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Role of Orally Induced Regulatory T Cells in Immunotherapy and Tolerance

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Abstract

Oral antigen administration to induce regulatory T cells (Treg) takes advantage of regulatory mechanisms that the gastrointestinal tract utilizes to promote unresponsiveness against food antigens or commensal microorganisms. Recently, antigen-based oral immunotherapies (OITs) have shown efficacy as treatment for food allergy and autoimmune diseases. Similarly, OITs appear to prevent anti-drug antibody responses in replacement therapy for genetic diseases. Intestinal epithelial cells and microbiota possibly condition dendritic cells (DC) toward a tolerogenic phenotype that induces Treg via expression of several mediators, *e.g.* IL-10, transforming growth factor-β, retinoic acid. Several factors, such as metabolites derived from microbiota or diet, impact the stability and expansion of these induced Treg, which include, but are not limited to, $FoxP3^+$ Treg, LAP^+ Treg, and/or Tr1 cells. Here, we review various orally induced Treg, their plasticity and cooperation between the Treg subsets, as well as underlying mechanisms controlling their induction and role in oral tolerance.

Graphical Abstract

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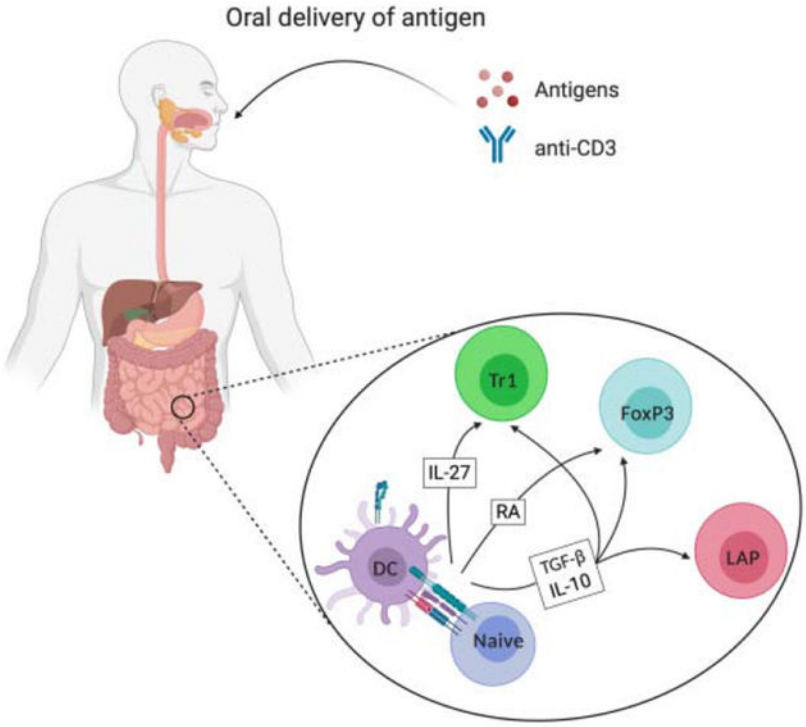

Keywords

Oral tolerance; Oral Immunotherapy (OIT); Regulatory T cell; LAP Treg; Tr1; Intestinal Immune System

1. Introduction

The small and large intestine are continuously exposed to a large variety of foreign antigens derived from food as well as commensal bacteria. Nevertheless, the intestinal immune system does not necessarily mount cellular or humoral immune responses to non-self-antigens due to regulatory mechanisms. One such mechanism is termed “oral tolerance”, referring to the natural development of induced tolerance to orally ingested stimuli in the gut-associated lymphoid tissue (GALT) [1]. Failure to establish oral tolerance leads to food allergy or the development of intestinal inflammatory diseases [2, 3]. While the colon is thought to respond to the massive saturation stimulation by bacterial or bacterially induced antigens, the immune system of the small intestine is distinct; it processes orally delivered antigens as well as systemic antigens, contains specialized lymphoid structures such as Peyer’s patches, has a distinct microbiome, distinct mechanisms of immune regulation, and uniquely interacts with the mesenteric lymph node (MLN) [4–10]. Hence oral tolerance mechanisms, including oral induction of regulatory T cells (Treg), take place in the small intestine.

Recently, the term oral immuno-therapy (OIT) was coined to define the oral delivery of antigen with the objective to suppress immune responses. Delivering a specific target antigen

or antibody that affects T cell function have been the two main methods used to achieve OIT. Alternatives routes of OIT include nasal, sublingual, subcutaneous and epicutaneous administration [11–15].

Oral tolerance was first demonstrated by Wells and Osbourne more than a century ago [16]. These investigators found that guinea pigs fed with corn-containing diet, but not corn-free diet, failed to show anaphylactic reactions against zein, a major protein of corn.

In 1946, another study reported that prior feeding of certain allergenic compounds to non-sensitive subjects induced a state of immunological tolerance against subsequent experimental dermal sensitization with the same compounds [17]. Subsequent studies confirmed the existence of an oral tolerance mechanism. For instance, rats developed tolerance to horse serum or pollen extract when fed with these antigen prior to non-oral exposure [18]. This was also demonstrated in other animal models that were fed bovine serum albumin [19] or sheep red blood cells [20]. However, induction of oral tolerance in humans was only demonstrated in the early 1990s, when adults fed with keyhole limpet hemocyanin followed by subcutaneous immunization with the same antigen were prevented from developing a subsequent delayed type hypersensitivity response [21]. Currently, OIT has been applied toward tolerance induction in autoimmunity and inflammatory diseases such as peanut allergy [22, 23], allergic asthma [24, 25], pollen allergy [26], hepatitis C infection [27], nonalcoholic steatohepatitis (NASH) [28], Pompe disease [29], rheumatoid arthritis [30, 31], type I diabetes [32, 33], hemophilia A and B [34–37] in clinical as well as preclinical studies.

The development of oral tolerance is thought to take place in both the small and large gastrointestinal (GI) tract, where the GALT plays a key role in regulating responses to ingested antigens [38]. Antigen can be acquired directly by phagocytes or can be delivered through goblet cell associated passages prior to capture by dendritic cells (DCs) in lamina propria (LP) [39]. Antigen uptake by a subset of regulatory DCs expressing CD103, which migrate from the gut mucosa to the MLN, concomitant secretory IgA production [40], forkhead box protein P3 (FoxP3) expressing Treg that produce transforming growth factor β (TGF-β) and IL-10, and expression of indoleamine 2,3-dioxygenase (IDO) or the vitamin A metabolite retinoic acid (RA) [41] are all implicated in this process.

For the last three decades, the role of T cells in oral tolerance induction has been studied in increasing detail. It was found that depletion of $CD4^+$ T cells abolished oral tolerance development [42], and that oral tolerance could be transferred from one animal to another by adoptive $CD4^+$ T cell transfer [43]. Moreover, a population of TGF-β secreting $CD4^+$ T cells termed Th3 were found to play a key role in oral tolerance [44], supporting the role of Treg in the induction of oral tolerance. Another conceptual advance in the oral tolerance field was the discovery of $CD4^+$ T cells expressing a membrane-bound form of TGF-β that contains latency-associated peptide (LAP) [1, 45, 46]. LAP^+CD4^+ T cells were found to have important immune regulatory functions in oral tolerance. Interestingly, TGF-β is required for the induction of both $FoxP3^+$ Treg and LAP^+ Treg [47, 48].

Here we review current understanding of oral tolerance mechanisms, applications of OIT in allergy, autoimmune disease and in inducing tolerance to protein replacement therapies for monogenic disorders. We highlight key regulatory cells that are induced by orally delivered antigen, circumstances leading to their induction and the suppressive mechanisms exerted by them. Understanding these mechanisms are critical to identify new strategies for modulating tolerance.

