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Oral tolerance to food protein

O Pabst¹ and AM Mowat²

Oral tolerance is the state of local and systemic immune unresponsiveness that is induced by oral administration of innocuous antigen such as food proteins. An analogous but more local process also regulates responses to commensal bacteria in the large intestine and, together, mucosally induced tolerance appears to prevent intestinal disorders such as food allergy, celiac disease, and inflammatory bowel diseases. Here we discuss the anatomical basis of antigen uptake and recognition in oral tolerance and highlight possible mechanisms underlying the immunosuppression. We propose a model of stepwise induction of oral tolerance in which specialized populations of mucosal dendritic cells and the unique microenvironment of draining mesenteric lymph nodes combine to generate regulatory T cells that undergo subsequent expansion in the small intestinal lamina propria. The local and systemic effects of these regulatory T cells prevent potentially dangerous hypersensitivity reactions to harmless antigens derived from the intestine and hence are crucial players in immune homeostasis.

THE NEED FOR PERIPHERAL TOLERANCE TO ANTIGEN IN THE INTESTINE

During lymphocyte differentiation, a virtually unlimited repertoire of B- and T-cell receptors is generated to recognize all kinds of different antigens. As a result, a large number of developing lymphocytes will have receptors that can react to self-antigens, as well as innocuous foreign antigens such as food and commensal bacteria. Deleterious immune responses against self-antigens are normally prevented by the process of negative selection or central tolerance, in which developing T and B cells in the thymus and bone marrow are eliminated from the repertoire. Additionally, some T cells recognizing self-antigen with high affinity will express the transcription factor Foxp3 (forkhead box P3) and become natural regulatory T cells (nTregs). Both central tolerance of T cells and nTreg differentiation require the interaction of the T-cell receptor with its cognate antigen in the thymus. Consequently, both of these mechanisms are unsuited for preventing responses against antigens such as those coming from the intestine and that are not present or expressed at the sites of lymphocyte differentiation. Thus, additional layers of peripheral tolerance are needed to ensure tolerance to antigens such as foods and commensal organisms.

MUCOSALLY INDUCED TOLERANCE TO ANTIGEN IN THE INTESTINE

The intestine is exposed continuously to vast amounts of foreign antigenic material. As well as ingesting > 100 g of foreign protein

per day in our diet, the intestine is colonized by a dense community of commensal microbes, referred to as the microbiota. The density of these microbes increases along the gastrointestinal tract, reaching up to 10^{12} bacteria per gram of gut content in the colon. At the same time, the mucosal barriers are thin and very vulnerable to pathogenic infection, meaning that the intestinal immune system has to discriminate between generating protective immunity against harmful antigens and tolerance against harmless materials. Active immune responses directed against the microbiota can result in inflammatory bowel diseases such as Crohn's disease and ulcerative colitis. The incidence of Crohn's disease and ulcerative colitis is increasing throughout the world and in some countries can affect up to 0.6% of the population.¹ Failure to induce tolerance to food protein is thought to result in food allergy and celiac disease, which is the most prevalent food-induced pathology.² The ability of orally administered antigen to suppress subsequent immune responses, both in the gut and in the systemic immune system, was first described 100 years ago and has been studied widely since.^{3,4} This phenomenon is referred to as "oral tolerance" and its effects on systemic immunity have led to attempts to exploit it therapeutically to prevent or treat autoimmune diseases (reviewed in ref. 5). However, there is an important difference between tolerance to gut bacteria and tolerance to food proteins: whereas tolerance to food protein induced via the small intestine affects local and systemic immune responses, tolerance to gut bacteria in the colon does not attenuate systemic responses. Such differences are frequently

¹Institute of Immunology, Hannover Medical School, Hannover, Germany. ²Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, Scotland. Correspondence: O Pabst (Pabst.Oliver@MH-Hannover.de) or AM Mowat (Allan.Mowat@glasgow.ac.uk)

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overlooked in the field and the term oral tolerance is often used interchangeably to describe tolerance to food protein and bacteria. In our opinion, this arbitrary terminology is misleading and has hampered a better understanding of the underlying processes. Here we will use “oral tolerance” to describe tolerance to orally administered soluble antigens and will discuss its role in the broader context of “mucosally induced tolerance” that can be induced by other antigens in different parts of the intestine.

