

Cellular Components and Mechanisms of Oral Tolerance Induction

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ABSTRACT: Oral tolerance can be defined as an inhibition of specific immune responsiveness to subsequent parenteral injections of proteins to which an individual or animal has been previously exposed via the oral route. Multiple mechanisms of tolerance are induced by oral-fed antigens, but induction of regulatory CD4 T-cells expressing the transcription factor Foxp3 and the membrane-bound TGF- β stands out as the major players in oral tolerance. Oral antigen administration suppresses several animal models of autoimmune disease, including experimental autoimmune encephalomyelitis, uveitis, thyroiditis, myasthenia, arthritis, and diabetes, but also nonautoimmune inflammatory conditions such as asthma, atherosclerosis, graft rejection, allergy, and stroke. However, human trials have produced mixed results, and a great deal remains to be learned about the mechanisms of oral tolerance before it can be successfully applied to people. In this review, we highlight the cellular components involved in oral tolerance induction. A deep knowledge of these intricate cell interactions will pave the way for a successful application of antigen tolerance to treat autoimmune and nonautoimmune inflammatory diseases.

KEY WORDS: Oral tolerance, gut mucosa, innate and adaptive immune cells

ABBREVIATIONS: AchR, acetylcholine receptor; B2M, beta2-microglobulin; CCL, chemokine (C-C) ligand; CCR, chemokine (C-C) receptor; CD, cluster of differentiation; CFA, complete Freund adjuvant; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; DC, dendritic cell; DNCB, dinitrochlorobenzene; DSS, dextran sodium sulfate; EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis; Foxp3, forkhead box P3; FVIII, factor VIII; GA, glatiramer acetate; GALT, gut-associated lymphoid tissue; GARP, glycoprotein A repetitions predominant; GF, germ-free; GITR, glucocorticoid-induced tumor necrosis factor receptor-related protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; HCV, hepatitis C infection; HLA, human leukocyte antigen; IBD, inflammatory bowel disease; IEL, intraepithelial lymphocytes; ILF, isolated lymphoid follicles; IFN, interferon; Ig, immunoglobulin; IL, interleukin; ILC, innate lymphoid cells; IPEX, syndrome immunodysregulation polyendocrinopathy and enteropathy associated with the X chromosome; IRBP, interphotoreceptor binding protein; KGF, keratinocyte growth factor; LAP, latency-associated peptide; LPS, lipopolysaccharide; MAdCAM-1, mucosal addressin cell adhesion molecule-1; MBP, myelin basic protein; MHC, major histocompatibility complex; MIP-1, macrophage inflammatory protein 1 beta; mLN, mesenteric lymph node; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NLR, NOD-like receptors; NOD, nonobese diabetes; OVA, ovalbumin; pCons, pConsensus; PLP, proteolipid protein; PP, Peyer's patches; RAG, recombination-activating gene; RALDH, retinoic acid-catalyzing enzyme; RANTES, regulated on activation, normal T-cell expressed and secreted; Reg, regenerating islet-derived; ROR, RAR-related orphan receptor; S-Ag, S antigen; SCID, severe combined immunodeficiency; SLE, systemic lupus erythematosus; SPF, specific pathogen free; TAChR, Torpedo californica acetylcholine receptor; TCR, T-cell receptor; TGF, transforming growth factor; Th, T helper; TLR, toll-like receptor; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNF, tumor necrosis factor; KLH, keyhole limpet hemocyanin; Tr1, T regulatory 1; Treg, regulatory T-cell

I. INTRODUCTION

Oral tolerance has classically been defined as the specific suppression of cellular and/or humoral immune responses to an antigen by prior administration of the antigen by the oral route.¹ It presumably evolved as

an analog of self-tolerance to prevent hypersensitivity reactions to food proteins and bacterial antigens present in the mucosal microbiota.

Oral tolerance is of paramount importance because it contributes to the balance between exogenous antigens that come from diet and commensal

bacteria and the self-components of the body at the mucosal surface. The mucosa of the small intestine alone is estimated to be 300 m² in humans, and there are 10¹² lymphoid cells per meter of human intestine.² The number of bacteria colonizing the colonic mucosa can reach 10¹² microorganisms/g of stool,^{3,4} and these antigens are crucial for the maturation of the immune system in the postweaning period, contributing to the establishment of oral tolerance rather than inflammatory immune response in order to keep homeostasis. Thus, oral tolerance is a form of peripheral tolerance that evolved to treat external agents that gain access to the body via a natural route as internal components that then become part of self.

Although oral antigens can suppress animal models of autoimmunity (e.g., experimental autoimmune encephalomyelitis, uveitis, diabetes, colitis), human trials have produced mixed results. Oral tolerance has been tested in human autoimmune diseases including multiple sclerosis (MS), arthritis, uveitis, and diabetes, and in allergy, contact sensitivity to dinitrochlorobenzene (DNCB), and nickel allergy. Although positive results have been observed in phase II trials, no effect was observed in phase III trials of CII in rheumatoid arthritis or oral myelin and glatiramer acetate (GA) in MS.⁵ Thus, a successful use of oral-fed antigens to treat inflammatory conditions in humans is the “Holy Grail” of modern immunology, and a deep knowledge of the cellular components of the gut involved in oral tolerance induction is certainly a shortcut for reaching this goal. In this review, the cellular interactions following an oral-fed antigen to induce tolerance are highlighted.

II. ORAL TOLERANCE

A. Historical Aspects

In the beginning of the twentieth century, Wells and Osbourne⁶ demonstrated for the first time the phenomenon of immunological tolerance through oral-fed antigens by showing that guinea pigs fed a corn-containing diet were not anaphylactically sensitized to zen, a major protein of corn. Subsequently, several researchers showed that when mammals are exposed

to foreign macromolecules by the digestive route, delivered either by gastric intubation or ingested with maternal milk, they became immunologically tolerant to these proteins.^{7,8} Moreover, rats fed horse serum, pollen extracts, or bovine serum albumin were made tolerant to those antigens^{9,10}; also, mice were tolerized by feeding on sheep red blood cells.¹¹ In the context of allergic disease, Vaz and colleagues¹ contributed significantly to the field by showing that intense and prolonged states of specific immunologic tolerance with profound specific IgE reduction could be induced in adult mice by a single exposure to OVA. Thus, these studies demonstrated that, even though the vast majority of the macromolecules ingested as food are degraded within the gut, antigenically intact materials are absorbed in amounts sufficient to induce significant modifications in the immune responsiveness of the organism.

The demonstration of transferable cellular suppression associated with oral tolerance is a recurrent theme reported by many investigators.¹² Mowat et al.¹³ reported that high doses of ovalbumin-induced tolerance were not abrogated by cyclophosphamide, which is believed to block active suppression, and that such tolerance affected antibody responses. Low doses of ovalbumin induced a state of tolerance that could be reversed by cyclophosphamide and primarily affected cell-mediated responses. Thus, this study already appeared to delineate active suppression versus anergy depending on the dose of antigen (discussed subsequently). Moreover, Hanson and Miller¹⁴ reported that two components of oral tolerance following oral administration of ovalbumin: tolerance were observed in both cyclophosphamide-treated and untreated animals, but tolerance from the cyclophosphamide-treated animals could not be transferred.