2. Subsets of orally induced regulatory T cells

In the intestine, crosstalk among several cells occurs in order to induce a naturally tolerogenic environment. Unlike thymus derived (t)Treg in other organs, which have a self-antigen TCR repertoire, intestinal Treg display a peripheral (pTreg) TCR repertoire responsive to resident and non-resident microbiota and dietary antigens, thus playing a crucial role in controlling pro-inflammatory responses [49–52]. Both tTreg and pTreg are located in the intestine, but it appears that the intestinal environment prefers the latter [49, 50], with dietary antigens driving the vast majority of small intestinal pTreg induction [53]. In accordance, pTreg, but not tTreg, are required for oral tolerance, as demonstrated by the development of oral tolerance even in the absence of thymus-derived Treg [54]. Development of orally induced tolerance includes diverse pTreg subsets such as $FoxP3^+$ Treg and $FoxP3^-$ Treg (Figure 1 and Table 1). Both cell types, however, exhibit a certain degree of functional plasticity to adapt to a specific microenvironment, with overlapping function and cooperation, thus giving rise to each other in order to establish tolerance. Not only do $FoxP3^+$ Treg display remarkable heterogeneity in expression of phenotypic and molecular signatures [55], they may lose FoxP3 expression with concurrent loss of suppressive function [56]. $FoxP3^-$ cells may also transiently express FoxP3 during activation [57].

2.1 $FoxP3^+$ Treg

The transcription factor FoxP3 is crucial to the development, maintenance and function of $CD4^+CD25^+$ Treg. Mutations in FoxP3 are associated with immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) in humans [58] and fatal autoimmunity in scurfy mice, which lack the FoxP3 gene [59]. Differentiation of naive T cells toward $FoxP3^+$ Treg depends, among other factors, on TGF-β and RA [60–62]. RA is able to induce FoxP3 by binding to the nuclear RA receptors (RAR) and the retinoid X receptor (RXR), which function as transcription factors, binding to RA response elements (RAREs) to regulate gene expression [63–66]. A combination of SMAD3 phosphorylation and inhibition of IL-6 receptor expression (and other putative mechanisms) leads to enhanced TGF-β-induced Foxp3 expression and Treg conversion [66]. Conversely, in the presence of IL-6 and TGF-β caused by inflammatory conditions, naive T cells differentiate into a Th17 phenotype [67]. Thus, integration of TGF-β and other cytokine signals determine the outcome of $CD4^+$ T cell differentiation. How TGF-β drives differentiation into LAP^+ Treg (discussed below) as opposed to $FoxP3^+$ Treg is less clear. Characteristics of $FoxP3^+$ Treg include expression of the co-inhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA4), inducible T cell co-stimulator (ICOS), glucocorticoid-induced tumor necrosis factor receptor family related gene (GITR), low levels of CD45RB, integrin α4β7,

αvβ8, glycoprotein A repetitions predominant (GARP), production of anti-inflammatory cytokines such as IL-10, TGFβ and IL-35, as well as regulation by IL-2 and ATP [68] (Figure 1).

FoxP3$^+$ Treg are present in every organ of the body, constituting 5–10% of peripheral CD4$^+$ T cells in mice and human [69]. However, these cells may represent a much higher proportion in the intestine. For instance, FoxP3$^+$ cells represent approximately 30% of CD4$^+$ T cells in the colonic LP and 20% in the LP of the small intestine [70, 71]. The role of FoxP3$^+$ Treg in maintenance of intestinal homeostasis was demonstrated by Sakaguchi and colleagues, who observed the development of spontaneous autoimmune disease, including gastritis, when the CD25$^+$ subpopulation of CD4$^+$ T cells was depleted [72]. In line with these results, Powrie et al showed that transfer of CD4$^+$CD25$^+$ T cells in mice with colitis prevented intestinal inflammation [73], which was confirmed by other studies [74, 75]. Importantly, failure to generate FoxP3$^+$ Treg in the gut-draining lymph nodes abrogates oral tolerance [76].

Because modulation of FoxP3 expression occurs at transcriptional, epigenetic, and posttranslational levels, FoxP3$^+$ Treg show broad heterogeneity [77]. FoxP3 expression is regulated by intronic enhancers: non-coding sequences 1 to 3 (CNS1-CNS3) that recruit proteins to the FoxP3 locus. Studies in Treg have shown that TGF-β is able to trigger FoxP3 induction in pTreg through CNS1, although this may not assure full Treg function [64, 78]. CNS1-deficient mice have dysbiotic microbiota and enhanced Th2-type inflammatory response in the colon [79, 80]. CNS3 regulates the expression of FoxP3 in tTreg [64]. To elicit the full Treg signature, additional genes besides FoxP3 are required, such as IKAROS family zinc finger 4 (IKZF4), interferon regulatory factor 4 (IRF4), SATB homeobox 1 (SATB1), lymphoid enhancer binding factor 1 (LEF1), and GATA binding protein 3 (GATA3) [81]. FoxP3 expression may be lost under inflammatory conditions, resulting in a loss of suppressive function. These cells can actively contribute to inflammation through secretion of pro-inflammatory cytokines such as IFN-γ and IL-2 [82–84]. Such “ex-FoxP3” cells can acquire a Th17 phenotype [83, 85, 86] or convert into Th2-like cells in a IL-4 dependent manner in the gut [87]. Altogether, these reports suggest that induced FoxP3 Treg do not necessarily exhibit lineage stability (Figure 2).

2.2 LAP$^+$ Treg.

TGF-β is central to the induction and suppression mechanism of LAP$^+$ Treg. Of the three different TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3), TGF-β1 is the most important in regulating immune responses [68]. TGF-β is first produced as pre-pro-TGF-β, which is transformed into pro-TGF-β dimers through homodimerization. Pro-protein convertase Furin cleaves the pro-TGF-β dimer into the carboxy-terminal dimer, or mature TGF-β; and amino-terminal dimer, LAP [88]. LAP remains non-covalently associated to mature TGF-β, thus preventing binding to the TGF-β receptor, forming a latent TGF-β complex (hence its name “latency-associated peptide”) [89]. Although surface LAP expression may be induced by TGF-β in a FoxP3-independent manner [90], both activated FoxP3$^+$ Treg and FoxP3$^-$ Treg can express LAP [91, 92]. Furthermore, LAP$^+$ cells are able to induce FoxP3 expression through a TGF-β-dependent mechanism [93]. The relationship between

FoxP3$^+$ Treg and LAP$^+$ Treg remains incompletely understood. Both require TGF-β for induction and suppression and share the ability to produce IL-10, so it is plausible that there is plasticity between these cells (Figure 2). In addition, surface LAP expression has also been described in CD8$^+$ T cells with suppressive properties [94].

LAP$^+$ Treg were first reported to aid in the induction of peripheric tolerance in a murine model of experimental autoimmune encephalomyelitis (EAE) [95, 96]. Recently, these cells have been implicated in tolerance to oral antigen or anti-CD3 administration in animal models of EAE and autoimmune diabetes [1, 97]. The small intestine is the site for LAP$^+$ Treg induction following oral antigen delivery [5] [34, 35, 37, 46, 98–100]. Administration of anti-LAP resulted in an increase in IL-17 and IFN-γ production, as well as abolishment of oral tolerance induction by anti-CD3 immunotherapy [101]. LAP$^+$ Treg were found to be important in the suppression of inhibitory antibody formation against coagulation factor VIII and IX (FVIII and FIX) in oral tolerance for treatment of the inherited bleeding disorder hemophilia A and B in murine and canine pre-clinical models [5, 9, 34, 35, 37, 46], illustrating their utility in prevention of anti-drug antibody formation. Oral tolerance induction using FVIII domains expressed in tobacco chloroplasts revealed an increase in the frequency of circulating LAP-expressing Treg in tolerized hemophilia A mice, thus highlighting their potential use as an important cellular biomarker in human clinical trials for plant-based oral tolerance induction [35].

TGF-β activation involves release of mature TGF-β from LAP, either via DC or Treg in a cell intrinsic manner [102–104]. Receptor binding of mature TGF-β leads to phosphorylation of SMAD2 and SMAD3, which then form a trimeric structure with SMAD4. Subsequently, the SMAD complex translocates to the nucleus, where it may activate or repress gene expression [105]. TGF-β activation may also occur through GARP [106–108], which is expressed at a low level in resting Treg. It is known that GARP tethers LAP/TGF-β complex to the cell membrane, so that TGF-β activation might occur at the right time and the right place within close proximity of effector T cells, thereby allowing suppression in a TGF-β dependent manner [88, 109]. This mechanism would explain the need for cell-contact for induced Treg to exert their function.

