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Antigen-Specific Regulatory T Cell Responses to Intestinal Microbiota

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Abstract

The mammalian gastrointestinal tract can harbor both beneficial commensal bacteria important for host health, but also pathogenic bacteria capable of intestinal damage. It is therefore important that the host immune system mount the appropriate immune response to these divergent groups of bacteria—promoting tolerance in response to commensal bacteria and sterilizing immunity in response to pathogenic bacteria. Failure to induce tolerance to commensal bacteria may underlie immune-mediated diseases such as human inflammatory bowel disease. At homeostasis, regulatory T (Treg) cells are a key component of the tolerogenic response by adaptive immunity. This review examines the mechanisms by which intestinal bacteria influence colonic T cells and B cell IgA induction, with an emphasis on Treg cells and the role of antigen-specificity in these processes. In addition to discussing key primary literature, this review highlights current controversies and important future directions.

Introduction

The mammalian gastrointestinal tract is adapted to harbor trillions of bacteria¹, many of which provide beneficial functions to the host, including protection from infection by pathogenic organisms^{2, 3} and nutrient metabolism^{4, 5}. However, the close physical proximity of these bacteria to the host represents a unique challenge to the immune system, as it must be able to discriminate pathogenic bacteria from those normally resident in the gut. Inappropriate tolerance to pathogens may facilitate infection^{6, 7}, whereas immune reactivity against harmless commensal microbiota is thought to underlie the pathogenesis of human inflammatory bowel disease^{8, 9} (IBD). Thus, the generation of appropriate immune responses to bacteria is crucial for intestinal health.

Although intestinal homeostasis requires multiple arms of the immune system¹⁰, here we will focus on the role of T cell responses to commensal bacteria. The absence of adaptive immune T cells leads to a failure of gut homeostasis in murine models, with bacterial translocation and colitis¹¹. Similarly, intestinal pathology without obvious pathogenic infection also happens in humans with HIV that is associated with decreased CD4⁺ T cell

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counts^{12, 13}. Adaptive immune cells are therefore not only required to target pathogenic bacteria^{14, 15}, but also recognize and control normal intestinal bacteria during homeostasis.

However, the predominant T cell responses to intestinal bacteria during homeostasis are likely inhibitory responses to limit inflammation and immune-mediated gut pathology, rather than effector responses to eliminate bacteria. It is now generally accepted that tolerance to intestinal bacteria requires CD4⁺ regulatory T (Treg) cells, as first suggested in adoptive transfer experiments in rodents¹⁶. Treg cells are defined by the transcription factor Foxp3, which is required for Treg cell function and development¹⁷. In humans, patients with Treg cell deficiency (IPEX; immune dysregulation, polyendocrinopathy, enteropathy, X-linked) exhibit diarrhea and at times colitis amongst other autoimmune disease manifestations¹⁷. Thus, Treg cell-mediated tolerance to gut bacteria is crucial for maintenance of immune homeostasis and prevention of IBD.

We will review our current understanding of the reciprocal interactions of T cells and intestinal bacteria during homeostasis. Specifically, we will discuss the roles of intestinal bacteria in shaping tolerogenic Treg cell responses via non-antigen-specific factors as well as address how antigen-specificity appears to be important for intestinal Treg cell development and function. Although other immunoregulatory T cell subsets will not be addressed in this review, it is important to note that they make important contributions to gut tolerance¹⁸⁻²¹. We will also explore the function of Treg and effector T cells during homeostasis and their roles in modulating antigen-specific IgA induction. Since bacteria-dependent immunopathology in the small intestine is less common in humans, we will focus on Treg cell:bacteria interactions in the colon. Throughout, we will discuss discrepancies in conclusions formulated from primary data as well as identify unanswered questions in the field.

Developmental Origin of Colonic Treg Cells

It is now well-established that the colonic Treg cell population is affected by intestinal bacteria¹⁰. For example, germ-free mice show a several-fold reduction in the frequency of Treg cells compared with conventionally housed specific pathogen free (SPF) mice^{22, 23}. This process does not require a complex microbiota, as introduction of individual bacterial isolates or defined consortia into germ-free mice is sufficient to induce colonic Treg cells^{22, 23}.

