

Immune mechanisms of allergen-specific sublingual immunotherapy

Sublingual immunotherapy has been shown in some clinical studies to modulate allergen-specific antibody responses [with a decrease in the immunoglobulin E/immunoglobulin G4 (IgE/IgG4) ratio] and to reduce the recruitment and activation of proinflammatory cells in target mucosa. Whereas a central paradigm for successful immunotherapy has been to reorient the pattern of allergen-specific T-cell responses in atopic patients from a T helper (Th)2 to Th1 profile, there is currently a growing interest in eliciting regulatory T cells, capable of downregulating both Th1 and Th2 responses through the production of interleukin (IL)-10 and/or transforming growth factor (TGF)- β . We discuss herein immune mechanisms involved during allergen-specific sublingual immunotherapy (SLIT), in comparison with subcutaneous immunotherapy. During SLIT, the allergen is captured within the oral mucosa by Langerhans-like dendritic cells expressing high-affinity IgE receptors, producing IL-10 and TGF- β , and upregulating indoleamine dioxygenase (IDO), suggesting that such cells are prone to induce tolerance. The oral mucosa contains limited number of proinflammatory cells, such as mast cells, thereby explaining the well-established safety profile of SLIT. In this context, second-generation vaccines based on recombinant allergens in a native conformation formulated with adjuvants are designed to target Langerhans-like cells in the sublingual mucosa, with the aim to induce allergen-specific regulatory T cells. Importantly, such recombinant vaccines should facilitate the identification of biological markers of SLIT efficacy in humans.

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Allergen-specific immunotherapy has been used in humans for almost a century with the aim to redirect inappropriate immune responses in atopic patients (1, 2). It has proven to be efficacious to treat type I allergies to a variety of allergens (3–8). While the parenteral (subcutaneous) route of immunization is still a reference, local routes (e.g. intranasal, oral) have been considered as an alternative with mixed results, both in terms of efficacy and tolerance (9). Of note, a specific form of oral tolerance induction, i.e. sublingual immunotherapy (SLIT) is raising a lot of interest as a noninvasive procedure, as it has been shown to be efficacious, provided that high doses of allergen (i.e. 50–100-fold the subcutaneous dose) are administered (10–25). In a recent meta-analysis, encompassing 22 clinical studies evaluating SLIT in 979 patients with allergic rhinitis to house dust mite, pollens (from grass, parietaria, olive, ragweed, cupressus) and cat dander, it was concluded that SLIT significantly reduces both symptoms and medication requirements (26). Importantly, it is now widely admitted that SLIT is much safer than subcutaneous immunotherapy (SCIT), with no evidence of anaphylactic shock recorded after more than 500 million doses administered to humans (12, 17, 27, 28). Whereas SLIT has

been successfully used to treat allergic patients, our understanding of the immunological mechanisms involved has been limited. We review herein recent scientific advances, which provide some clues on effector/regulatory immune mechanisms elicited during successful allergen-specific immunotherapy in general, and SLIT in particular. Based on such improved biological foundations, we comment on arising opportunities to design second-generation sublingual allergy vaccines relying upon well-characterized recombinant allergens, capable of controlling T-cell polarization following vaccine-mediated and subsequent natural exposure to the allergen.

Immunomodulation during allergen-specific immunotherapy

As summarized in Fig. 1, allergen-specific immunotherapy, whether it be SCIT or SLIT, is known to reduce both immediate as well as late-phase allergen-induced symptoms, by acting both on humoral, as well as on cellular immune mechanisms involved in allergic inflammation (4, 29–33). Schematically, three categories of immunological changes are induced by active immunotherapy, encompass-