Our early studies of oral tolerance in autoimmune models have found active suppression to be an important mechanism, and we have identified regulatory cells generated following oral tolerance that act via the secretion of antigen-nonspecific regulatory cytokines triggered by the fed antigen.¹⁵ Such cells were first characterized in the Lewis rat model of experimental autoimmune encephalomyelitis (EAE) orally tolerized to low doses of guinea pig

MBP. The regulatory cells identified in that model were CD8⁺ T-cells¹⁶; they transferred suppression *in vivo* and also suppressed *in vitro*; and acted via the secretion of transforming growth factor (TGF- β) following antigen-specific triggering.¹⁷ Further studies demonstrated that the epitopes of guinea pig MBP triggering CD8⁺ T regulatory cells following orally administered MBP were different from the encephalitogenic determinant.¹⁸ Moreover, TGF- β -secreting regulatory cells could be found in Peyer's patches 24–48 hours after one feeding of low doses of MBP, and these cells did not proliferate in response to 1 mg of MBP even though they release TGF- β upon *in vitro* stimulation.¹⁹

When similar studies were extended to a mouse system, it was found that CD4⁺ T-cells were also responsible for active suppression, both *in vivo* and *in vitro*.^{20,21} Thus, when a low dose of MBP was administered orally to SJL/J mice, Th1 but not Th2 immune responses were suppressed. In fact, interleukin (IL)-4 and TGF- β were significantly increased in mice fed with low doses of myelin basic protein (MBP). Furthermore, if animals were fed MBP and then immunized intraperitoneally with the same antigen, one enhanced the production of IL-4 and IL-10, as well as TGF- β . McGhee and colleagues also found that exposure of soluble antigens to the gut preferentially generated Th2-type responses as judged by increased IL-4 and IL-5 production.²² Thus, in the gut immune responses to soluble antigen are preferentially of a Th2 type and involve the generation of cells that secrete TGF- β . When cells from mice fed and immunized with MBP were further studied *in vitro*, it was found that both CD4⁺ and CD8⁺ T-cells secreted TGF- β , whereas only CD4⁺ T-cells secreted IL-4 and IL-10.²⁰ However, it was clear in the SJL model that a population of TGF- β -secreting CD4⁺ T-cells were also generated and amplified in the gut following the feeding and subsequent immunization with MBP.

CD4⁺ T-cells were then cloned from the mesenteric lymph nodes of MBP-fed mice, and it was found that the majority of T-cell clones produced active TGF- β in addition to varying amounts of one or the other of the Th2-type cytokines (IL-4 and IL-10). However, it appeared that the TGF- β

clones were different from classic Th2-type cells, as there was a general correlation between the secretion of IL-4 and IL-10 in an individual clone, whereas this was dissociated for TGF- β and IL-4/IL-10. We named these cells Th3 cells. Mucosal-derived CD4⁺ T-cell clones were further characterized for their epitope specificity, MHC restriction, and TCR use. Sequence analysis of their cDNA revealed that they used V α 1 or V α 3, and V β 4 or V β 17, all of which were also used by encephalitogenic Th1 cells. Most interestingly, one of these mucosal Th3 clones used identical TCR V α and V β chains as one of the encephalitogenic Th1 clones. The regulatory T-cell clones generated in this study had striking similarities to the encephalitogenic CD4⁺ Th1-cell clones in terms of their specificity, TCR use, and MHC restriction. However, they could be distinguished from the encephalitogenic CD4⁺ Th1-cell clones by the fact that they produced suppressive cytokines (TGF- β , IL-4, and IL-10) following antigen-specific activation. The clones inhibited the proliferation and cytokine production of MBP-specific Th1 cells, and they suppressed the development of MBP-induced EAE and proteolipid protein-induced EAE; this suppression was abrogated by *in vivo* injection of anti-TGF- β antibodies. This demonstrated that these clones were able to mediate bystander suppression *in vivo* mediated by TGF- β production.²⁰

The identification of the master transcription factor regulator Foxp3 in 2003 by Sakaguchi et al.²³ (discussed subsequently) shed light on how a CD4⁺ T-cell, using identical variable TCR chains to an effector CD4⁺ cell, can become committed to a regulatory phenotype. This is particularly important in mucosal sites due to the necessity of constant regulation. In fact, CD4⁺Foxp3⁺ regulatory T-cells (known as Tregs) have been shown to be crucial for oral tolerance, and they suppress not only in a cell-cell contact manner but also through the release of TGF- β and IL-10.²⁴ Because Th3 cells were described before the Foxp3 era, and Foxp3⁺ T-cells share many suppressive properties with Th3 cells, it is possible that these cells may give rise to each other depending on the environment where they encounter the cognate antigen.

A. High versus Low Dose of Antigen in Oral Tolerance Induction

The phenomenon of oral tolerance can be explained by different mechanisms, which are primarily dependent on the dose, type, and administration of the antigen.^{13,25} Anergy or clonal deletion occurs mainly with high doses of antigen, whereas low and repeated doses of the antigen promote active induction of Tregs.²⁶ Once oral tolerance is induced, various aspects of antigen-specific immune responses Th1 and Th2 are inhibited, such as production of immunoglobulins (IgM, IgG)^{1,27,28} and synthesis and release of immunoregulatory cytokines, such as TGF- β , IL-10, and IL-4.^{26,28}

A major conceptual advance in the oral tolerance field is the fact that TGF- β plays a central role in oral tolerance by being a primary link between distinct populations of Treg cells that are induced by feeding. Thus, conversion of CD4⁺CD25⁻ into CD4⁺CD25⁺ T-cells by the expression of Foxp3 involves TGF- β .²⁴ A membrane-bound form of TGF- β (containing latency-associated peptide, or LAP) has also been described, and CD4⁺LAP⁺ T-cells mediate suppression in a TGF- β -dependent mechanism.^{29–31} Thus, it appears that TGF- β -producing cells not only are crucial for oral tolerance but may be master regulators of most of the mechanisms triggered by antigen feeding.

III. CELLULAR COMPONENTS OF ORAL TOLERANCE

A broad range of cells are involved in oral tolerance induction. Both innate and adaptive immune systems play a crucial role in this process. In order for oral tolerance to develop, oral-fed antigens must encounter a healthy intestinal environment, which is primarily controlled by an adequate balance between the gut microbiota and the gut immune system. Changes in this balance will cause inflammation and thus break tolerance. In this section, we will review the current known aspects of cellular interactions in the gut responsible for intestinal homeostasis and tolerance development.

A. Gut Mucosa

Although it is generally assumed that our major surface of contact with the external milieu is the skin, the area of the intestinal mucosa exceeds several fold that of the skin. The mucosa of the small intestine alone is estimated to be 300 m² in humans.² Moreover, mucosal surfaces, differently from the skin, are more permeable to antigens, and much of the contacts with foreign antigenic materials occur at the mucosal sites. The microbiota in the small intestine is an additional source of natural antigenic stimulation, and the number of bacteria colonizing the human intestinal mucosa is approximately 10¹² microorganisms/g of stool.^{3,4} It is also known that in the intestine lodges the most abundant lymphoid tissue in the body. There are 10¹² lymphoid cells per meter of human small intestine.^{32,33} The number of immunoglobulin (Ig)-secreting cells located in the murine and human gut exceeds by several fold the number found in all other lymphoid organs together.^{24,32} Thus, it is reasonable to assume that antigenic contact initiated in the intestinal mucosa has an important impact in the activation of the immune system and in the consequent induction of oral tolerance.

The three major consequences of oral antigen administration have been reported. They include the local secretion of noninflammatory IgA in the gut mucosa; the induction of systemic humoral and cellular immunity; and the development of systemic and/or local immunological tolerance, which prevent the potentially damage caused by active immunity when the offending antigen is encountered on subsequent occasions. The first two of these situations comprise the protective immune response to invasive pathogens, whereas dietary proteins and commensal bacteria predominantly induce immunological tolerance.

There are several distinctive features of the gut immune system that participate in the tolerogenic environment. Consistent evidence has demonstrated that the organized structures of the GALT (gut-associated lymphoid tissue) play an important role in the immune recognition of particulate antigens. These structures include Peyer's patches (PP),

which are macroscopic lymphoid aggregates in the submucosa along the length of the small intestine and isolated lymphoid follicles (ILFs), which are organized lymphoid structures also present in the small intestine. However, the involvement of PP and ILF in the induction of oral tolerance is debatable.³⁴ One study demonstrated that inhibition of PP development during gestation-impaired oral tolerance induction to protein but not haptens in adult mice.³⁵ On the other hand, some studies reported that oral tolerance could be induced in the absence of PP^{36,37} and even in ligated small bowel loops regardless of the presence or absence of PP in the respective gut fragment.³⁸ Another important component of the GALT is the intraepithelial lymphocytes (IELs), which regulate intestinal homeostasis by maintaining epithelial barrier function, responding to infection and controlling adaptive and innate immune responses.³⁹ In the mouse small intestine, there is one IEL for every 10 intestinal villus epithelial cells. The majority of IELs are CD8⁺ T-cells, which express either $\alpha\beta$ or $\gamma\delta$ T-cell receptors (TCRs). Of note, it has been reported that depletion of $\gamma\delta$ T-cells impairs induction of oral tolerance.⁴⁰⁻⁴²