ORAL TOLERANCE: SCOPE AND EFFECTS

Oral tolerance has been demonstrated extensively in rodents using many different model antigens including purified proteins, cellular antigens, and small haptens, and it has also been shown in humans.^{6–8} The effects of oral tolerance are measured typically as reductions in systemic delayed-type hypersensitivity, T-cell proliferation, and cytokine production. Serum antibody responses can also be suppressed, particularly immunoglobulin E (IgE) and T helper type 1-dependent IgG2a production, as can mucosal T-cell and immunoglobulin A responses. Moreover, oral tolerance has been shown to suppress immunopathology in experimental models of autoimmune encephalitis,⁹ collagen-induced arthritis,¹⁰ type 1 diabetes,¹¹ and others (see ref. 5 for review). Thus, oral tolerance attenuates a broad range of immune responses and appears to play a central role in immune homeostasis.

As the basic aspects of oral tolerance have been reviewed exhaustively over the years,^{4,5,12} here we will concentrate on aspects that have attracted particular interest recently. First, we will discuss the routes by which antigen is taken up in the intestine and where tolerance is induced. Second, we will discuss the mechanisms that underlie local and systemic tolerance to orally administered antigens, before presenting a framework that might underpin future mechanistic studies.

MECHANISMS OF ANTIGEN UPTAKE IN THE INTESTINE

The intestinal immune system can be divided into inductive and effector sites. Inductive sites include the gut-associated lymphoid tissues (GALT) such as Peyer’s patches (PP) and isolated lymphoid follicles and the gut-draining mesenteric lymph nodes (mLNs); the lamina propria (LP) and epithelium constitute the main effector sites, harboring large populations of activated T cells and antibody-secreting plasma cells.¹³ As we discuss below, the LP may also contribute to the induction of tolerance, as a site of antigen uptake and loading of the migratory dendritic cells (DCs) that encounter naïve T cells in the mLNs (**Figure 1**).

There is considerable evidence that the organized structures of the GALT (PP and isolated lymphoid follicles) are critical for the immune recognition of particulate antigens such as bacteria and viruses. This reflects the special ability of the so-called microfold cells (M cells) present in the epithelium of PP/isolated lymphoid follicles to actively transport material from the gut lumen into the lymphoid areas of the GALT¹⁴ (**Figure 1**). It is less clear if M-cell-mediated antigen uptake into GALT also plays a role in oral tolerance to soluble antigens. Targeting protein antigen directly to M cells has been reported to facilitate tolerance induction,¹⁵ and one study observed that inhibition of PP development