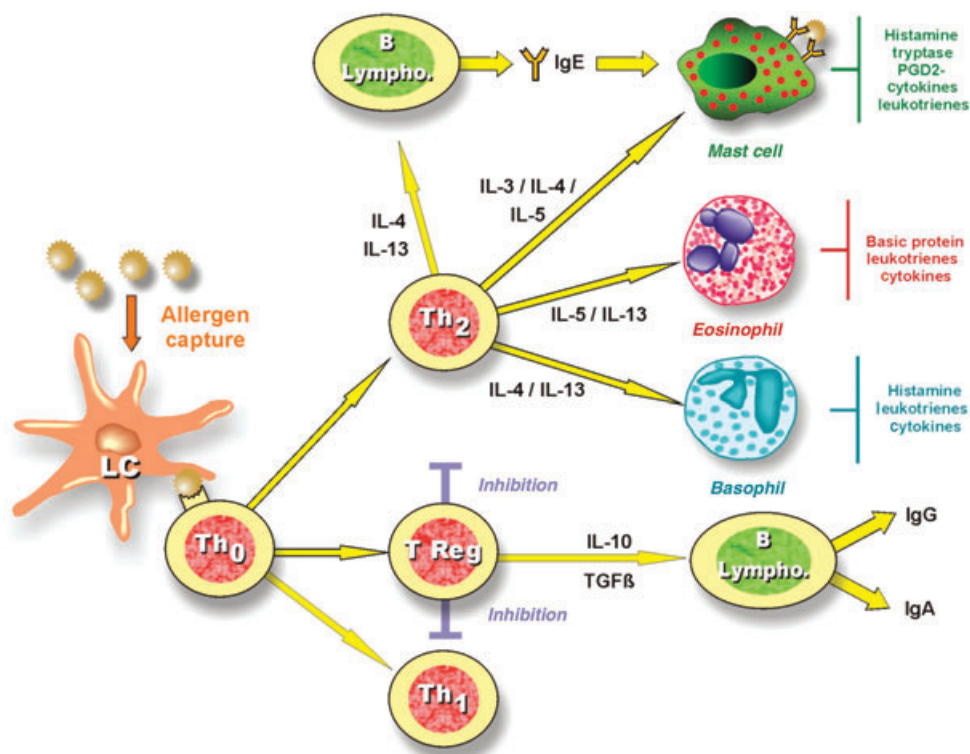


Figure 1. Humoral and cellular immune mechanisms involved in type I allergy and immunotherapy: an integrated view. Allergen-specific immunotherapy deals with the cause of type I allergies, by inducing long-term reorientation of an existing and inappropriate immune response (associated with Th2 cytokine production, high IgE levels, activation of mast cells, basophils and eosinophils leading to release of proinflammatory mediators such as histamine, eicosanoids and proteases). CD4⁺ T helper cells (e.g. Th1, Th2 or T regulatory) stimulated following allergen exposure or desensitization are critical in controlling these various components of the immune system through the production of distinct cytokines. IL-4 and IL-13 induce IgE class switching, IL-5 supports eosinophil recruitment and activation, and IL-13 increases mucus production. Regulatory T cells inhibit both Th1 and Th2 responses through the production of IL-10 and TGFβ. While atopic patients exhibit allergen-specific Th2 CD4⁺ cells, healthy people rather mount T reg responses when exposed to allergens. Thus, the purpose of allergen-specific immunotherapy is to restore tolerance by shifting T-cell responses from Th2 to T Reg.

sing (i) modulation of allergen-specific antibody responses; (ii) reduction in recruitment and activation of proinflammatory cells; and (iii) changes in the pattern of allergen-specific T-cell responses (Table 1). Although such biological changes are usually better documented with SCIT, there are, in this regard, no clear-cut qualitative differences between SCIT and SLIT, suggesting that immune mechanisms at play are similar (Table 1).

With respect to allergen-specific antibody responses, SCIT often induces an initial increase in seric immunoglobulin E (IgE) levels, prior to a subsequent downregulation in the following months (34–39). In patients allergic to grass pollen, SCIT prevents the seasonal rise in IgE antibodies associated with natural exposure to allergens (37, 39). Moreover, successful SCIT protocols resulting in clinical improvement of patients often elicit allergen-specific IgG responses (mostly IgG1 and IgG4) and in a few reported cases, IgA responses (Table 1) (5, 40–44). Similarly, SLIT was shown to increase allergen-specific IgG4 levels compared with placebo (10, 11, 13), with a more

limited impact on specific IgE responses (18). A decrease in the IgE/IgG4 ratio has been observed in a number of SLIT studies (11, 24, 45), with some exceptions (46). A meta-analysis of six SLIT studies with detailed analysis of antibody responses concluded on a consistent increase in allergen-specific IgG4 levels (26). Such changes in the IgE/IgG4 ratio were found to correlate with a decrease in the late-phase skin reaction to the allergen and with the overall clinical efficacy of the vaccine in some studies (23, 24) but not all (13, 18). In a recent phase I/II trial with grass pollen tablets, SLIT was shown to elicit allergen-specific seric IgAs in a dose-dependent fashion (47) and a small upregulation of IgA responses was also observed when SLIT was used in house dust mite allergic patients (45). Altogether, allergen-specific IgG (and IgA) antibodies induced by immunotherapy are thought to contribute to the positive clinical response through distinct and non exclusive mechanisms: (i) these antibodies can compete with IgEs for binding to the allergen, thereby preventing both basophil or mastocyte degranulation (38–40), as well

Table 1. Modifications of immunological parameters during allergen-specific immunotherapy (SCIT and SLIT) in humans

Parameters	Allergens used	Comments	Reference
Allergen-specific antibody responses (IgE, IgG1, IgG4, IgA)	Venoms, pollens (ragweed, birch, grasses, Parietaria, olive), dust mites Used as: – natural biological extracts, allergoids – recombinant protein (Bet v 1) associated with an adjuvant (e.g. CaPO₄, Alum, MPL) for SCIT – natural biological extracts, and allergoids without any adjuvant for SLIT	Subcutaneous immunotherapy <i>Immunoglobulin isotypes:</i> SCIT induces an initial increase, followed by a subsequent decrease of specific IgEs, as well as an increase in allergen-specific IgG1 and IgG4. An rise in IgG4/IgE ratio has been documented with hypoallergenic forms of recombinant Bet v 1 (peptide fragments and trimer). In some studies, but not all, changes in affinity of IgG4 antibodies for the allergen during immunotherapy have been shown. A monophosphoryl lipid A (MPL) adjuvanted vaccine reduces seasonally boosted IgE responses and induces strong IgG1 and IgG4 responses against grass-pollen allergens. SCIT elicits allergen-specific IgA responses detectable in the blood <i>Functional characterization of antibodies:</i> IgG4 exhibit some blocking activity. In some but not all studies, a correlation was found between IgG (mostly IgG4) concentrations and clinical outcomes. IgG4 antibodies reduce allergen T-cell responses by inhibiting binding of allergen-IgE complexes to antigen presenting cells Sublingual immunotherapy <i>Immunoglobulin isotypes:</i> SLIT against house dust mite or grass pollen increases specific IgEs and IgG4, in association with an improvement of the respiratory function. Several studies with SLIT report induction of IgG4 responses, without any increase or decrease of IgEs (i.e. $\nearrow$ of IgG4/IgE ratio). For example, SLIT with a <i>Parietaria judaica</i> extract in children reduces rhinitis symptoms and both skin and conjunctival allergen reactivity in parallel with increased specific IgG4 seric antibodies. In contrast, preseasonal rush SLIT against <i>P. judaica</i> rhinoconjunctivitis or SLIT in asthmatic children sensitized to mites had no detectable impact on specific IgE or IgG4 levels, despite improvement of symptom and drug scores. A phase I/II study conducted with grass pollen (<i>Phleum pratense</i>) tablets documented a dose dependent induction of allergen specific IgG4 and IgA responses. A meta-analysis conducted on six SLIT studies concluded that the combined standardized mean differences (SMD) between treated patients and controls for changes in allergen-specific IgE was 0.22 ($P = 0.06$), whereas SMD was 0.6 ($P < 0.00001$) for allergen-specific IgG4	5, 34–40, 44, 83 40–44, 48, 49 10, 11, 14, 23, 24, 26, 45, 47
Recruitment and activation of proinflammatory cells (mast cells, eosinophils and basophils)	Pollens (ragweed, grasses, Parietaria), dust mites Used as: – natural biological extracts, allergoids associated with an adjuvant (e.g. CaPO₄, Alum) for SCIT – natural biological extracts, allergoids recombinant protein (Phl p 1, Bet v 1) without any adjuvant for SLIT	Subcutaneous immunotherapy <i>Impact on cell recruitment:</i> SCIT reduces the recruitment into the skin and mucosa (nose, lung) of inflammatory cells including mast cells, eosinophils, basophils, neutrophils <i>Impact on proinflammatory mediators:</i> a decrease in seric eotaxin was found to be associated with successful IT. SCIT decreases the release of inflammatory mediators (e.g. histamine, kinins, PGD₂) by basophils and mast cells Sublingual immunotherapy <i>Impact on cell recruitment and activation:</i> Rush SLIT to Parietaria induces a reduction in neutrophils and eosinophils recruitment as well as ICAM-1 expression after eye or nasal challenge. In correlation with a reduction in both symptom and drug intake scores. Sublingual challenge with increasing doses of recombinant Phl p 1 or Bet v 1 shows limited reactivity with mast cells <i>Impact on proinflammatory mediators:</i> SLIT in children with grass pollen allergy decreases nasal eosinophil cationic protein levels and prevents increase in tryptase following allergen-specific challenge. In children allergic to <i>D. pteronyssinus</i>, SLIT reduces seric ECP and prolactin levels (the latter likely associated with a reduced activation of T lymphocytes). A long-term immunological effect of SLIT is to reduce residual signs of persistent inflammation (decrease in soluble ICAM-1, in mucosa, which correlates with a decrease in metacholine responsiveness)	50–60 63, 65, 66 61–65