One mechanism by which intestinal bacteria can influence Treg cell numbers is by inducing expansion of pre-existing thymic Treg (tTreg) cells. Classically, tTreg cells are generated in response to antigen recognition at an immature stage of T cell development prior to their release into the periphery²⁴. Although it may be possible that colonic bacterial antigens are transported and presented in the thymus to induce tTreg cells, there is currently no evidence that this occurs. A more compelling possibility is that some self-antigen reactive tTreg cells exhibit cross-reactivity with foreign antigens²⁵, resulting in expansion of those bacterial-reactive clones in the intestines. Consistent with these possibilities, a marked overlap between the tTreg and colonic Treg TCR repertoires was observed in one study²⁶.

Another mechanism is that intestinal bacteria can induce the peripheral differentiation of Treg (pTreg) cells from naïve T cells. There are several lines of data suggesting that pTreg cells comprise the majority of the colonic Treg population. First, the use of markers reported to identify tTreg versus pTreg cells suggest that gut bacteria induce pTreg cells. High expression of the transcription factor Helios and cell surface protein Nrp-1 have been associated with tTreg cells²⁷⁻²⁹. Although the utility of these markers remains controversial³⁰⁻³², colon lamina propria (cLP) Treg cells contain a much lower proportion of pTreg cells in germ-free compared with SPF mice based on these markers^{22, 27-29, 33}. In addition, treatment of SPF mice with broad-spectrum antibiotics results in the loss of cLP pTreg cells based on Helios levels²². Second, deletion of the conserved noncoding sequence 1 (CNS1) in the *Foxp3* locus results in a marked decrease in pTreg, but not tTreg, cell induction^{34, 35}. Notably, CNS1-deficient mice show fewer intestinal Treg cells at the time of weaning^{34, 35}. Of the remaining intestinal Treg cells, there is a lower proportion of Nrp-1^{lo} cells, consistent with loss of pTreg cells in CNS1-deficient mice²⁸. Third, our analysis of colonic Treg TCRs showed that they were unable to induce tTreg cell selection³⁶. The different conclusion from the aforementioned TCR repertoire analyses²⁶ may relate to the different analytical approaches used to assess tTreg cell selection—intrathymic injection of TCR-expressing thymocytes³⁶ vs comparison with thymic TCR repertoires²⁶. The conclusions may also be affected by the different TCR β chains used^{26, 36} or the use of a limited TCR α /fixed TCR β model²⁶, as effects on Treg cell selection has been reported for changes in a single TCR chain (TCR α)³⁷. The ability of two of these colonic TCRs (CT2/CT6) to facilitate pTreg cell induction from naïve T cells in the periphery in normal mice was confirmed via the generation of transgenic (Tg) lines³³. Altogether, these data suggest that the majority of colonic Treg cells arise via pTreg cell differentiation from naïve T cells.

pTreg cells specific for intestinal bacteria appear to be important for colonic homeostasis. For example, treatment of experimental colitis with Treg cells was more effective using co-transfer of normal Treg cells plus naïve Foxp3⁻ conventional T cells (Tconv) cells that became pTreg cells, than by co-transfer of Treg cells alone³⁸. In conjunction with TCR repertoire analysis, it was suggested that the induced pTreg cells prevented colitis by providing additional TCR specificities not present in the transferred Treg cell population, which were mostly tTreg in origin³⁹. Similarly, mice that have decreased induction of pTreg, but not tTreg, cells through genetic deletion of the CNS1 *Foxp3* enhancer region, eventually develop spontaneous colitis characterized by plasmacytic enteritis and high CD4⁺ T cell expression of Th2 cytokines³⁴. As in other tissues, tTreg cells specific for self-antigens such as those in intestinal epithelial cells are also involved in maintaining homeostasis in the gut^{16, 22, 40}, and may expand specifically in the context of certain types of intestinal perturbations such as sterile injury⁴¹. However, the above data suggest that tTreg cells are insufficient to maintain intestinal tolerance and that pTreg cells with a unique set of bacterial antigen specificities are required for homeostasis.

