

REVIEW ARTICLE

Adaptive T-cell responses regulating oral tolerance to protein antigen

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Abstract

The term oral (or mucosal) tolerance has been classically defined as the suppression of T- and B-cell responses to an antigen by prior administration of the antigen by the oral route. In recent years, it has become clear that both innate and acquired regulatory immune responses are essential for the development of oral tolerance. As such, mucosal microenvironmental factors such as transforming growth factor- β , prostaglandins but also dietary vitamin A create conditioning of an adaptive regulatory T-cell response that suppresses subsequent antigen-specific responses. Particular resident subsets of antigen presenting dendritic cells are pivotal to convey conditioning signals next to the presentation of antigen. This review discusses the primary mechanisms of adaptive regulatory T-cell induction to ingested soluble protein antigen. However, we also discuss the limitations of our knowledge with respect to understanding the very common food hypersensitivity Celiac disease caused by an aberrant adaptive immune response to the food protein gluten.

The mucosal surfaces of the gastrointestinal tract have developed as the largest surface area of the body that is in contact with the external environment. Not only do most pathogens enter through this site, the intestinal mucosa is continuously exposed to a large variety of harmless antigens, such as dietary proteins and constituents of commensal bacteria. To regulate this high antigenic pressure, the human gut holds approximately 50×10^9 lymphocytes, a large proportion of all immune cells in the body. These intestinal immune cells reside in the organized gut-associated lymphoid tissue (GALT), are scattered in between intestinal epithelial cells, or are found throughout the gut lamina propria, three compartments that mediate distinct immune responses. GALT, which comprises Peyer's patches, lymphoid follicles, and the appendix, are inductive sites where naïve T and B cells are found. In contrast, cells within the lamina propria and epithelial layer have effector and memory function (1). As such the GALT and draining mesenteric lymph nodes the primary sites for intestinal adaptive immune regulation (Fig. 1A).

Antigen-bearing antigen presenting cells (APC) that are equipped with a large diversity in pattern recognition receptors efficiently traffic to the areas of naïve B or T cells to convey specific costimulatory signals that determine T- or B-cell differentiation and migratory capacity (Fig. 1B). A close interaction between innate epithelial cells and APC ensures precise sensing of the nature of an antigen. Upon APC-T-cell interaction in GALT or mesenteric lymph nodes (MLN), these signals are translated into a tailored differentiation of the adaptive immune response leading to elimination of pathogenic antigens but active tolerogenic responses to harmless antigens. Next to regulating T-cell responses, this regional mucosal compartmentalization is of pivotal importance for the formation of IgA antibodies that protect against mucosal penetration by commensal microbiota (2).

Intriguingly, once an adaptive tolerogenic T-cell response to a soluble protein antigen has developed, not only does the effector immune response provide tolerance in the intestinal tissue but also peripheral immune responses to the same

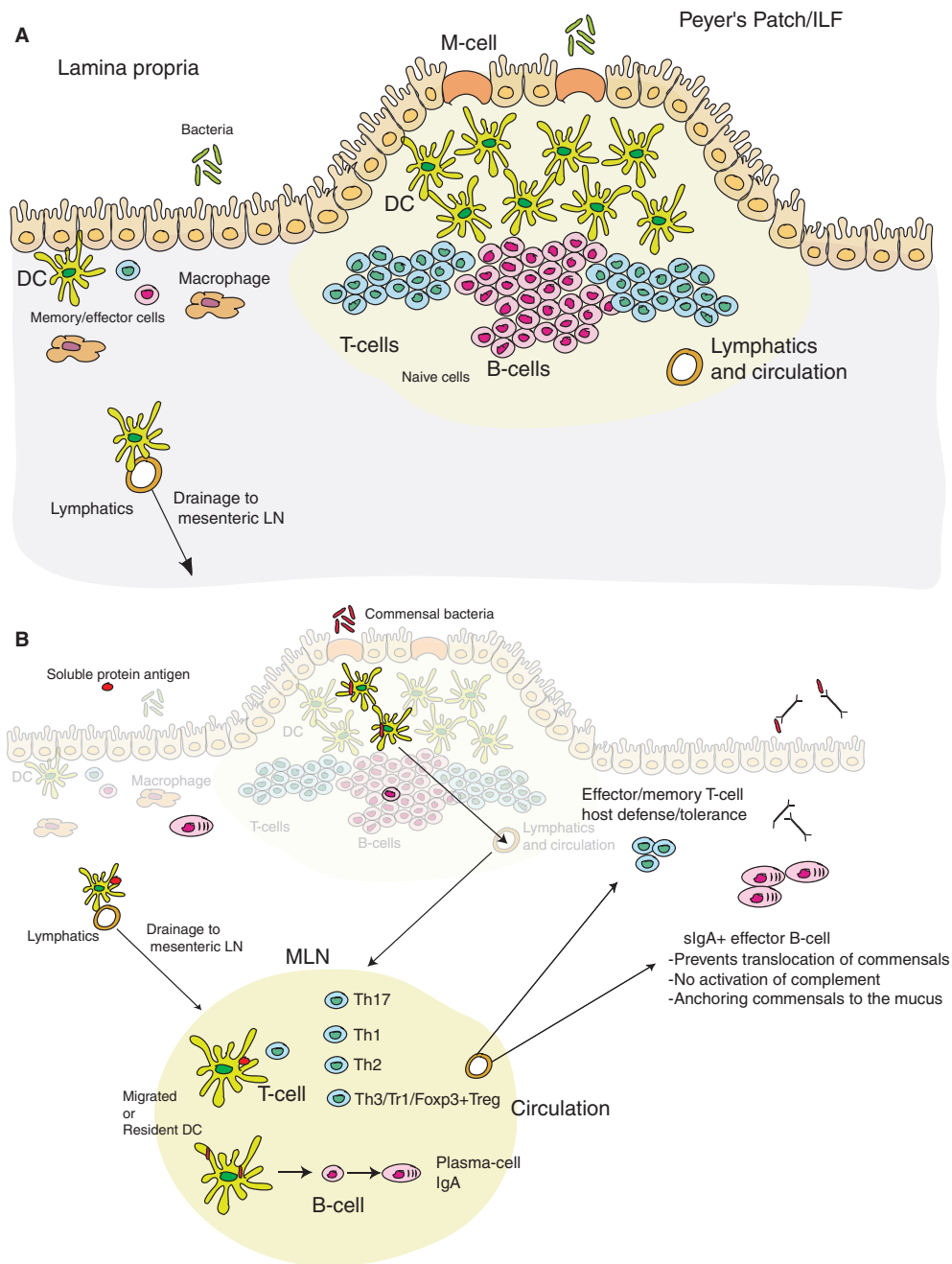


Figure 1 Structure of gut-associated lymphoid tissue (GALT). (A) GALT is comprised of Peyer's patches (PP), isolated lymphoid follicles, and the appendix (not shown). These are inductive sites where naive B and T cells differentiate upon antigen recognition. In contrast, cells within the lamina propria have effector or memory function. (B) The mesenteric lymph nodes (MLN) drain the small intestine. In both PP and MLN antigen carrying dendritic cells (DC)