Table 1. Continued

Parameters	Allergens used	Comments	Reference
T-cell responses and cytokine patterns	Venoms/PLA2, dust mites, pollens (ragweed/Amb a 1, grasses), cat dander/Fel d 1 Used as: – natural biological extracts, allergoids, purified natural allergen (Amb a 1) or peptides associated with an adjuvant (e.g. CaPO4, Alum, CpGs) for SCIT – natural biological extracts, without any adjuvant for SLIT	Subcutaneous immunotherapy <i>Impact on the Th1/Th2 ratio:</i> SCIT downregulates allergen-specific Th2 responses (both in terms of cytokine production and proliferation) with or without stimulation of allergen-specific Th1 cells. Rapid <i>in vitro</i> allergen-induced Th-2 lymphocyte apoptosis has been observed in one study. Increase in signaling lymphocytic activating molecules (SLAMF) gene expression during pollen IT. In some studies, Th2 responses are increased during the titration phase, and the Th2/Th1 ratio is only significantly decreased during the maintenance phase. Modification of Th2/Th1 ratio correlates with clinical improvement, decrease in late-phase skin reactions and allergen-specific IgE levels. Association between allergens (e.g. Amb a 1) and oligo CpG immunostimulatory sequences, used as a mix or as conjugate vaccines was found to decrease Th2 and to enhance Th1 responses <i>Modification of local T-cell responses:</i> decrease of Th2 cytokines (IL-4/IL-5/IL-3) and increase of Th1 cytokines (IFN-γ, IL-12), at local levels (e.g. in the skin following intradermal grass pollen challenge). Decrease in recruitment in target organs of Th2 CD4⁺ T cells expressing the CXCR1 receptor <i>Impact on regulatory T cells:</i> Induction of CD4⁺, CD25⁺ T regulatory lymphocytes and Tr1 IL-10 producing cells, in the blood. An increase in IL-10 mRNA and protein is detected in skin biopsies from allergen challenged subjects following wasp venom immunotherapy. Some of these regulatory T cells exhibit a CD62L⁺ CCR5⁺ profile suggesting a capacity to migrate to peripheral tissues. Early or sustained increase of regulatory cytokines (IL-10 and TGF-β, respectively) is proposed to correlate with successful immunotherapy. <i>Studies conducted with peptides:</i> studies conducted with peptides (Fel d 1, PLA A2) demonstrate a skewing of the Th1/Th2 ratio towards the former, induction of SOCS2 and of IL-10-producing cells. Injections of small (16 to 17-mer) Fel d 1 peptides inhibit the cutaneous late-phase reaction to whole cat dander, as well as allergen-stimulated production by PBMCs of both Th1 (IFN-γ) and Th2 (IL-4, IL-13) cytokines, concomitant with an increase in IL-10 production Sublingual immunotherapy <i>Impact on the Th1/Th2 ratio:</i> in adults with seasonal rhinoconjunctivitis, SLIT with a grass pollen extract had no detectable effect on sublingual infiltration by CD3⁺ T cells or local production of IL-12. No stimulation of T-cell proliferation or cytokine production in children allergic to grass pollen, despite a positive rescue on medication use. A decrease in seric IL-13 and T-cell proliferative responses (associated with an effect on symptoms) has been observed without any detectable change in Th1/Th2 ratio, in children treated with <i>D. pteronyssinus</i> extracts <i>Impact on regulatory T cells:</i> In a preliminary report, induction of circulating IL-10 producing peripheral T cells in house dust mite allergic patients	53, 66–80, 82, 112 81, 112 83, 84, 90, 112, 148 4, 79, 82 23, 46, 86 87

as allergen capture and presentation to T lymphocytes by Fc ϵ RI⁺ and CD23⁺ antigen-presenting cells (APCs) (48, 49); and (ii) such antibodies may act as blocking antibodies by engaging low-affinity Fc receptors for immunoglobulins (e.g. Fc γ RII) expressed by B lymphocytes, basophils, or mast cells. As Fc γ RII receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIM), they transduce, as a consequence, negative signals preventing cellular activation and release of soluble proinflammatory mediators following co-aggregation with Fc ϵ RI receptors (42, 43).

