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Review

Mechanisms underlying the induction of regulatory T cells by sublingual immunotherapy

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ABSTRACT

Background: Sublingual immunotherapy (SLIT) is used for the treatment of type 1 allergies, such as allergic rhinitis. SLIT leads to tolerance against allergens possibly via the redirection of allergen-specific T helper 2 cells to T helper 1 cells and the generation of peripheral regulatory T (Treg) cells. However, the detailed mechanisms remain unclear. Systemic tolerance to orally administered antigens (oral tolerance) has been extensively investigated. Recent studies have recognized the central role of Treg cells and classical dendritic cells (cDCs) in oral tolerance development.

Highlight: This review focuses on recent advances in the understanding of the underlying mechanisms of SLIT compared with those of oral tolerance. The sublingual administration of soluble protein antigens has been reported to induce antigen-specific Treg cells in oral mucosa-draining submandibular lymph nodes in mice. The generation of Treg cells is critical for SLIT efficacy because the transfer of SLIT-induced Treg cells confers tolerance against the antigens. A large number of oral cDCs with the $CD103^{-}CD11b^{+}$ phenotype exert retinoic acid-producing activity and convert naïve $CD4^{+}$ T cells into $Foxp3^{+}$ Treg cells *in vitro* in a transforming growth factor-β-dependent and retinoic acid-dependent manner. Oral $CD103^{-}CD11b^{+}$ cDCs transport sublingual antigens to submandibular lymph nodes and induce antigen-specific Treg cells. Sublingual antigens enter the mucosa most likely by crossing the sublingual ductal epithelium and are captured by oral antigen-presenting cells, especially macrophages.

Conclusion: Oral $CD103^{-}CD11b^{+}$ cDCs are specialized for the induction of Treg cells in mice; thus, targeting their human counterpart may enhance the therapeutic effects of SLIT.

Contents

Abbreviations: ALDH, aldehyde dehydrogenase; APC, antigen-presenting cell; cDC, classical dendritic cell; cDC1, type 1 cDC; cDC2, type 2 cDC; IgA, immunoglobulin A; ILF, isolated lymphoid follicle; IRF, interferon regulatory factor; LC, Langerhans cell; LP, lamina propria; ManLN, submandibular lymph node; MesLN, mesenteric lymph node; PP, Peyer's patch; RA, retinoic acid; SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; TGF, transforming growth factor; Th, T helper; Treg, regulatory T

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1. Introduction

Allergen-specific immunotherapy, which induces tolerance to allergens by rebalancing the immune system, is a causal treatment of allergic disorders. The pioneering clinical trials were conducted by Noon and Freeman in 1911 [1,2]. At that time, allergy was not considered a hypersensitive reaction to allergens but a sensitive reaction to toxins, which were innocuous to normal individuals. Noon aimed to induce neutralizing antibodies against "pollen toxin" for the treatment of hay fever and thus selected the subcutaneous route of vaccination. Although the theoretical basis was incorrect considering the present knowledge, allergic symptoms were successfully reduced. The use of subcutaneous immunotherapy (SCIT) has rapidly increased, remaining as the standard method of treatment. However, SCIT requires repeated injections of allergens and has been associated with severe adverse effects, such as systemic anaphylaxis [3]. Alternative routes of administration have been investigated, particularly mucosal routes, which do not require injections. The sublingual route was first attempted by Scadding and Brostoff in 1986 [4]. Now, sublingual immunotherapy (SLIT) is proven to be a safe and effective treatment for type 1 allergies, such as allergic rhinitis and asthma [5,6]. SLIT is widely used in European countries and is increasingly used worldwide including North America, South America, and Asia. Despite its clinical efficacy, the underlying mechanisms of SLIT remain poorly understood.

