Update on Immune Mechanisms Associated with Sublingual Immunotherapy: Practical Implications for the Clinician

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Sublingual immunotherapy (SLIT) is established as a safe and efficacious treatment for patients with type I respiratory allergies. The ability of SLIT to elicit antigen (allergen)-specific tolerance is linked to the peculiar biology of oral antigen-presenting cells. In the absence of danger signals, Langerhans cells, myeloid dendritic cells, and macrophages located in oral tissues, tonsils, and draining cervical lymph nodes are biased toward the induction of T_{H1} and IL-10—producing CD4^{+} regulatory T cells, thus supporting tolerance as opposed to inflammation. Sublingual administration does not lead to any detectable systemic exposure of intact allergens nor to IgE neosensitization. Oral tissues contain limited numbers of mast cells located in submucosal areas, thereby explaining the well-established safety profile of SLIT, with mostly local but rare systemic reactions. The induction of CD4^{+} regulatory T cells and blocking anti-inflammatory IgGs or IgAs are considered important for tolerance induction after SLIT. Specific molecular signatures associated with tolerogenic dendritic cells were recently reported during the onset of SLIT efficacy in the peripheral blood of patients exhibiting clinical benefit. Collectively, these observations confirm the induction of strong allergen-specific suppressive/tolerogenic immune responses during SLIT and pave the ground for the identification of biomarkers of efficacy. Practical implications of this emerging scientific knowledge are presented (1) to support the rational design of second-generation sublingual vaccines based on purified allergens, vector systems and/or adjuvants and (2) to help the clinician in decision making during his/her practice. © 2013 American Academy of Allergy, Asthma & Immunology (J Allergy Clin Immunol: In Practice 2013;1:228-41)

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When exposed to allergens, patients with type I respiratory allergies mount inappropriate immune responses characterized by T_{H2} cytokine production, high IgE levels, as well as recruitment and activation of basophils, eosinophils (Eos), and mast cells (MCs) in target mucosae.\(^1\)\(^6\) Innate and adaptive effector mechanisms involved in allergic inflammation are induced by allergen-specific CD4^{+} T helper cells through the production of IL-4, IL-5, IL-13, and possibly IL-9 and IL-17. In contrast to symptomatic treatments, allergen immunotherapy acts on the cause of the disease by modulating such CD4^{+} T-cell responses, with the aim to restore a balance in favor of T_{H1} and CD4^{+} regulatory T (Treg)-cell responses.\(^2\)\(^-\)\(^6\) Interestingly, the latter recapitulates the asymptomatic immune responses often observed in healthy persons after natural allergen exposure.\(^1\)\(^,\)\(^7\)

Although the subcutaneous route of immunization has previously been considered as a reference for allergen-specific immunotherapy, sublingual immunotherapy (SLIT) is now established in Europe as a valid noninvasive alternative to treat type I allergic rhinoconjunctivitis, with or without mild asthma, both in adults and children.\(^8\)\(^-\)\(^15\) The capacity of SLIT to modulate disease in the long term, to prevent new allergen sensitization, and to provide relief in asthma and in other conditions such as food allergy and atopic dermatitis have all been documented to some level, raising considerable worldwide interest for SLIT.\(^16\)\(^-\)\(^27\)

Allergen-specific SLIT is often considered as an approach to elicit oral tolerance, with the notion that the induction of suppressive rather than effector (proinflammatory) immune responses has been selected during evolution as a general property of the mucosal immune system across the whole digestive tract.\(^28\)\(^\) The most recent scientific evidence to document the specificities of the oral immune system as it relates to SLIT safety and efficacy is reviewed here. The practical implications of this scientific knowledge, both in terms of new vaccine development and guidance for decisions to be made by the clinician in his/her daily practice, are subsequently discussed.

OVERVIEW OF THE ORAL/LINGUAL IMMUNE SYSTEM

A detailed mapping of the oral/licual immune system has been performed in both mice and humans,\(^28\)\(^,\)\(^29\) yielding comparable information. Collectively, these studies establish that immune cells found in oral tissues encompass both antigen-presenting cells (APCs), lymphoid cells, and a few proinflammatory cells (Figure 1).\(^28\)\(^-\)\(^30\) In addition to such resident immune cells dispersed throughout oral tissues, the human oral immune system also comprises organized lymphoid structures. The latter include the Waldeyer ring of tonsils and adenoids that form the nasopharyngeal-associated lymphoreticular tissues, as
well as proximal (superficial cervical, submaxillary, and internal jugular) lymph nodes that drain both oral and nasal mucosae.32-34

Antigen-presenting cells
Oral APCs encompass three subsets of dendritic cells (DCs) with a distinct tissue distribution, including (1) Langerhans cells (LCs) located in the oral epithelium itself, (2) a predominant subpopulation of myeloid APCs comprising bona fide myeloid DCs (MDCs) as well as macrophage-like cells located along the lamina propria (all referred to as MDCs in Figure 1), and (3) plasmacytoid DCs (pDCs) rather found in subepithelial tissues (Figure 1).29-32 In humans, LCs and MDCs are abundant in oral tissues, whereas only low numbers of pDCs are detected.30 All such APCs have a capacity to uptake antigens/allergens by either phagocytosis, macropinocytosis, or receptor-mediated endocytosis.

The functional characterization of oral/lingual DCs has confirmed their critical role in tolerance induction. Both MDCs and DCs have been purified from murine oral tissues and shown in vitro to polarize naive CD4+ T cells toward Th1 and IL-10—secreting Treg cells that exhibit a suppressive activity.30 The central role of oral DCs in mediating tolerance has also been corroborated in vivo in murine models that use delivery systems to enhance allergen capture by oral APCs.32-34 For example, sublingual administration, based on a favorable (ie, high) LC-to-MC ratio.39 The human oral immune system comprises limited numbers both in murine and human oral tissues.40 Nonetheless, IgE-dependent activation of local MCs is considered as the cause of all common adverse events associated with SLIT, such as oral or throat irritation. MCs appear to be closer to the mucosal surface in lingual tissues,39 in agreement with the observation that SLIT is often associated with tongue edemas. Interestingly, some differences in relative numbers of LCs and MDCs have been reported, depending on the site considered (eg, vestibulum, lingua, sublingua, gingiva, palatum). With the goal to target the allergen to APCs before it reaches proinflammatory cells, the vestibulum has been suggested as an interesting site for allergen administration, based on a favorable (ie, high) LC-to-MC ratio.39