In a number of studies, SCIT was shown to inhibit both the recruitment and activation in mucosa of proinflammatory cells involved in the allergic reaction (50–60). For example, successful SCIT has been associated with a decrease in the recruitment of mast cells, basophils and eosinophils in the skin, nose, eye and bronchial mucosa, following provocation or natural exposure to allergens (Table 1). Similarly, SLIT prevented the recruitment of eosinophils in the eye or in the nose after allergen challenge (61–65). SLIT with grass pollen extracts was shown to decrease local or systemic levels of eosinophil cationic protein (ECP), without any increase in tryptase (61, 62). Similarly, rush SLIT to parietaria reduced the recruitment of neutrophils and eosinophils to the nasal mucosa as well as the expression of the intercellular adhesion molecule-1 (ICAM-1) (66).

In the context of an emerging integrated picture of the physiology of immune responses, the aforementioned changes in immune parameters are assumed to be a direct consequence of an impact of immunotherapy on CD4⁺ T-cell responses (Fig. 1). It is well known that allergic patients usually mount strong allergen-specific CD4⁺ T-cell responses of the T helper (Th)2 type, characterized by the secretion of high amounts of interleukin (IL)-4, IL-5 and IL-13 cytokines (30, 37, 67). In this regard, a central goal for immunotherapy has been to reorient allergen-specific T-cell responses in atopic patients from a Th2 to Th1 profile [the latter being rather associated with the production of interferon (IFN)- γ and IL-12 cytokines] (53, 68–80). A number of studies have indeed correlated successful SCIT with the induction of Th1 responses and/or the decrease of Th2 cytokine production (thus, it is the Th1/Th2 balance which appears critical). Interestingly, in a recent SCIT study, the Th2/Th1 ratio was found to increase during the titration phase, prior to decreasing during the maintenance phase, in parallel with late-phase skin reactivity and allergen-specific IgE levels (70). In several SCIT studies with grass pollen, a switch from a Th2 to a Th1 profile was not consistently observed at a systemic level, but was rather detected locally (i.e. within the nasal mucosa or the skin) (53, 69, 81). This observation emphasizes the importance of documenting immune changes not only in peripheral blood, but also locally in target organs. More recently, it has been shown that SCIT induces a new subset of CD4⁺ Th cells, called regulatory T cells, which exhibit a capacity to downregulate both Th1 and Th2 responses through the production of IL-10 and/

or transforming growth factor (TGF)- β (see below) (50, 82–85). There is less evidence on the impact of SLIT on T-cell responses. In several studies conducted in children or adults with seasonal allergic rhinoconjunctivitis to grass pollen, no significant effect of SLIT on T-cell functions (i.e. cytokine production, proliferation) was observed (23, 46). SLIT does not induce any detectable changes in the numbers of dendritic cells (DCs) nor T lymphocytes in the epithelium or lamina propria of the oral mucosa (14). Immunization through the sublingual route was nevertheless shown in other studies to decrease the production of the Th2 cytokine IL-13 and the proliferation of PBMCs from patients allergic to house dust mite (86, 87). As of today, there is still no firm evidence that SLIT can induce regulatory T cells. A preliminary study suggests that SLIT increases IL-10 production in peripheral blood mononuclear cells (PBMCs) from house dust mite (HDM) allergic patients following *in vitro* stimulation with *Dermatophagoides farinae* antigens, but also with recall antigens (e.g. *Candida albicans*) or PHA, when compared with untreated allergic patients (88). That some IL-10-secreting T cells are not allergen-specific raises the possibility of a bystander immunosuppressive effect of SLIT. Of note, high-dose SLIT regimens with ovalbumin in mice induces oval-specific T cells producing TGF- β in the spleen of sensitized animals (L. Van Overtvelt, P. Moingeon, unpublished results). Further studies are needed to document unambiguously regulatory T cells induction during SLIT.

Regulatory T cells and allergy vaccines

Although both anergy and T-cell depletion are known to contribute to the establishment of peripheral tolerance against environmental antigens, it is now broadly admitted that antigen-specific T-cell populations with suppressive/regulatory function play a key role in controlling immune responses to both self- and nonself-antigens (89–95). These cells, termed regulatory T cells, are heterogeneous, and include both: (i) naturally occurring CD4⁺CD25⁺ T cells and (ii) cells induced in the periphery following antigen exposure (e.g. Tr1 cells, Th3 cells, and CD8⁺ regulatory T cells). These various subsets of regulatory T cells can be distinguished on the basis of their surface markers and the pattern of cytokines they produce (Table 2). Antigen/allergen presenting DCs play a critical role in the induction of T-cell-mediated tolerance, in that immature DCs in the absence of proinflammatory signals and possibly subsets of specialized DCs can both support the differentiation of regulatory T cells (96, 97). Regulatory T cells are usually anergic with a low spontaneous proliferation rate, and are highly dependent on exogenous IL-2. They can downregulate both Th1 and Th2 immune responses against viruses, bacteria, parasites and allergens (89–92), either by direct cell–cell contact (involving PD1, membrane-bound

Table 2. Characteristics of naturally occurring and induced CD4⁺ and CD8⁺ regulatory T cells

	Naturally occurring T _R cells CD4 ⁺ CD25 ⁺	Peripherally induced T _R cells Tr1 and CD4 ⁺ CD25 ⁺ /–	Peripherally induced T _R cells CD4 ⁺ Th3	CD8 ⁺ T Reg
Surface markers				
CD25	+++	– to ++	++	+
GITR	++	–	?	
ICOS	++	++	?	
CTLA-4	+++	+	++	–
Other markers	CD103, LAG3, CD122, neurophilin1	CD122, T1-ST2		
Foxp3	++	– (+/– after activation)	?	
CD45 RB low	+	+	+	+
CD45 RO	+	+	+	
Cytokine secreted				
IL-10	+/-	++++	+/-	++
TGF-β	+/-	++	++++	+/-
IL-4	–	+/-	+/-	
IL-5	–	++	?	
IL-2	–	+/-	–	
IFN-γ	+/-	++	+/-	
	Cell contact (CTLA-4, PD1) IL-10, TGF-β (<i>in vitro</i>)	IL-10, TGF-β Cell contact (CTLA-4, PD1)	TGF-β	IL-10, TGF-β
Suppressive mechanism(s)				
Differentiation/induction factor(s)	Immature or tolerogenic DCs TGF-β	Immature or tolerogenic DCs IL-10, IFN-α IL-4, anti-CD3/anti-CD46, VitD3/ dexamethasone, α4β7 ⁺ T _R	Immature or tolerogenic DCs TGF-β, IL-4, IL-10, α4β1 ⁺ T _R	Immature or tolerogenic DCs?
Induction by allergen-specific immunotherapy	++ (SCIT)	+++ (SCIT, possibly SLIT)	+++ (oral IT), likely SLIT -swallow	Unknown

TGF-β or CTLA4 molecules) or through the production of immunosuppressive cytokines such as TGF-β (Th3 cells), or IL-10 (Tr1 cells) (Table 2) (90–92, 98, 99).