The intestinal mucosa is continuously exposed to various foreign antigens derived from commensal microbes and food [7]. Nevertheless, the intestinal immune system is unresponsive to these innocuous foreign antigens; thus, acute allergic and inflammatory reactions are suppressed at the mucosa. The breakdown of this homeostatic tolerance may cause intestinal disorders, such as food allergies, celiac disease, and inflammatory bowel disease. Importantly, tolerance to innocuous antigens can be induced not only locally in the mucosa but also systemically. Systemic tolerance to orally administered antigens, known as oral tolerance, originates in the intestinal immune system and was first described by Wells and Osborne in 1911 [8]. They showed that guinea pigs fed a corn-containing diet became resistant to subsequent systemic anaphylaxis to zein, a key protein found in corn. Oral tolerance has been applied in various experimental models of allergy and autoimmune disorders including delayed-type hypersensitivity [9], rheumatoid arthritis [10], and type 1 diabetes [11]. Although it seems to be a promising approach, oral tolerance has not been successfully applied for clinical use in humans [12]. The mechanisms of oral tolerance have been extensively investigated compared with those of SLIT. Recent studies have highlighted the central role of regulatory T (Treg) cells and classical dendritic cells (cDCs) in oral tolerance development [13–15].

Similar to the intestinal mucosa, the oral mucosa is exposed to various foreign antigens derived from commensal microbes and food and has pro-tolerogenic properties [16]. Therefore, the effect of SLIT may be similar to that of oral tolerance. In this review, we describe the known mechanisms of oral tolerance, which would provide a framework to understanding how mucosal tolerance develops. In addition, we highlight the recent advances in the mechanisms underlying SLIT. The mechanisms described in this review could contribute to the understanding and improvement of SLIT.

2. Mechanisms of oral tolerance

The intestinal mucosa consists of three layers: the simple columnar epithelium, underlying connective tissue called the lamina propria (LP), and muscularis mucosa. The intestinal immune system can be divided into inductive and effector sites [7]. Inductive sites include the gut-associated lymphoid tissues, such as Peyer's patches (PPs), isolated lymphoid follicles (ILFs), and mesenteric lymph nodes (MesLNs). Effector sites consist of the intestinal LP and epithelium containing various activated lymphocytes. Among these anatomical sites, oral tolerance requires MesLNs as their surgical removal impedes the initiation of oral tolerance [17]. Moreover, oral tolerance requires the CCR7-dependent constitutive migration of cDCs from the LP to draining MesLNs [17]. PPs and ILFs are covered with the follicle-associated epithelium, which harbors M cells, a specialized antigen-sampling cell type. M cells sample luminal antigens and transport them to underlying immune cells. However, oral tolerance can be induced in the absence of PPs [18,19]. PPs and ILFs are important for immunoglobulin A (IgA) responses and regulating the commensal microbiota [20]. Luminal soluble antigens can enter the mucosa through the columnar epithelial cells [21]. The form of an intestinal luminal antigen may determine its uptake route and subsequent immune responses. Particulate antigens and bacteria are taken up by M cells and transported to PPs and ILFs, leading to local IgA responses, whereas soluble antigens are taken up by LP cDCs and transported to MesLNs, leading to oral tolerance [7].

Recent studies have demonstrated the central role of Treg cells in oral tolerance. Treg cells can suppress immune responses through multiple mechanisms including the contact-dependent suppression of effector cells and secretion of immunosuppressive cytokines, such as interleukin-10 and transforming growth factor (TGF)-β [22]. Treg cells were first described as $CD4^+CD25^+$ T cells. The transcriptional factor Foxp3 is required for the development and function of Treg cells, and its expression specifically marks Treg cells. Treg cells are divided into two major subsets: thymic Treg cells, which differentiate in the thymus, and peripheral Treg cells, which differentiate from naïve $CD4^+$ T cells in the periphery [23]. Thymic Treg cells are derived from precursors that recognize self-antigens with a high affinity, thus mediating the tolerance to self-antigens and protecting individuals from autoimmunity. Peripheral Treg cells may be needed for tolerance to foreign antigens. The lack of specific markers to distinguish these Treg cell subsets hinders the understanding of their respective contribution to immune tolerance. The oral administration of antigens has been reported to induce the conversion of antigen-specific naïve $CD4^+$ T cells into $Foxp3^+$ Treg cells in the gut-draining MesLNs [24,25]. Based on definitions, these $Foxp3^+$ Treg cells are peripheral Treg cells but not thymic Treg cells. Moreover, oral tolerance has been shown to depend on the generation of peripheral Treg cells in well-designed experimental settings [13,14].