Nevertheless, it is now well known that antigen uptake by dendritic cells (DCs) underlying the regular villus epithelium is critical for the development of tolerance by soluble antigens in the small intestine.⁴³ After taking up the antigen, CD103-expressing DCs migrate to the mesenteric lymph node (mLN) and induce Tregs, which through the expression of gut-homing molecules, such as CCR9 and $\alpha 4\beta 7$ integrin, migrate to the gut lamina propria and evoke tolerance. Importantly, the ability of CD103⁺ DCs to induce Tregs and the gut-homing molecules is directly linked to the production of retinoic acid from vitamin A by these DCs.⁴⁴⁻⁴⁷ Importantly, mLNs, which are the largest lymph nodes in the body and serve as a crossroads between the peripheral and mucosal recirculation pathways, have been shown to be crucial for oral tolerance induction because mice deficient for CCR7, a chemokine receptor for cell migration to lymph nodes, failed to induce oral tolerance.^{48,49} Furthermore, lymphotoxin- α -deficient mice lacking all lymph nodes and PP did not develop oral tolerance, but this was reversed by selective rescue of

mLN development.⁵⁰ Consistent with this, surgical removal of the mLN was shown to abrogate oral tolerance induction.⁴⁹ Thus, the combination of gut microbiota,⁵¹ T-cells,⁵² and DCs⁵³ set up a tolerogenic environment in the gut. Antigen uptake in the GALT, DC migration to the mLN, and induction of gut-homing imprinted Treg cells appear to be the pathway by which oral tolerance is induced. Major factors that condition the gut to be a tolerogenic environment are interleukin-10 (IL-10), retinoic acid, and (TGF- β , which serves as a switch factor for IgA, the predominant immunoglobulin of the gut.⁵⁴

1. Interactions between Intestinal Intraepithelial Lymphocytes and the Microbiota for Oral Tolerance Induction

The intestinal intraepithelial lymphocytes (IELs) constitute a large population of lymphoid cells located between the intestinal epithelial cells that form the gut mucosal barrier. Because of their location, IELs are chronically exposed to food and microbes, thus being the first line of defense against pathogens.⁵⁵ IELs are made up of heterogeneous activated T-cells, characterized by the high expression of activation markers, cytotoxic T-cell-related genes, and anti-inflammatory or inhibitory receptors.⁵⁶ “Natural” IELs comprise cell populations expressing the $\alpha\beta$ and $\gamma\delta$ TCR and are CD8 $\alpha\alpha$. “Induced” IELs are peripheral, mature TCR $\alpha\beta$ ⁺ CD4⁺, or CD8⁺ T-cells that acquire IEL characteristics upon migration to the gut.⁵⁶

As mentioned previously, one of the primary functions of gut IELs is to control intestinal homeostasis by maintaining gut epithelial barrier integrity. Disruption of this barrier leads to bacterial infiltration and inflammation, which impairs oral tolerance induction. Thus, interactions between IELs and the microbiota play an important role in the development of oral tolerance (Fig. 1, left). In fact, although the involvement of gut microbiota and oral tolerance to fed antigens is still debatable,^{57,58} several studies have clearly demonstrated that gut microbiota-immune cell interaction plays a crucial role in oral tolerance induction. It has been shown that oral tolerance to

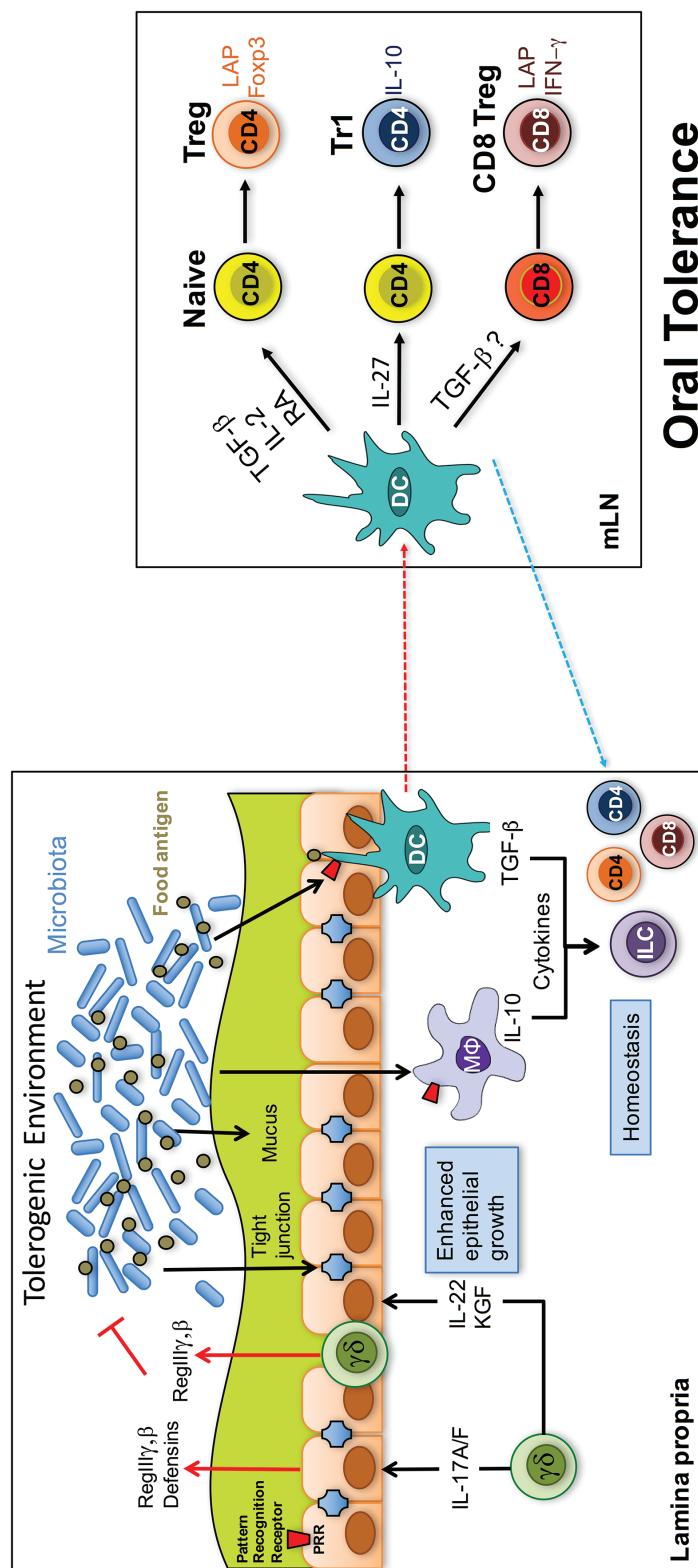


FIG. 1. Schematic for gut microbiota, epithelial cells, and innate and adaptive immune cell interactions involved in oral tolerance induction. An intestinal tolerogenic environment is maintained by a tolerogenic gut microbiota, which leads to healthy mucus layer formation, properly tight junction functioning, and a balance between epithelial and innate immune cells, IELs, particularly IEL-gd, can sense microbes and release AMPs, such as in the Reg protein family of C-type lectins (RegIII β , RegIII γ), and defensins, which protect against early invasion by intestinal-resident bacteria. By secreting IL-17A and IL-17F, $\gamma\delta$ T-cells from the LP induce intestinal epithelial cells to produce AMPs. LP $\gamma\delta$ T-cells also secrete IL-22 and KGF, which protect the intestinal barrier by enhancing epithelial cell renewal and prevent intestinal leakage and bacterial invasion. This balanced gut environment is crucial for oral tolerance induction. In a tolerogenic state, macrophages (M Φ s) and DCs secrete IL-10 and TGF- β , which may favor ILC homeostasis. Antigens coming from food reach DCs that in turn process and present these antigens to CD4 $^{+}$ T-cells upon migration to the mLN in a CCR7-dependent fashion. By releasing different cytokines, DCs induce regulatory T-cells, including CD4 $^{+}$ Foxp3 $^{+}$ LAP $^{+}$ cells (Treg cells), CD4 $^{+}$ Foxp3 $^{+}$ IL-10 $^{+}$ cells (Tr1 cells), and CD8 $^{+}$ IFN- γ $^{+}$ LAP $^{+}$ cells (CD8 Treg cells). These regulatory lymphocytes migrate to the gut and promote oral tolerance.