Our group has used colon Treg TCR Tg lines to study the process of pTreg cell differentiation to commensal bacterial antigens³³. Using adoptive transfer of peripheral naïve T cells from CT2/CT6 TCR Tg mice into wild type, lymphoreplete mice, it was found that pTreg cell generation was very efficient and resulted in over 80% of cells in the colon and mesenteric lymph nodes upregulating Foxp3 by three weeks after transfer. This was

associated with induction of Foxp3 in the most proliferated T cell population, and is in stark contrast with previous studies in non-mucosal tissues showing pTreg cells in the least-divided population^{42, 43}. These colonic TCR Tg data therefore demonstrate that pTreg cell generation can be the dominant outcome of naïve T cell activation.

TCR Tg cells from these two lines did not undergo Treg cell induction upon transfer in young mice, but only in mice around the age of weaning or thereafter³³. This was due to the lack of the appropriate bacteria in young mice, rather than an immature immune compartment incapable of inducing Treg cell selection, as fecal microbiota transplant from three-, but not one-, week old donors into one-week old recipients was sufficient to induce Treg cell development. The time-period around weaning is associated with major changes in the composition of the intestinal microbiota due to the switch to solid food and acquisition of a more adult-like microbiota⁴⁴. In fact, studies using Helios and Nrp-1 to identify Treg cell origin show a marked shift in the colon from tTreg to pTreg cells around weaning^{22, 23, 27, 33, 36}. In summary, there is now a substantial body of work supporting the notion that the majority of colonic Treg cells are generated by pTreg cell selection in response to colonic bacteria acquired around weaning.

Mechanisms of Colonic pTreg Cell Generation

The molecular mechanisms involved in colon pTreg cell development have been recently reviewed⁴⁵, which we will briefly summarize (Fig. 1). TGF β , an important factor for the generation of pTreg cells⁴⁶, can be induced by bacteria such as *Clostridium* from epithelial cells⁴⁷. Retinoic acid, a metabolite of dietary vitamin A, has been shown to augment the Foxp3-inducing effects of TGF β *in vitro*⁴⁸⁻⁵⁰. Indoleamine-2,3-dioxygenase (IDO) can be produced by dendritic cells and intestinal epithelial cells in response to gut bacteria to favor pTreg cell differentiation^{51, 52}. Short chain fatty acids (SCFAs) produced by gut bacteria from dietary components increase the percentage of Treg cells in the CD4⁺ T cell subset as well as the expression of IL-10 in Treg cells⁵³⁻⁵⁶. SCFAs can inhibit histone deacetylase and via epigenetic modification confer greater stability to Foxp3 gene expression and increase pTreg differentiation^{54, 55}. Polysaccharide A (PSA) from *B. fragilis* outer membrane vesicles (OMVs) was recently reported to tolerize dendritic cells through LC3-associated phagocytosis, which in turn increases IL-10 expression in gut Treg cells⁵⁷. Thus, a number of signals originating directly or indirectly from intestinal bacteria or the diet have been described to facilitate Treg cell differentiation or expansion in the gut.

While many of these mechanisms have been carefully studied on pTreg cell differentiation *in vitro*, their *in vivo* roles are not as well established. Recently, we have used bacteria-reactive colonic TCR Tg lines to begin to address these issues³³. For example, colonic TCR Tg cells expressing a dominant negative TGF β RII (dnTGF β RII) transgene to inhibit TGF β signaling showed only a 50% blockage of pTreg cell generation, whereas a previous study examining non-mucosal associated pTreg cell generation showed a 90% decrease⁴³. As the dnTGF β RII transgene is a hypomorph⁵⁸, a requirement for TGF β cannot be excluded. However, these data suggest that pTreg cell generation in the colon is not very sensitive to the level of TGF β signaling, contrary to predictions based on prior *in vitro* and *in vivo* data⁴³. Although it has been suggested that the level of TGF β is increased in the intestine⁵⁹, there is little evidence

to suggest that the normal level of TGF β *in vivo* is high enough to overcome the effect of dnTGF β RII^{33, 60}. Thus, one interpretation of these data is that TGF β is not a singular or “master” factor that specifies pTreg cell selection, and that other signals in the colon are also important.