Most intestinal LP cDCs express αE integrin (CD103) (Fig. 1). These $CD103^+$ cDCs transport orally administered soluble antigens to MesLNs in a CCR7-dependent manner [26]. Intestinal $CD103^+$ cDCs produce the vitamin A metabolite retinoic acid (RA), which acts as a cofactor in the TGF-β-mediated induction of $Foxp3^+$ Treg cells [24,25]. Intestinal $CD103^+$ cDCs highly express retinal dehydrogenase 2, which converts retinal to RA [24]. Moreover, RA induces the expression of gut-homing receptors α4β7 integrin and CCR9 on activated T cells [27]. TGF-β is ubiquitously expressed in cells and tissues and is produced as an inactive latent protein that exerts its function after being activated. TGF-β function may be better controlled at the level of its activation rather than at the level of its production [28]. Intestinal $CD103^+$ cDCs preferentially express αvβ8 integrin, which activates TGF-β, and the expression of αvβ8 integrin on these cDCs is required for Treg cell induction *in vitro* and *in vivo* [29,30]. Therefore, intestinal $CD103^+$ cDCs are specialized to induce gut-homing Treg cells.

Oral tolerance requires the gut homing of Treg cells after their generation in the MesLNs [14,31]. Moreover, MesLN-derived Treg cells undergo secondary expansion after arrival in the small

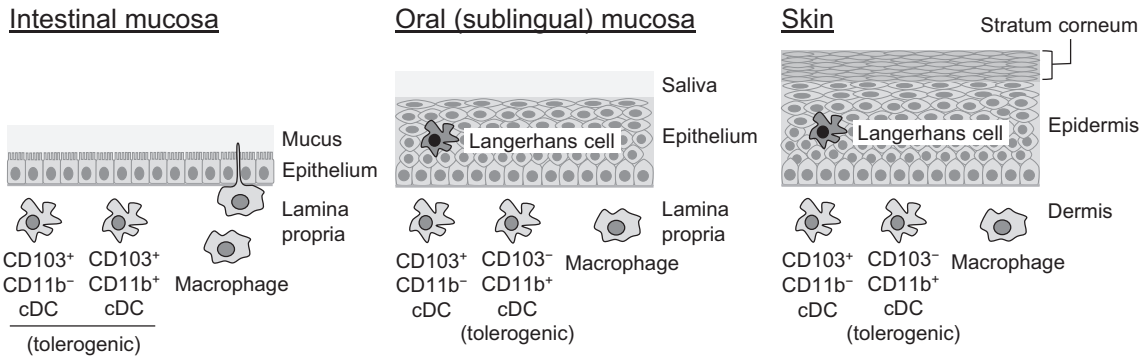

Fig. 1. Antigen-presenting cell (APC) subsets in the intestinal mucosa, oral (sublingual) mucosa, and skin of mice. In the intestine, classical dendritic cells (cDCs) and macrophages reside in the lamina propria (LP). Intestinal macrophages can form transepithelial dendrites to take up luminal antigens. Most intestinal cDCs express CD103 and are further classified into the $CD103^+CD11b^-$ and $CD103^+CD11b^+$ subsets. Skin APCs are classified as Langerhans cells (LCs) in the epidermis and cDCs and macrophages in the dermis. Similarly, oral APCs are classified as LCs in the epithelium and cDCs and macrophages in the LP. Skin and oral cDCs are further classified into the $CD103^+CD11b^-$ and $CD103^-CD11b^+$ subsets. Intestinal $CD103^+$ cDCs (especially the $CD11b^-$ subset) and skin and oral $CD103^-CD11b^+$ cDCs are retinoic acid (RA)-producing, regulatory T cell-inducing tolerogenic cDCs.

intestinal LP [14]. A proportion of these expanded Treg cells may exit the intestine and spread systemically, contributing to the systemic effects of oral tolerance [7].