traffic to B- and T-cell areas to convey specific costimulatory signals that determine T- or B-cell differentiation and migratory capacity. Upon DC-cell-T-cell interaction in GALT or MLN, these signals are translated into a tailored differentiation of the adaptive immune response leading to Th1, Th2, Th17 or regulatory T-cell differentiation (Th3, Tr1, Foxp3+Treg).

antigen are suppressed (3). Because of this "bystander" peripheral regulation, "oral tolerance" was defined as the suppression of T- and B-cell responses to an antigen by previous administration of the antigen by the oral route (3).

In spite of the robust mechanisms that maintain oral tolerance, hypersensitivity to food proteins is common in genetically susceptible individuals. Many food hypersensitivities are caused by immune responses that are IgE mediated

Box 1 Coeliac disease

Coeliac disease (CD) is one of the most common food hypersensitivities, affecting approximately 1% of the western world. In patients with CD, the ingestion of gluten proteins in wheat and similar proteins in barley and rye results in a chronic inflammation in the small intestine that is characterized by villous atrophy, crypt hyperplasia and increased numbers of infiltrating lymphocytes in both the epithelium and in the lamina propria. The only available treatment for the disease is a life-long gluten-free diet, resulting in a complete restoration of the normal intestinal architecture. Symptoms of CD include diarrhea, abdominal distension, malnutrition, fatigue, weight loss or, in young children, a failure to gain weight. However, symptoms and disease severity vary widely among patients, and some patients have a completely asymptomatic form of the disease. A small subgroup of CD patients become unresponsive to the gluten-free diet and develop refractory coeliac disease (RCD). RCD is a severe complication of the CD and patients with RCD have a high chance of developing an enteropathy associated T-cell lymphoma.

Loss of tolerance to gluten proteins in CD patients is associated with an inflammatory gluten-specific CD4⁺ T-cell response. Activated gluten-specific T cells that produce high amounts of IFN- γ can be isolated from the small-intestinal mucosa of CD patients but not of healthy individuals (95, 96). These gluten-specific T cells are exclusively restricted to the disease predisposing HLA-DQ2 and HLA-DQ8 molecules (95). More than 90% of the CD patients carry HLA-DQ2, whereas HLA-DQ2-negative patients usually express HLA-DQ8, demonstrating that these molecules play an important role in the presentation of gluten peptides in CD. Efficient binding of gluten peptides requires modification of the peptides by the enzyme tissue transglutaminase (TG2). This enzyme is constitutively expressed in the intestinal lamina propria and transiently activated upon tissue damage (97). TG2 introduces negative charges in certain gluten peptides by converting glutamine residues to glutamate. This process, termed deamidation, increases the affinity of gluten peptides for HLA-DQ2 and HLA-DQ8 molecules (98, 99).

Despite our understanding about the role of HLA-molecules and gluten-reactive T cells in the pathogenesis of CD, it is still unknown why gluten, out of so many food proteins, fails to induce tolerance and triggers an inflammatory immune response in CD patients.

(4) (1). However, also “non-IgE mediated food hypersensitivities” are distinguished in which the disease-inducing immune mechanisms have not been identified (4). One such non-IgE mediated food hypersensitivity is Coeliac disease (CD). CD patients develop an inflammatory response to the food protein gluten, resulting in severe intestinal pathology (Box 1).

The emphasis of this review lies on the role of the adaptive T-cell responses in the induction of mucosal tolerance to soluble protein fed and the unique properties of the mucosal microenvironment that regulate these responses.

Regulatory T cells and oral tolerance

The development of oral tolerance has been shown to depend on multiple mechanisms including clonal anergy, deletion as well as the generation of antigen-specific regulatory T (Treg) cells (5, 6). Originally, high doses of fed-antigen were thought to result in anergy or deletion of antigen-specific T cells, whereas low doses would favor the induction of Treg. However, it is now recognized that both mechanisms may occur simultaneously and cannot be fully distinguished. For instance, several types of Treg have characteristics of anergic T cells (7). As a result, the role of Treg in mucosal tolerance has been the focus of extensive research in the past decades and it is now widely accepted that suppression by Treg is an absolutely essential process for the establishment of mucosal tolerance.

Site of mucosal Treg induction

Induction of mucosal Treg differentiation from a naïve precursor T cell depends on the presentation of orally encountered antigens by an APC. Consequently, a crucial issue in the induction of mucosal tolerance is to understand the exact site where naïve T cells meet their cognate antigen

and differentiate into mucosal Treg. Fed proteins that reach the lumen of the gut can gain access to the mucosal immune system in at least three different ways (Fig. 1A.)

First, specialized microfold (M) cells can selectively bind antigens from the intestinal lumen. Microfold cells are mainly present in the follicle-associated epithelium that lines organized lymphoid follicles, which include Peyer's patches (PP) and isolated lymphoid follicles in the small intestine. Upon internalization by M cells, the intact antigen is effectively transferred to professional APC in the subepithelial dome region of the follicle. From this region, antigen-loaded APC may present the antigen to naïve T cells in the PP or enter the lymphatics to gain access to the gut-draining MLN (8).

Alternatively, luminal proteins can be acquired directly by resident lamina propria dendritic cells (DC) in several ways. It has been reported that M cells exist in the villous epithelium that is not associated with organized lymphoid follicles and in this way provide a mechanism by which lamina propria can sample luminal content. In addition, lamina propria DC that are situated underneath the epithelial monolayer can directly gain access to the luminal side of the epithelium, as it has been demonstrated that DC are able to open the tight junctions between adjacent epithelial cells and extend their dendrites through the epithelium to sample luminal content (9, 10). Moreover, DC constitutively endocytose apoptotic epithelial cells that have the ability to take up intestinal antigens and transport them to the MLN. Thus, under homeostatic conditions, lamina propria DC actively sample antigens from the gut lumen and constitutively migrate from the intestine to the draining MLN.