Another key mechanism involved in pTreg cell selection is reported to involve CNS1, a region in the *Foxp3* locus^{34, 35} that includes transcription factor binding sites downstream of TGF β and retinoic acid signaling^{61, 62}. Analysis of naïve T cell differentiation using CNS1-deficient colonic TCR Tg cells revealed a strong inhibition of Foxp3-induction at seven days, but a gradual upregulation of Foxp3 by two to five weeks, which could also be observed in polyclonal cells using Helios or Nrp-1³³. Thus, CNS1 appears to be important, but not essential, for pTreg cell generation.

Many more studies are required before the factors that affect the colonic Treg cell population are fully understood. One important question is whether these factors affect pTreg cell differentiation, versus expansion or gut homing of pre-existing Treg cells. In one paper, tTreg cell expansion, and not pTreg cell differentiation, was proposed to be the mechanism by which SCFA via Gpr43 affect intestinal Treg cells⁵³. However, this issue is not easily addressed via analysis of polyclonal Treg cell numbers, requiring an approach where the kinetics of Treg cell induction and expansion can be monitored³³. Another major issue with experimental manipulations in the study of intestinal Treg cell generation are potential effects on the microbiota, both in terms of population composition but also bacterial gene expression, which is currently not often assessed. For example, NOD2-deficiency can lead to an expansion of *B. vulgatus*⁶³, which could directly or indirectly affect pTreg cell generation to *B. vulgatus* or other intestinal species by altering synthesis of SCFA, TLR signals for innate and adaptive immune cells, antigen presentation, and so forth. We think that this issue can be minimized by restricting the experimental manipulation to transferred T cells, which constitute only a small fraction of T cells in the host. While the TCR transgenic transfer model is limited to the study of T cell intrinsic factors, it permits analysis of Foxp3 induction, proliferation, migration, and survival of T cells in a normal host during homeostasis.

Bacterial Species Specificity of Colonic T Cells

The above discussion suggests that tolerance to intestinal bacteria is dependent on induction of Treg cells by intestinal bacteria. In addition to mechanisms such as SCFA and TLR ligands that act through TCR-independent mechanisms, there is growing evidence that the development of colonic Treg cells is in response to species-specific bacterial antigens (Fig. 1). This notion is consistent with TCR repertoire analyses of colonic T cells during homeostasis. Our group found that colonic Treg cells utilize a different TCR repertoire than Treg cells from other secondary lymphoid organs³⁶, suggesting recognition of local colonic antigens. Another study showed that the colonic Treg cell repertoire was markedly affected by broad-spectrum antibiotics²⁶, consistent with bacterial antigens selecting the gut Treg cell population. Thus, these reports show that intestinal bacteria play an important role in shaping the TCR repertoire of colonic Treg cells during homeostasis. Another consideration is that the cLP may also contain pTreg cells with non-microbiota specificities and tTreg cells

that are either specific to intestinal self-antigens or cross-react with bacterial antigens. Treg cells are observed in the cLP, albeit in reduced number, in germ free mice²², indicating that non-microbiota-dependent Treg cells can home to and survive in colonic tissue.