Besides MCs and Eos, other potential proinflammatory cells include the above-mentioned resident Th1, Th2, and Th17 lymphocytes along the lamina propria, as well as innate lymphoid cells located in the tonsils,41 as discussed in the next section. Thus, although the oral immune system is clearly biased toward tolerance induction, sublingual administration of antigen(s) in association with specific adjuvants (eg, cholera toxin, lymphotxin) or presentation platforms (plasmid DNA) elicited in murine models systemic effector immune responses involving IgGs and cytotoxic CD8 T cells directed against infectious pathogens.42-43

Noteworthy, a specific inflammatory condition of the mouth, the oral allergy syndrome is induced in some patients by food allergens cross-reactive with aeroallergens.44 A better understanding is needed of the physiopathology of this syndrome that results in a loss of the tolerogenic bias of the oral immune system and the subsequent activation of proinflammatory/effecter immune mechanisms that should not be recruited during SLIT.

Lymphoid cells
In mice, few lymphoid (ie, B, CD8+ T, natural killer [NK] T, γδ T) cells are detected in oral tissues, with the exception of CD4+ T cells, 50% of which express the CD103 mucosal addressing marker.28 The human oral immune system comprises as well abundant CD4+ and fewer CD8+ resident T lymphocytes.29,30 These T lymphocytes are mostly located along the lamina propria, that is, in the vicinity of numerous APCs, and include both regulatory as well as effector (T\textsubscript{H}1, T\textsubscript{H}2, or T\textsubscript{H}17) CD4+ T cells, likely involved in defense against infectious pathogens. The latter hypothesis is consistent with the observation that patients with a deficit in oral T\textsubscript{H}17 cells are highly susceptible to Candida albicans infection.39 Altogether, T\textsubscript{H}1 and Treg cells differentiated from naive T cells in oral lymphoid organs are thought to be more critical than such resident CD4+ T cells for establishing SLIT-induced tolerance (Figure 1).

Proinflammatory cells
Proinflammatory cells such as MCs and Eos are found in limited numbers both in murine and human oral tissues.32,37 and are rather located in subepithelial areas (Figure 1), thus explaining why allergen reactivity in the mouth is estimated to be 100- to 1000-fold less than the one in the skin.40 Nonetheless, IgE-dependent activation of local MCs is considered as the cause of all common adverse events associated with SLIT, such as oral or throat irritation. MCs appear to be closer to the mucosal surface in lingual tissues,39 in agreement with the observation that SLIT is often associated with tongue edemas. Interestingly, some differences in relative numbers of LCs and MDCs have been reported, depending on the site considered (eg, vestibulum, lingua, sublingua, gingiva, palatum). With the goal to target the allergen to APCs before it reaches proinflammatory cells, the vestibulum has been suggested as an interesting site for allergen administration, based on a favorable (ie, high) LC-to-MC ratio.39

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Oral lymphoid organs
Those lymphoid organs are located in the gateway of both alimentary and respiratory tracts and as such are in contact with food and aeroallergens as well as infectious agents. Their role is to bring in close vicinity immune cells to orchestrate efficient and appropriate antigen-specific immune responses, whether it be proinflammatory or regulatory/suppressive, depending on the occurrence of danger signals.27 Tonsils contain B and T

Abbreviations used
APC- Antigen presenting cell
DC- Dendritic cell
Eos- Eosinophil
ILT- Immunoglobulin-like transcript
LC- Langerhans DC cell
MC- Mast cell
MDC- Myeloid dendritic cell
NK- Natural killer
pDC- Plasmacytoid dendritic cell
SLIT- Sublingual immunotherapy
TLR- Toll-like receptor
Treg- Regulatory T [cell]
lymphocytes, as well as MDCs and pDCs (Figure 1). Tonsils also contain innate lymphoid cells, that is, non-T, non-B lymphocytes recently described as both regulatory and effector cells that contribute to innate immune responses. Tonsilar innate lymphoid cells comprise NK and NK-like cells (expressing NKp44, NKp46, and CD56 markers), lymphoid tissue inducer cells, and CD127 (IL-7R) lymphoid cells. These innate lymphoid cells can directly sense bacterial components and exhibit a strong capacity to produce cytokines such as IL-2, IL-5, IL-13, and IL-22.

After stimulation of various TLRs, tonsil APCs secrete low levels of cytokines, with only a limited upregulation of surface molecules such as MHC class II, CD40, CD80, CD83, and CD86 required for antigen presentation and T-cell activation. Tonsil pDCs were confirmed in vitro to support the differentiation of Foxp3+ CD4+ Treg cells. Tonsils represent thus a tolerogenic environment, although this state of tolerance can be broken in the presence of proinflammatory cytokines such as IL-1β or IL-6. As of today, no evidence for an effect of tonsillectomy on SLIT efficacy has been reported, consistent with the fact that only palatine tonsils and adenoids are usually removed, whereas lingual tonsils (anatomically the most important) are preserved.

Like tonsils, draining cervical lymph nodes represent specialized microenvironments that act as inductive sites for both tolerogenic and inflammatory immune responses. In the absence of danger signals, the responses induced are rather anti-inflammatory, including antibodies (IgG2b in mice) and CD4+ Treg lymphocytes with a suppressive function on effector T lymphocytes.

**Oral bacterial flora**

The presence of commensal (or pathogenic) bacteria in mucosae can favor both tolerogenic or proinflammatory mechanisms, most particularly by engaging pattern recognition receptors at the surface of resident immune cells. Thus, differences between persons in terms of flora composition could in theory affect SLIT efficacy. Recently, a comprehensive study of human microbiota has been conducted by the Human Microbiome Project Consortium at various body sites in 242 healthy adults. The latter included buccal mucosa (cheek), saliva, keratinized gingiva, palate, tonsils, dorsal tongue, supra and subgingival plaques, as well as throat. After 16S ribosomal RNA sequencing and metagenomic typing, up to 800 species of commensal bacteria were found in the oral cavity and oropharynx. Beyond the observation that strains belonging to the Streptococcus genus (eg, S. mitis) are consistently the most prevalent at all oral sites, followed by Haemophilus, Prevotella, and Veillonella spp, this study shed light as well on interindividual differences in terms of strain composition of the oral microbial ecosystem. This variability was relatively lower in the mucosa.
oropharynx than in other body sites, such as skin or vagina. Nonetheless, considerable variation was found between persons for patterns of bacterial genes expressed in oral tissues, the latter reflecting host-specific enrichment in structural variants beyond strain variability. Interestingly, the uniqueness of each person’s oral microbial community was found to be stable over time in the 22-month follow-up of the study.50

This study represents a first step toward a better understanding of the role of microbiota in shaping local immune responses, with likely a significant polymorphism across the human population. Together with the observation that selected lactic acid-producing bacterial strains act as powerful adjuvants to enhance SLIT efficacy in murine asthma models52-54 (see section on “Implications for the Development of New Forms of SLIT”), this emerging scientific knowledge could eventually lead to new immunomodulatory strategies tailored to the oral/sublingual route to favor tolerance.