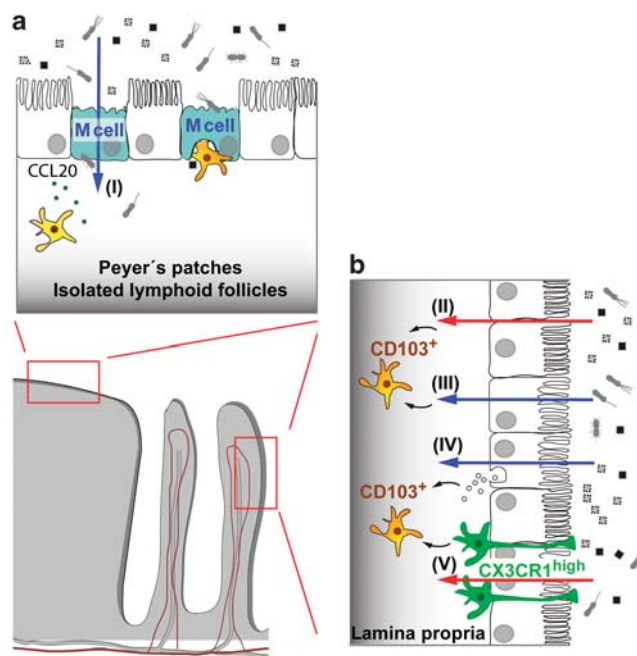


Figure 1 Mechanisms of antigen uptake in gut-associated lymphoid tissue (GALT) and lamina propria. (a) In organized tissues of the GALT such as Peyer’s patches and isolated lymphoid follicles, specialized microfold cells (M cells) in the epithelium overlying the lymphoid follicles mediate transcellular transport of particulate material including intestinal microbiota (I). This antigen is then passed on to dendritic cells (DCs) that lie either below the epithelium or in a “pocket” created at the basolateral surface of the M cell. Production of CCL20 by surrounding epithelial cells can attract further DCs via their expression of CCR6.⁸⁶ Some DCs in the GALT may also take up antigen directly from the lumen.⁸⁷ (b) CD103⁺ DCs in the lamina propria underlying normal villus epithelium also play a critical role in presenting antigen for the induction of tolerance. They may acquire soluble antigens that have diffused through epithelial tight junctions (II), or that have been transferred across epithelial cells by transcellular routes (III). Exosomes containing antigen derived from class II MHC⁺ enterocytes may be taken up by DCs (IV). CX3CR1^{high} macrophages have also been reported to capture luminal antigens by extending processes through the epithelial layer and they may pass this on to neighboring CD103⁺ DCs (V). There may also be a few M cells within villus epithelium⁸⁸ (not depicted).

during gestation reduced oral tolerance to protein but not haptens in adult mice.¹⁶ However, other studies reported normal oral tolerance induction in the absence of PP,^{17,18} and oral tolerance could be induced in spliced out intestinal loops, irrespective of the absence or presence of PP in the respective gut fragment.¹⁹ On balance, it seems that antigen uptake by PP and isolated lymphoid follicles might play a subordinate role in oral tolerance to proteins, but may be more important in the analogous process that controls immune responses to commensal bacteria (see below).

In contrast, there is mounting evidence that antigen uptake by DCs in the LP underlying regular villus epithelium is crucial for the induction of tolerance to soluble antigens in the small intestine (**Figure 1**). Despite the low pH in the stomach and the presence of proteolytic enzymes that can degrade nutrients in the upper gastrointestinal tract, some food components are resistant to degradation and immunogenic material enters the

intestinal lumen. Orally administered antigen has been detected in the gut epithelium and LP within minutes after feeding,²⁰ and we observed antigen-loaded CD11c⁺ cells in the LP 30–60 min after feeding mice labeled dextran or ovalbumin.²¹

How luminal antigen gains access to these DCs through the supposedly impermeable epithelial barrier remains something of a mystery. Material of low molecular weight, such as haptens and polypeptides, may pass directly across the epithelium by paracellular diffusion through pores in the tight junctions connecting epithelial cells. Conversely, larger molecular complexes can be taken across enterocytes by transcytosis after fluid phase uptake at the apical membrane (**Figure 1**, see ref. 22 for a recent review on intestinal permeability). Antigenic material may also reach the LP within exosomes derived from major histocompatibility complex class II-expressing enterocytes. Exosomes are formed when major histocompatibility complex class II loading compartments fuse with endosomes containing only partially degraded proteins. Notably, exosomes can be taken up efficiently by antigen-presenting cells and, when isolated from serum of antigen-fed mice, they are capable of inducing tolerance in recipient mice.²³ Recently, attention has focused on the idea that a population of LP myeloid cells expressing the chemokine receptor CX3CR1 may be able to sample the intestinal lumen by extending cellular processes across the epithelial barrier²⁴ without perturbing tight junctions and epithelial integrity.²⁵ Although it has been speculated that these CX3CR1^{high} cells might facilitate antigen uptake *in vivo* (**Figure 1**), the frequency of transepithelial protrusions varies markedly between mouse strains and between the various segments of the intestine.^{26,27} Finally, our own and other work has shown that the CX3CR1^{high} cells in LP do not appear to be *bona fide* DCs, as they do not migrate from LP to mLNs and cannot present luminal antigen to naive T cells.^{28–30} Nevertheless, they do seem to be loaded efficiently with antigen and hence may play an accessory role, by passing it on to neighboring migratory DCs for transport and presentation. As discussed below, CX3CR1^{high} cells may also be important for local differentiation of the Tregs needed to maintain oral tolerance. Furthermore, as the transepithelial dendrites from CX3CR1⁺ myeloid cells in the LP have been shown to interact with bacteria in the lumen, they may play a specific role in inducing tolerance to commensal bacteria.³¹ However, orally administered inert particles or nonpathogenic bacteria appear to localize preferentially in organized tissues of the GALT rather than the villous LP,^{32,33} indicating that the efficient uptake of particulate material usually requires follicle-associated epithelia and M cells. In conclusion, the nature of the antigen determines its route of uptake. Particulate material and microbiota mostly enter into GALT by M-cell-mediated transcytosis, whereas soluble antigens induce oral tolerance after being taken up by DCs in the LP and, to a lesser extent, the GALT.