Naturally occurring CD4⁺CD25⁺ regulatory T cells represent 5–10% of peripheral CD4⁺ T cells in healthy mice and humans. They are mainly thymus-derived, but can also be induced in the periphery following sustained antigen or TGF-β stimulation of CD4⁺CD25[–]-naïve T cells (99). These cells express the forkhead/winged helix transcription factor Fox p3, which appears to play a key role in supporting their regulatory function (100).

Type 1 regulatory (Tr1) T cells have a low-proliferative capacity, produce high levels of IL-10 and TGF-β and low levels of IL-2 and IL-4. Tr1 cells can be generated *in vitro* by stimulating naïve CD4⁺ T cells in the presence of IL-10, with/without IFN-α (101), vitamin D3 plus dexamethasone (102) or a combination of anti-CD46 plus anti-CD3 antibodies (103). CD4⁺CD25⁺ regulatory T cells expressing the α4β7 integrin can also convert naïve CD4⁺ T cells into Tr1 cells (104).

T helper type 3 (Th3) cells which produce TGF-β, IL-4 and IL-10 are induced following oral administration of the antigen (105). *In vitro*, CD4⁺CD25⁺ regulatory T cells expressing the α4β1 integrin have been shown to convert naïve CD4⁺ T cells into Th3 cells (104).

As of today, there is a growing evidence supporting the role of regulatory T cells in controlling the development of asthma and allergic disease in a variety of models

(Fig. 2), although it is not clear yet which of the various regulatory T cell subsets are the most important in this regard (85, 92, 94). A revised version of the hygiene hypothesis proposes that a limited exposure to infectious pathogens during infancy, most particularly telluric mycobacteria and parasites, may prevent the establishment of not only a Th1, but also a T reg repertoire, thereby explaining in part the observed increase in prevalence of allergies in developed countries (106). Several studies document an association between atopy and a deficit in T reg functions. For example, children born with a dysfunctional Fox p3 gene exhibit a deficit in CD4⁺CD25⁺ regulatory T cells and develop severe autoimmune diseases often associated with eczema, elevated IgE levels, eosinophilia and food allergy [the polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX) syndrome] (107). Moreover, for at least some atopic subjects with active disease, the suppressive activity of CD4⁺CD25⁺ regulatory T cells is significantly decreased *in vitro* when compared with nonatopic individuals, potentially explaining the loss of tolerance against allergens (108). In patients allergic to birch pollen, a functional deficit has also been observed in CD4⁺CD25⁺ regulatory T cells, which appears to peak during the pollen season, and impacts the capacity of such cells to inhibit Th2, but not Th1 responses (109). Moreover, DCs from children with allergic rhinitis can be impaired in their capacity to produce IL-10 (110).

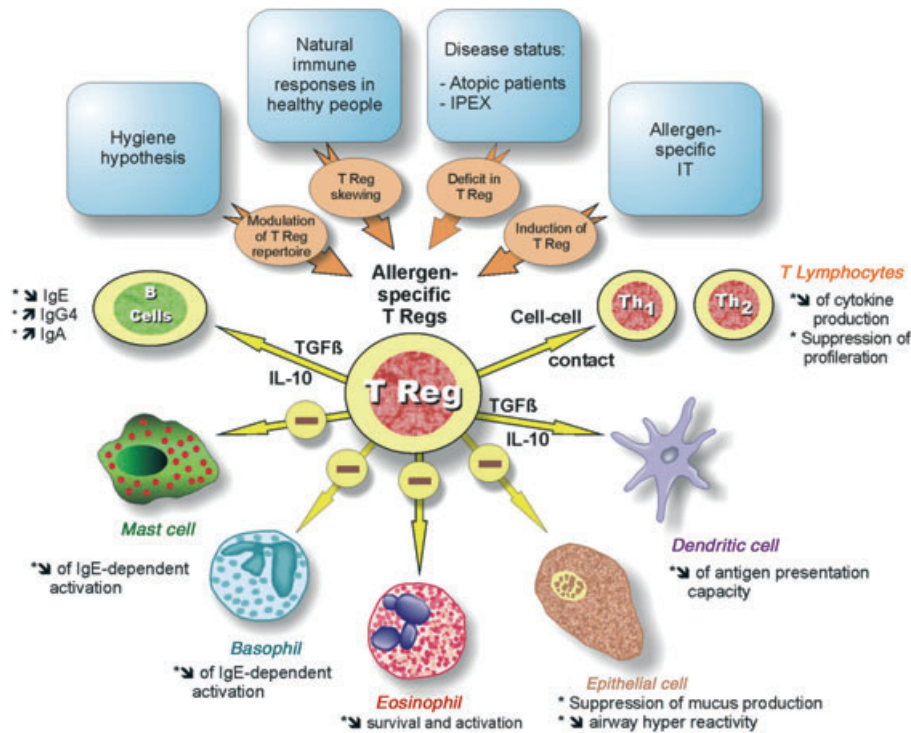


Figure 2. Regulatory T-cell dynamics in allergy. Regulatory T cells producing IL-10 and/or TGF- β are induced not only in atopic patients by successful immunotherapy, but also during natural allergen exposure in healthy people. As per the hygiene hypothesis, limited exposure to bacteria and parasites in developed countries may result in a poor establishment of a T reg repertoire during childhood, thereby contributing to an increase in the frequency of allergies. Certain atopic patients have a deficit in their T reg function, although several SCIT studies have shown that it is possible to induce allergen-specific regulatory T cells in atopic patients. Regulatory T cells can control and regulate all effector mechanisms activated during allergy and Th2 responses through the production of IL-10/TGF- β and/or cell-cell contact. IL-10 is a potent suppressor of total and allergen-specific IgEs, whereas it induces an antibody isotype switch towards IgG4. TGF- β also decreases IgE production and induces immunoglobulin isotype switch towards IgA. IL-10 and TGF- β act directly or indirectly on human airways to decrease both mucus production and airway hyper-reactivity.