As GARP is associated with FoxP3$^+$ expression [109], most reports suggest that GARP is found on LAP$^+$ Treg co-expressing FoxP3. However, Kuhn and collaborators developed an optimized *in vitro* model, in which CD4$^+$ T cells expressing LAP do not co-express FoxP3. In this report, the *in vitro* induction of CD4$^+$LAP$^+$ T cells was abrogated by the IL-6 cytokine, which prevents GARP expression by STAT3-dependent inhibition of the *Lrrc32* gene, which encodes the GARP protein. *In vivo* IL-6 and IL-6R deficiency induced an increase in CD4$^+$LAP$^+$ T cells, and in particular CD4$^+$FoxP3$^+$LAP$^+$ T cells, thereby enhancing oral tolerance induction [110]. These reports show the key role of LAP$^+$ Treg in oral tolerance induction, but they also raise questions about the plasticity and cooperation between LAP$^+$ Treg and FoxP3$^+$ Treg (Figure 2).

2.3 Tr1 cells

Type 1 regulatory T (Tr1) cells are a distinct subset of Treg that highly express IL-10 and that have been described in the context of mucosal antigen administration, including

tolerance induction by nasal antigen or anti-CD3 administration [111, 112]. Tr1 cells are identified by surface co-expression of CD49b and lymphocyte activation gene 3 (LAG3) [113] (Figure 1), and may also express CTLA-4, programmed cell death protein 1 (PD-1), ICOS, early response gene 2 (Erg-2), and GATA-3 [114]. Tr1 induction or cell therapy can be used to prevent autoimmune disease or transplant rejection, and a number of clinical trials with antigen specific, allospecific, or polyclonal Tr1 cells have been assessed or are in clinical development (NCT02327221, NCT03198234, NCT01346085, NCT01656135) [114]. In plant cell-based oral tolerance, orally delivered antigen resulted in Tr1 ($CD4^+LAG\text{-}3^+CD49^+$) cell expansion in LP, which locally upregulated IL-10 expression in a pre-clinical hemophilia B model [46]. However, its exact role in orally induced tolerance remains unclear.

Tr1 cell induction depends on IL-27 secreted by DCs, but not on FoxP3 expression [115, 116]. Although these cells may display transient expression of FoxP3 [117, 118], the transcription factor is not a prerequisite for the suppressive ability of Tr1 cells [119]. Tr1 cells induced by IL-27 and TGF-β produced by DC in lymph nodes or by IL-27 production by splenic macrophages has been observed in models of oral tolerance to food allergen [111, 120, 121]. IL-27 promotes Tr1 differentiation through induction of c-Maf, IL-21 and ICOS [122]. IL-27 also induces ligand-activated transcription factor aryl hydrocarbon receptor (AhR), which interacts with c-Maf and acts in synergy to induce Tr1 differentiation [123]. Tr1 cells induce immunosuppression mainly by producing the cytokine IL-10 and IL-21 [111, 123], which in turn inhibits IL-17 polarizing cytokines on DCs such as IL-1β, IL-6 and IL-23 (Figure 1) [124]. Besides high amounts of IL-10, Tr1 cells also secrete TGF-β upon TCR activation, thus exerting suppressive responses through release of both IL-10 and TGF-β [125].

3. Orally induced non-CD4 T cells with regulatory function

3.1 Regulatory $CD8^+$ T cells

The majority of cells involved in oral tolerance are thought to be $CD4^+$ T cells, but these may not be the only immune regulatory cells involved in oral immunotherapy. For example, it has been reported that $CD8^+$ T cells with regulatory activity may be induced upon interaction with intestinal epithelial cells [126]. Regulatory $CD8^+$ T cells express lower levels of FoxP3 compared to $CD4^+$ Treg in mice, rats and humans [127]. In mice, surface markers such as CD122(+) or CD28(−) have been used to identify regulatory $CD8^+$ T cells [128]. However, the complete definition of regulatory $CD8^+$ T cells remains undefined [129]. Patients with IBD show defects in regulatory $CD8^+$ T cells in the LP, which is associated with a breakdown of mucosal tolerance [130]. The regulatory role of $CD8^+$ T cells was also shown in tolerance induction by oral administration of myelin basic protein (MBP) in experimental autoimmune encephalomyelitis [131]. Suppression of autoimmune encephalomyelitis was observed in recipient mice that received adoptive transfer of $CD8^+$ cells from orally tolerized mice [131]. However, *in vivo* depletion of $CD4^+$ T cells but not of $CD8^+$ T cells completely abolished orally induced tolerance to ovalbumin (OVA) [42]. These reports imply that $CD8^+$ Treg participate in but may not be essential to the development of oral tolerance.

3.2 γδ T cells

Gamma-delta TCR (γδ)-expressing T cells, representing only 1–2% of cells in secondary lymphoid tissues [132], have also been implicated in oral tolerance. γδ T cells display less TCR diversity, but can rapidly respond to pathogens at first contact, as well as self-molecules in response to danger signals or cellular stress through pattern recognition receptors [132, 133]. γδ T cells play an important role in gut homeostasis, as they are constitutively activated by normal microbiota of the intestinal lumen [134]. Interestingly, there is a subset of regulatory γδ T cells that expresses LAP ($\gamma\delta^+LAP^+$ cells) in the Peyer's patches and small intestine. $\gamma\delta^+LAP^+$ cells themselves lack FoxP3 expression but can function as antigen presenting cells and are able to induce $CD4^+FoxP3^+$ T cells. $\gamma\delta^+LAP^+$-mediated induction of $FoxP3^+$ Treg was shown to lead to amelioration of disease in an induced colitis model [135]. Further, γδ T cell deficiency [136] or depletion of γδ T cells [137] compromised orally induced tolerance to OVA in mice, whereas adoptive transfer of $CD3^+\gamma\delta^+$ spleen cells from tolerogenic mice into naive recipients restored tolerance [136]. The abolishment of antigen-induced oral tolerance in γδ T cell deficient mice was shown to be related to low levels of IL-10 production when low antigen-dose, but not high dose, was applied [137]. Moreover, recently published data demonstrated that anti-CD3 induced oral tolerance is also impaired in γδ T cell-deficient mice. γδ T cells enhance production of the chemoattractant XCL1/lymphotactin in the small intestine, which recruits tolerogenic $XCR1^+$ DC to the mesenteric lymph node and consequently enhances Treg induction [138]. Importantly, the XCL1/XCR1 axis is required for orally induced tolerance by anti-CD3, but not by oral antigen administration [138] (Figure 2). Hence, these reports confirm that the mechanism of oral tolerance induction may vary somewhat depending on the approach.

3.3 Regulatory B cells

A possible regulatory role of B cells was already described in the 1970s [139], and more recently, B cells with immunosuppressive proprieties and intestinal immune tolerance have been termed regulatory B cell (Breg) or B10 cells, reflecting the production of IL-10 cytokine [140–142]. IL-10-producing Breg promote IgG4 isotype switching in human B cells, a non-inflammatory immunoglobulin isotype that suppresses IgE production [143]. For example, multiple studies on OIT based on sublingual immunotherapy (SLIT) have observed a significant increase in the level IgG4 in patients with food allergy [14, 144–147]. Although there was an initial increase of IgE levels, these subsequently declined, suggesting isotype switching. Discontinuation of therapy led to decrease of food-specific IgG4 levels in patients and loss of tolerance, suggesting that IgG4 plays a role in maintaining desensitization and sustaining immunological tolerance [148]. Furthermore, it is known that the production of antigen-specific IgA and secretory IgA (S-IgA) in the intestines prevents uptake of antigen across the epithelium, thereby preventing inflammatory responses [149]. Indeed, significant increases in allergen-specific IgA have been reported in SLIT [150, 151]. Oral administration of casein for treatment of cow's milk allergy resulted in increase of IL-10 producing $CD5^+$ B cells in MLN in casein tolerogenic mice. The adoptive transfer of mesenteric $CD5^+$ B cells from casein-tolerized mice suppressed allergic responses via induction of $FoxP3^+$ Treg in an IL-10 dependent manner [152]. Other reports also suggest that Breg can induce $FoxP3^+$ Treg expansion [153, 154] (Figure 2).