OVA cannot be induced in germ-free (GF) mice due to decreased T-cell numbers in the PP and reduced expression of secondary lymphoid-tissue chemokines that drive T-cell migration to lymphoid organs.⁵⁹ Accordingly, another study reported that the impaired development of oral tolerance to OVA in GF mice was associated with reduced frequency and number of CD4⁺CD25⁺ Treg cells in the PP and mLN of naïve GF mice as compared to specific-pathogen-free (SPF) mice.⁶⁰

Moreover, Treg cells from GF mice appear to have fewer suppressive capabilities because they produce less IL-10 and TGF- β ⁶⁰ indicating that the absence of gut microbiota affects not only Treg cell frequency and number but also function. Lambert et al. demonstrated that treatment with the antibiotic erythromycin prior to oral OVA exposure in mice prevented oral tolerance induction, an effect likely associated with a reduction in intestinal *Lactobacillus* populations. Erythromycin treatment also resulted in a significant decrease in tolerogenic DCs in the mLN, indicating that intestinal dysbiosis impairs oral tolerance induction by affecting tolerogenic DC development and function.⁶¹ However, it has been shown that transient depletion of gut microbiota by antibiotics enhances the development of oral tolerance to OVA by expanding Treg cells with high expression of glycoprotein A repetition-predominant (GARP)/LAP complex.⁶² Thus, the mechanisms by which the microbiota shape the gut immune system to maintain homeostasis remain largely elusive and need deep investigation.

2. Gamma-Delta T-Cells

Gamma-delta ($\gamma\delta$) T-cells are lymphocytes bearing a T-cell receptor composed of gamma and delta chains as opposed to alpha and beta chains found in conventional CD4⁺/CD8⁺ T-cells. Despite making up the majority of immune cells in niches associated with epithelial surfaces such as the intestine, only 1–2% of $\gamma\delta$ T-cells are present in secondary lymphoid tissues.⁶³ $\gamma\delta$ T-cells are considered the first line of defense against pathogens because they can rapidly respond to TCR signals in an MHC-independent manner⁶⁴ and to pattern recognition receptors signals

such as toll-like receptors.⁶⁵ Consistent with this, gut IEL $\gamma\delta$ T-cells are dramatically reduced in mice deficient for MyD88,⁶⁶ an adaptor protein downstream of the TLR signaling pathway, or NOD2, a member of the NOD-like receptor (NLR) family.⁶⁷ Moreover, as mentioned in Section I, the $\gamma\delta$ T-cell population attached to the intestinal lumen is constitutively activated even by normal commensal bacteria,⁶⁸ indicating the importance of $\gamma\delta$ T-cells in gut homeostasis maintenance (Fig. 1, left).

Regulatory properties of $\gamma\delta$ T-cells have also been described. For example, in inflammatory bowel disease models, $\gamma\delta$ T-cell-deficient mice develop spontaneous colitis and are susceptible to 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis.⁶⁹ Transfer of IEL $\gamma\delta$ T-cells ameliorates colitis in this model. In dextran sodium sulfate (DSS)-induced colitis in mice, IEL $\gamma\delta$ T-cells help preserve the integrity of damaged epithelial surfaces by the localized delivery of keratinocyte growth factor, a potent intestinal epithelial cell mitogen.⁷⁰ Furthermore, by secreting IL-22 in a retinoic acid-dependent fashion, $\gamma\delta$ T-cells play an important role in the attenuation of intestinal inflammation induced by DSS or *Citrobacter rodentium* infection in mice.⁷¹ Importantly, $\gamma\delta$ T-cells also contribute to gut epithelial barrier integrity directly by secreting antimicrobial products, such as the regenerating islet-derived (Reg) protein family of C-type lectins (RegIII β , RegIII γ), which protect against early invasion by intestinal-resident bacteria,⁷² or indirectly by secreting IL-17A and IL-17F, which then induce intestinal epithelial cells to secrete antimicrobial peptides.^{71,73,74} Moreover, $\gamma\delta$ T-cells produce keratinocyte growth factor (KGF) that protects the intestinal barrier by enhancing epithelial cell renewal and preventing intestinal leakage and bacterial invasion.⁷⁰

We recently showed that $\gamma\delta$ T-cells from Peyer's patches and small intestine lamina propria can express LAP, and that these LAP⁺ $\gamma\delta$ T (TCR $\gamma\delta$ ⁺LAP⁺) cells function as antigen-presenting cells that induce CD4⁺Foxp3⁺ regulatory T-cells, although TCR $\gamma\delta$ ⁺LAP⁺ cells do not themselves express Foxp3. Moreover, in a T-cell model of colitis induced by CD4⁺CD45RB^{high} cell transfer into immunodeficient mice (a rodent model for Crohn's disease), or in an

innate immune-mediated model of colitis induced by oral administration of DSS, transfer of TCR $\gamma\delta^+$ LAP $^+$ cells ameliorated the wasting disease by promoting the induction of Foxp3 Treg cells.⁷⁵ Again, these data point to a crucial role of $\gamma\delta$ T-cells in the control of gut homeostasis. Importantly, oral tolerance is impaired in mice depleted of $\gamma\delta$ T-cells⁴² and in $\gamma\delta$ T-cell-deficient mice.⁴¹ However, low-dose, not high-dose, antigen-induced oral tolerance was affected by the absence of $\gamma\delta$ T-cells, and this was associated with reduced levels of IL-10 in oral-fed OVA to TCR $\gamma\delta^{-/-}$ mice.⁴¹ Thus, $\gamma\delta$ T-cells appear to be crucial for oral tolerance induction, but the mechanisms underlying this effect remains to be determined.

3. Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are innate immune cells derived from a common lymphoid progenitor that belong to the lymphoid lineage. These cells are defined by the absence of antigen-specific B- or T-cell receptors because of the lack of the recombination-activating gene (RAG).⁷⁶ ILCs have an important role in protective immunity and the regulation of homeostasis and inflammation, which is related to their ability to rapidly secrete immunoregulatory cytokines that allow them to contribute early on in immune response to infection. They often reside at mucosal surfaces, where they are exposed to infectious agents in the environment (Fig. 1, left). ILCs can be divided into three groups based on the transcription factors they express and on the cytokines they secrete. Thus, ILC1s express T-bet and secrete IFN- γ and TNF- α ; ILC2s express GATA-3 and RAR-related orphan receptor alpha (ROR- α) and secrete IL-4, IL-5, IL-9, and IL-13; finally, ILC3s express the transcription factor ROR- γ t and produce IL-17 and IL-22.⁷⁷ More recently, regulatory ILCs were also described.⁷⁸

ILCs not only act as “first-line” effectors at mucosal barriers against pathogens, but they are also associated with numerous microbiota-related disorders, including inflammatory bowel diseases (IBD), cancer, allergy, and autoimmune disease.^{79–82} ILC3s were shown to sustain oral tolerance through

a microbiota-dependent interaction with macrophages by secreting granulocyte-macrophage-colony factor stimulation (GM-CSF) that promote the production of tolerogenic molecules by DCs such as retinoic acid (RA) and IL-10.⁸³ However, ILC3-derived GM-CSF can also promote colitis.^{84,85} Thus, a fine-tuning ILC3-derived GM-CSF is critical in regulating tolerance and inflammation, and further studies need to be done to better understand its role in oral tolerance induction.

4. CD8 $^+$ T-Cells

Classical reports on oral tolerance have suggested that CD8 $^+$ T-cells are the suppressor cells involved in its induction.^{86–88} These cells were first characterized in the Lewis rat model of experimental autoimmune encephalomyelitis (EAE) orally tolerized to low doses of guinea pig MBP. They transfer suppression *in vivo* and can suppress *in vitro*. Additional studies have demonstrated that the epitopes of guinea pig MBP-triggering regulatory CD8 $^+$ T-cells after feeding are different from the encephalitogenic determinant.¹⁸ Furthermore, evidence that CD8 $^+$ T-cells may participate in oral tolerance comes from studies showing that a population of regulatory CD8 $^+$ T-cells that produce IL-4 or IL-10 can be primed by feeding antigen, even when cytotoxic CD8 $^+$ T-cells are tolerized.^{89,90} Interestingly, we have described a novel subset of CD8 $^+$ T-cells that express the membrane-bound TGF- β (CD8 $^+$ LAP $^+$ cells) and exhibit regulatory functions both *in vitro* and *in vivo*. Mechanistically, CD8 $^+$ LAP $^+$ cells exert their regulatory activities by inducing and expanding Foxp3 regulatory T-cells and by inhibiting proliferation and IFN- γ production.⁹¹ (Fig. 1, right).