Studies of individual bacterial species transferred into germ-free mice support the concept that colonic Treg cells can be specific to bacteria. Both murine and human *Clostridium* species^{22, 47} (primarily from clusters IV, XIVa, and XVIII), but not all gut bacterial species, transferred to germ-free mice can induce increased frequencies of Treg cells. While the pro-Treg cell effects of *Clostridium* species may be both antigen specific and non-specific, *in vitro* suppression by Treg cells from colonized mice were enhanced by inclusion of *Clostridium*, but not germ-free isolates with CD11c⁺ antigen presenting cells⁴⁷. While this is consistent with *Clostridium* TCR-specific Treg cell function, it is possible that *Clostridium* isolates enhanced Treg cell function via TCR-independent effects in this assay. In addition to *Clostridium*, a recent report identified several *Bacteroides* species that selectively induced increased percentages of Nrp1^{lo} Treg cells in germ-free mice upon monocolonization⁶⁴, although this may not be true of all *Bacteroides* species²². While these mono-colonization data in germ-free mice do not provide direct proof that Treg cells recognize these bacteria in the context of a complex microbiota, they are consistent with the notion that certain bacterial species preferentially induce bacteria-specific pTreg cells.

Our studies of colon TCR specificities also support the existence of bacteria-specific colonic Treg cells. We screened 10 colonic Treg TCRs against a panel of murine bacteria *in vitro*. One Treg TCR, CT6, showed reactivity to a mouse unclassified *Clostridium* spp³⁶, which was unfortunately lost during cryopreservation. Subsequently, we have found that CT6 can recognize a human *C. symbiosum* isolate (unpublished). Another Treg TCR, CT7, was found to react to *Parabacteroides distasonis* and *B. uniformis*³⁶. However, recognition of *Clostridium* or *Bacteroides* spp. by these TCRs have not yet been confirmed *in vivo*.

On the whole, the bacterial species recognized by colonic Tconv and Treg cells *in vivo* in the setting of the complex microbiota in normal SPF mice remain unclear, in contrast with studies showing direct Th17 cell recognition of segmented filamentous bacteria (SFB) in the small intestine⁶⁵. The best characterized colonic TCR Tg model, CBir1, which recognizes a defined bacteria flagellin epitope, CBir1, is not reported to be activated during homeostasis⁶⁶. This is not an issue with the TCR Tg line, as analysis of polyclonal T cells using a tetramer with the CBir1 epitope shows that the majority of T cells are in the phenotypically naïve CD44^{lo} population⁶⁶. Interestingly, CBir1 flagellin falls within the *Clostridium* subphylum XIVa, which is associated with induction of Treg cells^{22, 47}. These *Clostridium* species are common constituents of mouse microbiota, and the epitope is clearly present in most microbiota⁶⁷ (and unpublished observations). Thus, the availability of the epitope in the lumen does not always equate with antigen presentation to T cells.

The relative lack of conclusive data on T cell specificity to gut bacteria is due to several issues. First, *in vivo* analysis of total Treg cell numbers or function is not proof of TCR recognition, as non-TCR-specific bacterial signals may be important. For example, SCFA have been shown to expand Treg cells in germ-free mice⁵³⁻⁵⁵. Similarly, purified PSA from *B. fragilis* has been shown to induce IL-10 on polyclonal Treg cells⁶⁸. Retinoic acid⁴⁸⁻⁵⁰ and

Ahr ligands⁶⁹ might be expected to act similarly in an antigen-independent fashion. Second, the study of individual bacteria introduced into germ-free mice may result in their abnormal exposure to the immune system due to the availability of niches which are normally filled by other bacterial species. T cell responses in mono-colonized mice would then need to be verified in more complex microbiota, requiring antigen-specific tools like transgenic mice or MHC tetramers. Third, culturing gut bacteria can be quite difficult. Most species require anaerobic techniques, and many remain unculturable. Fourth, the current protocols for *in vitro* culture may result in changes in bacterial antigen expression. Use of isolates with the same or similar taxonomy by 16S rDNA sequences may still miss isolate-specific changes in T cell epitopes. Finally, even if TCR recognition of *in vitro* grown bacteria is achieved, it remains possible that a different species actually provides the antigen to T cells *in vivo*. In our limited experience, we have observed overlap of TCR reactivity to bacteria of similar taxonomy. For example, CT7 TCR recognizes epitopes from *B. uniformis* and *P. distasonis*, whereas DP1 TCR reacts with *B. thetaiotaomicron*, *B. acidifaciens*, *B. vulgatus*, *B. sp. TP5*, and *P. goldsteini*³⁶. Thus, there are a number of hurdles in identifying the species responsible for induction of bacteria-specific Treg cells *in vivo*.