3.4 Innate lymphoid cells

Innate lymphoid cells (ILC) have been linked to maintenance of the integrity of intestinal epithelial barriers through commensal microbiota signals, and promoting adaptive immunity [155]. In response to colonization of the gut by Clostridia species, RORγt-expressing ILC (RORγt$^+$ ILC3) and T cells upregulate IL-22 production in the intestinal LP, thereby reducing sensitization to oral antigens [156]. RORγt$^+$ ILC3 have been associated with the success of oral tolerance to dietary antigen. In the small intestine environment, macrophages produce IL-1β in response to microbiota signals. IL-1β activates IL-2-producing RORγt$^+$ ILC3, which in turn triggers FoxP3$^+$ Treg expansion in the small intestine [157]. Furthermore, IL-1β induces GM-CSF production in ILC3 cells, which is a crucial cytokine for CD103$^+$ DC differentiation [157] (Figure 3). Thus, failure to produce IL-1β by macrophages results in reduced production of GM-CSF and IL-2 by RORγt$^+$ ILC3, which in turn impedes FoxP3$^+$ Treg expansion and impaired ability to induce oral tolerance. Similarly, genetic ablation of *il-2* or *csf2* in ILC3 cells decreases accumulation of intestinal Treg upon oral administration of antigen [157]. Thus, RORγt$^+$ ILC3-derived IL-2 plays a role in maintaining Treg homeostasis and promoting oral tolerance in the small intestine. Therefore, crosstalk between innate myeloid and lymphoid cells is an interesting field to be explored in OIT (Figure 2).

4. Other regulatory cells that likely assist in orally induced tolerance

4.1 RORγt-expressing Treg

The abundant microbial community found on intestine plays a prominent role in the regulation of oral tolerance. Retinoic acid-related orphan receptor gamma t (RORγt)-expressing Treg (RORγt$^+$ Treg) represent a substantial number among Treg in the intestine and are dependent on the microbiota for maintenance and thus might be sensitive to microbiota shifts [3, 53, 158, 159]. Clostridia and Helicobacter species are examples of bacteria that may induce RORγt$^+$ Treg differentiation in the gut [156, 160–162], and use of broad-spectrum antibiotics may potentially deplete RORγt$^+$ Treg [158]. RORγt is a transcriptional factor expressed in both Treg and Th17 cells. CD103$^+$ DC are able to generate RA through enzymatic activity of retinal dehydrogenases (RALDH) and, in synergy with TGF-β and IL-6, induces optimal differentiation of RORγt$^+$ Treg [50]. Furthermore, RORγt$^+$ Treg are associated with IL-10 production [163], which may assist FoxP3$^+$ Treg differentiation (Figure 2). While it has become evident that RORγt$^+$ Treg play a crucial role in induction and maintenance of tolerance and homeostasis in the intestine with regard to responses to bacteria, the role of these cells in orally induced tolerance is unknown.

4.2 GATA3$^+$ Treg

Although representing only a minor fraction of the overall Treg population, GATA3$^+$ Treg reside in the small intestine and colon and are involved in control of chronic inflammation [50]. Additionally, GATA3 has an important role in maintenance of FoxP3 expression and Treg function at sites of inflammation [164, 165]. GATA3$^+$ Treg require IL-2 for persistence, hence IL-2-deficient mice show a robust decrease of this subset in the small intestine [165]. Conversely, *in vivo* systemic administration of IL-2 induces an increase of Treg-expressing

GATA in the spleen [165]. In orally induced tolerance, *in vivo* administration of anti-IL-2 resulted in abolishment of conversion of antigen-specific FoxP3^{-} to FoxP3^{+} CD4^{+} T cells [166]. It is therefore plausible that GATA3^{+} Treg contribute to the generation of FoxP3 expressing cells in the intestine during oral tolerance induction (Figure 2).

5. Mechanisms of oral Treg induction

Orally induced Treg cells have a crucial role in maintaining tolerance to microbiota, diet and other harmless antigens [50]. Here, we describe mechanisms that mediate Treg differentiation during OIT.

5.1 Immune cells promoting Treg induction and expansion

CD103^{+} DCs are considered major drivers of tolerance in the intestine. Intestinal DC express the integrin αvβ8, which has a crucial role in activation of TGF-β from the latent form and in the generation of Treg [102, 167, 168]. Both DCs and macrophages in the LP express aldehyde dehydrogenase (ALDH), which mediates RA production from dietary vitamin A [60, 62]. RA production by CD103^{+} DCs in combination with TGF-β induces FoxP3^{+} expression in naive T cells and promotes tolerogenic environment in the intestine [60–62]. RA-produced by CD103^{+} DCs also mediates pTreg migration in the LP through upregulation of gut-homing receptors, CCR9 and α4β7 integrin [61, 63]. In fact, ablation of CCR9 or β7 integrin impairs oral tolerance development [76, 169]. CD103^{+} DCs also mediates differentiation of LAP^{+} Treg in gut draining lymph nodes, and their activation promotes expression of gut homing receptors [13, 46]. Finally, CD103^{+} DCs express high levels of the enzyme indoleamine 2,3-dioxygenase (IDO), which metabolizes tryptophan. IDO activity contributes to differentiation of FoxP3^{+} Treg and the suppression of Th1 and Th17 cells [170, 171] (Figure 3).

Oral tolerance involves antigen uptake by oral administration, capture by intestinal DC and presentation to T cells in the Peyer's patches or in MLN. However, free antigen can also enter the blood vessels in the LP and reach the liver, where it is captured by plasmacytoid DC (pDC) [172]. pDC are important mediators of innate immune responses against pathogens but also play an important role in immune regulation [173]. [173]. Although pDC play a major role in TLR7 and 9 mediated sensing of viral RNA and DNA, leading to a robust type I interferon (IFN-I) response in the periphery, studies reveal that pDC mediate an immunoregulatory profile in the gut, thus suppressing T cell responses [174, 175]. Factors produced in mucosal sites, such as IL-10 and TGF-β inhibit IFN-I production, but do not block the ability of pDC to induce Treg differentiation [174, 176]. Thus, pDCs may contribute to oral tolerance development through enhancing the induction of Treg differentiation [172, 177, 178]. pDC express TGF-β and ALDH enzyme, which have a key function in Treg induction as mentioned above. Studies by Uto and colleagues demonstrated that pDC induce oral tolerance through expansion of FoxP3^{+} Treg in a food allergy model [179] and their frequencies are increased in plant-based oral tolerance [46]. Similarly, oral administration of *Lactobacillus gasseri* enhances accumulation of pDCs and Tr1 cells in the intestinal LP in a food allergy model [180].

The intestine is also populated with other subsets of DC, including $XCR1^+$ and $CD103^-$ DC, which also express enzymes required for RA formation [181]. Further studies are required to better understand the role of these cells in OIT.

In addition to the critical role of DCs in oral Treg induction, there is evidence that regulatory lymphocytes impact each other's induction. $FoxP3^+$ Treg generated outside the intestinal immune system are necessary for the generation of intestinal Treg. For example, Edwards and colleagues demonstrated that ablation of Treg or specific deletion of TGF-β in $FoxP3^+$ Treg decreased *de novo* generation of $FoxP3^+$ Treg in the GALT of OVA-fed mice and impaired oral tolerance induction [166]. Induction of antigen specific Treg from $CD4^+FoxP3^-$ effector T cells occurs by conversion in the presence of antigen and $FoxP3^+$ Treg. This process of infectious tolerance is thought to require interaction of FoxP3+ Treg and the effector T cell with a common antigen presenting cell (APC) [182]. Enhanced consumption of essential amino acids by Treg and APC has also been associated with induced Treg generation and inhibition of T cell proliferation [183]. The role of infectious tolerance in Treg cells induction during OIT remains to be further elucidated.

5.2 Diet-derived mediators

Early contact with dietary antigens induces pTreg development and accumulation in the intestinal LP [53]. However, continuous generation of pTreg requires constant contact with dietary antigens, which preserve the accumulation of these cells in the LP [3, 53, 158]. Furthermore, depletion of dietary antigens increases susceptibility to intestinal allergy by inducing enhanced accumulation of Th1 cells and decreased number of pTreg in the intestine in an experimental model of food allergy [53]. Thus, the immune response to newly introduced dietary antigens is suppressed by pTreg in the LP, which are generated with a continuous exposure to antigens derived from diet.

Diet-derived metabolites, including vitamin A, D3 and B3 influence various immune functions that include Treg. Vitamin A (retinol) is taken up through diet and converted to RA in a two-step enzymatic reaction. Retinol is metabolized to retinal by alcohol dehydrogenase (ADH), which is ubiquitously expressed, and then the retinal dehydrogenases (RALDH) catabolize the final conversion of retinal to RA [184, 185]. RA plays a crucial role in maintenance of mucosal tolerance in the intestine through induction of $FoxP3^+$ Treg [53, 185]. Specifically, RA mediates Treg differentiation through binding to the nuclear RA receptors (RAR) and the retinoid X receptor (RXR), which promotes FoxP3 expression [63, 64, 79]. *In vivo* administration of RA promotes expansion and intestinal homing of $FoxP3^+$ Treg in OVA immunized mice [63]. Furthermore, vitamin A-deficient diets decrease expansion of $FoxP3^+$ Treg and LAP^+ Treg and promote Th17-mediated inflammation, while vitamin A supplementation enhances accumulation of Treg in the GALT [3, 186].