The role of CD8 $^+$ T-cells has also been examined using genetically engineered CD8- or β 2m-deficient mice^{92–94} and in mice treated with anti-CD8 antibodies.^{21,93,95,96} In all these studies, oral tolerance was induced normally, indicating that there is no absolute requirement for CD8 $^+$ T-cells in the induction or maintenance of systemic tolerance. However, Lui et al.⁹⁷ recently found that CD8 $^+$ T-cells can cross-differentiate into MCH-I-restricted CD4 $^+$ T-cells, which are enriched in the mesenteric lymph node and

large intestine lamina propria. In the mLN, converted CD4⁺ T-cells can differentiate into CD4⁺Foxp3⁺ regulatory T-cells, migrate to the gut lamina propria, and promote tolerance. Nevertheless, whether CD8⁺ T-cells can contribute to individual aspects of the tolerant state or play discrete roles in different tissues, such as the mucosa itself, remains to be established.

5. Intestinal Macrophages and Dendritic Cells

A crucial step for oral tolerance induction is antigen sampling in the gut. Antigen sampling relies on various mechanisms, such as Peyer's patches (PP)-associated microfold (M) cells, epithelial cell-mediated uptake, and protrusion of dendrites by CX3CR1-expressing macrophages and DCs.⁹⁸ CX3CR1-expressing cells are particularly important because CX3CR1 conditional deletion in either macrophages or DCs result in a reduction of 60% in OVA-specific Treg cells at 48 hours, but not 7–8 days, after oral administration of OVA,⁹⁹ suggesting that CX3CR1⁺ macrophages and CX3CR1⁺ DCs have an important role in initiating oral tolerance induction (Fig. 1, left). However, CX3CR1⁺ macrophages do not migrate to the mLN to induce tolerance. It has been proposed that CX3CR1⁺ macrophages, after sampling gut antigens, can transfer them to migratory CD11c⁺CD103⁺ DCs via connexin 43 gap junctions.¹⁰⁰ DCs then migrate to the mLN and induce antigen-specific Treg cells. These Treg cells are granted the ability to migrate back to the gut, as described previously, and promote tolerance.

Interestingly, another crucial role of CX3CR1⁺ macrophages is to further reactivate and induce proliferation of mLN-generated antigen-specific Treg cells. This effect has been shown to be strictly dependent on IL-10 secreted by CX3CR1⁺ macrophages.¹⁰¹ Thus, CX3CR1-expressing macrophages in the gut lamina propria, by sampling antigens and transferring them to migratory tolerogenic DCs, and by further providing IL-10 to newly generated Treg cells, contribute to oral tolerance induction.

Dendritic cells (DCs) play a major role in oral tolerance induction. Several subtypes of gut DCs have been described and are located in small and

large intestine lamina propria as well as in PPs and the mLN. By analyzing the transcriptional profile of gut DC subtypes involved in the conversion of Treg cells and oral tolerance induction, Esterhazy et al. defined two major groups of DCs: the resident and the migratory DCs.⁹⁹ Resident CD11c⁺CD11b⁺ DCs and migratory CD11c⁺CD11b⁺CD103⁺ DCs are dependent on the transcription factor IRF4 whereas resident CD8 α ⁺CD11c⁺ DCs and migratory CD11c⁺CD11b⁺CD103⁺ DCs are dependent on IRF8. Resident DCs are poorly tolerogenic, and the IRF8-dependent migratory CD11c⁺CD11b⁺CD103⁺ DCs are the most potent tolerogenic subset, expressing TGF- β and the retinoic acid-catalyzing enzyme RALDH. The primary factor responsible for DC migration to the mLN is the chemokine receptor CCR7, which binds to the chemokines CCL19 and CCL21, which are highly expressed in lymph nodes. Consistent with this, mice deficient for CCR7 failed to induce oral tolerance.¹⁰² Importantly, lymphocytes from both the IEL and the lamina propria compartments have been shown to secrete the chemokine XCL1, which binds to its receptor XCR1 expressed on CD103⁺ DCs from the gut lamina propria, resulting in CCR7 upregulation on these DCs and migration to the mLN.¹⁰³ Once in the mLN, CD11c⁺CD103⁺ DCs present antigens to cognate CD4⁺ T-cells, and by releasing TGF- β and RA, turn these CD4⁺ T-cells into Treg cells.¹⁰⁴ RA is also crucial for induction of the gut-homing molecule CCR9, which binds to CCL25 in the small intestine lamina propria, and α 4 β 7, which binds to the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed on the high endothelial venules of the gut lamina propria.⁴⁷ The induced Treg cells then migrate back to the gut, where they promote tolerance (Fig. 1).

6. Regulatory T-Cells

As described previously, Treg cells were first described in the 1970s, when they were considered to be mainly CD8⁺ and referred as “suppressor” T-cells. The recent wave of research on CD4⁺CD25⁺ Treg cells rehabilitated the old concept of suppressor cells and brought important new insights into

the tolerance field as a whole and into oral tolerance studies as a consequence. Initially reported by Nishizuka and Sakaguchi,¹⁰⁵ and further explored by Sakaguchi et al.,^{106–108} studies on animals thymectomized as neonates showed that autoimmune pathology, characterized by gastritis, oophoritis, and orquitis, arises as a result of the ablation of a subpopulation of thymic T-cells. These cells can restore immunoregulatory function in diseased mice upon adoptive transfer; they have been characterized as CD4⁺ T-cells that express the high-affinity receptor for IL-2 (IL-2R α or CD25)¹⁰⁹ and the transcription factor Foxp3.²³ In fact, a mutation of the gene encoding Foxp3 results in manifestation of a disease known as IPEX, or “syndrome immunodysregulation polyendocrinopathy and enteropathy associated with the X chromosome,” which is characterized by autoimmune enteropathy, psoriasiform or eczematous dermatitis, nail dystrophy, and autoimmune skin conditions such as alopecia universalis and bullous pemphigoid.¹¹⁰

Regulatory T-cells can be divided into two main classes: natural, or nTregs, and induced, or iTregs. nTregs are generated in the thymus and express CD25 and Foxp3. These cells are anergic and therefore do not proliferate nor secrete IL-2 as effector T-cells; rather, they depend for survival on IL-2 produced by other lymphocytes.¹¹¹ The regulatory mechanism of Treg cell action involves cell-cell contact more than the secretion of cytokines.¹¹¹ This evidence was corroborated by experiments using two-photon intravital microscopy. It was shown that the contact between T-cells and antigen-loaded DCs is shorter in the presence of Treg cells than in their absence.¹¹² Molecules that contribute to the regulatory function of Treg cells include CTLA-4, GITR, CD39, CD73, LAP, and its anchoring molecule glycoprotein A repetitions predominant (GARP or LRRC32).²⁴

In addition to nTregs, CD4⁺CD25⁺Foxp3⁺ naïve T-cells can be converted into functional CD4⁺CD25⁺Foxp3⁺ iTreg in the periphery (Fig. 1, right). Importantly, only iTregs are crucial for oral tolerance induction because oral tolerance can be induced in mice devoid of naturally occurring Treg cells.¹¹³ Consistent with this, recent work has shown that, under normal conditions, the vast majority of

small intestinal iTreg cells are induced by dietary antigens from solid foods, have a limited life span, are distinguishable from microbiota-induced iTreg cells, and repress underlying strong immunity to ingested protein antigens.¹¹⁴ Such intestinal iTreg cells are likely to be induced in mLNs by specialized DCs that migrate from the gut to the mLNs through a mechanism dependent on TGF- β and retinoic acid.¹¹³ However, in the presence of TGF- β and IL-6, as in an inflammatory process, naïve CD4⁺CD25⁺Foxp3⁺ T-cells can be differentiated into IL-17-producing CD4⁺ T (Th17) cells and favor inflammation,^{115,116} Thus, TGF- β has the paradoxical effect of inducing distinct subsets of T-cells with opposite effects on the immune response.