In addition to active uptake, a fraction of dietary protein antigens may cross the epithelial layer and reach the circulation. A majority of antigens that enter the circulation will reach the liver via the portal vein, a route that has previously been associated with oral tolerance induction (11). It has been suggested that the site and mechanism of uptake may in

part be dependent on the nature of the antigen (12). It should be noted that many studies are based on the use of the soluble egg white protein ovalbumin (OVA). This finding is of particular importance as the uptake of dietary proteins appears to be widespread throughout the intestinal epithelium, whereas it has been suggested that PP are more involved in the initiation of mucosal immune responses to bacteria (8). Moreover, in oral tolerance models using haptens, a prominent role for the liver is discerned (13).

It was originally assumed that M cells in the PP epithelium provide the main mechanism of entry for most fed antigens. Indeed, functionally suppressive antigen-specific Treg can be isolated from PP as early as 24 h after protein feed, demonstrating that antigen-uptake through PP contributes to the induction of oral tolerance (14). However, at 48 h after protein feed, antigen-specific Treg also differentiate in the MLN (14). Several studies have shown that oral tolerance can be normally induced in the absence of PP, indicating that functional MLN are sufficient for oral tolerance induction to soluble protein antigen (15). The presence of mucosa-draining tissue is critical for oral tolerance, as deficiency of both PP and lymph nodes as occurs in lymphotoxin α -deficient mice causes loss of oral tolerance. Reciprocally, treatment of pregnant lymphotoxin α -deficient mice with an agonistic anti-lymphotoxin β -receptor antibody resulted in reconstitution of MLN, which was sufficient to restore oral tolerance (15). A more recent study by Worbs et al. (16) demonstrated that surgical removal of the MLN impairs the induction of oral tolerance, emphasizing that intact PP alone are not sufficient to maintain oral tolerance. Using CCR7-deficient mice, which have defective migration of DC from the intestine to the MLN, the authors demonstrate that oral tolerance relies on the uptake of fed antigens by small-intestinal DC and subsequent CCR7-dependent migration to the MLN (16). Moreover, although intestinal antigens have been shown to enter the circulation and reach peripheral lymph nodes (17), transplantation experiments of the small bowel together with the MLN have demonstrated that antigen transport via the circulation is not sufficient to induce proliferation of antigen-specific T cells (16). Taken together, the uptake of antigen by lamina propria and PP DC and the subsequent induction of Treg in PP and the MLN represent the initial events in the induction of oral tolerance to soluble protein.

Regulatory T cells

Treg are defined by their functional capacity to suppress an effector T-cell response. Suppression can consist of inhibition of effector-cell proliferation as well as blockade of inflammatory cytokine release. Many different CD4⁺ Treg-cell subsets have been described, all of which are able to inhibit the responses of effector T cells. On the basis of their origin, a distinction between thymus-derived Treg and peripherally induced or adaptive Treg can be made. Thymus-derived CD4⁺CD25⁺ forkhead box P3 (Foxp3)⁺ Treg play a fundamental role in maintaining self-tolerance and preventing auto-immunity (18). In addition, thymus-derived Treg contribute to tolerance to nonself-antigens as was substantiated

by their ability to inhibit immune responses directed against commensal bacteria in the intestine and, consequently, to prevent the onset of colitis (19, 20). Although these findings indicate that thymus-derived Treg contribute to the maintenance of intestinal homeostasis, they are dispensable for oral tolerance to soluble protein antigens. Using mice that contain a monoclonal population of CD4⁺ T-cells specific for the model food protein OVA and that were crossed on a RAG-1 knockout background, Mucida et al. (21) demonstrated that tolerance to OVA was effectively induced in the absence of thymus-derived Treg.

In contrast to thymus-derived Treg, Treg that are induced after feeding protein are essential for mucosal tolerance (14, 22–24). Such peripherally induced or adaptive Treg can develop outside the thymus under specific microenvironmental conditions. Several subsets of induced Treg have been defined, including transforming growth factor (TGF)- β -producing Th3 cells that depend on TGF- β for their regulatory capacity (3), interleukin (IL)-10-producing type 1 regulatory T (Tr1) cells that mediate suppression through IL-10 (25) and induced Foxp3⁺ Treg (21). However, these subsets may not be mutually exclusive as for example Th3 and Foxp3⁺ cells have similarity with Tr1 cells by their secretion of IL-10. All subsets have extensively been studied for their role in oral tolerance (see Table 1 for a detailed overview of Treg studied during oral tolerance). As a result of the various experimental settings and the lack of exclusive cell markers identifying the specific Treg-cell subsets, it is difficult to determine what the relative contribution of each cell type is to the induction of mucosal tolerance. Moreover, multiple subsets may act in synergy to achieve suppression of peripheral chronic inflammation. As such, orally induced Foxp3⁺ Treg were essential for establishing tolerance to allergic airway inflammation, for suppressing IL-4 production and lymphoid neogenesis in chronic inflammation. However, IL-5 production and eosinophilia were controlled by Foxp3-independent, IFN- γ -dependent mechanisms (22). Crucially, Foxp3-independent regulation may contribute to the conditioning of the tolerogenic microenvironment of the gut and thereby influence the conversion of Foxp3⁺ Treg in response to orally encountered proteins.

Mucosal microenvironment

Although it was long recognized that the mucosal environment is immune privileged by its capacity to induce tolerance, only recently, progress has been made in identifying local factors in the mucosal microenvironment that drive this process (Fig. 2). Resident DC in the intestine have an intrinsic non-inflammatory activation state that preferentially drives tolerogenic T-cell responses. This fact is best illustrated by the report that Flt3 ligand-induced DC expansion enhanced oral tolerance, but when activation of the cells was elicited with IL-1, tolerance was lost. In particular, increased levels of costimulatory molecule expression CD80 and CD86 were associated with this process (26). This association is corroborated by the lack of functional mucosal Treg-cell induction in Fc γ RIIB-deficient mice that exhibit enhanced costimulatory molecule expression on DC in mucosa-draining tissue (27).