BEYOND THE GUT: DISSEMINATION OF INTESTINAL ANTIGEN

As we have discussed, one of the characteristic features of oral tolerance to soluble antigens is that it can involve the entire animal. This is difficult to explain in the context of current

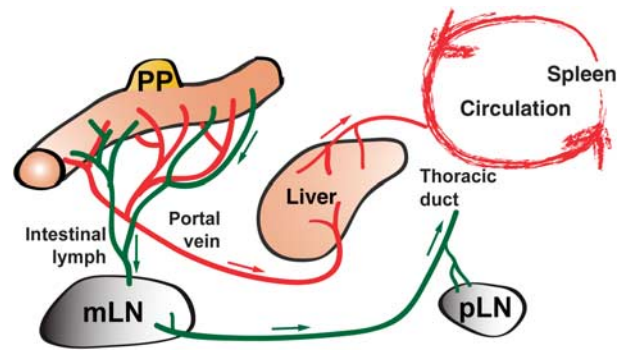


Figure 2 Routes by which intestinally derived antigen might disseminate and generate widespread systemic tolerance. Antigen taken up into Peyer's patches (PP) or lamina propria may enter the bloodstream via the portal vein, first reaching the liver before it becomes distributed into the wider circulation. Systemic tolerance may then occur because of antigen presentation in the liver by sinusoidal endothelial cells, tolerogenic conventional dendritic cells (DCs) or plasmacytoid DCs (pDCs), or because antigen reaching peripheral lymph nodes (pLNs) will be presented by resident DCs in the absence of costimulation. Free antigen taken up into afferent intestinal lymph will pass through the mesenteric lymph nodes (mLNs) and eventually enter the bloodstream via the thoracic duct.

views on anatomical compartmentalization within the mucosal immune system, as antigen uptake and recognition restricted to the LP, GALT, and mLNs should limit the resulting effects to the intestinal mucosa. One explanation for this could be that orally administered antigens can disseminate systemically via blood and lymph (**Figure 2**). Food protein can be detected in the blood of mice and humans soon after eating.^{34,35} This does not go unnoticed by the immune system, as feeding antigen can induce simultaneous expression of the activation marker CD69 and behavioral changes in T cells in mLNs and peripheral LNs.^{36,37} Furthermore, serum from protein-fed mice can induce antigen-specific tolerance in naive recipients, indicating the presence of tolerogenic material.³⁸ This raises the important question of how and where this absorbed antigen can contribute to oral tolerance induction.

One possible site is the liver. The portal vein drains blood from the intestine to the liver (**Figure 2**) and injection of antigen directly into the portal vein is well known to induce antigen-specific tolerance.³⁹ Conversely, diverting the blood flow away from the liver by portocaval shunting prevents the induction of oral tolerance.^{40,41} The liver contains several subsets of specialized antigen-presenting cells that may contribute to tolerance induction. Liver sinusoidal endothelial cells efficiently sample circulating antigen, can act as antigen-presenting cells, and have been shown to induce tolerance rather than active immunity. Antigen presentation by Kupffer cells and conventional liver DCs also favors tolerance over immunity (reviewed in ref. 39), whereas plasmacytoid DCs are particularly abundant in the liver and have been implicated in the induction of systemic tolerance to orally administered proteins and haptens.²⁰ Antigen reaching beyond the liver into peripheral LNs and spleen might be expected to induce tolerance in these sites, as it will be presented by resident DCs in the absence of costimulation, leading to the induction of anergy or Tregs.