PHARMACODYNAMICS OF SUBLINGUAL IMMUNIZATION

Fate of the allergen after sublingual administration

In the classical sublingual-swallow procedure used for SLIT, the allergen extract is administered on an empty mouth, kept under the tongue for 1 to 2 minutes, and then swallowed.14 Detailed biodistribution studies in mice have shown that even in such a short time frame, allergens bind immediately to epithelial cells after sublingual exposure, likely as a consequence of electrostatic interactions with the negatively charged glycocalyx at the surface of epithelial cells.31 Tissues under the tongue are highly vascularized, with blood vessels draining directly into the jugular vein. As a consequence, the sublingual route was initially selected to administer small synthetic molecules (such as the vasodilator glyceryl trinitrate, the morphinic analgesic buprenorphine, or epinephrine) with the goal to obtain a peak plasma release within 5 to 10 minutes.2,55 However, larger molecules such as peptides or glycoproteins are not directly adsorbed into the blood after sublingual administration, in contrast with what is observed after intravenous or even subcutaneous administration, as shown in animal experiments (Figure 2). Pharmacokinetics studies conducted in humans with the use of radiolabeled allergens such as Der p 2 or Par j 1 administered sublingually confirmed both the absence of direct systemic absorption of allergens and the adhesion of significant amounts of the allergen(s) to the oral mucosa for several hours,56,57 allowing it to be probed efficiently by the immune system.

Subsequently, the fate of the allergen can be summarized as follows (Figure 1): allergens cross the mucosa within 15 to 30 minutes and are then captured and processed by APCs.30 This uptake occurs in the upper layers of oral tissues, with the involvement of LCs in the mucosa itself, and of MDCs/macrophages at the level of the lamina propria.30,31 After capture, these APCs migrate within 24 to 48 hours to draining cervical lymph nodes and tonsils, where they present allergen-derived peptides to naive CD4⁺ T cells.32 Because of the peculiar biology of oral APCs, CD4⁺ T-cell responses of the Th1 and Treg cell types are elicited within a few days, with a subsequent migration of those cells to the bloodstream, and then to the mucosa.30 As discussed below, those CD4⁺ T cells are thought to be critical in controlling both antibody responses as well as cellular recruitment of inflammatory cells in target mucosa.2-6

Altogether, the magnitude of SLIT efficacy in eliciting tolerance is not related to any plasmatic release of the allergen(s), but rather to numbers of APCs carrying peptides derived from allergen(s) to stimulate resting T cells in the oral lymphoid organs.2,58 As confirmed by murine experiments, the latter is directly linked to the level of allergen uptake by APCs in the
upper layers of oral tissues, which can be considered as a limiting step for SLIT efficacy.\(^2\)\(^{,31}\)

**Rationale for high-allergen dosing**

Whereas doses in the range of 5 to 25 \(\mu\)g of major allergens are administered during both subcutaneous immunotherapy (SCIT) and SLIT, the latter relies on a daily administration, with eventually 50- to 100-fold more allergen in terms of cumulated doses used during therapy to reach a comparable level of efficacy.\(^14\)\(^,23\) An explanation is that no adjuvant or delivery system is used during SLIT, in contrast to SCIT protocols which, in Europe, are based on an association between the allergen(s) and calcium phosphate or aluminum hydroxide. As a consequence, high-dose regimens are likely necessary to allow the capture of sufficient amounts of allergen by oral sentinel DCs to elicit a tolerogenic T-cell response. Interestingly, both murine and human studies have suggested that reiterated exposure to high-allergen doses during immunotherapy favors the orientation of CD4\(^+\) T-cell responses toward IFN-\(\gamma\) and IL-10–secreting cells,\(^59\)\(^-\)\(^71\) consistent with immune changes observed during SLIT (see “Effect on allergen-specific T-cell responses”).

**Contribution of allergen swallowing to tolerance induction**

Whether allergen absorption in the gastrointestinal tract also contributes to SLIT efficacy remains an open question, in light of the known capacity of the intestinal immune system to elicit T\(_{H3}\) regulatory cells as well as secretory IgAs.\(^2\) Noteworthy, the degradation of allergens by local proteolytic enzymes is likely limited during SLIT, because capture and processing by oral APCs occurs within minutes. In contrast, oral absorption leads to exposure to gastrointestinal fluids for several hours and thus to a substantial degradation of the allergen before reaching the immune cells. It can be inferred that the epitopic repertoire derived from an allergen given sublingually is likely broader than the one obtained after oral administration, implying that allergen exposure to the oral/lingual mucosa is more efficient than intestinal exposure to induce tolerance. Consequently, given the fast-occurring allergen uptake during SLIT, the sublingual-spit procedure could be an alternative to sublingual-swallow for those few patients with severe local reactions or gastrointestinal adverse events induced during SLIT.

**Adverse events in relationship with the specificities of the oral immune system**

One advantage of SLIT when compared with other routes, most particularly the subcutaneous one, is its excellent safety profile established in both adults and children.\(^14\)\(^,15\) Side effects associated with SLIT are usually local, encompassing oral itching, tongue edema, ear pruritus, throat irritation, and nasopharyngitis. These side effects observed in 40% to 75% of patients usually occur within minutes, mostly at the beginning of the treatment, as a consequence of a local inflammation caused by IgE-mediated activation of MCs.\(^52\)\(^,65\) It is thus recommended that the first administration is made under medical supervision, with the patient monitored for 30 minutes.\(^14\)\(^,23\) Local reactions commonly observed during SLIT are mild and transient and usually do not persist with continued treatment. Systemic adverse events are rare, and as of 2012, only 11 cases of anaphylaxis (all nonfatal) have been published, with an estimated 1 billion SLIT doses administered worldwide.\(^65\) As a comparison, a recent survey of North American allergists reported for SCIT a rate of three life-threatening anaphylactic events for every 100,000 injection visits.\(^64\)

This favorable safety profile for the sublingual route can be explained by the limited, if any, release of intact allergens into the bloodstream, unlikely to stimulate systemic proinflammatory immune responses. The rare systemic adverse events observed are often linked with overdosing or poor quality of the allergen extracts.\(^65\) Obviously, SLIT is not appropriate after recent oral surgery, tooth extraction, or any condition that may lead to substantial allergic exposure to blood immune cells.\(^14\)\(^,25\) Because of the bias toward tolerance induction of the oral immune system, even in allergic patients, it is now established that SLIT does not elicit de novo IgE responses to allergens for which the patient is naive before treatment,\(^65\)\(^,66\) which constitutes another advantage over SCIT as it relates to safety.