Table 1 Antigen-induced regulatory T cells in oral tolerance

Type	Cytokine	Organ	Model	Reference
TGF- β -producing	TGF- β , IL-10, IL-4	MLN	WT mice	(5)
	TGF- β , IL-10, IL-4	Spleen	MBP-TCR Tg mice	(100)
	TGF- β	PP	WT mice	(101)
IL-10-producing	IL-10, TGF- β , IL-4	PP	WT mice	(54)
CD25 ⁻ (Foxp3 [?])	?	PP and MLN	DO11.10 transfer	(14)
CD25 ⁻ Foxp3 ⁺		PP, MLN, Spleen	DO11.10, DO11.10 transfer (OVA/CTB)	(102)
CD25 ⁺ (Foxp3 [?])		PP and MLN	DO11.10 transfer	(14, 23)
	TGF- β , IFN- γ , IL-10	PP	WT mice	(103)
	IL-10, TGF- β	MLN, inguinal LN, Spleen	DO11.10	(104)
	TGF- β	Celiac LN (liver draining) MLN	DO11.10 transfer	(105)
CD25 ⁺ Foxp3 ⁺		PP, MLN, Spleen	DO11.10, DO11.10 transfer (OVA/CTB)	(102)
CD25 ⁺ Foxp3 ⁺		MLN+Spleen	T/Bmc RAG1 ^{-/-}	(21)
Foxp3 ⁺ (PCR)		PP	DO11.10 transfer	(106)
Foxp3 ⁺		PP, MLN, lamina propria	OT-II RAG1 ^{-/-} transfer	(32)
Foxp3 ⁺		MLN	DO11.10 SCID	(49)
Foxp3 ⁺		MLN	DO11.10 RAG2 ^{-/-} transfer	(48)

Foxp3, forkhead box P3; IL, interleukin; MBP, myelin basic protein; MLN, mesenteric lymph nodes; PP, Peyer's patch; RA, retinoic acid; TCR, T cell receptor; Tg, transgenic; TGF, transforming growth factor; WT, wild-type; DO11.10 and OTII mice have a tg TCR specific for ovalbumin; RAG1/2^{-/-} indicates that the mice are backcrossed on a recombination activation gene deficient background.

However, next to a difference in activation state, particular subsets of mucosal DC exist that can have intrinsic regulatory properties when compared with non-mucosal DC. For example, DC isolated from MLN and PP very efficiently induce the expression of the gut-homing receptors $\alpha 4\beta 7$ and CCR9 on proliferating T cells *in vitro* (28, 29) and stimulate B cells to secrete immunoglobulin A (IgA) (30). Moreover, mucosal DC promote the conversion of naive T cells into Foxp3⁺ Treg (31, 32) and are less able to induce the differentiation of Th17 cells (31). Recent advances in the field of DC development have shown that these intrinsic capacities can be coupled to phenotypically definable CD11c⁺ CD103⁺ and CX3CR1⁺ subpopulations in the intestinal lamina propria, which are derived from pre-cDCs and Ly6C⁺ monocytes, respectively (33) (Fig. 3). Although CD103⁺ cells emigrate from the lamina propria to the mesenteric lymph nodes and have imprinting capacities, CX3CR1⁺ cells do not emigrate to the mesenteric lymph nodes or efficiently induce a T-cell response (34). As such, these CX3CR1⁺ cells share more characteristics with lamina propria macrophages than DCs (33). The role of CD103⁺ DC in mediating oral tolerance is further discussed below (See Retinoic acid).

Accumulating evidence suggests that the function of mucosal DC is conditioned by the local microenvironment (35). As mucosal DC continuously migrate from the lamina propria to the MLN, they are influenced by local factors at both sites. For the lamina propria, it has been demonstrated that one source of such local factors are intestinal epithelial cells (IEC). Intestinal epithelial cells are in close contact with lamina propria DC and have been shown to release molecules that condition the DC into a noninflammatory state, such as the cytokine thymic stromal lymphopoietin (TSLP) (36), TGF- β , and the vitamin A-derivative retinoic acid (RA) (37).

The local environment of the mucosa-draining lymph nodes also has unique characteristics that are important for the induction of mucosal tolerance as well as the generation of gut-homing T cells (38). Studies that have focussed on the induction of mucosal tolerance via the nasal mucosa have demonstrated that the regional nose-draining lymph nodes are essential for the establishment of tolerance via this route (39). Similar to data obtained from oral tolerance experiments, nasal tolerance was completely abrogated upon surgical removal of the nose-draining lymph nodes (39). Transplantation experiments revealed that only replacement with nose-draining lymph nodes but not with peripheral or gut-draining lymph nodes could restore tolerance, in spite of the fact that all transplanted lymph nodes were fully functional (38, 39). It is currently unclear whether intrinsic capacities of the gut-draining MLN are important for oral tolerance in a similar way. However, evidence that the MLN as well have unique functions that are not shared by peripheral lymph nodes came from a recent study on the imprinting of gut-homing receptors. Hammerschmidt et al. (40) demonstrated that the induction of CCR9 expression on T cells *in vivo* depends on the presence of both resident stromal cells in the MLN as well as steady-state migratory DC.

Taken together, both the activational state of resident DC as well as specific subsets of functionally distinct DC maintain mucosal tolerance. However, this occurrence is fully dependent on the various factors derived from local APC, epithelial cells or lymph node stromal cells that together condition the microenvironment and enforce intestinal homeostasis. In particular, the role of TGF- β , IL-10, retinoic acid, and cyclooxygenase-2 derived prostaglandins play an important role in conditioning the tolerogenic microenvironment of the gut.