In contrast to this evidence that dissemination of fed antigen is important for oral tolerance, we found that the transport of antigen from the LP into the mLNs by CD103⁺ DCs is the key event for inducing the systemic consequences of oral tolerance.⁴² The constitutive migration of DCs into draining LNs requires the chemokine receptor CCR7.⁴³ Consequently, genetic deficiency in CCR7 prevents the recognition of fed antigen by T cells in the mLNs and impairs the induction of oral tolerance.⁴⁴ Other reports have also emphasized a central role for the mLNs in oral tolerance induction. Oral tolerance is abrogated in lymphotoxin α -deficient mice lacking all LNs and PP, but can be restored by selective rescue of mLN development.¹⁸ Moreover, surgical removal of mLNs prevented the induction of oral tolerance in wild-type mice.⁴⁴ The reason for these discrepant findings on the relative roles of the mLNs vs. more widespread dissemination of antigen to the liver and elsewhere is unknown. However, the concentration of antigen reaching the circulation may vary considerably depending on the nature and dose of antigen, as well as the health status of the animals. Therefore, subtle differences in housing conditions and experimental models might explain some of the differences. Nevertheless, we consider that the mLN is the crucial site involved in oral tolerance induction,⁴² and recently there has been considerable progress in understanding the molecular mechanisms involved.

MIGRATORY CD103⁺ DCs AND LOCAL CONDITIONING CREATE A TOLEROGENIC ENVIRONMENT IN THE MLNS

The integrin chain α_E (CD103) is expressed by a major population of migratory DCs present in the LP. These CD103⁺ DCs that carry antigen from the intestine do not travel beyond the mLNs and are largely excluded from entering the bloodstream and other lymphatic organs.⁴⁵ CD103⁺ DCs from the LP and mLNs have a unique ability to induce the expression of gut-homing molecules on T cells,⁴⁶ and are particularly potent in inducing the generation of Foxp3⁺ Tregs.⁴⁷ Both of these properties are linked to the ability of CD103⁺ DCs to metabolize dietary retinoids (Vitamin A) to retinoic acid (RA). RA is sufficient to induce the gut-homing molecules CCR9 and $\alpha_4\beta_7$ integrin on activated T cells *in vitro*, and the ability of CD103⁺ DCs to induce these homing molecules is blocked by antagonists of RA synthesis and/or signaling.⁴⁸ Moreover, CD103⁺ DC-derived RA can act as a cofactor in the transforming growth factor- β (TGF- β)-mediated conversion of naive CD4 T cells into Tregs.⁴⁷ CD103⁺ DCs can themselves activate TGF- β by virtue of their expression of the $\alpha_V\beta_8$ integrin, and selective lack of $\alpha_V\beta_8$ on these DCs abrogates the induction of Tregs and oral tolerance.^{49,50}

The special properties of mucosal CD103⁺ DCs are not shared by CD103⁺ DCs from other tissues and seem to be imprinted by the gut tissue itself.⁵¹ In particular, gut epithelial cells promote a tolerogenic phenotype in DCs via the production of thymic stromal lymphopoietin, TGF- β , and RA.^{52,53} RA seems to play a crucial role in defining the properties of CD103⁺ DCs, not only mediating their effects on T cells, but also helping to induce and maintain their unique phenotype.⁵⁴ DCs isolated from the gut of mice fed an retinoid-free diet have reduced levels of RA-producing

enzymes and RA can induce its own generation by DCs *in vitro*.^{55,56} The sources of RA in the intestine may include RA produced by epithelial cells that metabolize retinoids taken up from the diet or from the bile.⁵⁷ Other external factors present in the gut may also contribute to the production of RA by local DCs, such as ligands for Toll-like receptor 2,⁵⁸ granulocyte-macrophage colony-stimulating factor, and interleukin-4 (IL-4).⁵⁹ Whether CD103 itself plays any role in the function of these DCs is unclear, although the fact that wild-type Tregs cannot prevent colitis in CD103-deficient recipients could support this idea.⁶⁰