Interestingly, allergen-specific IL-10-secreting Tr1 cells are highly represented in healthy individuals in comparison with allergen-specific IL-4-secreting Th2 cells, suggesting that regulatory T cells are predominant during natural immune responses to environmental allergens in nonatopic donors (111). Natural Tr1 responses are also involved in establishing phospholipase A2 tolerance in beekeepers exposed to multiple stings (112, 113). In contrast, this pattern of T-cell responses is reversed in allergic individuals with a heavy skewing towards Th2 relative to T reg responses (111, 114). A functional role of regulatory T cells has been demonstrated *in vivo* in mice, on the basis of adoptive transfer experiments showing that allergen-specific CD4⁺CD25⁺ cells or Tr1 clones inhibit allergen-induced Th2 responses and IgE production, as well as airway eosinophilia (90–92).

Regulatory T lymphocytes can control an established allergic response via distinct mechanisms (Fig. 2): IL-10 and TGF- β decrease IgE production and enhance IgG4 and IgA production, respectively. Both cytokines lower the release of proinflammatory mediators by downregulating IgE-dependent activation of basophils and mast

cells and by decreasing survival and activation of eosinophils. IL-10 and TGF- β also inhibit the production of Th2 cytokines such as IL-4 and IL-5 (50, 90, 113). In addition, regulatory T cells exhibit a direct inhibitory effect on Th1 and Th2 T cells, through cell–cell contact, or by decreasing the antigen presenting function of DCs.

Recently, several successful immunotherapy studies conducted through the subcutaneous route in patients with either grass pollen, house dust mite or venom allergy have shown an induction of various types of regulatory T cells including CD4⁺CD25⁺ regulatory T cells (84, 90, 115) or IL-10-producing CD4⁺CD25⁺ Fox p3-Tr1 cells (82, 83). Importantly, the suppressive activity of regulatory T cells induced in the course of immunotherapy appears to be allergen specific (83). Collectively, these studies suggest the importance of stimulating allergen-specific T regs during immunotherapy. They also prove that it is feasible to elicit regulatory T cells from a pool of CD4⁺CD25⁺-naïve T-cell progenitors in allergic patients, some of which may present a deficit in their T reg function and repertoire. Early IL-10 production (within days) and sustained TGF- β levels (over a year after

immunization) have been proposed as potential biological correlates for successful immunotherapy (90).

Oral mucosa and immune responses

SLIT and induction of peripheral tolerance

Sublingual immunotherapy takes advantage of an important physiological mechanism (i.e. oral tolerance), which has been evolutionarily conserved to ensure immune tolerance to various antigenic stimuli from the environment, especially from food and commensal bacteria (116). During SLIT, as for immunization at any mucosal surface, the allergen is captured locally (i.e. within the oral mucosa) by Langerhans-like DCs following either phagocytosis, macropinocytosis or receptor-mediated endocytosis. Subsequent to allergen capture, DCs mature and migrate to proximal draining lymph nodes (e.g. submaxillary, superficial cervical and internal jugular), as a consequence of changes in expression of surface receptors (e.g. the CCR7 chemokine receptor) involved in adhesion and trafficking (Fig. 3) (117, 118). Those lymph nodes represent specialized microenvironments favoring the induction of mucosal tolerance through the production of blocking IgG antibodies (IgG2b in mice) and the induction of T lymphocytes with suppressive function (119, 120). Importantly, the magnitude of CD4⁺ T-cell responses elicited within lymph nodes is directly proportional to the number of allergen-carrying DCs that migrate to lymph nodes, which clearly represents a limiting step (121). Eventually, as a consequence of the circulation of allergen-specific activated effector T cells throughout the body and the persistence of memory cells, a local (i.e. sublingual) administration of the allergen during desensitization results in both systemic and mucosal protective immune responses.

Dendritic cells in the sublingual mucosa exhibit morphological characteristics of Langerhans cells, including the presence of intracytoplasmic Birbeck granules (122). Interestingly, Langerhans-like cells from the oral mucosa constitutively express both low- (CD23) and high- (FCεRI) affinity receptors for IgEs, which may facilitate IgE-mediated allergen capture in atopic individuals (122). Perhaps, more importantly, upon engagement of such IgE receptors, oral Langerhans-like cells produce IL-10, TGF-β and upregulate indoleamine 2-dioxygenase (IDO), a rate-limiting enzyme-metabolizing tryptophan, thereby resulting in a decrease in T-cell proliferation (122, 123). As discussed above, there is still no formal evidence of T reg induction via the sublingual route. Nevertheless, on the basis of its aforementioned characteristics, the immune system in the oral mucosa appears prone to induce active tolerance mechanisms against allergens and antigens from the environment. Consistent with this, there is preliminary evidence that SLIT elicits IL-10-producing T cells in humans (88) and antigen-specific TGF-β⁺ T cells in murine (P. Moingeon, T. Batard, R. Fadel, F. Frati, J. Sieber, L. Van Overtvelt, unpublished results). The latter