**IMMUNE CHANGES INDUCED BY SLIT**

Allergen-specific SLIT reduces immediate as well as late-phase associated symptoms, via humoral and cellular mechanisms similar to the ones involved during SCIT.\(^2\)\(^-\)\(^6\) Those immune mechanisms are presented in Figure 3 as well as in Table I, in relationship with the time of occurrence during treatment.

**Effect on allergen-specific T-cell responses**

Changes in the polarization of allergen-specific CD4\(^+\) T cells are considered to be central to the efficacy of specific immunotherapy. Given the dramatic differences in patterns of cytokines produced by the distinct subsets of CD4\(^+\) T cells, such changes are thought as well to explain in large part the effect of allergen immunotherapy on allergen-specific antibody responses and on the recruitment and activation of proinflammatory innate cells.\(^2\)\(^-\)\(^6\) Several studies have now reported that successful SLIT is associated with the decrease of allergen-specific CD4\(^+\) T\(_{H2}\)-cell responses (via either apoptosis and/or anergy) paralleled with the induction of T\(_{H11}\) cells (immune deviation).\(^67\)-\(^69\) In addition, SLIT also elicits IL-10—producing CD4\(^+\) Treg cells (immuno-suppression)\(^67\)\(^,68\)\(^,70\)\(^-\)\(^75\) (Figure 3).

Various subsets of Treg cells are known to establish and maintain antigen (or allergen)-specific tolerance, which can be distinguished on the basis of surface markers and patterns of cytokines they produce. These suppressive cells can downregulate established allergen-specific T\(_{H2}\) responses either by direct cell–cell contact (involving PD-1, membrane-bound TGF-\(\beta\) or cytotoxic T lymphocyte–associated antigen 4 molecules) or through the production of immunosuppressive cytokines such as TGF-\(\beta\) or IL-10 (for T\(_{H3}\) or Tr1 cells, respectively).\(^1\)\(^-\)\(^2\)\(^,5\)

In murine experiments, T cells induced during SLIT were purified from draining lymph nodes and confirmed to be Tr1 (Foxp3\(^+\), producing both IFN-\(\gamma\) and IL-10) cells with a strong suppressive activity on third-party–activated T cells.\(^80\) In mice treated sublingually with an allergen conjugated to the cholera toxin B subunit, CD25\(^+\) Foxp3\(^+\) Treg cells were induced in wild-type animals but not in B-cell–deficient animals.\(^76\)\(^,77\) In humans, SLIT in adults or children with mite, grass, or tree pollen allergens also results in the induction of either CD25\(^+\) Foxp3\(^+\) Treg cells or IL-10—producing Foxp3\(^+\) Tr1 cells.\(^67\)\(^,68\)\(^,71\)\(^-\)\(^75\) Several studies further suggest that SLIT in patients with mite allergy induces TGF-\(\beta\)–producing T cells,\(^72\)\(^,78\) possibly T\(_{H3}\) cells, as previously shown for SCIT.\(^79\) It remains however, as of today, difficult to draw a simple and
coherent picture of the kinetics of induction of such T_{H}1 and Treg-cell responses, occurring within weeks and/or months, likely as a consequence of differences in dosing regimens and treatment protocols (Table I).

Noteworthy, several clinical studies have failed to document any induction of Treg cells, or even changes, in peripheral T-cell responses during SLIT. Although the effect of SLIT on CD4{sup+} T-cell responses is often documented in peripheral blood, other studies further stress the importance of local mucosal changes. In this regard, the induction of Foxp3{sup+} CD4{sup+} T cells within the oral mucosa was reported after SLIT. Collectively, these studies suggest that various types of Treg cells are induced during SLIT, including T1 and Foxp3{sup+} regulatory cells, which can be raised from naive CD4{sup+} T-cell progenitors even in allergic patients. In patients with grass pollen allergy, the need for a 2- to 4-month pretreatment period before the pollen season is 9-12 is possibly due to the time needed to mount a robust Treg-cell response. As discussed in the “In Search of Biomarkers for SLIT Efficacy” section, however, the link between changes in patterns of allergen-specific CD4{sup+} T-cell responses and clinical improvement remains to be established.

**Effect on allergen-specific antibody responses**

The modulation of allergen-specific CD4{sup+} T-cell responses has a profound effect on antibody responses, as a consequence of alterations in immunoglobulin isotype switching linked with cytokines produced (Figure 3 and Table I). During SLIT, an increase in allergen-specific IgE sige titers, before a progressive decline (after 6 months to 1 year of treatment) or a significant blunting of recall responses during allergen re-exposure are commonly observed. Such a boost of existing IgE responses is well tolerated and occurs in most patients, including those with documented clinical benefit. One explanation is that this upregulation of specific IgEs is detected in peripheral blood, but not in nasal secretions (my unpublished results).

In parallel, allergen-specific seric IgG_{4} responses are induced within 2 weeks and last for more than a year after treatment cessation. A debate has been heated as to whether such specific IgG_{4}s induced in peripheral blood correlate with clinical efficacy. Evidence is now clear that this is not the case, but that seric induction of IgGs rather represents a pharmacologic marker of allergen exposure during treatment. Consistent with this, a current hypothesis is that more than IgG_{4} titers, clinical efficacy is better correlated with peculiar functional properties (ie, a blocking activity) which may be the hallmark of high-affinity antibodies. Such blocking antibodies are thought to inhibit IgE-allergen interactions, leading as a consequence to a decrease in histamine release by basophils. They also inhibit CD23-dependent IgE-facilitated allergen binding to B lymphocytes or other APCs (thereby affecting allergen presentation to T cells). These antibodies could also act by engagement of FcγRIIB (CD32) inhibitory receptors to downregulate B-cell, MC, and basophil activation.