Intestinal $CX3CR1^+$ macrophages sample luminal bacteria through transepithelial dendrites (Fig. 1) [32,33]. In addition, intestinal $CX3CR1^+$ macrophages efficiently take up luminal soluble protein antigens independently of the transepithelial dendrites [34]. However, they do not migrate from the LP to draining MesLNs and are unable to stimulate naïve T cells [34]. Intestinal $CX3CR1^+$ macrophages can contribute to oral tolerance by transferring fed antigens to neighboring cDCs [35]. Nevertheless, a recent study involving the depletion of either macrophages or cDCs showed that macrophages are dispensable, whereas cDCs are required for the induction of Treg cells and oral tolerance [15]. It is possible that intestinal macrophages may promote the secondary expansion of Treg cells by producing interleukin-10, contributing to the full establishment and maintenance of oral tolerance [7,14].

Recently, cDCs have been subdivided into interferon regulatory factor (IRF) 8-dependent type 1 cDCs (cDC1s) and IRF4-dependent type 2 cDCs (cDC2s) [36]. Intestinal $CD103^+$ cDCs consist of $CD103^+CD11b^-$ cDC1s and $CD103^+CD11b^+$ cDC2s; $CD103^+CD11b^-$ cDC1s are superior in inducing Treg cells (Fig. 1) [15]. The depletion of IRF8-dependent cDC1s partially prevents the generation of Treg cells but does not affect oral tolerance [15]. Therefore, cDC subsets play a hierarchical, though redundant, role in the induction of Treg cells and oral tolerance.

3. Mechanisms of SLIT

The oral mucosa structurally resembles the skin rather than the intestinal mucosa (Fig. 1). The oral mucosa consists of two main layers, the stratified squamous epithelium and underlying LP, which are the equivalents of the epidermis and dermis of the skin, respectively [37]. The epithelium of the sublingual mucosa is thin and not keratinized, making it an attractive site for drug delivery [38]. The oral mucosa has a unique distribution of antigen-presenting cells (APCs), which is variable depending on the mucosal sites. The numbers of $CD207^+$ Langerhans cells (LCs) in the epithelium and major histocompatibility complex class II^+ APCs in the LP are lower in the sublingual mucosa than in the buccal mucosa and dorsal surface of the tongue [39]. The submandibular lymph nodes (ManLNs), which are localized above the sublingual and submandibular glands in mice, drain the oral mucosa [40]. Mucosa-associated lymphoid tissues and M cells have not been detected in the oral mucosa. The oral mucosa is covered with saliva, which is secreted from salivary glands. These salivary glands are important IgA effector sites [41]. In the sublingual compartment, the sublingual duct opens to the sublingual caruncle and sublingual fold. Therefore, the oral/sublingual mucosa has its own unique anatomical characteristics, which would contribute to the mechanisms underlying SLIT.

SLIT has been successful for the treatment of allergic rhinitis and asthma [5,6]. Considering that oral tolerance can suppress various allergic and autoimmune disorders both locally in the mucosa and systemically, it is possible that the efficacy of SLIT may not be limited to type 1 allergies of the respiratory tract. Indeed, we and others have found that SLIT can suppress delayed-type hypersensitivity in mice [42,43]. Therefore, SLIT may have therapeutic potential in a broad range of allergic and autoimmune disorders.

Previous studies indicated that the redirection of allergen-specific T helper (Th) 2 cells to Th1 cells and the generation of Treg cells are vital for the therapeutic effects of SLIT [5]. We demonstrated that the transfer of $CD25^+$ Treg cells could confer tolerance against sublingual soluble antigens in naïve recipients [43]. Furthermore, we demonstrated that SLIT could induce the conversion of naïve $CD4^+$ T cells into $Foxp3^+$ Treg cells and interferon-γ-producing Th1 cells in ManLNs but not MesLNs, suggesting that although sublingual antigens may be swallowed, they are mostly absorbed into the sublingual mucosa and not (or to a much lesser extent) into the intestinal mucosa [43]. Collectively, SLIT acts on the sublingual mucosa to induce antigen-specific $Foxp3^+$ Treg cells in draining ManLNs, which are capable of suppressing hypersensitivity (Fig. 2).