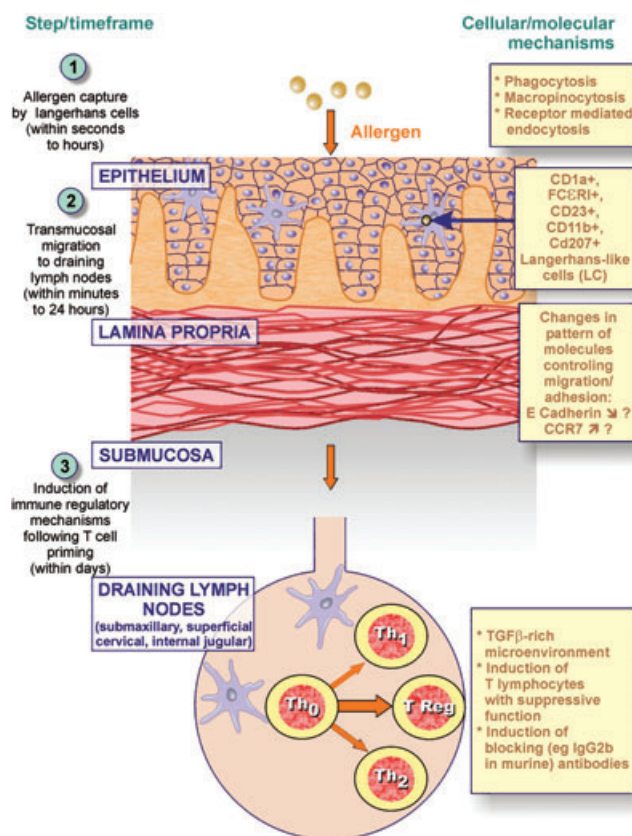


Figure 3. Oral mucosa as an immunocompetent site. Macromolecules such as allergens exhibit a limited direct absorption through the oral mucosa. This mucosa is rich in Langerhans-like dendritic cells expressing constitutively high-affinity receptors for IgE (FCεRI). Following engagement of such receptors, DCs produce IL-10 and TGF-β and upregulate IDO. These cells are specialized in antigen or allergen capture and are instrumental in stimulating T lymphocytes following migration to regional lymph nodes. Such lymph nodes, including submaxillary, superficial cervical and internal jugular lymph nodes represent a favorable environment for IgG responses (blocking antibodies of the murine IgG2b isotype) as well as for the stimulation of T cells with suppressive functions. For those reasons combined, immune responses elicited following oral immunization are biased towards tolerance induction. The oral mucosa contains few or no mast cells, eosinophils and basophils, and the integrity of the lamina propria is likely key in limiting contact of allergens with such cells in submucosal tissues or in the blood, thereby explaining the excellent safety profile of SLIT.

observation is consistent with the fact that in a SLIT-swallow protocol, allergen exposure directly to the gut immune system is expected to stimulate a Th3 response (105).

Pharmacokinetics and pharmacodynamics of the sublingual route

During SLIT, the vaccine is kept under the tongue for 1–2 min and then swallowed (as per the sublingual/

swallow procedure). When the vaccine is immediately swallowed, clinical efficacy is substantially decreased (22, 124). On the basis of human studies demonstrating that a limited therapeutic effect is observed when the vaccine is spat out, it has been proposed that sublingual-swallow immunotherapy is more efficient than sublingual-spit because it enhances the duration of contact of the allergen with the oral mucosa (124). Whereas immunomodulation is initiated within minutes following contact with the oral mucosa, allergens absorbed in the gastrointestinal tract also probably contribute to the efficacy of SLIT.

The tissue under the tongue is highly vascularized, with blood vessels draining directly into the jugular vein. As a consequence, small synthetic drugs administered sublingually are quickly absorbed and enter the bloodstream without passing through the intestine and the liver (125, 126). For example, the sublingual route is commonly used in humans to administer the vasodilator nitroglycerin (glyceryl trinitrate), as a treatment for angina pectoris, leading to a plasmatic peak within 5 min with an overall bioavailability of approximately 70% (125). Similarly, sublingual administration of the opioid analgesic buprenorphine yields a bioavailability of up to 55% through the sublingual route, in comparison with 15% obtained after direct ingestion (126). In contrast with these observations made with small synthetic molecules, biodistribution studies in humans demonstrated that there is no significant direct absorption of peptides or proteins – including allergens – through the sublingual mucosa (127–130). Following administration of the radiolabeled *Parietaria judaica* pollen allergen (Par j 1), either as an orosoluble tablet or a solution kept in the mouth for 1–1.5 or 20–30 min, respectively, no direct absorption into the blood was detected (129, 130). Only after swallowing and contact with the gut mucosa, the radiolabeled Par j 1 allergen got distributed rapidly in the blood, with a plasmatic peak detectable within 2 h (129). Such biodistribution studies also suggested that significant amounts (e.g. 20% of the administered dose) of the allergen can persist for 2 h on the sublingual mucosal surface, even though patients were allowed to rinse their mouth extensively (129, 130). Consistent with this observation, a limited proteolytic activity against allergens is found in saliva, whereas in contrast exposure to gastrointestinal fluids results in complete allergen degradation (131–133).

As for SCIT, conventional SLIT protocols rely upon a build-up phase (with a gradual increase in the dosing during 4–6 weeks) and a maintenance phase (with administration of the maximum dose one to three times a week over several years). In the course of such immunization schemes, it has been proposed that both downregulation of proinflammatory cells and upregulation of blocking IgG antibodies as well as IL-10 production occur quickly (i.e. within days) following immunotherapy (16). In contrast, a detectable impact on Th1 and Th2

responses and adaptive immunity is rather thought to occur within months (16). Whereas immunological changes can be detected shortly following immunotherapy, a recent study has suggested that SLIT is efficacious on symptoms and medication after at least 1 year of therapy, with maximum benefit being observed only after 2 years (134). It should be emphasized, however, that several rush and ultrarush protocols for SLIT have demonstrated that it can induce a decrease in skin reactivity and a readily detectable clinical benefit within weeks or even days (16, 66, 135). Whereas little is known on the duration of SLIT-induced immunomodulation, several independent studies have suggested that SLIT in children with rhinoconjunctivitis to either grass pollen or HDM, prevents asthma as well as new sensitizations (20, 136, 137). Given that in some of these studies, a follow-up of up to 10 years after the initiation of SLIT was performed, it is likely that immune memory mechanisms are established following SLIT.

Why high doses for SLIT?