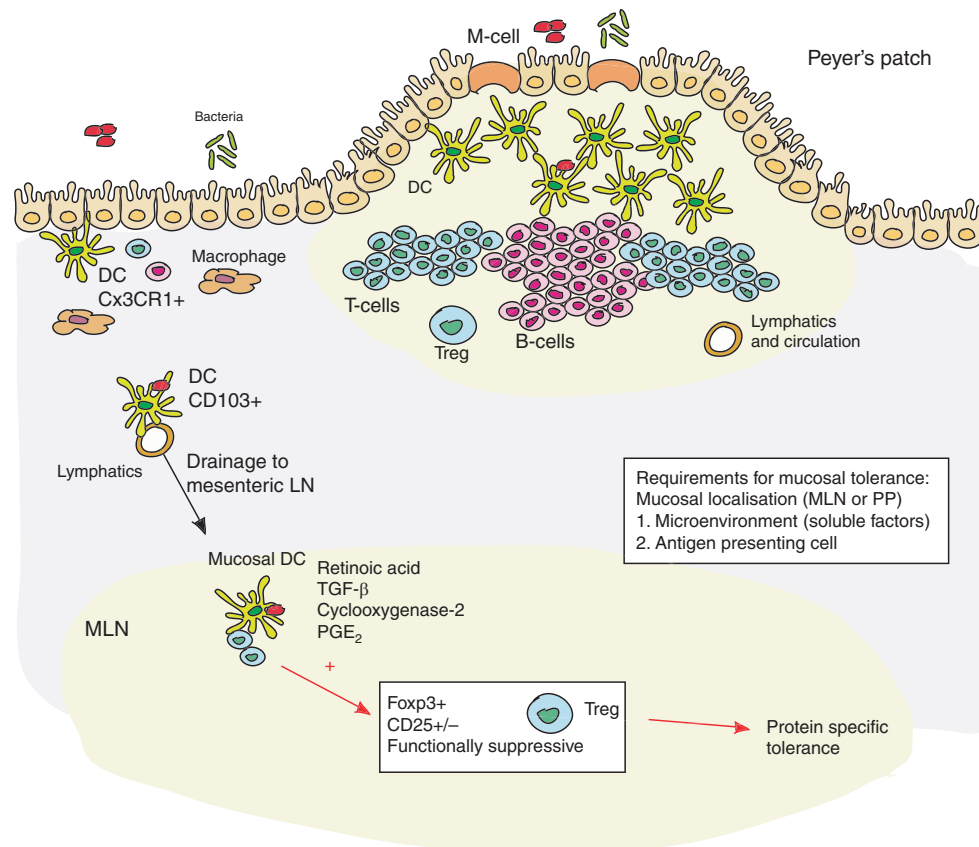


Figure 2 Mucosal Treg differentiation is driven by factors within the mucosa associated lymphoid tissue. Fed proteins that reach the lumen of the small intestine can be taken up in several ways. First, proteins enter by selective uptake through microfold cells (M cells) that overlie Peyer's patches (PP). Second, luminal proteins may directly be acquired by DC in the lamina propria outside of the gut associated lymphoid tissue (GALT) tissues. Third, a fraction of the dietary protein may cross the epithelium and reach the liver via the portal vein (not shown). Both PP and mesenteric lymph nodes (MLN) have the capacity to support mucosal Treg differentiation.

TGF-β

The anti-inflammatory cytokine TGF-β is abundantly expressed by nonhematopoietic as well as hematopoietic cells in the gut-microenvironment, and is a critical factor in maintaining intestinal homeostasis. TGF-β has strong immunosuppressive effects on lymphocytes: it inhibits T-cell proliferation in the absence of exogenous IL-2 (41) and suppresses the differentiation of naive T cells into effector Th1 and Th2 cells through direct downregulation of T-bet and GATA3 expression (42, 43). In addition to its immunosuppressive properties, TGF-β plays an important role in epithelial cell differentiation and IgA class switching in the intestine.

The key role of TGF-β in oral tolerance is best illustrated by the finding that neutralization of TGF-β impairs the development of tolerance to fed proteins (21, 44). In addition, TGF-β-producing Th3 cells can be isolated from the PP

However, in the absence of PP the presence of the MLN is sufficient to sustain oral tolerance. Differentiation of functionally suppressive Tregs occurs within 48–72 h post protein feed and requires antigen presentation by specialized DC as well as the presence of mucosal regulatory factors. In particular, CD103⁺ DC contribute to Treg differentiation by their secretion of retinoic acid. However, also retinoic acid, TGF-β, and cyclooxygenase-2 derived prostaglandins derived from hematopoietic and non-hematopoietic cells contribute to this process.

and MLN of orally tolerized mice (5). These Th3 cells are antigen-specific and suppress experimental autoimmune encephalomyelitis in a TGF-β-dependent manner (5).

More recently, TGF-β-dependent Treg have been identified that express latency-associated peptide (LAP) and TGF-β on the cell surface (45). Suppression by these LAP⁺ Treg is cell-cell contact dependent and can be blocked with anti-TGF-β antibodies. LAP⁺ Treg have been implicated in the suppression of colitis (45). However, it remains to be determined whether LAP⁺ Treg are also induced after protein feeding and what the exact relationship is between LAP⁺ Treg and Th3 cells.

In addition to functioning as an effector molecule of Th3 cells and LAP⁺ Treg, TGF-β can mediate immune tolerance by the induction of Foxp3⁺ Treg. *In vitro* studies have revealed that the activation of naive T cells in the presence of TGF-β induces the *de novo* expression of Foxp3 and the acquisition of suppressive capacity (46). In addition to the induction of

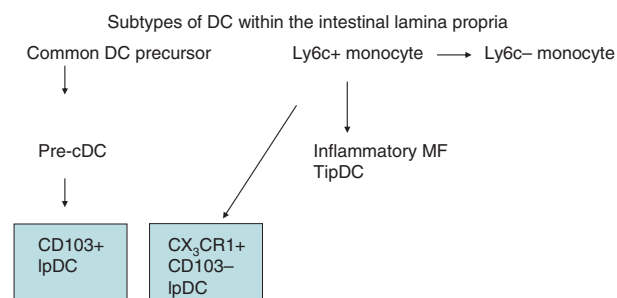


Figure 3 Subtypes of dendritic cells within the small intestinal lamina propria. Intestinal Macrophages and DC all stem from myeloid precursors in the bone marrow. These myeloid precursors give rise to monocytes, some populations of macrophages, and common DC precursors. Two monocyte subsets, Ly-6C+ and Ly-6C-, leave the bone marrow to enter the blood. Common DC precursors give rise to preclassical dendritic cells (pre-cDCs). Pre-cDCs circulate in blood and populate lymphoid organs and tissues. As such, Pre-cDC may give rise to CD103+ lamina propria DCs (IpDCs). Ly-6C+ monocytes can become CX3CR1+ IpDCs in nonlymphoid tissues. During inflammation, Ly-6C+ monocytes give rise to monocyte-derived DCs, for example, tumor necrosis factor (TNF) and inducible nitric oxide synthase (iNOS)-producing dendritic cells (TipDCs), inflammatory macrophages.

Foxp3⁺ Treg, TGF- β plays an important role in the maintenance of Foxp3⁺ Treg in the periphery (47). Multiple studies have shown that the development of tolerance to oral proteins is associated with the *de novo* induction of Foxp3⁺ Treg (21, 32, 48, 49). An important role in this process can be attributed to TGF- β as neutralization of the anti-inflammatory cytokine during induction of oral tolerance resulted in a reduction in Foxp3 expression in MLN and the spleen (21).