Although these findings suggest that intestinal CD103⁺ DCs are imprinted with their unique functions in the mucosa itself, the situation appears to be more complex *in vivo*. Our own group and that of Molenaar *et al.*⁶¹ have found that the full ability of intestine-derived DCs to drive gut tropism in T cells requires cooperation with nonhematopoietic stromal cells in the mLNs.⁶² Stromal cells from mLNs express higher levels of RA-producing enzymes than peripheral LN stromal cells. Moreover, peripheral LNs fail to support the induction of gut-homing molecules on T cells, even when transplanted into the intestinal mesentery, where they are populated by gut-derived DCs.⁶² Similarly, stromal cells from mLNs but not from skin-draining LNs support the generation of Foxp3⁺ Tregs (S Cording, B Wahl, J Hühn, O Pabst, unpublished observations). Indeed, LN stromal cells of the mLNs and skin-draining LNs possess unique transcriptome profiles.⁶³ Thus, tissue-specific differences in stroma help generate a uniquely tolerogenic environment in mucosal LNs, and additional imprinting of CD103⁺ DCs in this environment is needed for them to play their full role in regulating intestinal immune responses.

MECHANISMS OF ORAL TOLERANCE

As with other models of peripheral tolerance, a number of different mechanisms have been implicated in oral tolerance, including active regulation by Tregs, as well as clonal deletion and clonal anergy of T cells. In this context, Weiner *et al.*^{4,64,65} suggested that the mechanism of oral tolerance was determined by the feeding regime used, with single high doses of antigen favoring clonal deletion or anergy, whereas multiple low doses of antigen were linked to T-cell-mediated suppression. However, more recent work has found that the frequencies of Foxp3⁺ Tregs in oral tolerance were highest when a high dose of antigen was used.⁶⁶ Moreover, there is little direct evidence that clonal deletion and/or anergy contribute to oral tolerance in the presence of a polyclonal T-cell repertoire, although the direct entry of free antigen to the mLNs could account for such effects. Nevertheless, the influence of antigen dose on the mode of suppression needs to be re-evaluated.

The fact that CD103⁺ DCs and the mLNs seem to have special abilities to drive the generation of Tregs, and both are required for the induction of oral tolerance, suggests that Tregs should play a central role in oral tolerance. Indeed, the induction of T cells with active suppressor function was first shown in oral tolerance several decades ago.¹² Although most of this work pre-dated the discovery of Foxp3 and molecularly defined subsets of Tregs, it has been substantiated subsequently using

more modern techniques. Transfer of CD4⁺CD25⁺ CD4 T cells (which are highly enriched in Foxp3⁺ Tregs) can transfer oral tolerance to naïve animals, and depletion of CD25⁺ cells *in vivo* abrogates oral tolerance.⁶⁷ A number of different Tregs have been implicated in oral tolerance, including IL-10-producing Tr1 T cells and Th3 T cells,¹² in addition to the Foxp3⁺ Tregs that are currently the focus of most attention. As much of the literature on “Tr1” cells and “Th3” T cells in the intestine was published before the Foxp3 era, it is not always possible to define the exact nature of the Tregs that have been described. However, it is known that Foxp3⁺ and Foxp3⁻ populations of IL-10-secreting Tregs are present in the mucosa⁶⁸ and, judged by their secretion of TGF- β , the TH3 cells described by the Weiner group are likely to be similar to, if not identical to, what we now know as Foxp3⁺ Tregs.

The best characterized population of Tregs found in other forms of tolerance expresses Foxp3. Foxp3⁺ Tregs come in two distinct flavors, natural Foxp3⁺ Tregs (nTregs) and induced Foxp3⁺ (Tregs (iTregs)).⁶⁹ nTregs are selected in the thymus as a consequence of their reactivity to self, whereas iTregs are generated from naïve CD4 T cells in the peripheral immune system. Whereas nTregs are stable *in vivo*,⁷⁰ iTregs can differentiate into other helper T cells under inflammatory conditions.⁷¹ Still, under noninflammatory conditions, Foxp3⁺ Treg-mediated immune regulation is a robust phenomenon and may confer longlasting oral tolerance. Interestingly, oral tolerance to ovalbumin could not be induced in a mouse model in which nTregs but not iTregs could be generated, suggesting that this model required peripheral conversion of naïve CD4 T cells into iTregs.⁷² This observation is consistent with the findings described above showing that the mLN provides a specialized microenvironment in which CD103⁺ DCs drive the selective differentiation of iTregs, as well as our own observation that depletion of ovalbumin-induced Tregs abolished oral tolerance.⁷³