In addition to IgGs, allergen-specific IgA antibodies have been detected in the serum of patients in a few clinical studies. In murine asthma models, the efficacy of SLIT conducted with allergens formulated in vector systems is consistently associated with a strong induction of IgAs in respiratory mucosa. These IgAs may act as powerful anti-inflammatory antibodies, competing with IgEs for allergen binding, most particularly because they are produced at the level of mucosal surfaces. In this regard, the induction of such secretory IgAs is expected to be significantly higher after SLIT compared with SCIT.

**Effect on the innate immune system**

As a consequence of changes elicited during SLIT in the cytokine milieu produced by CD4{sup+} T cells or after direct contact with induced Treg cells, the recruitment and activation of proinflammatory innate cells (ie, MCs, basophils, Eos, neutrophils) is significantly reduced in the skin, nose, eye, as well as in bronchial and oral mucosae after provocation or natural exposure.
<table>
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<tr>
<th>Parameters</th>
<th>Short-term SLIT (hours to &lt;1 wk)</th>
<th>Mid-term SLIT (1 wk to 6 mo)</th>
<th>Long-term SLIT (6 mo to several years)</th>
<th>Reference citations</th>
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<tr>
<td>T cell responses</td>
<td>• Not significantly affected in such a short time frame</td>
<td>• Induction of T&lt;sub&gt;H&lt;/sub&gt;1, TGF-β, or IL-10 producing Treg cells (Tr1 or Foxp3&lt;sup&gt;+&lt;/sup&gt;) in peripheral blood</td>
<td>• Induction of T&lt;sub&gt;H&lt;/sub&gt;1, TGF-β, or IL-10 producing Treg cells (Tr1 or Foxp3&lt;sup&gt;+&lt;/sup&gt;) in peripheral blood</td>
<td>67-75, 78, 83, 84</td>
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<td>• Induction of Foxp&lt;sup&gt;+&lt;/sup&gt; T cells in oral mucosa</td>
<td>• Induction of Foxp&lt;sup&gt;+&lt;/sup&gt; expression in memory Treg cells in association with epigenetic modifications</td>
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<td>• In an allergen chamber study, no correlation between changes in allergen-specific CD&lt;sup&gt;+&lt;/sup&gt; T-cell responses in peripheral blood and SLIT efficacy after 4-mo SLIT</td>
<td>• Decrease in Th2 cytokines (IL-5, IL-9, IL-13) in serum or PBMC culture</td>
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<td>• T&lt;sub&gt;H&lt;/sub&gt;2 CD4&lt;sup&gt;+&lt;/sup&gt; T cells: not significantly decreased in peripheral blood</td>
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<td>Antibody responses</td>
<td>• Not significantly affected in such a short time frame</td>
<td>• Boosting (by 1.5- to 5-fold) of existing specific IgE levels (in the absence of neosensitization); upregulation of IgEs detected in serum but not in mucosal fluids</td>
<td>• Decrease in seric-specific IgE titers</td>
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<td>• 1.5- to 5-fold increase in seric specific IgG&lt;sub&gt;4&lt;/sub&gt; and IgAs (serum, mucosa)</td>
<td>• 1.5- to 5-fold increase in seric-specific IgG&lt;sub&gt;4&lt;/sub&gt; levels that revert to baseline 1 year after treatment cessation</td>
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<td>• Induction of blocking antibodies (high-affinity IgG&lt;sub&gt;4&lt;/sub&gt; and IgAs)</td>
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<td>Innate immune parameters</td>
<td>• During rush venom immunotherapy performed via the SC route:</td>
<td>• No detectable effect of SLIT on peripheral blood basophils</td>
<td>• Decrease in basophil activation</td>
<td>74, 85, 88, 99</td>
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<td>- Downregulation of basophil activation</td>
<td>• Two markers of regulatory (tolerogenic) DCs (ie, C1Q and Stabilin) were upregulated in peripheral blood of patients exhibiting clinical benefit</td>
<td>• After 1 year of SLIT, evidence for monocyte-derived DCs from the blood with low expression of CD86, reduced production of IL-12, and increased IL-10 secretion</td>
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<td>- Induction of ILT3, ILT4, cAMP in monocytes</td>
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<td>- IL-10 production by monocytes and T cells</td>
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<td>Candidate biomarkers of efficacy</td>
<td>• None established</td>
<td>• Ratio-specific/total IgEs and peripheral blood CD4&lt;sup&gt;+&lt;/sup&gt; Treg cells: not confirmed as biomarkers of efficacy</td>
<td>• None established</td>
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<td>• Hypothesis: reduced basophil responsiveness to allergens</td>
<td>• Hypothesis: modified patterns of memory CD4&lt;sup&gt;+&lt;/sup&gt; T cells (decrease in Th2 cells, upregulation of Th1 and Treg cells)</td>
<td>• Hypothesis: changes in adaptive immunity such as</td>
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<td></td>
<td></td>
<td>- Induction of specific IgG&lt;sub&gt;4&lt;/sub&gt; or IgAs with blocking activity</td>
<td>- Induction of specific IgG&lt;sub&gt;4&lt;/sub&gt; (or IgAs) with blocking activity</td>
<td>74, 86, 87, 92, 99</td>
</tr>
</tbody>
</table>

cAMP, Cyclic adenosine monophosphate; PBMC, peripheral blood mononuclear cell.
to allergens.\textsuperscript{2-6,89,90} (Figure 3). In addition, recent studies suggest as well a significant effect of SLIT on monocyte-derived APCs in peripheral blood. SLIT in children with house dust mite allergy after a year resulted in a decrease of the capacity of monocyte-derived DCs to mature and to produce IL-12 while upregulating IL-10 secretion,\textsuperscript{91} consistent with their acquisition of a tolerogenic phenotype and reduced capacity to activate T lymphocytes. Another study reported the increase in APCs expressing programmed cell death-ligand 1 and IL-10—producing CD14\textsuperscript{+} (monocytes) and CD19\textsuperscript{+} B lymphocytes during SLIT.\textsuperscript{91} Finally, a 4-month course of SLIT in patients with allergy to grass pollen was shown to elicit monocyte-derived DCs expressing high levels of C1Q and Stabilin-1, that is, two markers of DC regulatory cells.\textsuperscript{92} This induction was detectable in peripheral blood by quantitative PCR only in those patients exhibiting clinical responses, further corroborating the hypothesis that the induction of regulatory/suppressive immune responses is critical for tolerance induction via the sublingual route.\textsuperscript{92}

**IN SEARCH OF BIOMARKERS FOR SLIT EFFICACY**

Most clinical studies that documented immune changes during SLIT were small open studies that did not allow the correlation of those changes with clinical improvement at an individual patient level. Consequently, as of today, no biomarker of SLIT efficacy has been established yet, which could be used as a tool to support decision making by the clinician.