The GALT has been recognized as a very efficient site for the conversion of naive T cells into Foxp3⁺ Treg (31, 32, 49). Although the efficient induction of Foxp3 may seem evident in a microenvironment that harbors high concentrations of TGF- β , it should be noted that TGF- β is secreted in a biologically inactive form and needs to be activated to exert its suppressive activities. Latent TGF- β can be activated by several molecules, including the integrins $\alpha_4\beta_6$ expressed by IECs and $\alpha_v\beta_8$ by DC (50, 51). Local mucosal DC are thought to play a key role in maintaining intestinal homeostasis by activating latent TGF- β . It has been demonstrated that mucosal DC are significantly better than splenic DC at inducing the expression of Foxp3 in naive T cells (32, 49). This difference is in part dependent on the ability of mucosal DC to release bioactive TGF- β as mucosal DC lacking the TGF- β -activating integrin $\alpha_v\beta_8$ failed to induce the differentiation of Foxp3⁺ cells *in vitro* (50, 51).

IL-10

IL-10 is a cytokine with broad anti-inflammatory properties that is produced by many other cell types including epithelial cells, monocytes, macrophages, DC, NK cells, B cells, CD8⁺ T cells, intraepithelial lymphocytes, and different subsets of

CD4⁺ T-cells, including Th1 cells, Foxp3⁺ Treg, Tr1 cells, Th3 cells, Th17 cells, and follicular helper T cells (52, 53). High numbers of IL-10-producing T cells can be detected in the intestinal microenvironment, especially in PP, small intestinal intraepithelial lymphocytes, and colonic lamina propria lymphocytes (53). Despite its abundant presence in the intestines, the role of IL-10 in oral tolerance is controversial. Several studies have reported that antigen feeding results in an increased IL-10 production, mainly in the PP, but also in MLN, the spleen, and serum (5, 54). It has been suggested that antigen-specific Tr1 cells that depend on IL-10 production are induced when oral administration of antigen is combined with IL-10 (55). However, multiple studies have demonstrated that oral tolerance can be normally induced in the absence of IL-10 (56), indicating that in steady state conditions, IL-10 is not absolutely necessary for the establishment of tolerance to soluble protein.

Although IL-10 seems to be dispensable for the induction of tolerance to dietary proteins, its role in promoting tolerance to intestinal microbiota is well established. IL-10-deficient mice develop spontaneous colitis (57), and reciprocally exogenous IL-10 was shown to restore tolerance in mice with colitis. The mechanism by which IL-10 protects from colitis is poorly understood. Although both IL-10⁺Foxp3⁻ Tr1 cells and IL-10⁺Foxp3⁺ Treg are detected in the small intestine, all IL-10-producing T-cells in the colonic lamina propria express Foxp3 (58). Specific deletion of IL-10 in Foxp3⁺ cells resulted in the development of spontaneous colitis, demonstrating that IL-10 is required for the suppressive function of Foxp3⁺ Treg in the colon (59). However, in a different model it has recently been demonstrated that IL-10 derived from lamina propria macrophages is crucial for the prevention of colitis and may act by supporting the function of Foxp3⁺ Treg in the inflamed intestine (60). Thus, it is likely that multiple cell populations contribute to homeostasis in the large intestine through their production of IL-10.

Although it is well recognized that the intestines are a preferential site for the generation of IL-10-producing T-cells, the local factors that mediate the differentiation of these cells remain largely unclear. An important role is attributed to tolerogenic APC in mucosal tissues, as MLN-derived CD8 α ⁺ plasmacytoid DC (61), CD11b⁺ DC from PP (62) and CD11b⁺F4/80⁺ lamina propria macrophages (63) have been shown to induce efficiently the expression of IL-10 in responding T cells. The ability of mucosal APC to induce IL-10-producing T cells has been associated with their own secretion of IL-10 (62, 63), which in turn may be dependent on conditioning by the tolerogenic environment of the gut. However, recently, several very different pathways have been reported to lead to IL-10-producing T cells. Importantly, IL-10 production is not only restricted to the differentiation of IL-10-producing Tr1 cells from naive T cells, as IL-10-producing T cells with suppressive activity can also derive from effector T cells. This finding adds to the complexity of studying these cells because the suppressive capacity of IL-10-secreting effector T cells may thus be very transient. Particular levels of antigen encounter may account for this

effector T cell-derived IL-10, as high and sustained levels of antigen induce Th1-derived IL-10 through stimulation with IL-12 (64). Further clarification of the mechanisms that contribute to the induction of IL-10-producing Tr1 cells under homeostatic conditions in the small intestine requires further investigation.

Retinoic acid

Another key factor that contributes to the tolerogenic mucosal microenvironment is RA, a metabolite of vitamin A. Retinoic acid is metabolized from the dietary vitamin A in a two-step reaction involving alcohol dehydrogenase (ADH) and retinal dehydrogenase (RALDH) enzymes. Vitamin A deficiency is a cause of increased inflammation. Prolonged insufficiency compromises epithelial barrier function and causes a loss of mucus-producing goblet cells, resulting in an increased severity of disease during infections. Despite these defects, vitamin A-deficient mice do not show significant inflammation in the small intestine, and oral tolerance develops normally in these mice (65).

Recently, RA was identified as a cofactor that enhances TGF- β -mediated induction of Foxp3 by mucosal DC (31, 32, 49). Dendritic cells from the MLN and the small-intestinal lamina propria have a significantly greater capacity to induce Foxp3 expression than nonmucosal DC, a quality that is associated with their ability to catalyze retinal into RA (32, 49). In addition to the induction of Foxp3, the induction of the gut-homing receptors $\alpha 4\beta 7$ and CCR9 as well as the stimulation of intestinal B cells to secrete IgA is also dependent on the ability of mucosal DC to convert vitamin A into RA.

Following up on these findings, it was shown that mucosal DC that express CD103 can induce Foxp3⁺ Treg in the absence of exogenous TGF- β and RA (49) (Fig. 4). In contrast, CD103⁻ mucosal DC were not able to induce Foxp3 expression in the absence of exogenous TGF- β and, when TGF- β was provided, the induction was much weaker than that observed with CD103⁺ DC (49). This difference in function was explained by the observation that CD103⁺ DC from MLN express higher levels of Aldh1a2, the gene encoding RALDH2, than CD103⁻ MLN-DC (49).