It is well established that SLIT requires more allergen (at least 50–100 times) than SCIT to reach the same level of efficacy (15). One explanation for this is that current immunotherapy protocols rely upon an adjuvant (e.g. calcium phosphate or aluminum hydroxide) for subcutaneous but not sublingual administration. Although it has been suggested that reaching a threshold cumulative dose of allergen is important for a successful SLIT, it is also possible that current vaccines do not target immune cells efficiently, most particularly DCs. Thus, high-dose regimens likely facilitate capture of sufficient amounts of allergens by sentinel DCs within the oral mucosa, which clearly represents a critical step to induce a strong and long-lasting T-cell response (121). Interestingly, numerous studies suggest that low or high doses of antigens exhibit a radically distinct effect on T lymphocyte stimulation, with low doses inducing Th2 responses, whereas higher doses of antigen rather stimulating Th1 CD4⁺ T cells (138, 139). Moreover, exposure to high doses of allergen during immunotherapy may promote the trafficking of Th1 over Th2 cells by modulating surface expression of adhesion molecules and chemokine receptors selectively on those cells (140). The influence of antigen dosage on regulatory T cells is presently unclear: initial studies suggested that small doses of antigen administered orally were efficient at eliciting IL-10⁺ TGF- β ⁺ Th3 cells in the gut (105). However, regulatory T cells are poorly reactive with antigen *in vitro*, raising the hypothesis that a strong antigenic stimulation may be necessary to induce proliferation and activation of such T cells. Importantly, high doses of antigen will provide a strong initial burst of T lymphocyte stimulation, leading to the establishment of T-cell-mediated immune memory and long-term tolerance (141).

SLIT safety profile

On the basis of extensive clinical experience, it is now firmly established that SLIT is very well tolerated both in adults and young children (22, 26, 27). Side effects are usually local, encompassing oral itching, swelling, and irritation, and occur usually within minutes, thus, likely implying an IgE-mediated reaction. As inflammatory markers such as tryptase or ECP are not increased locally during SLIT, IgE-mediated reactions occurring in the mouth following sublingual exposure to the vaccine are usually rather limited (62). Allergen-challenge experiments performed in humans with recombinant allergens (e.g. rPhl p 1, rBet v 1) have confirmed that the sublingual mucosa is at least 10 times less reactive than the nasal mucosa or the skin (142). This is likely explained by the fact that only limited numbers of proinflammatory cells (such as mast cells) are present in the oral mucosa. Thus, in contrast to SCIT, allergens exposed to the oral mucosa are not in direct contact with basophils from the blood or mast cells in tissues.

While SLIT efficacy clearly correlates with allergen dosage, high-dose regimens do not appear to enhance dramatically the frequency of systemic or local adverse events, in contrast to SCIT (28). Importantly, high doses of allergens have been shown *in vitro* to be less efficient than low doses in inducing the release of proinflammatory mediators by mast cells (143). In a phase I dose-finding study with sublingual grass pollen tablets, high-dose regimens were very well tolerated provided that a short-dose escalation was conducted prior to reaching the maintenance dose (144).

Implications for the development of second-generation sublingual vaccines

Based on our current understanding of immune mechanisms underlying allergen-specific immune responses, optimizing vaccines to control the type of T lymphocytes, and thus the pattern of cytokines induced during natural exposure to the allergen or vaccination, is critical. Second-generation vaccines could possibly be more efficacious if specifically designed to elicit a strong allergen-specific regulatory T-cell response without exacerbation of the Th2 allergic response, thereby limiting adverse reactions. For this, the sublingual route appears mostly appropriate given that FCεRI⁺ IL-10/TGF-β-producing sentinel DCs present in the oral mucosa are likely to be prone to induce tolerance, as opposed to immunostimulation.

While the first generation of sublingual vaccines currently used is based on natural biological extracts, new vaccines are being developed which rely upon selected recombinant allergens (145, 146). There is, as of today, considerable interest in developing hypoallergenic versions of target allergens in which IgE-binding epitopes

have been knocked down by site-directed mutagenesis or disruption of the three-dimensional structure of the molecule (145, 146). Such vaccines exhibit a reduced allergenic activity while retaining a capacity to elicit blocking IgG antibodies and T-cell recognition. While this approach looks promising for vaccines administered parenterally, a more appropriate strategy for SLIT is rather to rely upon recombinant allergens presented in the most native conformation (147), to allow IgE-mediated targeting and capture of allergens by FCεRI⁺ DCs in the oral mucosa of atopic patients.

Strategies based on biological or synthetic adjuvants and formulations to improve allergen presentation to oral Langerhans cells should be investigated to enhance SLIT efficacy, reduce allergen dosing, and simplify immunization schemes. T reg adjuvants specially designed for the sublingual route may (i) target the allergen to Langerhans-like cells; (ii) trigger the production of IL-10 and/or TGF-β by antigen-presenting cells while enhancing their capacity to migrate to local lymph nodes to ensure T cell priming; and (iii) facilitate the polarization of T-cell responses towards regulatory T cells by engaging surface receptors such as ICOS, CD46 or specific Toll-like receptors (e.g. TLR4).

Among many advantages, such molecularly defined vaccines will facilitate the identification of immunological correlates of clinical efficacy. Such biological surrogate markers would be extremely useful to ease the development of future vaccines, most particularly with the long-term goal of making vaccines tailored to patient-specific allergen sensitization patterns determined on the basis of component-resolved diagnosis. Importantly, whereas in the past the immunological follow-up of immunotherapy trials focused on humoral (IgE/IgG4) responses, tools and methods should rather be developed to monitor in detail allergen-specific CD4⁺ T-cell responses [e.g. using human leukocyte antigen (HLA) class II-peptide soluble tetramers, quantitative Elispot measurement of Th1/Th2/T reg cytokine-producing T cells, real-time PCR analysis of early events in T-cell polarization, etc.].

Altogether a better understanding of immune mechanisms involved in allergen-specific immunotherapy, combined with the power of molecular engineering and innovative antigen delivery systems should offer the opportunity to rationally design second-generation recombinant vaccines specifically tailored for the sublingual route. The development of such second-generation vaccines should help to confirm (or not) current working hypotheses, to improve vaccine efficacy, and to evaluate simpler administration schemes.

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