Biomarkers can be defined as biological parameters that can be objectively measured to monitor or predict disease severity or treatment efficacy. Herein, I focus on the identification of biomarkers useful as readouts of efficacy after SLIT implementation, for which recent progress has been made. This topic is discussed below in the framework of the duration of SLIT, discriminating candidate biomarkers that can be considered during either short- (ie, days), mid- (up to 6 months), or long-term (6 months to several years) courses of immunotherapy (Table I).

**Markers of short-term efficacy**

Short-term efficacy biomarkers (documented within a week or less of SLIT) are currently unknown, even if rush and ultrarush protocols for SLIT are associated with a decrease in skin reactivity and a readily detectable clinical benefit within days.\textsuperscript{93} A recent study conducted in a cohort of patients with grass pollen allergy suggested that SLIT is efficacious at decreasing symptoms as early as within a week.\textsuperscript{94} Such observations are unlikely to be correlated with significant modulation of T- or B-cell responses in such a time frame. The most detailed studies investigating short-term efficacy markers have been conducted in the context of venom immunotherapy with the use of the subcutaneous route. In such a setting, alterations of immune parameters are seen within hours, which include a decrease in allergen activation of basophils (possibly through a downregulation of the signaling machinery), IL-10 production by T lymphocytes or monocytes, induction of immunoglobulin-like transcript 3 (ILT3) and ILT4 molecules or intracellular cyclic adenosine monophosphate in monocytes, as well as enhanced tryptophan degradation in serum.\textsuperscript{5,95,96}

**Biomarkers for mid-term efficacy**

Changes in adaptive immunity parameters have been reported in many SLIT studies conducted between 1 week and 6 months (Table I). Of particular interest, candidate biomarkers of mid-term efficacy have been investigated in the context of a double-blind placebo-controlled randomized SLIT trial conducted in a cohort of 89 patients with grass pollen allergy.\textsuperscript{94} In that study conducted in an allergen challenge chamber over 4 months, individual clinical responders were distinguished from nonresponders. The grass pollen sublingual tablet was highly efficacious in reducing symptoms as early as after 1 month of treatment, compared with the placebo group, with a clinical benefit sustained during the 3 subsequent months of treatment.\textsuperscript{95} In such a time frame, no effect on the activation of peripheral blood basophils was noticed.\textsuperscript{97} In addition, changes in phenotype and function of grass pollen-specific CD4\textsuperscript{+} T cells were limited, with no differences between clinical responders and nonresponders detected in peripheral blood with respect to T_{H1}, T_{H2}, T_{reg}, or regulatory cytokine production, Treg markers, or T-cell proliferation.\textsuperscript{95} These results do not preclude that functionality relevant CD4\textsuperscript{+} Treg cells were induced in target mucosae, but they imply that monitoring in transit Treg cells in peripheral blood cannot be used as a marker of SLIT efficacy. This explanation likely applies to the other studies that failed to document changes in T-cell responses or induction of Treg cells during SLIT.\textsuperscript{70,76}

Interestingly, no decrease in proallergic T_{H1}2 cells (ie, a subset of CD45RO\textsuperscript{CD27} terminally differentiated CD4\textsuperscript{+} T cells with a capacity to produce high levels of IL-5)\textsuperscript{98} was observed over the 4-month SLIT protocol, even in patients with confirmed clinical responses.\textsuperscript{98} Together with the well-known fact that IgE levels in serum are unchanged in this time frame, a conclusion is that SLIT brings clinical benefit irrespective of any major depletion or downregulation of immune cells mediating T_{H1}2 responses, including IL-5—producing CD4\textsuperscript{+} memory T cells as well as IgE-secreting memory B lymphocytes.

Although the ratio between specific and total IgEs has been proposed as a potential marker of SLIT efficacy,\textsuperscript{74,99} this hypothesis was not confirmed in this challenge chamber study, based on IgE measurements performed before or during SLIT.\textsuperscript{76} Interestingly, clinical responders were found to encompass both immunoreactive patients with strong grass pollen-specific IgE, IgG, and IgG4 responses induced during SLIT, as well as nonimmunoreactive responders with no detectable antibody responses to distinguish them from either patients receiving placebo or active nonresponders (manuscript in preparation). Altogether, although allergen-specific IgG4 responses could contribute to tolerance induction in a subset (ie, 15% to 20%) of patients, seric antibody responses cannot be used as a marker of mid-term efficacy for SLIT.

Among all the biological parameters tested, only the upregulation of tolerogenic (ie, regulatory) monocyte-derived DC markers such as C1Q and Stabilin-1 was selectively detected in the peripheral blood of patients with a confirmed clinical response to the treatment.\textsuperscript{92} That study raised the intriguing possibility that molecular changes at the level of blood DCs can be used as an early signature of the polarization of allergen-specific adaptive immune responses, persisting in peripheral blood in contrast to alterations of CD4\textsuperscript{+} T-cell patterns. These candidate biomarkers of early efficacy for SLIT are currently being validated in larger cohorts of patients.

**Long-term efficacy biomarkers**

Several clinical studies have now documented the carryover effect (ie, the sustained efficacy) of SLIT, as well as its preserved efficacy after treatment cessation, leading to a true disease-modifying effect.\textsuperscript{11,18,19} As of today, no candidate biomarker of
long-term efficacy has been identified, which could support the decision to stop or resume SLIT according to measured levels of protective immune responses. When considering SLIT-induced immunomodulation over a 6-month to multiyear time frame, a reasonable assumption is to anticipate an effect at the level of adaptive immune responses, which rely on the induction and maintenance of long-lived allergen-specific memory T and B lymphocytes.