A FRAMEWORK FOR THE GENERATION AND MAINTENANCE OF ORAL TOLERANCE

Given the considerable amount of foreign protein antigen taken up with our daily diet and the knowledge that oral tolerance can be maintained for several months after a single encounter with antigen^{12,74} one would envision that the generation of iTregs in response to intestinal antigens might be a continuously ongoing process. Pathways by which this can occur *in vivo* are suggested by our recent work showing that the induction and maintenance of oral tolerance may reflect a multi-step process involving both lymphoid organs and the mucosal tissues (**Figure 3**).⁷³ Key to this is our observation that oral tolerance was reduced in mice lacking β_7 integrin or its ligand MadCAM-1 (mucosal vascular addressin cell adhesion molecule 1), whose interaction is critical to enable gut homing of T cells.⁷⁵ Importantly, neither gene deficiency impaired the initial generation of iTregs in the mLNs, and defective oral tolerance in β_7 -integrin-deficient but not MadCAM-1-deficient mice could be rescued by adoptive transfer of β_7 -competent T cells. Thus, oral tolerance requires the β_7 -integrin-dependent gut homing of iTregs after their initial generation in the mLNs.⁷³ In support of this idea, the group

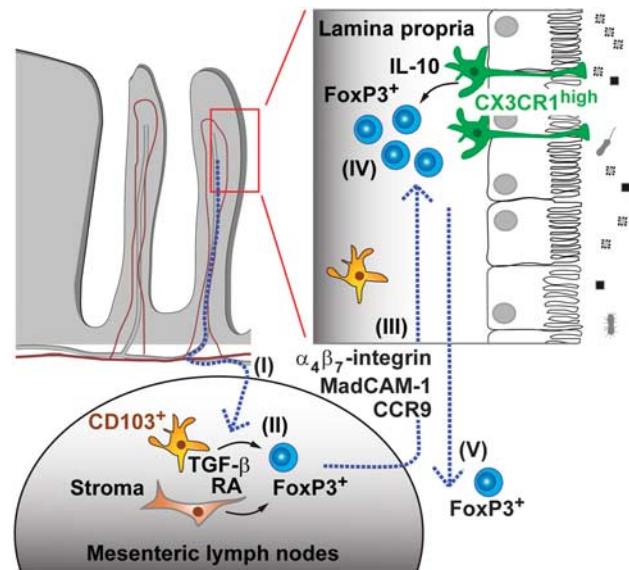


Figure 3 A multistep model of oral tolerance to soluble antigens. Oral tolerance is initiated by the migration of antigen-loaded CD103⁺ dendritic cells (DCs) from the lamina propria (LP) into the draining mesenteric lymph nodes (mLNs; I). In the mLN, retinoic acid (RA) produced by DCs and local stromal cells induces the expression of gut-homing receptors $\alpha_4\beta_7$ integrin and CCR9 on antigen-specific T cells and favors the transforming growth factor- β (TGF- β)-dependent differentiation of Foxp3⁺ induced regulatory T cells (iTregs; II). These committed Tregs home back to the intestinal lamina propria (III), where they undergo secondary expansion under the influence of interleukin-10 (IL-10) produced by CX3CR1^{high} macrophages in the lamina propria (IV). In a putative fifth step (V), some of the Tregs may exit from the mucosa via lymph and/or bloodstream, disseminating throughout the immune system, and promoting the systemic consequences of oral tolerance. Foxp3, forkhead box P3; MadCAM-1, mucosal vascular addressin cell adhesion molecule 1.

of Rodrigo Mora recently observed defective oral tolerance in CCR9-deficient mice.⁷⁶ CCR9 along with β_7 integrin targets T cells to the small intestine⁷⁷ and, as we found in β_7 -integrin-deficient mice, defective oral tolerance in CCR9-deficient mice could be restored by transfer of wild-type T cells.⁷⁶ However, in our hands, CCR9-deficient mice developed normal oral tolerance to ovalbumin (independent unpublished observations in the Mowat and Pabst labs), suggesting there may be differences in individual strains of CCR9-deficient mice, or that differences in the composition of the microbiota may influence the impact of CCR9 on oral tolerance. There are controversial reports on the capacity to induce oral tolerance in germ-free mice devoid of live intestinal bacteria.^{78,79} Yet, no doubt, the intestinal microbiota has profound effects on the gut immune system; for example, distinct bacterial species may favor or disfavor Foxp3⁺ Treg induction.⁸⁰ Thus, it is conceivable that differences in microbiota composition may also affect oral tolerance.