Peripherally induced regulatory T-cells may comprise not only those cells expressing the transcription factor Foxp3. In 1994, we described a subtype of CD4 T-cells (Th3 cells) originating during oral tolerance induction to myelin basic protein (MBP) that secretes high amounts of TGF- β , IL-4, and IL-10 that suppress experimental autoimmune encephalomyelitis (EAE), a rodent model for multiple sclerosis.²⁰ Th3 regulatory cells form a unique T-cell subset, which primarily secretes TGF- β , provides help for IgA class switch, and has suppressive properties for Th1, Th2, and Th17 cells. Th3 cells are distinct from Th2 cells, because CD4⁺ TGF- β -secreting cells with suppressive properties have been generated from interleukin (IL)-4-deficient animals. Th3 cells are triggered in an antigen-specific fashion, but suppress in a non-antigen-specific manner and thus mediate “bystander suppression” when they encounter the fed antigen at the target organ.¹¹⁷ In addition to the secreted form of TGF- β , an active membrane-bound form was described. Murine CD4⁺CD25⁺Foxp3⁺ Treg cells have been reported to express LAP and TGF- β on the surface after activation and to exert regulatory function by the membrane-bound TGF- β ^{29–31} Blockage of TGF- β by anti-TGF- β monoclonal antibodies curtails the ability of these cells to suppress CD4⁺CD25⁺ T-cell proliferation and B-cell immunoglobulin production *in vitro*.²⁹ We also have detected LAP on CD4⁺CD25⁺Foxp3⁺ cells in normal mice and this cell compartment increases after oral tolerance

induction.^{30,118} We therefore believe that Th3 cells are CD4⁺CD25⁺Foxp3⁺LAP⁺ cells that exist in the peripheral immune compartment and that are triggered by TCR signaling in the gut by oral antigen.

Another subtype of peripherally induced Tregs is type 1 regulatory T (Tr1) cells. Tr1 cells are memory CD4⁺ T lymphocytes that express the surface markers CD49b and LAG-3, but are Foxp3 negative (Figure 1, right). Upon activation, Tr1 cells secrete high amounts of IL-10 and TGF- β , variable levels of IFN- γ , IL-5, and GM-CSF, and no IL-17. Tr1 cell suppressive properties are related to the release of IL-10 and TGF- β upon TCR activation by a cognate antigen,¹¹⁹ and like Th3 cells Tr1 cells can mediate bystander suppression to other antigens.¹²⁰ Interestingly, Tr1 cells are better induced upon nasal rather than oral tolerance, which preferentially gives rise to Th3 cells, as discussed previously. Indeed, we have shown that nasal administration of anti-CD3 in mice induced suppressive Tr1 cells that were dependent on IL-27 produced by upper-airway dendritic cells.¹²¹ Moreover, we recently showed that nasal administration of anti-CD3 monoclonal antibodies ameliorated disease in a progressive model of multiple sclerosis in mice by inducing Tr1 cells that suppress the astrocyte inflammatory transcriptional program.¹²² Thus, the site of antigen exposition is crucial to the subtype of regulatory T-cells to be expanded and to the mechanism of tolerance induction.

B. Systemic Tolerance after Oral-Fed Antigens

One of the important characteristics of oral tolerance is that it can spread out to the entire animal. This is somehow difficult to understand because antigen uptake and recognition are restricted to the gut lamina propria, GALT, and mLNs. It is possible that orally administered antigens can reach the circulation and activate the immune system. In fact, food proteins can be found in the blood of mice and humans soon after eating.^{123,124} Moreover, food antigens can simultaneously activate T-cells in the mLN and in the peripheral lymph nodes,^{125,126} and serum from protein-fed mice can induce antigen-specific tolerance in naïve recipients,¹²⁷ suggesting

that tolerogenic material is found in the circulation after protein feeding. Of note, antigens that reach secondary lymphoid organs may induce systemic tolerance because they are sampled by dendritic cells and presented to T-cells in a noninflamed environment. However, how proteins from the diet that reach the bloodstream are involved in oral tolerance induction remains elusive.

Another explanation for systemic tolerance after oral-fed antigens comes from the tolerogenic environment of the liver. Blood draining the intestine via the portal vein reaches the liver, where oral antigen metabolism occurs; intestinal venous drainage through the liver is important for oral tolerance. In fact, antigens injected directly into the portal vein induce tolerance,¹²⁷ and preventing antigens from reaching the liver by portocaval shunting blocks oral tolerance induction.¹²⁸ Oral antigens are presented to T-cells within the liver by DCs and Kupffer cells, rendering these lymphocytes tolerogenic.¹²⁹ Plasmacytoid dendritic cells, which are abundant in the liver, have also been shown to induce systemic tolerance to fed proteins and haptens.¹³⁰ Antigen uptake is also performed by liver sinusoidal endothelial cells, which leads to CD8⁺ T-cell tolerance.¹³¹ Early studies demonstrated that portal administration of transplantation antigens is very effective in inducing graft tolerance.¹³² Treg cells were induced in liver-draining celiac lymph nodes during induction of oral tolerance to OVA, and expression of the α E-integrin CD103 on OVA-specific T-cells in the celiac lymph nodes may favor homing to the epithelium of the intestine.¹³³ Moreover, IL-10 transgenically expressed in mouse liver enhanced oral tolerance, whereas systemic administration of IL-10 had only a modest effect.¹³⁴ Ectopic expression of MBP in mouse liver induced antigen-specific Treg cells and suppressed EAE.¹³⁵ Interestingly, antibody titers to intestinal microbiota are elevated in humans with chronic liver disease who undergo portocaval shunts,¹³⁶ suggesting a role for the liver in tolerance to intestinal microbiota. Thus, the tolerant environment of the gut and the liver may together contribute to the systemic tolerance induced by fed antigens.

IV. THERAPEUTIC APPLICATIONS OF ORAL TOLERANCE IN AUTOIMMUNITY AND INFLAMMATORY CONDITIONS

Although oral tolerance as a treatment for autoimmunity and nonautoimmune inflammatory diseases have shown mixed results in clinical trials, several studies have demonstrated the effectiveness of orally administered autoantigens in animal models of autoimmune and inflammatory diseases. Importantly, we have found that oral administration of anti-CD3 monoclonal antibodies induces oral tolerance in several autoimmune and inflammatory diseases, such as streptozotocin-induced and NOD autoimmune diabetes,^{137–139} type 2 diabetes in the Ob/Ob mouse,¹⁴⁰ lupus-prone SNF1 mice,¹⁴¹ and atherosclerosis in mice.¹⁴² In this section, we describe the animal models in which oral tolerance induction has been shown to have positive effects.

A. Experimental Autoimmune Encephalomyelitis

The first studies to show that orally administered myelin antigens could suppress experimental autoimmune encephalomyelitis (EAE) were performed using the Lewis rat. EAE was suppressed by low doses of oral MBP and MBP fragments¹⁴³ and by high doses of MBP given in bicarbonate.¹⁴⁴ High doses of MBP can suppress EAE via the mechanism of T-cell clonal anergy,¹⁴⁵ whereas multiple lower doses prevent EAE by transferable active cellular suppression.¹⁴⁶ In the nervous system of low-dose-fed animals, inflammatory cytokines such as TNF- α and IFN- γ are downregulated and TGF- β is upregulated.¹⁴⁷ Oral MBP partially suppresses serum antibody responses, especially at higher doses. Administration of myelin to sensitized animals in the chronic guinea pig model or larger doses of MBP in the murine EAE model is protective and does not exacerbate disease,^{148,149} and long-term (6-month) administration of myelin in the chronic EAE model is beneficial.¹⁵⁰

Several studies have demonstrated suppression of EAE in murine models. Both conventional and TCR transgenic animals have been used, and oral MBP, proteolipid protein (PLP), and myelin oligodendro-

cyte glycoprotein (MOG) have been administered with successful induction of oral tolerance.^{19,151,152} In these models, MBP regulatory clones have been described,²⁰ and such cells have also been induced in MBP TCR transgenic mice.¹⁵³ Both CD4⁺ and CD8⁺ T-cells have been shown to mediate active suppression,²¹ and all mechanisms of tolerance, including anergy and deletion, have been demonstrated. Moreover, orally administered MOG reduced Th17 cells and overall inflammation by decreasing proinflammatory cytokines, such as IL-1 α , IL-6, IL-9, IL-12p70, and the chemokine macrophage inflammatory protein (MIP)-1 β , regulated on activation, normal T-cell expressed and secreted (RANTES), eotaxin, and KC in the central nervous system.¹⁵²