Dysregulation of pTreg Cell Differentiation in the Colon

The current data suggest that bacteria-reactive Treg cells are often generated from naïve T cells. Gut perturbations may therefore affect T cell differentiation. Models of intestinal inflammation such as IL-10-deficiency are associated with increases in Th1 and Th17 cell frequencies and a decrease in Treg cell frequencies^{70, 71}. On a monoclonal level, CBir1 TCR Tg cells, specific to commensal-derived flagellin, largely remain naïve in healthy hosts but have been shown to develop into stable Th1 effector cells after *Toxoplasma* infection or into Th17 cells after DSS-mediated mucosal injury and colitis⁶⁶. Adoption of effector, rather than regulatory, T cell fate may be particularly relevant to the induction of immune responses against commensal antigens, which has been implicated in the pathogenesis of IBD^{8, 72, 73}. Consistent with this hypothesis, CT2/CT6 TCR sequences typically found in the Treg cell subset in normal mouse colons can be found in colonic effector Foxp3⁻ T cell subsets in genetic backgrounds such as *IL-10*^{-/-} and dnTGFβRII mice that lead to spontaneous colitis³⁶. Treatment of mice with IL-10R antibodies and DSS to induce a transient colitis resulted in differentiation of a portion of the transferred naïve CT2/CT6 cells into RORγt⁺ Th17 T cells (unpublished, Chai, J.N. & Hsieh, C.S.). In addition, transfer of naïve CT2/CT6 T cells into a *Rag1*^{-/-} lymphopenic mice resulted in Tbet⁺ Th1 differentiation³³.

The specific signals that disrupt the normal process of pTreg cell development to gut bacteria have not been extensively studied. In a polyclonal setting, expansion of already existing effector cells is likely during inflammation. The inflammatory milieu with altered cytokine balance can also promote differentiation of naïve T-cells into effector phenotypes. Finally, intestinal changes that lead to bacterial antigens being acquired and presented by different APC subsets could affect pTreg vs effector T cell differentiation⁷⁴. Further work in this area could have therapeutic implications for intestinal inflammatory diseases.

Treg Cells Co-expressing Canonical Effector T Cell Transcriptions Factors

While certain environments described above skew T cell development away from Treg towards effector subsets, normal Foxp3⁺ Treg cells can upregulate effector transcription factors such as Tbet, GATA3, and ROR γ t to provide additional functional capacity⁴⁵. However, Treg expression of GATA3, the canonical transcription factor for Th2 cells, and Tbet for Th1 cells, appears to be independent of intestinal bacteria at homeostasis. GATA3 is expressed in about 15-20% of Treg cells in the colon and small intestine⁷⁵. GATA3⁺ Treg cells are equally abundant in germ-free versus SPF mice⁷⁶ and express Helios, suggestive of tTreg lineage^{76, 77}. Tbet⁺ Treg cells are even less frequent at around 5% in the mesenteric and peripheral lymph nodes and small intestine lamina propria⁷⁸. Thus, Treg cells co-expressing GATA3 or Tbet do not appear to play an integral part in the response to commensal bacteria during homeostasis.

By contrast, the colon is normally highly enriched in Treg cells that express the canonical Th17 transcription factor ROR γ t (30-80%), which is expressed in only 10-20% of Treg cells in the small intestine^{76, 77, 79, 80}. These cells are likely to be bacteria-I nduced pTreg cells, as they are: Helios^{lo} and Nrp-1^{lo}, dramatically decreased in germ free and antibiotic-treated mice, and can be induced in germ-free mice to SPF percentages by several commensal species belonging to different phyla^{76, 77}. ROR γ t⁺ Treg cells express high levels of IL-10 and CTLA-4⁷⁶, consistent with activated Treg cells with increased immunoregulatory capability⁸⁰.