Oral APCs can be classified as LCs in the epithelium and dendritic cells and macrophages in the LP [37]. We reconsidered the classification of oral APCs based on recent advances in defining mononuclear phagocytes, including the identification of FcγRI (CD64) as a specific marker of macrophages [44,45]. We analyzed murine lingual and sublingual tissues by flow cytometry [43]. Oral APCs can be divided into $CD64^+$ macrophages and $CD64^-CD11c^+$ cells. $CD64^-CD11c^+$ cells include $CD207^+$ LCs and $CD207^-$ cDCs. These cDCs are further subdivided into a minor population of $CD103^+CD11b^-$ cDCs and a major population of $CD103^-CD11b^+$ cDCs. Furthermore, $CD103^+CD11b^-$ cDCs and $CD103^-CD11b^+$ cDCs are classified as cDC1s and cDC2s, respectively, based on their marker expression. $CD19^-B220^+PDCA\text{-}1^+$ plasmacytoid dendritic cells are absent in the oral mucosa. The composition of oral APCs resembles that of skin APCs (Fig. 1) [46].

Intestinal $CD103^+$ cDCs promote the generation of $Foxp3^+$ Treg cells via the production of RA [24,25]. Therefore, we used a flow cytometry-based assay to measure aldehyde dehydrogenase (ALDH) activity as a functional marker of RA-producing ability

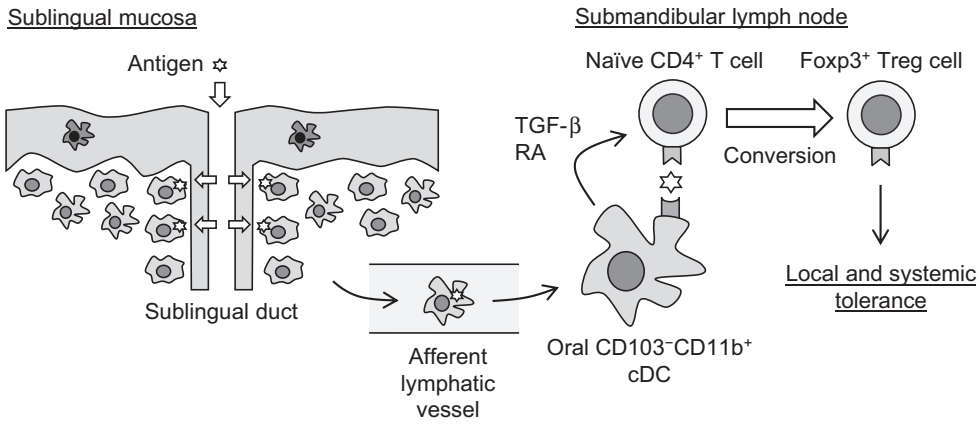

Fig. 2. Induction of regulatory T (Treg) cells by sublingual immunotherapy (SLIT). Sublingually administered antigens enter the mucosa most likely by crossing the sublingual ductal epithelium and are captured by oral macrophages. Oral CD103$^-$CD11b$^+$ classical dendritic cells (cDCs) transport the antigens into draining submandibular lymph nodes (ManLNs) and induce antigen-specific Foxp3$^+$ Treg cells in a transforming growth factor (TGF)-β-dependent and retinoic acid (RA)-dependent manner. These induced Treg cells can suppress inflammation both locally in the mucosa and systemically.

[43]. Among oral CD45$^+$ hematopoietic cells, ALDH activity was restricted to CD103$^-$CD11b$^+$ cDCs. In comparison with small intestinal CD103$^+$ cDCs, oral CD103$^-$CD11b$^+$ cDCs more efficiently induced Foxp3$^+$ Treg cells *in vitro*. The *in vitro* induction of Foxp3$^+$ Treg cells by oral CD103$^-$CD11b$^+$ cDCs was dependent on TGF-β and RA, which is similar to the induction by intestinal CD103$^+$ cDCs. Interestingly, intestinal CD103$^+$ cDCs and oral CD103$^-$CD11b$^+$ cDCs upregulated ALDH activity and the mRNA expressions of retinal dehydrogenase 2 and β8 integrin following migration to draining lymph nodes, indicating that the microenvironment of the lymph nodes may be critical for their Treg cell-inducing ability. Oral CD103$^-$CD11b$^+$ cDCs were found to transport sublingual antigens to ManLNs in 8–16 h. Moreover, sublingual antigen-primed migratory CD103$^-$CD11b$^+$ cDCs in ManLNs induced antigen-specific Foxp3$^+$ Treg cells *ex vivo*, which is consistent with the results of *in vitro* Treg cell induction and *in vivo* antigen transportation. Therefore, we established that oral CD103$^-$CD11b$^+$ cDCs could be responsible for Treg cell induction in ManLNs by SLIT (Fig. 2). Nevertheless, it is possible that other APCs could redundantly contribute to the induction of Treg cells and SLIT efficacy. Further studies with the depletion of each APC subset are needed to clarify their respective contributions to SLIT.