The second crucial finding to arise from our own studies was that the mLN-derived iTregs in antigen-fed mice appeared to undergo secondary expansion after arrival in the small intestinal LP (**Figure 3**). Both the expansion of iTregs and the induction of oral tolerance required the chemokine receptor CX3CR1, as they were abrogated in CX3CR1-deficient mice. This correlated with reduced production of IL-10 by myeloid cells in the LP

and could be restored with IL-10-producing wild-type macrophages.⁷³ This suggestion that IL-10 produced in the mucosa is needed to drive or maintain local Foxp3 iTregs is supported by work from Murai *et al.*,⁸¹ which showed that IL-10 production by CD11b⁺ myeloid cells in LP is needed to maintain Foxp3 expression by iTregs in the colon. Thus, secondary maintenance of iTregs by IL-10-producing gut-resident macrophages may be common to both classical oral tolerance to proteins in the small intestine and in tolerance to commensal bacteria in the colon.

On the basis of these findings, we propose that both forms of mucosally induced tolerance are initiated in the draining LNs, but are then expanded and sustained in the LP. In the case of oral tolerance induced in the small intestine, cooperative production of RA by antigen-loaded, gut-derived CD103⁺ DCs and mLN stromal cells leads to the imprinting of gut-homing molecules on specific T cells. Some of these also begin to differentiate into Foxp3⁺ iTregs. These “primed” iTregs then leave the mLN and home to the small intestine, where they undergo secondary expansion and are sustained by IL-10-producing CX3CR1^{high} myeloid cells. Clearly, this can explain the ability of oral tolerance to maintain an inhibitory environment that can prevent hypersensitivity in the mucosa itself. Interestingly, intestinal antigen may also sustain Tregs in the gut. The T-cell receptor repertoire of Tregs present in the small intestinal LP showed a higher overlap with the Treg repertoire of gut-draining mLNs than skin-draining LNs,⁸² and many Tregs present in the colon seem to arise only after response to microbiota-derived antigen.⁸³ Thus, there is a concept emerging that intestinal antigen not only triggers the generation of iTregs but might subsequently allow for the maintenance of an antigen-specific Treg pool in the intestine. As Tregs can produce TGF- β and may even convert into follicular helper T cells within GALT,⁸⁴ these processes may also account for the production of noninflammatory immunoglobulin A responses against intestinal antigens, something that plays a particularly crucial role in maintaining the local symbiotic relationship between the host and its microbiota. Less obvious is how these local processes could suppress immune responses systemically. As a potential explanation, we speculate that a fraction of Tregs that have undergone secondary expansion in the LP may exit the tissue and enter the circulation either via the draining lymphatics, or directly via the bloodstream. In this respect, it is interesting to note that Foxp3⁺ Tregs constitute a significant population in skin-draining afferent lymphatics.⁸⁵ Escape from the LP in this way could allow iTregs generated initially in the intestinal lymphoid tissues to act more generally, either by directly inhibiting systemic effector T cells or by fueling generation of further iTregs in systemic tissues by the process of infectious tolerance. Further experiments should explore these processes in more detail. It will also be important to understand why this dissemination of iTreg-dependent tolerance only seems to occur with soluble antigens, whereas tolerance to microbiota is limited to the intestinal tissues.

CONCLUSIONS

Oral tolerance is a multifaceted process and multiple cellular and molecular processes may be needed to ensure durable tolerance

to harmless intestinally derived antigens, both in the mucosa itself and in the systemic immune system. The chain of events we propose here suggests that tolerance involves stepwise and anatomically defined processes of induction, maintenance, and migration of Tregs that ensure immune regulation in all appropriate tissues. The model also highlights how these processes are dependent on the unique properties of the intestinal immune system and these may allow the identification of new targets for modulating tolerance.

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DISCLOSURE

The authors declared no conflict of interest.

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