The process by which ROR γ t⁺ Treg cells are generated appears to be dependent on certain bacteria. A large study of single bacterial isolates from the human gastrointestinal tract revealed that only certain species induced ROR γ t expression in Treg cells⁷⁷. Consistent with this observation, TCR repertoire analyses by our group using a fixed TCR β model suggest that Foxp3⁺ ROR γ t⁺ Treg cells in the colon use TCRs largely distinct from ROR γ t⁻ Treg cells and Th17 cells, although some overlap with Th17 cells was observed⁷⁹. However, another report suggested that TCR usage could be shared between ROR γ t⁺ Treg and other subsets⁸⁰. The different results obtained between these two reports may be due to fixed TCR β -chain versus paired-chain TCR repertoire analysis in a fully polyclonal population, as well as sequencing of colon versus splenic and lymphoid organ T cells. Nonetheless, this latter analysis is consistent with the observation that non-bacterial ovalbumin in an oral tolerance model induced OT-II cells to become ROR γ t⁺ Treg cells in the small intestine⁷⁶, suggesting that the developmental signals do not need to be directly linked to the T cell epitope. While additional studies are required to resolves these issues, the selectivity of certain gut bacteria to induce the ROR γ t⁺ Treg cell population⁷⁷ favors, but does not prove, the hypothesis that generation of these cells occurs in an antigen-specific context.

The microbiota-derived signals that drive generation of ROR γ t⁺ Treg cells are not well established. It has been suggested that T cells first upregulate Foxp3 and then subsequently ROR γ t in both polyclonal and monoclonal T cell populations^{77, 79}. The development of ROR γ t after pTreg cell generation suggests that this may occur upon interaction with microbial-induced factors in the colon. Such factors may include IL-6 and IL-23, although the studies were not entirely consistent^{76, 77}. The microbial-derived signals that generate

ROR γ ^t Treg cells *in vivo* therefore remain to be established. In addition, the temporal sequence of Foxp3 and then ROR γ ^t induction may only apply to a subset of cells. For example, it may be speculated that cells expressing TCRs that overlap with Th17 cells⁷⁹ could arise from upregulating ROR γ ^t first and then Foxp3.

Although ROR γ ^t is the canonical Th17 transcription factor, it is not clear that ROR γ ^t Treg cells primarily exist to inhibit Th17-mediated inflammation. For example, the frequency of ROR γ ^t-expressing Treg cells^{76, 77, 79, 80} in a tissue is inverse that of Th17 cells, which are found predominantly in the small intestine and not the colon⁸¹. In addition, Treg cell-specific knockout of ROR γ ^t has been reported to result in Th2-mediated or Th1/Th17-mediated colitis^{76, 77}, and not purely Th17-mediated disease. Future studies are therefore required to determine the specific mechanisms by which ROR γ ^t expression facilitates Treg cell suppression of specific subsets of T effector cells in the colon.

Function of Colonic Treg Cells

Intestinal Treg cells likely employ a number of mechanisms for regulating effector T cell responses to gut bacteria, including cell surface proteins and cytokines (reviewed in ¹⁷). One likely mechanism is the inhibition of antigen presenting cell (APC) function by CTLA-4, a transmembrane protein constitutively expressed in Treg cells⁸². CTLA-4 binding to CD80/86 can prevent CD28 on effector T cells from engaging and costimulating TCR activation⁸³. Treg cell CTLA-4 may also directly remove CD80/CD86 from APC membranes⁸⁴. Treg cell-mediated suppression via CTLA-4 appears to be important clinically, as an important side effect of CTLA-4 antibody therapy for human malignancy is the development of colitis⁸⁵.