Niacin or vitamin B3, which is known to have anti-inflammatory properties [187], binds to GPR109A on DCs in the LP. This interaction promotes RALDH expression, which mediates formation of RA and enables DCs to induce $FoxP3^+$ Treg and IL-10 production in T cells [188]. Genetic ablation of GPR109A leads to impaired oral tolerance and enhanced susceptibility to colitis in a murine model [188]. The receptor of vitamin B9 or folic acid, which is derived from diet and bacterial commensal metabolites, is also

highly expressed on Treg and its known to enhance Treg survival through upregulation of the anti-apoptotic BCL-2 [189]. Mice fed with folic acid-deficient diet have decreased accumulation of FoxP3$^+$ Treg expressing IL-10 in the colon, which is associated with an enhanced susceptibility to intestinal inflammation. Furthermore, folic acid supplementation enhances Treg accumulation in the colon by inhibiting apoptosis [189].

5.3 Microbiota

The GI compartment is colonized by a large number of commensal microbiota, which are a large source of foreign antigens and are known to promote oral tolerance [61, 62, 160, 188]. Commensal microbiota produce several factors, such as polysaccharide A, short chain fatty acids and RA, which regulate innate and adaptive immune cells, and their dysbiosis has been associated with an increased incidence of food allergy [190]. For example, Zhou and colleagues demonstrated that gut-microbiota induce IL-1β production by intestinal macrophages. IL-1β activates IL-2 producing ILC3, which in turn triggers FoxP3$^+$ Treg expansion in the small intestine [157]. Furthermore, IL-1β induces GM-CSF production in ILC3 cells, which is a crucial cytokine for CD103$^+$ DC differentiation. Thus, the initial crosstalk between myeloid cells and ILC3 plays a key role in the expansion of Treg cells and the induction of oral tolerance induced by dietary antigens. However, further studies are necessary to identify additional interactions between these cells. Colonization by certain species of commensal bacteria such as Clostridia and Bacteroides enhances accumulation of intestinal Treg, thereby promoting immune homeostasis in the intestine [5, 158, 190, 191]. Treg numbers are decreased in the small intestine of germ-free mice compared to specific pathogen-free (SPF) mice [160, 192]. However, restoration of microbiota induces an enhanced expansion of Treg and restores immune homeostasis in germ-free mice [160]. Song and colleagues found that intestinal microbiota mediated formation of gut bile-acid metabolites from the diet, which are essential for Treg expansion in the intestine [192]. Thus, immune cells activated by microbiota triggers Treg differentiation and its function in the intestine during oral tolerance induced by dietary antigens.

Bacteria can also influence the suppressive phenotype of Treg. For instance, colonization of *Bacteroides fragilis*, a human symbiont, promotes accumulation of IL-10-producing FoxP3$^+$ Treg in the intestine [70]. Other species of commensal microbiota, like Clostridia also facilitate differentiation of Treg and expression of immunosuppressive molecules ICOS, CTLA4, LAG-3, PD-1, IL-10 and TGF-β [49, 70, 193]. Expression of these surface molecules inhibits T cell activation and promotes maintenance of intestinal homeostasis [68]. Thus, there is a mutual relationship between commensal bacteria and intestinal Treg, which together promotes intestinal homeostasis. However, the exact mechanisms by which microbiota-derived signals induce functional Treg still need to be determined. Commensal bacteria also play a key role in Treg induction to plant cell-derived antigens. Plant cells provide natural bioencapsulation through their cell wall, which protects antigens from degradation by stomach acids. However, once plant cells reach the small intestine, bacterial enzymes are required to at least partially degrade cell wall components so that antigens are released and can be delivered to the immune system. While such enzymatic activity is most abundant in the colon, there is recent evidence for presence of several bacterial orders in the small intestine, in particular in the duodenum, including producers of

cellulase, β-N-acetylhexosaminidase, amino-acid N-acetyltransferase, β-glucosidase, xylan 1,4-β-xylosidase, and pectinesterase [5]. These are distinct from bacteria that predominantly produce these enzymes in the colon. In addition, plant cells are a source of vitamins that can affect immune function such as provitamin A carotenoids, a precursor of retinol/retinoic acid.

6. Mechanisms of suppression

Treg preserve homeostasis in the intestinal tract through multiple suppressive mechanisms: production of inhibitory cytokines IL-10, IL-35 and TGF-β; apoptosis or anergy of effector T cells, perforin and granzyme-dependent cytolysis of target cells; suppression of DC maturation and function through expression of PD-1, CTLA4, LAG3; and apoptosis of effector T cells by deprivation of IL-2. Of these, inhibitory cytokines have been studied the most in intestinal Treg.

6.1 Cytokines mediating Treg suppressor activity

IL-10 is an anti-inflammatory cytokine produced by many cells, including FoxP3$^+$ Treg, LAP$^+$ Treg and Tr1 cells. IL-10 exerts its function by inhibiting Th1 and Th17 responses and downregulating MHC-II expression in monocytes [194–196]. Expression of IL-10 in Treg is regulated by STAT3 [194] and is crucial for the maintenance of intestinal homeostasis. Studies in humans and mice have shown that mutations of IL-10R leads to development of severe colitis and enhances accumulation of Th17 in intestine [193, 195, 197]. Furthermore, absence of IL-10R or STAT3 in APCs in the LP exacerbates intestinal inflammation [198, 199]. Th17 cells also express IL-10R, and mutations in this receptor enhance Th17 cell proliferation in the intestine, so that IL-10 produced by Treg plays a key role in suppression of Th17. Indeed, IL-10RA or STAT3 deficiency in Treg induced increased activation and accumulation of Th17 cells in the intestine in an animal model of colitis [194, 195]. The relevance of IL-10 for the function of orally induced Treg is evident from the observation that OIT with anti-CD3 leads to induction of IL-10 producing LAP$^+$ Treg, which prevents T cell activation and protects against inflammatory conditions [98, 99]

TGF-β has been broadly described as an immunomodulator of effector T cell activation with key roles in OIT [200]. TGF-β is responsible for FoxP3$^+$ and LAP$^+$ Treg induction and differentiation and most Treg types are able to produce this cytokine (Figure 1). Oral administration of anti-CD3 enhances regulatory functions by induction of TGF-β produced by LAP$^+$ Treg, which contributes to protection against inflammation in murine models of EAE and colitis [98, 99]. Depletion of LAP$^+$ Treg or TGF-β enhances T cell proliferation and exacerbates inflammatory diseases [98, 99, 200].

IL-35 is an immunoregulatory cytokine secreted specifically by FoxP3$^+$ Treg, and its role in Treg suppressive activity has been broadly studied [201, 202]. The importance of IL-35 for Treg was initially documented by Collison and colleagues (2007), who demonstrated that deletion of Ebi3, the IL-27B subunit of IL-35, reduced Treg activity *in vitro* and impaired intestinal homeostasis *in vivo* [201]. More recently, Wei et al (2017) found that expression of IL-35 depends on FoxP3, while IL-10 production by Treg depends on the c-Maf and Blimp-1 transcription factors [191, 202]. Furthermore, deletion of IL-35 producing Treg or

genetic deletion of Blimp-1 in Treg enhances production of IL-17 and IFN-γ and worsens colitis [202].

6.2 Induction of anergy and effector T cell apoptosis

Antigen dose is an important factor for suppressive mechanisms. Low antigen dose favors active suppression with Treg induction whereas a high dose favors anergy or deletion of antigen-specific T cells [203, 204]. In oral tolerance induction using low antigen dose, the suppressive effect was associated with IL-10 secreting cells in Peyer's patches [205]. In EAE, TGF-β-secreting regulatory cells were found in Peyer's patches after low doses of orally administered MBP, whereas this was not observed after high dose oral administration [206]. Benson and colleagues also demonstrated that oral administration of MBP reduces antigen-specific TCR expression in $CD4^+$ T cells in the peripheral LNs of a mouse model of EAE [207]. In human food allergy studies, high dose oral desensitization along with omalizumab (anti-IgE monoclonal antibody) reduced Th2 responses, which was associated with anergy/depletion of allergen-specific T cells [208]. Patients with peanut-allergy achieved successfully OIT by inducing an anergic Th2 cell phenotype, while no induction of antigen-specific Treg was observed [209].

