange circle denotes tolerant subjects. ** $P < .01$ and *** $P < .0001$.

FIG 2.

Change from baseline in mean antibody levels for TG-specific humoral responses. Phl p 5-specific IgE (**A**) and IgG₄ (**B**) responses from serum collected at baseline and at various time points from dual SLIT-treated subjects (*squares*, n = 20) or placebo-treated subjects (*circles*, n = 10) is shown. **P* < .05. GPS, Grass allergy season; mgA, milligrams of antigen-specific antibody.

FIG 3.

Decreased basophil activation from subjects receiving dual immunotherapy. Expression of CD203c levels on basophils stimulated with TG, DM, and oak from subjects before therapy (*Pre*) and after (*Post*) 24 months of dual SLIT (A–C) or placebo (D–F) is shown *** $P < .001$. NS, Not significant: $P = .08$; *MFI*, mean fluorescence intensity.

FIG 4.

Memory Treg cells from dual SLIT-treated subjects suppress allergen-specific T-cell responses. Treg cell-suppressive activity of total mixed Treg cells from dual SLIT-treated subjects (A), memory Treg cells from dual SLIT-treated subjects (B), total mixed Treg cells from placebo-treated subjects (C), or total Teff cell proliferation (D) before (*pre*) and after (*post*) treatment is shown. * $P < .05$. NS, Not significant.

FIG 5.

Epigenetic regulation of Foxp3 in Treg cells from subjects treated with dual SLIT. **A**, CpG methylation of purified memory or naive Treg cells from subjects before (*Pre*) and 12 months after (*Post*) therapy. **B**, Analysis of Foxp3 transcript from memory Treg cells. Foxp3 transcription levels are shown as relative fold increase over expression of the housekeeping gene β -glucuronidase. * $P < .05$.

FIG 6. Increased memory Treg cells in tolerized subjects. Longitudinal analysis of memory CD4⁺CD25^{high}CD127^{lo}CD45RO⁺CD62L⁻Foxp3⁺ Treg cells from tolerant (*circles*, n = 7) and desensitized (*squares*, n = 9) subjects after dual SLIT is shown. **P* < .05. *mo*, Months after baseline.

FIG 7.

Expression profiles of Treg cell markers in tolerant subjects. **A** and **B**, Expression of Treg cell markers on memory or naive Treg cells purified from dual SLIT-treated subjects (Fig 7, A) or placebo-treated subjects (Fig 7, B) before (*pre*) and 12 months after (*post*) treatment. * $P < .05$ for PD-1, IL-10, and Foxp3. **C**, Baseline expression levels from memory Treg cells from subjects who became tolerized ($n = 7$) or desensitized ($n = 9$). *Gzm B*, Granzyme B; *MFI*, mean fluorescence intensity.