Our results are in line with the results of a previous study on skin APCs showing that the production of RA was restricted to dermal CD103$^-$CD11b$^+$ cDCs and that these cDCs induced Foxp3$^+$ Treg cells *in vitro* in a TGF-β-dependent and RA-dependent manner [47]. Together, these results suggest that the presence of RA-producing, Treg cell-inducing cDCs is not limited to the intestinal mucosa and that CD103 is not a universal marker for these tolerogenic cDCs (Fig. 1). SCIT may share underlying mechanisms with SLIT, both involving Treg cell induction by CD103$^-$CD11b$^+$ cDCs.

The sublingual route of administration has been used for the delivery of certain small-molecule drugs, such as nitroglycerin and nifedipine [48]. These drugs are rapidly absorbed through the mucosa and enter the systemic circulation, bypassing the gastrointestinal tract and first-pass metabolism in the liver and exerting systemic effects within seconds to minutes. On the other hand, biodistribution studies with radiolabeled allergens in humans have consistently found that direct absorption through the oral mucosa is absent or negligible [49,50]. This direct absorption could cause systemic anaphylaxis in sensitized individuals. Another biodistribution study with mice showed that sublingually administered ovalbumin, a model soluble protein antigen, crossed the epithelial barrier within 15–30 min and was captured by APCs in the LP within 30–60 min [51]. We recently reported that sublingual antigens including soluble antigens and particulate materials could be transported across sublingual ductal epithelial cells to oral APCs around the duct (Fig. 2) [52]. The sublingual duct is composed of pseudostratified or simple columnar epithelium, indicating that it may be more efficient than the sublingual mucosal epithelium for transporting antigens. In addition, we found that sublingual antigens could be captured by oral macrophages in the mucosa after 1–8 h [43]. However, oral macrophages lack the ability to stimulate naïve CD4$^+$ T cells and do not transport sublingual antigens into draining ManLNs. One possible explanation is that oral macrophages transfer the sublingual antigens to oral cDCs in the mucosa in a similar manner to intestinal macrophages [35].

SLIT has been associated with increased abundance of Foxp3$^+$ cells in biopsies from the sublingual mucosa, indicating that SLIT may induce local Treg cells to suppress local allergic reactions in the mucosa [53]. Considering that oral CD103$^-$CD11b$^+$ cDCs have RA-producing ability and that RA induces gut-homing receptors on responding T cells, it is possible that SLIT induces gut-homing Treg cells in addition to oral mucosa-homing Treg cells. These Treg cells may undergo secondary expansion in the mucosa, which has been shown to establish oral tolerance [7,14].

4. Conclusion

Recent studies have highlighted the central role of Treg cells, which are induced by tolerogenic cDCs in draining lymph nodes, in the development of tolerance against both orally and sublingually administered antigens. A distinct difference between the intestinal and oral immune systems is the phenotype of tolerogenic cDCs (Fig. 1). They are characterized by the presence of CD103$^+$ cDCs (especially the CD11b$^-$ subset) and CD103$^-$CD11b$^+$ cDCs in the intestinal and oral mucosa, respectively. The gene expression profiles and functions of human CD141$^+$ cDCs and CD1c$^+$ cDCs resemble those of mouse cDC1s and cDC2s, respectively [36]. Human oral CD1c$^+$ cDCs are commonly detected in biopsies from the sublingual mucosa [53]. Therefore, targeting oral CD1c$^+$ cDCs, presumably the equivalents of mouse oral CD103$^-$CD11b$^+$ cDCs, may be a promising strategy for improving the clinical efficacy of SLIT.

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Ethical approval

Ethical approval is not required for this review.

Conflict of interest

The authors declare no conflict of interest.

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