6.3 Bystander suppression

Bystander suppression is a mechanism in which antigen specific Treg generate a nonspecific antigen effect [210]. This suppression mechanism requires colocalization of tolerogen and the unrelated antigen [211, 212]. Hemagglutinin immunized mice showed decreased specific T cell proliferation when mice were also given OVA OIT [210]. More recent studies demonstrated that antigen-specific Treg cells induced during OVA administration are recruited into the airway mucosa and control lung inflammation in an airway allergy mouse model [212]. This mechanism of suppression is also associated with an enhanced production of IL-10 and TGF-β, which aids in inhibition of the unrelated T cell activation [210].

6.4 Other suppressive mechanisms

In order to preserve immune homeostasis in the intestine, Treg modulate microbiota diversity by induction of IgA production. For example, Kawamoto et al. (2014) demonstrated that transfer of Treg, but not T naive cells, enhanced the diversity of bacterial species in *Cd3e* deficient mice. Furthermore, transferred $FoxP3^+$ Treg increased germinal center (GC) formation and induced IgA production in the small intestine [213]. Several studies have shown the role of granzyme in controlling effector T cells [214, 215]. Treg can regulate inflammation by inducing cytolysis of effector T cells through production of granzyme A and B [215]. However, the role of granzyme-dependent cytolysis of target cells in the maintenance of oral tolerance remains unknown.

7. Examples of OIT-induced Treg in treatment of disease

OIT can be used to alleviate inflammatory disease in an antigen-nonspecific fashion by non-specific enhancement of Treg [1, 97, 216]. This can be accomplished by supply of IL-2, which is critical for Treg development, expansion and maintenance of suppressive function [49, 217, 218]. Several studies have shown that low dose subcutaneous injections of IL-2

leads to an increase in number and function of Treg, thereby ameliorating autoimmune disease in patients [12, 15]. Bonnet and colleagues found that low dose IL-2 enhanced accumulation of $FoxP3^+$ Treg in the GALT and prevented food allergy in two different experimental animal models of allergy [216]. An alternative approach is oral administration of anti-CD3 [98, 99]. The mechanism by which systemic anti-CD3 mediates tolerance is not well understood but is thought to include a combination of apoptosis and anergy of T cells. While the use of anti-CD3 for Treg induction may appear paradoxical, given its role in depleting T cells, it is thought that $CD4^+FoxP3^+$ Treg express CD3 at lower levels as compared to $CD4^+FoxP3^-$ effector T cells, thus rendering them less susceptible to anti-CD3 mediated apoptosis [219]. The tolerogenic microenvironment caused by anti-CD3 treated apoptotic T cells and phagocytic macrophages is also thought to lead to TGF-β production inducing FoxP3 in $CD4^+$ T cells, thus rendering them suppressive. TGF-β can further skew DC into a tolerogenic phenotype [220]. Humanized anti-CD3 treatments like otelixizumab, teplizumab and visilizumab are being evaluated for transplant rejection and autoimmunity (NCT01287195, NCT00129259, NCT04270942). In contrast to systemic delivery, oral anti-CD3 treatment does not cause depletion of T cells [98], although it does increase the incidence of LAP^+ Treg in the GALT [101], which limits inflammation by regulating T cell activation via TGF-β and IL-10 production [98, 99]. Nasal administration of anti-CD3 or antigen also enhances expansion of different types of Treg, including LAP^+ Treg and Tr1 cells [11, 100]. Illustrating the importance of TGF-β in the anti-CD3 approach, blockage of TGF-β abolished oral tolerance induction and increased inflammation in autoimmune disease [11, 100].

Antigen-specific treatment by oral administration in OIT has been broadly explored as a means of tolerance induction, as it can elevate numbers and improve function of antigen-specific Treg, such as increase of hypomethylation of *FoxP3* gene and thus enhanced expression [221, 222]. OIT seeks to expand pTreg in order to alleviate various autoimmune and allergic conditions, as well as prevent anti-drug antibody formation. For example, peanut OIT mitigates food allergies in part through induction of antigen specific $FoxP3^+$ Treg and reduction of basophil activation in patients with peanut allergy [221, 222]. Recently the Food and Drug Administration (FDA) approved an oral immunotherapy termed (AR101, Palforzia) for the reduction of allergic reaction incidence in patients with peanut allergy [223]. Peanut OIT increases the migration of Treg toward the intestinal epithelium and enhances the suppressive capacity of Treg in allergic patients submitted to peanut OIT.

Another application of antigen-specific oral administration is the prevention of anti-drug antibodies known as “inhibitors” that form in a proportion of hemophilic patients as a result of intravenous coagulation factor replacement therapy [34, 35, 224, 225]. In order to orally induce Treg while avoiding expensive production of recombinant proteins and protecting antigen from degradation in the stomach prior to reaching the small intestine, a plant cell-based approach was developed [34, 226]. Chloroplasts of transgenic tobacco or lettuce plants expressing high levels of the mature sequence of human FIX for tolerance induction in hemophilia B or the heavy chain and C2 domain of the human FVIII for hemophilia A were expressed as N-terminal fusions to the cholera non-toxic B subunit (CTB) to target the GM1 receptor of intestinal epithelial cells for enhanced transmucosal delivery. The plant cell wall provides natural bioencapsulation that can resist breakdown by stomach

acids. However, enzymatic activities provided by commensal bacteria release antigen into the small intestine, where up-take and translocation to the immune system was observed in LP and Peyer's patches [5, 46]. As a result, antigen was delivered to intestinal DCs, including $CD103^+$ DCs, and increased frequencies of $CD103^+$ DCs and pDCs were observed in mesenteric lymph nodes of hemophilic mice upon challenge with intravenous antigen [5, 34, 35, 37, 46]. Activated $CD103^+$ DC migrated to draining lymph nodes and induced Treg expansion and migration. Uptake of orally administered coagulation factor induced IL-10 and TGF-β producing LAP^+ T cells, $FoxP3^+$ Treg, and T cells expressing gut-homing receptors [5, 34, 35, 37, 46]. Adoptive transfer studies demonstrated that suppression of antibody formation was achieved by induced $FoxP3^+$ and LAP^+ Treg, with LAP^+ Treg providing most of the suppression [5, 46]. Ultimately, this resulted in suppression of inhibitor formation against FVIII and FIX in murine and canine models of hemophilia, and also in suppression of IgE formation and anaphylactic reactions against FIX [34, 36, 37].

However, there are some limitations in OIT including dosing frequency and lack of long-term tolerance. In plant cell-based therapy, prolonged oral delivery is required for long-lasting tolerance as an increase in inhibitor formation was observed once oral delivery was discontinued [34, 46]. As the use of adjuvant allows lower and shorter dosing regimens, further approaches could consider the use of adjuvant as an alternative to overcome the long-term tolerance issue.

8. Conclusions and future perspectives

In the past decade, there has been an increase in knowledge regarding OIT, as well as mechanisms that mediate the development of this process. Three distinct Treg subsets have been shown to majorly contribute to OIT: $FoxP3^+$ Treg, LAP^+ Treg and Tr1 cells. These cells share several key characteristics and functions, such as the ability to produce IL-10 and TGF-β, which are critical mediators of immune suppression in OIT. TGF-β is also a key cytokine for $FoxP3^+$ and LAP^+ Treg differentiation. $CD103^+$ DC and pDC have been shown to be key drivers of Treg differentiation and expansion in the intestine. Crosstalk between these cells and ILC, as well as between Treg subsets in the intestinal compartment is integral for the success of OIT. Future research may therefore focus on further understanding of this cellular crosstalk in order to improve the OIT approach and achieve lasting tolerance. Although the underlying mechanisms of OIT have not been fully elucidated, it is recognized as a promising strategy, which has resulted in several clinical trials including peanut allergy (FDA approved AR101), type 1 diabetes (NCT00223613, ISRCTN76104595 NCT02547519, NCT03364868), and arthritis (NCT00000435), demonstrating the importance of OIT in the improvement of allergic and inflammatory conditions [227, 228]. Other routes of antigen administration known to induce tolerance have been used for ongoing clinical trials in T1D and in multiple sclerosis, such as intradermal (NCT01536431, NCT01973491) and subcutaneous (NCT00435981, NCT0072341, NCT00529399) administration of the antigen. These routes of antigen administration have been successful in induce $FoxP3^+$ Treg and enhance IL-10 production. However, more studies are required to address the mechanisms of tolerance induced by these pathways and the differences with OIT. In summary, critical questions

remain not only regarding mechanisms, but also optimal antigen dose selection, route of administration and responsiveness to the treatment. These will aid in identifying new targets for treatment and development of new treatment protocols that are safer and more effective.