Changes in T-cell polarization, including an induction of Treg cells, have been reported during long-lasting SLIT regimens. However, as of today, no firm correlation between those changes and clinical benefit has been established at an individual patient level.68,72-75,78 As a matter of fact, allergen-specific Treg measurement in peripheral blood is unlikely to be used as a readout of long-term SLIT efficacy, given the known difficulty to standardize T-cell assays, and further, because the magnitude and quality of local T-cell responses (at the level of target mucosae), more than patterns of circulating CD4+ T cells, are critical to explain tolerance.83,84 In contrast, it will be interesting to document a potential decrease in the pool of circulating allergen-specific Th1/Th2 cells during sustained SLIT, which could deplete or down-regulate allergen-specific memory T and B cells. In support of this hypothesis, it is well documented that specific IgE levels are usually decreasing after 6 months to a year of SLIT, even if the clinical significance of such changes in seric IgE titers is unclear.6-8

For allergen-specific IgG4 induction, a 1-year SLIT course in patients with mite allergy induced such antibodies only in a subset (ie, 15%) of patients, irrespective of clinical benefit. Specific IgG4s reverted back to pretreatment levels within a year after treatment cessation, despite preserved clinical benefit.66 The latter results are in agreement with SCIT studies conducted in patients with grass pollen allergy, concluding that after treatment cessation, IgG4 titers decrease significantly, whereas an IgE-blocking activity persists, which correlates with clinical efficacy.86,87,100 I also recently documented a similar correlation at a cohort level between the induction of blocking antibodies and clinical benefit in patients with grass pollen allergy after 6 months of SLIT (manuscript in preparation). As suggested earlier, although all these studies focused on IgG4 as a source of blocking antibodies, a valid hypothesis is that allergen-specific secretory IgAs also contribute to establish long-term tolerance at mucosal surfaces.

**IMPLICATIONS FOR THE DEVELOPMENT OF NEW FORMS OF SLIT**

**Allergens in a native conformation**

The only commercially available products for SLIT are aqueous allergen extracts obtained from natural sources, administered at high doses in the absence of adjuvant.101 There is currently a major interest in developing second-generation allergy vaccines that are based on recombinant allergens.102,103 To this aim, hypoallergens (ie, forms of the allergen for which the IgE-binding capacity has been abrogated by point mutation or structural alteration) represent the most common approach for the development of subcutaneous vaccines.102 When considering the sublingual route, however, a more preferred approach is to rely on allergens in a natural conformation.101 The rationale behind this is to allow IgE binding and to address the allergen onto oral APCs that bear Fc receptors for IgEs, which as specified above, further reveal the tolerogenic phenotype of oral APCs.31 This concept has been tested in a phase I/II study conducted in patients with birch pollen allergy receiving sublingually recombinant Bet v 1103 at daily doses that range between 12.5 and 50 µg in a pre-coseasonal scheme. SLIT was well tolerated with only expected local reactions, and it reduced significantly symptom and medication scores compared with placebo, although no dose–effect relationship was noticed over the range of doses tested (my unpublished results). Importantly, the recent availability of such pharmaceutical-grade recombinant allergens should greatly facilitate the combination with vector systems or adjuvants.104

**Vector systems and adjuvants for the sublingual route**

Two classes of immunopotentiators can be used in association with the allergen with two distinct, nonmutually exclusive goals: (1) vector systems, to improve targeting of the allergen(s) onto LCs and/or CD11b+ myeloid DCs/macrophages located in upper layers of oral tissues before it reaches MCs and (2) adjuvants, to provide powerful signals to oral APCs via specific receptors to control the cytokine milieu in which the allergen is presented to resting T cells (Figure 1). Expected benefits of combining the allergen(s) to such immunopotentiators are multiple, including improving SLIT overall efficacy at decreasing symptoms (both in terms of size of the effect, faster onset, and longer duration of clinical improvement), simplifying administration schemes, and decreasing allergen dosing (thereby reducing local reactions).104

That vector systems can dramatically improve allergen uptake and, as a consequence, enhance SLIT efficacy in downregulating allergic inflammation has been established in murine ovalbumin-induced asthma models, using various vector systems.32-34 The latter include positively charged carbohydrate polymers, based on maltodextrin or chitosan, yielding mucoadhesive particles on which the allergen can be adsorbed, to bind efficiently to negatively charged epithelial cells. Besides enhancing the duration of allergen contact with the mucosa, those particulate vector systems also facilitate phagocytosis of the allergen(s) by oral APCs.32-34 In addition, the adenylate cyclase protein from Bordetella pertussis was successfully used as a vector after conjugation with the allergen to target it efficiently to oral APCs expressing the CD11b surface receptor.32 More generally, vectors targeting lectins or other surface receptors that allow endocytosis by APCs can also be considered.

The interest of combining allergens with adjuvants was also broadly documented in murine SLIT models.1 Several compounds affecting DC function to favor the induction of Treg and Th11 responses were identified, such as dexamethasone plus 1,25-dihydroxy vitamin D3, bacterial probiotic strains (eg, Lactobacillus plantarum, Lactobacillus helveticum, Bifidobacterium bifidum) as well as TLR2 (Pam3CSK4) or TLR4 (synthetic lipid A analogs) ligands.52,54,105,106 All those adjuvants were able to enhance allergen-specific tolerance in murine models of chronic allergic inflammation through at least two distinct mechanisms, that is, inhibition of the nuclear factor κB pathway of signaling (dexamethasone) and engagement of TLR2 and/or TLR4 (all other compounds). Interestingly, although all those molecules and bacteria share a property to act as Treg or Th11/Treg adjuvants, strict Th11 adjuvants did not bring any benefit when administered via the sublingual route.104 As of today, the only adjuvants tested to improve SLIT efficacy in allergic patients...
include the T141 adjuvant monophosphoryl lipid A (a well-known TLR4 ligand) and bacille Calmette-Guérin (BCG). Monophosphoryl lipid A has been shown to enhance allergen-specific IgG responses and to reduce reactivity to a nasal allergen challenge in patient with grass pollen allergy.107 Bacille Calmette-Guérin did not bring any obvious clinical benefit in children with mite allergen asthma, although noticeably it was administered via the intradermal route, whereas the allergen extract was administered sublingually.108