We showed that oral administration of a recombinant *Lactococcus lactis* strain that produces and releases LPS-free heat shock protein 65 (Hsp65) prevented the development of MOG-induced EAE in mice. This effect was associated with reduced IL-17 and increased IL-10 production in the mLN and spleen as well as with increased numbers of natural and inducible CD4⁺Foxp3⁺ Treg cells and CD4⁺LAP⁺ (Th3) cells in the spleen, mLN, and spinal cord.¹⁵⁴ Importantly, because Hsp65 is not an antigen involved in the pathogenesis of EAE and Hsps are hyperexpressed during inflammation, it is plausible that the inhibition of immune responses to Hsp65 in EAE promotes a bystander suppression of reactivity to other antigens involved in the pathological processes. Bystander suppression is a phenomenon associated with oral tolerance that spreads its inhibitory effect toward other neighbor antigens probably via the action of antigen-presenting cells.^{155,156} This cross-suppressive event is also called an indirect effect of oral tolerance,¹⁵⁷ and it precludes the need to identify the target antigen when a therapeutic use of oral tolerance protocols is planned. Thus, EAE can be suppressed by oral-fed specific or unspecific antigens.

Although the investigation of oral tolerance has classically involved the administration of antigens, we found that oral administration of anti-CD3 monoclonal antibodies induces oral tolerance through a mechanism dependent on Treg cell generation.¹⁵⁸ By giving hamster antimouse CD3 (2C11 clone) to SJL mice and immunizing them with PLP emulsified in

CFA to induce EAE, we found that oral anti-CD3 suppresses both clinical and pathological features of EAE. Similar results were observed when C57BL/6 mice were fed anti-CD3 and immunized with MOG_{35–55} plus CFA.^{118,158,159} Mechanistically, oral administration of anti-CD3 induced both CD4⁺CD25⁺LAP⁺ and CD4⁺CD25[–]LAP⁺ Tregs in the mLN and spleen.³¹ LAP-expressing cells are crucial for oral tolerance induced by anti-CD3 because oral tolerance is prevented by intraperitoneal injection of anti-LAP monoclonal antibodies.¹¹⁸ Induction of Tregs by oral anti-CD3 is seen with lower doses rather than higher doses, suggesting that this effect is not simply related to higher doses of anti-CD3 to overcome degradation of the antibody in the gut. Indeed, we isolated biologically active anti-CD3 from intestinal eluates of animals orally dosed with anti-CD3¹³⁷ and visualized anti-CD3 being taken up by gut epithelial cells and binding to gut DCs in intestinal loop experiments.¹⁵⁵ Studies are in progress to test possible factors that may enhance oral tolerance induced by anti-CD3, including the combination of anti-CD3 and antigens, and by anti-CD3 with proinflammatory cytokine-blocking monoclonal antibodies.

B. Colitis

Transforming growth factor-beta (TGF- β) plays a crucial role in the development of animal models of colitis, including TNBS colitis, colitis in the IL-2-deficient mouse model following systemic administration of keyhole limpet hemocyanin (KLH) in adjuvant, and in the model of Th1 colitis in severe combined immunodeficiency (SCID) mice. It has been shown that TNBS colitis can be prevented by oral administration of TNBS, which acts via the induction of TNBS-specific TGF- β responses.¹⁶⁰ Protected animals had low IFN- γ and high TGF- β levels, and tolerance could be transferred by mLN cells.¹⁶¹ Transfer of CD4⁺CD45RB^{high} T-cells into SCID or recombination-activating gene (RAG)-deficient mice is another experimental model of Th1-type colitis characterized by inflammatory infiltrate containing macrophages and CD4⁺ T lymphocytes that extends diffusely from the cecum to the rectum, leading to mucosal thickening, increased IFN- γ secretion

by local T-cells, diarrhea, and weight loss. Histological and immunological findings in these mice are similar to those seen in patients with Crohn's disease.¹⁶² The ability of CD4⁺CD45RB^{low} T-cells to protect against disease induced by transfer of these inflammatory CD4⁺CD45RB^{high} T-cells into SCID mice is inhibited by anti-TGF- β antibodies,¹⁶³ as is the protective effect of feeding antigen on the colitis induced by local administration of contact-sensitizing agents to normal animals.^{160,164} CD4⁺CD25[–]LAP⁺ (Th3) T-cells also inhibit colitis in a TGF- β -dependent fashion.³⁰ In addition, colitis can be suppressed by orally administered OVA, which acts via bystander suppression in an OVA-induced colitis model.¹⁶⁵

As mentioned earlier for the EAE mouse model, genetically modified *Lactococcus lactis* that produces and releases *Mycobacterium* Hsp65 also completely prevented DSS-induced colitis in C57BL/6 mice. This protection was associated with reduced proinflammatory cytokines, such as IFN- γ , IL-6, and TNF- α ; increased IL-10 production in colonic tissue; and expansion of CD4⁺Foxp3⁺ and CD4⁺LAP⁺ regulatory T-cells in the spleen and mLN. This effect was dependent on IL-10 and toll-like receptor 2.¹⁶⁶ IL-10 in immunoregulation of gut responses has been shown to be essential for Treg cell function in inhibiting intestinal inflammation¹⁶⁷ and for stimulation of TGF- β -producing T-cells.¹⁶⁸ Importantly, it is already known that inflammatory bowel diseases (IBDs) are autoimmune diseases that result from a breakdown in immune tolerance to the microbiota, causing an inflammatory process in the intestine. Gut inflammation generates a detrimental environment for oral tolerance induction because immune cells in such conditions are primed to "attack." Thus, it is important to consider, before treatment with an IBD therapy using oral tolerance to fed antigens, the reestablishment of gut homeostasis. In this case, patients will benefit from the immunoregulation induced by orally fed antigens, resulting in a long-term improvement of the symptoms.

C. Diabetes

Oral insulin has been shown to delay and, in some instances, prevent diabetes in the nonobese diabetic

(NOD) mouse model. Such suppression is transferable, primarily with CD4⁺ T-cells.¹⁶⁹ Immunohistochemistry of pancreatic islets of Langerhans isolated from insulin-fed animals demonstrates decreased insulinitis associated with decreased IFN- γ , as well as increased expression of TNF- α , IL-4, IL-10, TGF- β , and prostaglandin E2.¹⁷⁰ Oral insulin suppressed diabetes in a viral-induced model of diabetes, in which LCMV was expressed under the insulin promoter and animals were infected with it to induce diabetes.⁹⁰ Protection was associated with cytokine shifts—IL-4/IL-10, TGF- β —in the islets. It has also been shown that expression of TGF- β in the pancreatic islets protects NOD mice from diabetes and that TGF- β seems to alter antigen-presenting cell properties, polarizing islet antigen responses toward a Th2 phenotype.¹⁷¹ Oral administration of a B-chain of insulin, a 30–amino acid peptide, slowed the development of diabetes and prevented diabetes in some animals.¹⁷² This effect was associated with a decrease in IFN- γ and an increase in IL-4, TGF- β , and IL-10 expression. Oral administration of recombinant glutamic acid decarboxylase from plant sources or a plant-based CTB-insulin fusion protein¹⁷³ suppressed the development of diabetes in NOD mice.¹⁷⁴

Recently, we showed that oral administration of anti-CD3 delayed the onset and reduced the incidence of diabetes in NOD mice, inducing changes in both effector and regulatory T-cell compartments. The therapeutic effect was associated with decreased T-cell proliferation, reduced IFN- γ and IL-17 production, and increased TGF- β and IL-10 production *in vitro*. *In vivo* transfer experiments demonstrated that oral anti-CD3 decreased the diabetogenicity of effector T-cells and increased the function of Treg cells. Oral OKT3, a monoclonal antibody specific for human CD3, had equivalent effects in transgenic NOD mice expressing the human CD3 epsilon chain, which serves as a preclinical model for testing human anti-CD3.¹⁷⁵ These results suggest that oral anti-CD3 has the potential for treating autoimmune diabetes in humans.