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Highlights:

- Oral antigen-based oral immunotherapies (OITs) alleviate allergies and autoimmune diseases.
- OIT induces expansion of several subtypes of regulatory T cells (Treg) such as FoxP3$^+$ and LAP$^+$ Treg and Tr1 cells.
- Orally induced Treg exhibit overlapping function and cooperation between each other.
- Tolerogenic dendritic cells are drivers of Treg cells expansion and differentiation in the small intestine.
- TGF-β, RA, IL-27 and IDO determine differentiation of orally induced Treg.
- Role of intestinal microbiota in oral Treg induction remains to be defined.

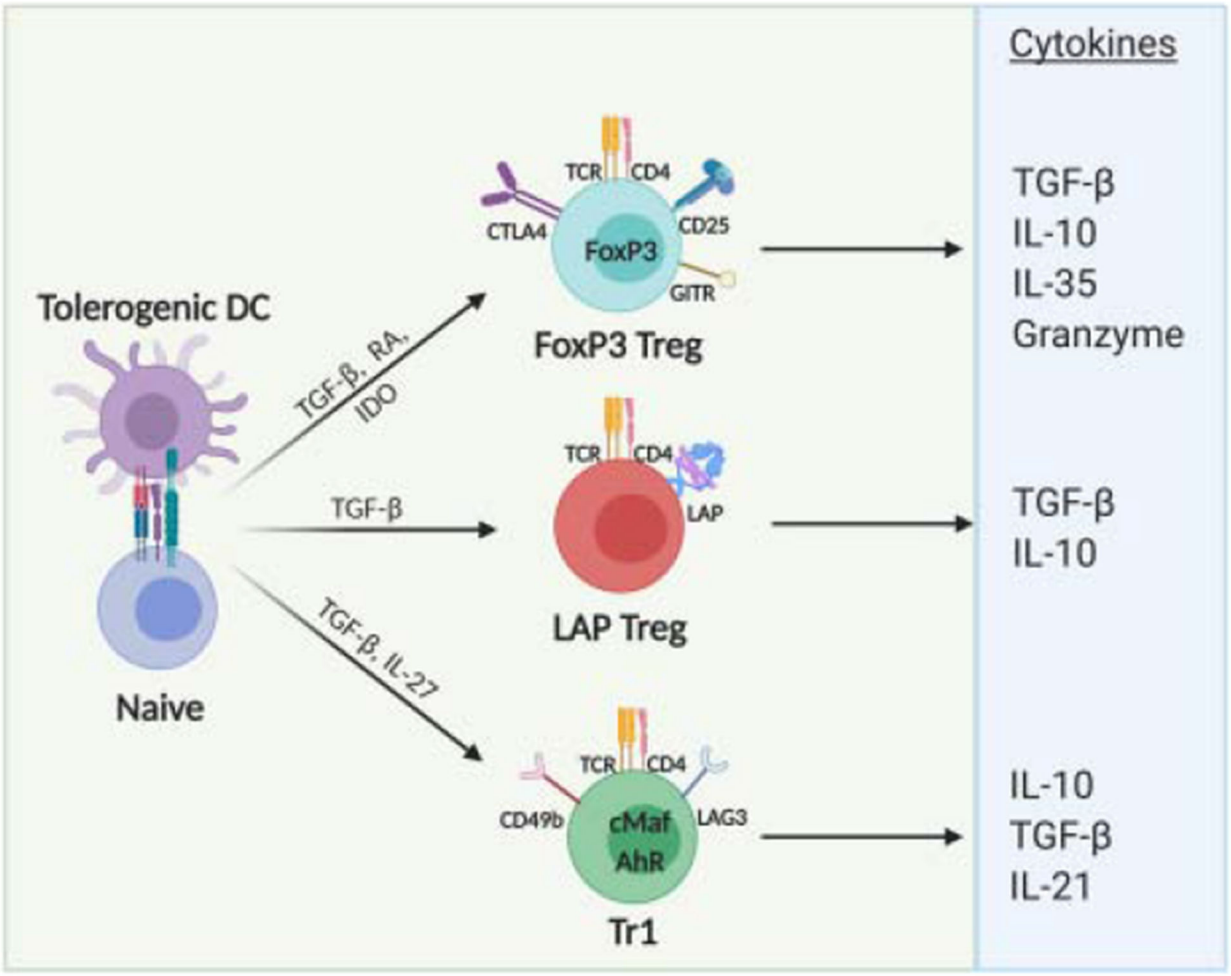

Figure 1: Types of orally induced CD4$^+$ regulatory T cells.

Interactions of tolerogenic DCs in the MLN or in PP with naive T cells may result in the oral induction of three major types of CD4$^+$ regulatory T cells: FoxP3$^+$ Treg, LAP$^+$ Treg and Tr1 cells. TGF-β and RA drive differentiation of FoxP3$^+$ pTreg, which express TCR, CD4, CD25, FoxP3, GITR and CTLA-4, among other markers. FoxP3$^+$ Treg produce suppressive cytokines such as TGF-β, IL-10, IL-35 as well as granzyme, which are critical to suppression of immune responses in OIT. Induction of LAP$^+$ Treg is not completely understood, although TGF-β plays a key role. LAP$^+$ Treg express TCR, CD4 and LAP, among other markers, on the surface. These cells secrete TGF-β as wells as IL-10, which are responsible for immunosuppressive proprieties. Tr1 cells express TCR, CD4, CD49b, LAG-3, among other markers, on the cell surface, and c-Maf and AhR transcription factors. Tr1 cell differentiation depends on IL-27 secreted by DCs and are characterized by high IL-10 secretion, but also secrete TGF-β.

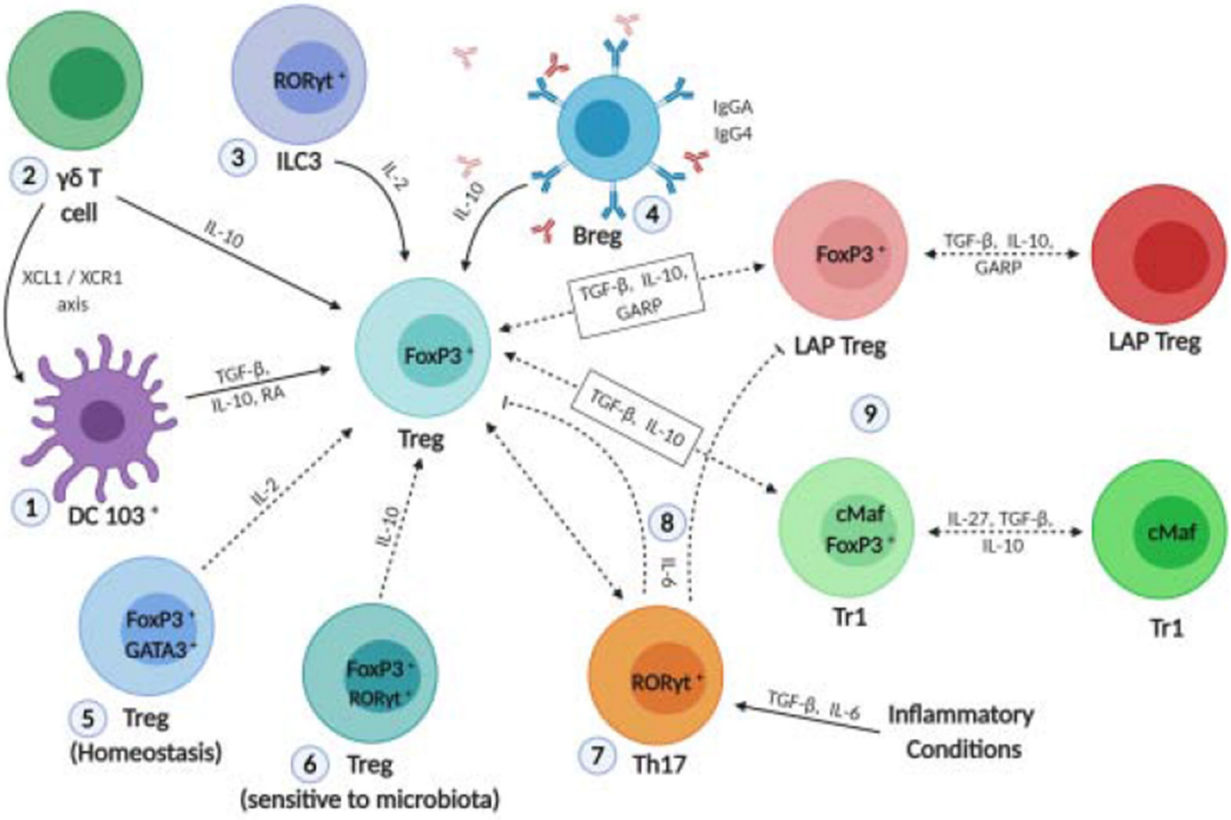

Figure 2: Overlapping function, cooperative interaction and plasticity among cell types during establishment of immune regulation upon oral antigen delivery.