The exact mechanisms by which RA enhances the capacity of TGF- β to convert naive T cells into Foxp3⁺ Treg remain largely unclear. Recent progress has clarified that RA elicits its effects in multiple ways and can affect naive T cells both directly and indirectly. An important strategy by which RA enhances the differentiation of Treg is by interference with the function of proinflammatory cytokines, which are known to suppress the induction of Foxp3 in naive T cells (66). Not only does RA inhibit the production of inflammatory cytokines by effector or memory T cells (67–70), it also interferes with the negative effects of those cytokines on naive T cells (31, 70–73). Mechanisms by which RA protects naive T cells from this suppression by cytokines involve the downregulation of IL-6 receptor (72) and regulation of the Foxp3 promoter region by RA receptor α (73). Besides these cytokine-dependent mechanisms, RA can enhance the conversion of naive T cells into Foxp3⁺ Treg independent of secreted

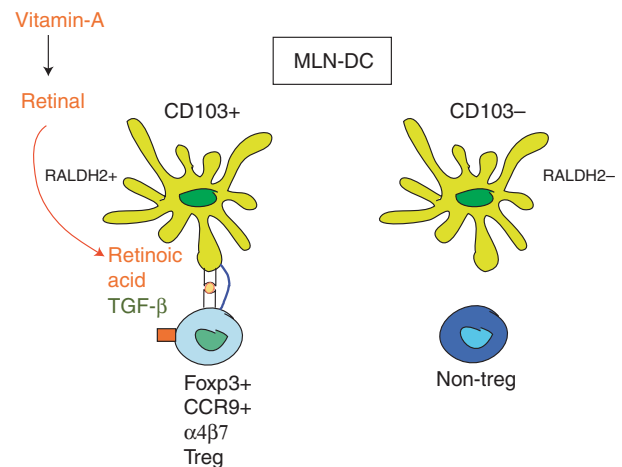


Figure 4 Functional characteristics of CD103⁺ MLN-DC. MLN CD103⁺ DC have distinct characteristics from CD103⁻ MLN-DC. Majority of CD103⁺ MLN DCs are thought to be a tissue-derived migratory population that plays a central role in presenting orally derived soluble antigen to CD8⁺ and CD4⁺ T cells. CD103⁺ DC contain the enzyme retinaldehyde dehydrogenase 2 (RALDH2), which converts retinal into retinoic acid. Retinoic acid acts on T cells by inducing the mucosal homing receptors $\alpha 4\beta 7$ and CCR9. Moreover, in the presence of TGF- β , retinoic acid promotes conversion of naive T cells into Foxp3⁺ Treg. In contrast, CD103⁻ MLN DCs, which appear to derive from blood precursors do not have this same capacity.

inhibitory cytokines (71). It has been demonstrated that RA counteracts the inhibitory effects of costimulation on Foxp3⁺ Treg-cell differentiation (74). Another suggestion of how RA stimulates Treg-cell differentiation is via its capacity to enhance TGF- β signaling, as RA increases the expression of the TGF- β -dependent Smad3 (72). However, it has recently been demonstrated that despite the increase in Smad3 expression, the enhancement of Foxp3 expression by RA was largely Smad independent (71).

It must be taken into account that apart from mucosal DC other cells including intestinal epithelial cells (37) and MLN stromal cells (40, 75) have been shown to express vitamin A converting enzymes. This epithelial cell or stromal cell-derived RA might be transferred to DC, as it has been demonstrated that DC can acquire RA from other sources, store it and in this way gain the tolerogenic characteristics of mucosal DC (76). Alternatively, contact between epithelial cells or stromal cells and DC may increase the expression of RALDH enzymes and CD103 in the DC in an RA-dependent manner, resulting in an enhanced ability to induce Foxp3⁺ Treg-cell differentiation (37).

Reciprocal to its role in Treg-cell induction, RA has been shown to inhibit the generation of Th17 cells, depending on the local cytokine environment. Dendritic cells stimulated in the presence of TGF- β induce Treg and in the presence of TGF- β and the proinflammatory cytokine IL-6 induce Th17 cells (31). However, it was recently suggested that the effect of RA depends on the dose as at a low concentration, RA

was shown to stimulate instead of inhibit the differentiation of Th17 cells (77). Taken together, these results demonstrate that RA plays an important role in controlling the balance between effector and regulatory CD4⁺ T-cell populations in the small intestine.

The ability to enhance the reciprocal TGF- β -mediated induction of Foxp3 and Th17 cells is not restricted to mucosal DC as it has been demonstrated that lamina propria macrophages can also perform this action (63). The induction of Foxp3⁺ cells by lamina propria macrophages was not only dependent on TGF- β and RA but also on IL-10, suggesting that TGF- β , RA, and IL-10 may all act synergistically in inducing Foxp3⁺ Treg-cell differentiation (63).

Cyclooxygenase 2

Cyclooxygenase-2 (COX-2) is the inducible isoform of the COX enzymes that catalyze the formation of prostaglandins from arachidonic acid. Although the COX-1 isoform is constitutively active in most tissues, COX-2 is induced by proinflammatory cytokines and microbial agents and has therefore long been recognized as a proinflammatory enzyme. However, in the intestines, the expression of COX-2 is constitutive and contributes to the maintenance of mucosal homeostasis. Although COX-2-deficient mice do not show any spontaneous inflammation of the gut, these mice have an increased susceptibility to chemically induced colitis that is associated with an inability to produce the COX-2-dependent metabolite prostaglandin E₂ (PGE₂) (78). In agreement, a deficiency in EP4, one of the G-coupled PGE₂ receptors, also causes increased susceptibility to chemically induced colitis, whereas deficiency in other prostaglandin receptors did not have this effect (79). Treatment of mice with the selective COX-2 inhibitor NS-398 results in loss of tolerance to fed proteins (48, 80). This selective inhibition of COX-2 during protein feeding was associated with enhanced T-cell-derived IL-4 production and defective induction of Treg in the gut-draining MLN. Crucially, IL-4 is directly responsible for the loss of oral tolerance as treatment of mice with anti-IL-4 antibodies prior to COX-2 inhibition and protein feed restores oral tolerance. Indeed, multiple studies have shown that IL-4 directly inhibits Foxp3⁺ Treg differentiation (48). Thus, COX-2 promotes mucosal Treg-cell differentiation through downregulation of IL-4-producing Th2-like cells. Concomitantly, the COX-2-dependent metabolite PGE₂ can also directly induce Foxp3 expression in T cells (48). Taken together, these studies demonstrate a crucial role for COX-2-dependent arachidonic acid metabolites in maintaining both small intestinal and colonic homeostasis.