**Sublingual immunoglobulins**

Batard et al109 recently observed in a murine asthma model that immunoglobulins (ie, rat monoclonal IgG1s) administered sublingually in the absence of allergen(s) decrease both airway hyperresponsiveness and bronchoalveolar Eos infiltrates. Surprisingly, this anti-inflammatory activity is observed irrespective of the antigen specificity of the antibodies and does not correlate with a downregulation of circulating CD4\(^+\) T\(_{h}^\dagger\) cells nor with the induction of Treg lymphocytes.109 The therapeutic potential of sublingual immunoglobulins in asthma is consistent with the known anti-inflammatory effect documented in humans after intravenous administration of polyclonal immunoglobulins.110 Immunoglobulins are thought to mediate such anti-inflammatory properties through numerous antigen-specific or nonspecific mechanisms, raising the possibility of immunointervention at the level of oral immune cells expressing Fc receptors for IgGs (most particularly the inhibitory FcγRIIB receptor) to establish tolerance at mucosal surfaces.109

**PRACTICAL IMPLICATIONS FOR THE CLINICIAN**

**Patient selection for SLIT**

SLIT is currently indicated in those patients with mild-to-severe allergic rhinoconjunctivitis, or with or without mild asthma, poorly controlled with symptomatic treatments.14,23 Patients are classically selected on the basis of a positive skin prick test and/or a positive IgE in vitro assay (using as a threshold of >0.7 kU/L specific IgEs). The value of selecting patients on the basis of measurable levels of seric IgE titers was recently emphasized to offer SLIT only to patients likely to exhibit symptoms during natural allergen exposure.24 Importantly, in a cohort of patients allergic to grass pollens, SLIT was found to be equally efficacious in decreasing symptoms of patients, irrespective of whether they were sensitized to few (eg, group 1 and group 5) or multiple (ie, major and various minor) grass pollen allergens (manuscript in preparation). One theoretical drawback in treating patients with IgE sensitization to only a restricted pattern of allergens is less efficient.112 The efficacy of SLIT in patients with severe immunodeficiency or malignancy has not been documented. Even if SLIT is an allergen-specific immunomodulatory approach, little is known of the effect of immunotherapy on autoimmune diseases.113 Numerous embryofetal toxicology studies failed to show any effect of high doses of allergen(s) given orally on either fetal development or teratogenicity (my unpublished results). A recent prospective study concluded on the safety of SLIT in pregnant women,114 but the influence of pregnancy on oral tolerance induction remains to be investigated. The common practice both in Europe and in the United States is that SLIT can be continued, but is not usually initiated during pregnancy.14,23-115 It is also unclear as to whether significant amounts of the allergen(s) pass into the milk of lactating mothers. Collectively, caution should be exercised by clinicians when considering the use of SLIT in patients with an impaired immune system, in pregnant women, or in nursing mothers.

**SLIT in polysensitized patients**

A critical issue for the allergist is that most (ie, 55% to 75%) patients with allergic rhinoconjunctivitis are polysensitized; that is, they are sensitized to several non-cross-reactive allergens.116-118 Interestingly, several studies that used single allergen(s) based on closely related extracts (ie, a single or five-grass mix extract, or a birch pollen extract, or a mixture of Dermatophagoides pteronyssinus and Dermatophagoides farinae mix extracts) have unambiguously confirmed that SLIT is safe and efficacious in reducing symptom scores in both monosensitized and polysensitized patients.119-122 A trial that compared SLIT with either a birch or a grass pollen extract, or a combination of the two extracts in patients sensitized to the two allergens, concluded that SLIT with single extracts provides a clear symptom improvement, with a potential cumulative benefit when treating with the two-allergen extracts.123 Although it is generally admitted that simultaneous delivery of multiple unrelated allergens can be clinically effective, more studies are needed to document the efficacy of allergen immunotherapy, most particularly SLIT, when performed with a combination of more than two extracts.116,117 Although the practice in the United States allows for treating polysensitized patients jointly with an average of eight distinct allergen extracts,16 the recommendation in Europe is rather to perform single allergen SLIT with the most clinically relevant single allergen.14,115 Mixing several allergen extracts brings two potential issues, that is, (1) a risk of some allergen degradation when mixing heterologous extracts that contain a proteolytic activity and (2) an antigenic competition because of an excess of proteins provided in a single administration to allow optimal processing by the immune system.115 It is not known exactly how many complex allergen extracts can be handled at once by the oral immune system, but, as a comparison, antigenic competition has been evidenced in studies that evaluated the immunogenicity and efficacy of injectable pediatric vaccines combining proteins from six distinct infectious pathogens.124 This potential issue has also been raised in a recent SLIT study conducted in the United States, showing that immunomodulation with a single allergen (timothy grass pollen) might be impaired when it is combined with nine additional allergen extracts.125 In this context, for patients with symptoms caused by a limited number of allergens, a common practice in Europe is to treat them sequentially with single extracts.
administered separately during the day (waiting 10 to 15 minutes between the two administrations). 115

CONCLUSION AND PERSPECTIVES

A better understanding of immune mechanisms involved in allergen-specific sublingual immunotherapy confirms the pertinence of the sublingual route to induce antigen (or allergen)-specific tolerance in humans. It is now well documented that the oral/lingual immune system is prone to induce tolerance, as a default response to antigen(s) in the absence of danger signals. This unique property, likely selected during evolution to prevent inappropriate inflammatory or effector responses to numerous harmful molecules, appears mostly linked to the peculiar tolerogenic phenotype of oral DCs, programmed to orient immune responses toward a T<sub>reg</sub>/T<sub>reg</sub>-cell profile. Importantly, this tolerogenic bias of the oral immune system is fully preserved in all allergic patients.

The absence of a detectable systemic exposure of intact allergens explains in part the excellent safety profile of SLIT, with mostly local but only rare systemic adverse reactions. Together with the observation that no IgE neosensitization is elicited during SLIT, those features corroborate the safer profile of the sublingual route of immunotherapy compared with subcutaneous administration.

Importantly for the clinician, the safety and efficacy of SLIT in treating allergic rhinoconjunctivitis associated with major aeroallergens have been well documented by multiple large-scale placebo-controlled clinical studies. In light of the specific properties of the oral immune system, additional applications of SLIT to the treatment of asthma, food allergy, atopic dermatitis, and possibly autoimmune diseases are being, or will undoubtedly be, considered. In this regard, the combination of molecular engineering and innovative antigen delivery systems provides the opportunity to rationally design second-generation sublingual vaccines. Although some of those applications are to be considered only in the long term, biomarkers of SLIT efficacy easy to quantify in the blood may soon be available to help clinicians to select and monitor their patients.

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