The success of OIT depends on crosstalk between several cell types in the GALT. 1) CD103$^+$ DCs mediate differentiation of LAP$^+$ Treg and produce RA, which in combination with TGF-β and IL-10 induces FoxP3$^+$ expression in naive T cells. 2) γδ$^+$ T cells induce FoxP3$^+$ Treg by IL-10 production. XCL1-produced by γδ T cells influence migration of tolerogenic XCR1$^+$ DC to the mesenteric lymph node and consequently Treg induction. 3) Microbiota and production of IL-1β by macrophages in turn induce production of IL-2 by RORγt$^+$ ILC3 followed by FoxP3$^+$ Treg expansion. 4) IL-10-producing Breg promote class switching to suppress IgE production and maintain desensitization, while sustaining immunological tolerance. Breg are able to induce FoxP3$^+$ Treg in an IL-10-dependent manner. 5) GATA3$^+$ Treg reside in the small intestine and colon and have an important role in the maintenance of FoxP3 expression and Treg function by IL-2 production. 6) RORγt$^+$ Treg are dependent on microbiota for maintenance and are sensitive to microbiota shifts. RA, TGF-β and IL-6 induce optimal differentiation of RORγt$^+$ Treg. 7) GARP may induce both Foxp3$^+$ Treg and LAP$^+$ Treg. TGF-β and IL-10 may induce Foxp3$^+$ Treg, LAP$^+$ Treg and Tr1 cells. In addition, LAP$^+$ Treg and Tr1 cells may transiently express the Foxp3 transcription factor. In inflammatory conditions, the presence of IL-6 and TGF-β may induce differentiation of naive T cells into Th17 phenotype, instead of FoxP3$^+$ Treg. Moreover, Treg may lose Foxp3 expression under inflammatory conditions. Such “ex-FoxP3” cells may acquire a Th17 phenotype. IL-6 cytokine abolishes Foxp3$^+$ Treg and LAP$^+$ Treg induction by preventing GARP expression.

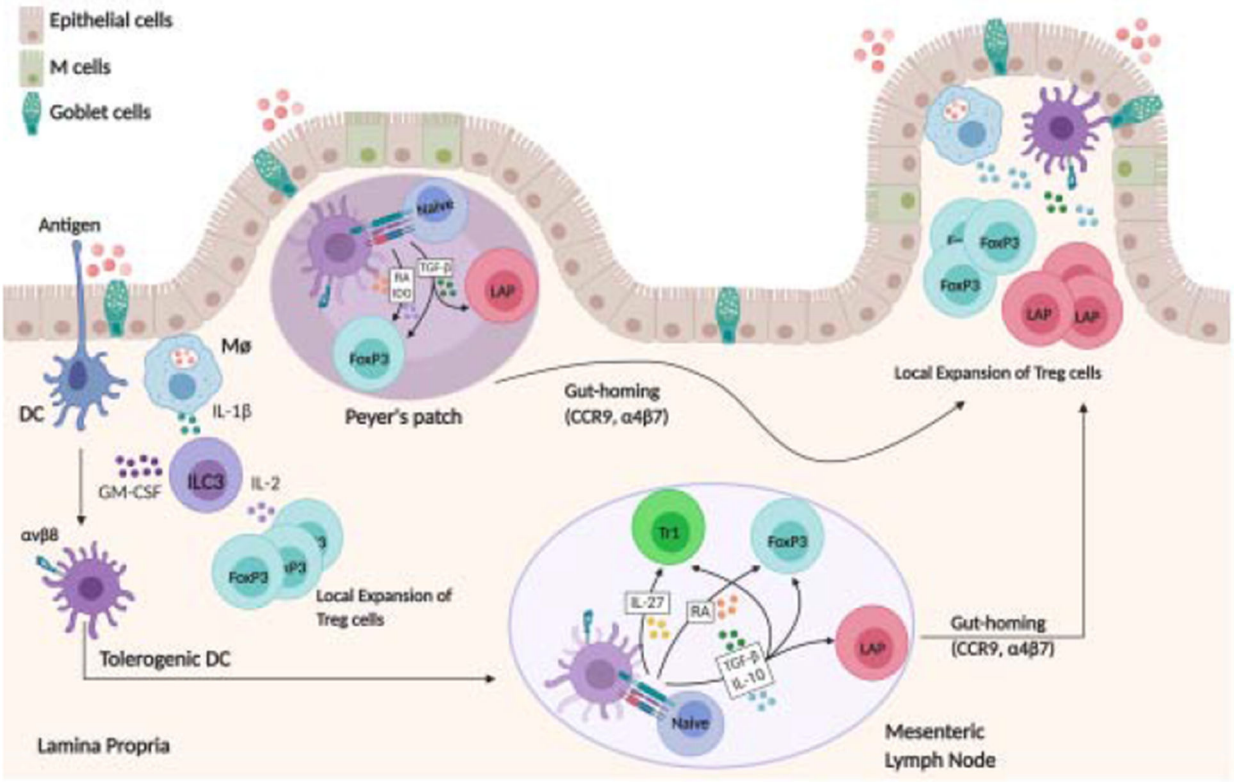

Figure 3. Schematic overview of oral tolerance development/oral Treg induction.

Upon ingestion and the digestive process, antigen reaches the small intestine and can be taken up directly by phagocytes or pass through goblet cell associated passages prior to capture by DC in LP. Microbiota induced IL-1β-secreting intestinal macrophages can induce $CD103^+$ DC differentiation by stimulating GM-CSF-producing ILC3. Moreover, IL-1β-secreting intestinal macrophages induce IL-2 production by ILC3, which in turn stimulates $FoxP3^+$ Treg expansion. Antigen is presented to T cells in the Peyer's patches or in the MLN through tolerogenic $CD103^+$ DCs, which express the integrin αvβ8 able to activate TGF-β from the latent form and induce Treg. $CD103^+$ DCs produce IDO and RA, which in combination with TGF-β induces FoxP3 Treg differentiation. TGF-β by itself can induce LAP^+ Treg differentiation. Tr1 differentiation is triggered by IL-27 producing tolerogenic DCs. $CD103^+$ DCs also mediate upregulation of gut-homing receptors, CCR9 and α4β7 integrin, allowing the recently primed Tregs to home back to the LP, where they undergo local expansion to induce oral tolerance.

Table 1.

Summary of types of regulatory T cells induced during OIT

	FoxP3+	LAP+	Tr1
Classical Inductors	TGF-β	TGF-β	TGF-β
	RA		IL-27
	IDO		
Transcription factors	FoxP3		cMaf
			AhR
Extracell markers	CD25	LAP	LAG-3
	CTLA4		CD49
	GITR		
Cytokines produced	TGF-β	TGF-β	TGF-β
	IL-10	IL-10	IL-10
	IL-35		IL-21
	Granzyme		
OIT inductors	Dietary antigens	Vitamin A	IL-2 (s.c.)
	Vitamin A	Microbiota	Anti-CD3 (Nasal)
	Vitamin B9	Anti-CD3 (Nasal or oral)	
	Microbiota		
	Low dose of IL-2 (i.p. or oral)	FVIII or FIX encapsulated in chloroplasts	
	FVIII encapsulated in chloroplasts		