Intriguingly, Newberry et al. (80) have demonstrated that administration of COX-2 inhibitors simultaneous with protein feed resulted in pathological changes in proximal small intestine that resemble human CD, including villus blunting and increased proliferation of epithelial crypt cells resulting in crypt expansion. Moreover, a decrease in villus height was also observed in HLA-DQ8 transgenic (Tg) mice that were immunized with gliadin and treated with the COX inhibitor

indomethacin (81). Thus, these findings indicate that COX-2 derived prostaglandins can act as cofactors for induction and maintenance of orally induced Treg and subsequent mucosal tolerance.

What is so particular about gluten?

In view of the known antigen, the strong HLA association and our understanding of the mechanisms that maintain oral tolerance, one could expect that the pathogenesis of CD can easily be resolved. The contrary is true. One reason may be that gluten is a very particular food antigen. The structure of gluten itself may already provide several indications that could explain a failure of tolerance induction for this particular food protein. It has been demonstrated that antigens that induce oral tolerance are generally soluble, whereas antigens that are particulate in nature are likely to induce immunity (82). In contrast to most food proteins, gluten proteins are insoluble in water and contain disulfide and hydrogen bonds, resulting in assembly of stable gluten aggregates (83). Moreover, these proline-rich aggregates are resistant to degradation by gastrointestinal enzymes causing large gluten complexes to reach the gut lumen (83). Furthermore, incomplete degradation may increase immunostimulatory capacities of gluten proteins. It has been demonstrated that the gluten peptides can activate the innate immune system and may thus contribute to the development of gluten-induced enteropathy independently of their capacity to bind HLA-DQ2/DQ8 and activate gluten-specific T cells. Maiuri et al. (84) demonstrated that the gluten p31-43 peptide elicits the production of the proinflammatory cytokine IL-15 by lamina propria macrophages and DC in biopsies of CD patients. More recently, it was demonstrated that gluten peptides can even trigger an IL-15-mediated innate immune response in individuals without CD (85). Since the first publication by Maiuri et al., evidence is accumulating that gluten can induce the maturation of DC (86, 87), thereby restructuring previously tolerogenic DC to stimulate the differentiation of proinflammatory Th1 cells. In agreement with these findings, it was demonstrated that DC isolated from the coeliac mucosa have a nontolerogenic phenotype, as indicated by high surface expression of CD80, CD86, and CD83, increased expression of interferon (IFN)- α and decreased levels of IL-10 and TGF- β transcripts. However, despite all these findings, it remains clear that most HLA-DQ2 positive individuals are normally tolerant to gluten. This tolerance is likely to develop at the first introduction of gluten into the diet. Therefore, many studies have focussed on identifying an age that is associated with a lower risk for disease development (88). Alternatively, there is evidence that suggests that disease development is associated with a particular inflammatory trigger. For instance, Cammarota et al. (89) reported the onset of CD in two patients with chronic hepatitis C that received treatment with IFN- α .

Loss of the tolerogenic intestinal microenvironment in CD

The small-intestinal mucosa of CD patients is characterized by the presence of high levels of proinflammatory cytokines,

such as IFN- α , IL-15, IL-18, and IL-21, that may interfere with the tolerogenic microenvironment of the gut (90). Perhaps surprisingly, the expression levels of the anti-inflammatory cytokines TGF- β and IL-10 are not decreased in the mucosa of patients with active CD. Several groups have performed immunohistochemical staining of intestinal biopsies and observed an intense TGF- β staining in the epithelium and lamina propria of CD patients that was comparable with controls (91). In addition, the inflamed intestine of untreated CD contains high levels of IL-10-producing T cells that was similar to or even increased in comparison with treated or healthy controls (92, 93). Despite the fact that these anti-inflammatory cytokines are not decreased in active CD, a relative lack due to the massive increase in proinflammatory cytokines or defective signaling may contribute to the loss of gluten tolerance in CD. For example, exogenous IL-10 was shown to suppress the gluten-specific inflammatory T-cell response in *ex vivo* culture of biopsies from CD patients, suggesting that the IL-10 levels in the CD mucosa are insufficient to downregulate ongoing inflammation and that administration of exogenous IL-10 may contribute to reset the balance between tolerance and immunity (93). Moreover, IL-10 is associated with disease remission as IL-10-producing gliadin-specific Tr1-cell clones have been isolated from the mucosa of treated CD patients on a gluten free diet. These Tr1-cell clones inhibited the proliferation of gliadin-specific T-cell clones (94) and these findings may implicate the involvement of IL-10-producing Tr1 cells in response to dietary proteins.

It has recently been demonstrated that TGF- β signaling is impaired in CD patients and that intestinal T cells isolated from active CD patients are unresponsive to exogenous TGF- β signals (91). Moreover, it was found that this defective TGF- β signaling is the result of the high level of IL-15 in the coeliac mucosa, which directly impedes TGF- β -signaling through inhibition of the Smad3 pathway (91). Thus, IL-15 mediated inhibition of TGF- β -signaling might be an important mechanism that causes abrogation of oral tolerance to gluten and promotes the development of intestinal

inflammation in CD. To decipher the exact mechanisms that lead to gluten hypersensitivity, there is a need for mouse models to study the disease. In recent years, progress has been made in this area using transgenic expression systems as well as transfer models. Despite their limitations such models may not only advance the field of CD but also reveal fundamental aspects of mucosal tolerance to nonsoluble food proteins.

Concluding remarks

The immunosuppressive microenvironment of the gut is controlled by a complex network of immunological interactions between epithelial cells, antigen-presenting cells, and lymphocytes that allow the induction of oral tolerance to food proteins. Over the last decades, the increased identification of tissue-specific factors in the intestines has emphasized the redundancy in mechanisms that contribute to this local tolerogenic environment. This extreme redundancy explains why oral tolerance is so robust and difficult to inhibit when aiming at mucosal vaccine delivery. Reciprocally, this complexity enhances the difficulty of identifying the mechanisms that underlie loss of oral tolerance in diseases such as CD. Although pivotal tolerogenic mechanisms are unraveled using the model protein ovalbumin, there is a strong need to study disease associated antigens. There is a risk that such antigens may differ in uptake, processing, and presentation and therefore mediate tolerance via alternative mechanisms.

Note

The authors wish to apologize to their colleagues for not always citing all original publications due to space limitations.

Conflict of interest

The authors have no conflict of interest.

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