D. Uveitis

Oral administration of the S antigen (S-Ag), a retinal autoantigen that induces experimental autoimmune

uveitis (EAU), or of S-Ag peptides prevented or markedly diminished the severity of S-Ag-induced disease, as measured by ocular inflammation.¹⁷⁶ S-Ag-induced EAU was also suppressed by feeding a human leukocyte antigen (HLA) peptide.¹⁷⁷ Feeding interphotoreceptor binding protein (IRBP) suppressed IRBP-induced disease and was potentiated by IL-2.¹⁷⁸ Oral feeding of retinal antigen not only prevented acute disease but also effectively suppressed second attack in chronic-relapsing EAU, demonstrating that oral tolerance may have practical clinical implications in uveitis, which is predominantly a chronic-relapsing condition in humans.^{179–181}

Other investigators have found that oral administration of bovine S-Ag peptides was very efficient in preventing EAU but could only inhibit mild disease if feeding was delayed until after immunization and relatively high feeding doses were administered.¹⁸² More recently, Huber et al. showed that in a relapsing-remitting EAU in rats, oral administration of specific peptides prior to or after EAU onset suppressed EAU and prevented relapses, an effect associated with an increase in Treg cells.¹⁸³

E. Myasthenia Gravis

Although myasthenia gravis is an antibody-mediated disease, oral administration of the Torpedo californica acetylcholine receptor (TAChR) to Lewis rats prevented or delayed disease onset. Levels of anti-AChR antibodies in the serum were lower in orally tolerized animals than in control animals. The effect was dose-dependent, and large doses of antigen (at least 5 mg of AChR) plus soybean trypsin inhibitor¹⁸⁴ were required, suggesting that anergy may be the primary mechanism. Purified AChR was found more effective than an unpurified mixture.¹⁸⁵ Ongoing myasthenia was suppressed by an AChR recombinant fragment.¹⁸⁶ Moreover, oral administration of the T-cell epitope alpha146-162 of the TAChR alpha-subunit¹⁸⁷ or a syngeneic rat recombinant fragment of the extracellular domain of the acetylcholine receptor (AChR) alpha-subunit (Ralpha1-205),¹⁸⁸ suppressed ongoing experimental autoimmune myasthenia gravis (EAMG) in rats. The proposed mechanism for this effect was a shift from Th1 to Th2 regulation,

as evidenced by downregulated mRNA expression levels of IFN- γ and TNF- α , upregulated IL-10, changes in anti-AchR IgG isotypes, and diminished Th1 signaling via CD28/CTLA-4:B7. However, in both cases TGF- β was not involved.

F. Systemic Lupus Erythematosus

Like myasthenia gravis, systemic lupus erythematosus (SLE) is an autoantibody-mediated autoimmune disease in which a dysregulated immune system leads to tissue damage in multiple organs. Oral tolerance has been shown to be effective in animal models of SLE. Skaggs et al.¹⁸⁹ showed that oral administration of six different forms of the pConsensus (pCons) peptide induced better animal survival rates, which were associated with less cumulative proteinuria and serum anti-dsDNA antibody levels than controls. Moreover, the SLE-prone mice fed pCons had increased serum TGF- β , implying a protective role for pCons-induced regulatory T-cells in the mechanism of oral tolerance induction. Furthermore, a H2A histone fragment termed IIIM1 given orally to MRL/lpr mice resulted in prolonged animal survival, decreased lymphadenosis, and reduced levels of pathogenic or abnormal lymphoid cells in lymph nodes and spleen. Moreover, the researchers found that IIIM1 induced the production of an additional peptide, a fragment of alpha-1-antitrypsin termed UBE, and that a relatively low dose of UBE reduced proteinuria and hematuria in MRL/lpr mice. This effect was associated with a significant reduction in serum IL17, IL12, and anti-dsDNA antibodies in UBE-treated mice.¹⁹⁰

We found that oral administration of anti-CD3 attenuates lupus development and arrests ongoing disease in lupus-prone SNF1 mice. Oral anti-CD3 induced Th3 cells that secreted high levels of TGF- β and suppressed effector T-cells *in vitro* in a TGF- β -dependent fashion. Animals treated with oral anti-CD3 had less glomerulonephritis and diminished levels of anti-dsDNA autoantibodies. Oral anti-CD3 led to a downregulation of IL-17⁺CD4⁺ICOS⁺CXCR5⁺ follicular helper T-cells, CD138⁺ plasma cells, and CD73⁺ mature memory B-cells.¹⁴¹ Thus, oral administration of anti-CD3 may be a promising therapy for SLE treatment.

V. ORAL TOLERANCE TRANSLATION TO HUMAN DISEASES

Although it is clear that oral-fed antigens can suppress autoimmunity and inflammatory diseases in animals, several factors need to be carefully considered for oral tolerance to be effective in human disease: (1) the antigen dose and dosing frequency are crucial; (2) a clear immunological marker or immunological effect has to be established as a parameter for dosing in each disease; (3) mucosal adjuvants that enhance induction of tolerance have been described and may be required; (4) purified proteins are more effective oral tolerogens than protein mixtures; (5) in some instances, a nasal antigen may be more effective; (6) combination therapy using conventional anti-inflammatory and immunosuppressive drugs may be required; and (7) early therapy after disease onset is required. Taking all these factors into account, oral tolerance as treatment of allergy and autoimmune diseases, has been moving forward. Studies have successfully demonstrated that oral administration of peanut to peanut-allergic children/adolescents leads to a substantial increase in dose threshold, conferring protection against peanuts. This effect is accompanied by a reduction in skin prick tests, decreased levels of IL-5 and IL-13, and an increased ratio of FOXP3^{high}:FOXP3^{intermediate} CD25⁺CD4⁺ T-cells in treated patients compared to placebo controls.^{191,192} Moreover, two single-blind, randomized, placebo-controlled phase IIa studies of oral anti-CD3 antibodies in patients with resistant chronic hepatitis C infection (HCV)¹⁹³ or nonalcoholic steatohepatitis (NASH)¹⁹⁴ reported positive effects on disease and immunological markers, including an increase in regulatory T-cells. Interestingly, a recent study by Sherman et al., using two different hemophilia mouse strains, reported that oral administration of planT-cell extracts expressing factor VIII (FVIII) antigens induced unresponsiveness to FVIII challenge.¹⁹⁵ This is an important approach that can be rapidly translated into the clinic to treat hemophilic patients because it does not require large amounts of FVIII, which is highly expensive; also, it can be delivered as bioencapsulated antigen in planT-cells and consumed as a vegetable.

Mucosal tolerance has been reported to help prevent rejection in maternal-donor renal allografts. It is known that a large number of maternal lymphocytes are present in breast milk, and a 1984 report suggested that breast-fed recipients may do better after a maternal donor-related renal transplant than non-breast-fed recipients.¹⁹⁶ We observed that neonatal administration of guinea pig MBP in the Lewis rat does not induce oral tolerance and in fact makes the animals more susceptible to EAE induction as adults.¹⁹⁷ This raises the possibility that neonatal exposure to antigen may be a factor in the development of autoimmune disease in adulthood, which indeed has been postulated for diabetes in terms of exposure to cow's milk as a risk factor for development of diabetes. Interestingly, we fed insulin to neonatal NOD mice and found not an enhancement of diabetes but better protection against its development.¹⁹⁸ We did not observe protection with oral MOG given to neonates, but MOG did not exacerbate EAE. Thus, worsening of autoimmunity by neonatal exposure may be considered a possible risk factor for the development of autoimmune disease in adulthood.

Many aspects of the mechanisms involved in mucosal tolerance induction need to be further investigated. Cell-surface molecules and cytokines associated with inductive events in the gut that generate and modulate oral tolerance are not completely understood. Important areas of investigation include the gut microbiota, cytokine milieu, antigen presentation and costimulation requirements, routes of antigen processing, form of antigen, role of the liver, effect of oral antigens on antibody and IgE responses and on cytotoxic T lymphocytes, and the role of $\gamma\delta$ T-cells and ILCs. As the molecular events associated with the generation and modulation of oral tolerance are better understood, we hope that the ability to successfully apply mucosal tolerance for the treatment of human autoimmune and other diseases will be soon achieved.

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