Treg cells also secrete pro-tolerogenic cytokines such as TGF β ⁸⁶. Deletion of TGF β 1 from T cells results in spontaneous autoimmunity, albeit delayed in comparison with a germ-line knockout⁸⁷. While one might have predicted that Treg cell-derived TGF β 1 was primarily affected by CD4^{Cre}-mediated deletion, Foxp3^{Cre}-mediated deletion of TGF β 1 did not result in development of spontaneous autoimmunity or colitis⁸⁸. However, an effect of TGF β 1 was observed in the T cell transfer model of colitis into lymphopenic hosts⁸⁷, suggesting that certain experimental conditions could reveal a role for Treg cell-derived TGF β . Thus, these data demonstrate that while TGF β production by Treg cells may play a role, it is not essential for preserving normal gut homeostasis.

IL-10 is another important immuno-regulatory cytokine in the gut. Genetic polymorphisms of IL-10 and IL-10R in humans are associated with development of IBD^{89, 90}, and IL-10 deficient mice develop spontaneous colitis⁹¹. T cells are an important source of IL-10⁹², with important contributions from both Treg and non-Treg cells⁹³. Notably, of the CD4⁺ T cell population, colonic Treg cells exhibit the greatest production of IL-10 based on the use of reporters⁹⁴, and the expression of IL-10 is associated with co-expression of ROR γ ^t⁷⁶. Induction of IL-10 in gut Treg cells has been reported to occur with microbiota-derived factors such as *B. fragilis*-derived PSA via a T cell intrinsic TLR2-dependent mechanism⁶⁸. In summary, there is considerable evidence that gut bacteria induce IL-10 expressing pTreg

cells in the colon, and that Treg cell production of IL-10 is necessary to preserve gut homeostasis.

However, none of the Treg cell-derived factors such as CTLA-4 or regulatory cytokines like IL-10 appear to be directed at effector T cells in an antigen-specific manner. Rather, Treg cell inhibition of antigen-specific effector cells could occur via effects on APCs. A recent study using multiplex quantitative imaging showed that Treg cells cluster with CD11c⁺MHCII^{hi} APCs in lymphoid tissue and that CTLA-4 expression is increased in clustered versus non-clustered Treg cells⁹⁵. Expression of the TCR was required on Treg cells for Treg cell/APC clustering. Thus, these data provide evidence that TCR activation of gut bacteria-specific pTreg cells generate a local microenvironment that is anti-inflammatory via CTLA-4 and IL-10 interactions with dendritic cells, thereby inhibiting effector cells specific to bacterial-antigens that may also be presented on that APC.

Antigen-Specificity of IgA

The specificity of gut IgA responses may provide important clues for understanding T cell responses in the colon. IgA is known to be induced against toxins and pathogenic bacteria⁹⁶, but it has become clear that IgA is also induced by normal intestinal bacteria during homeostasis⁹⁷. Unexpectedly, this recognition may actually facilitate the uptake and presentation of bacteria or other antigens within the intestinal lumen to the adaptive immune system⁹⁷. Thus, IgA responses may reflect adaptive immune responses to intestinal bacterial antigens.

One important issue, though, is that IgA specificity cannot be directly equated with T cell antigen-recognition, as both T-dependent (TD) and T-independent (TI) IgA responses occur^{98, 99}. Some studies have suggested that the majority of IgA in the gut is TD, as the level of free IgA in T cell-deficient mice is 1/5th that of mice with T cells¹⁰⁰. In addition, sequencing studies showed that a large portion of gut IgA has somatic hypermutation^{97, 101}, supporting a substantial role for T follicular helper (Tfh) cells in gut IgA induction. By contrast, studies assessing IgA reactivity to bacteria using flow cytometry of T cell-deficient mice have varied in their interpretation as to whether TI IgA represents a major¹⁰² versus minor¹⁰³ part of the B cell response to commensal bacteria. However, the flow cytometry assay does not assess reactivity to secreted bacterial antigens or outer-membrane vesicles (OMVs), which could be important targets of TD IgA. Thus, the relative contribution of TD versus TI IgA in response to commensal bacteria during homeostasis is incompletely understood.

TD IgA to surface bacterial antigens seen by bacterial flow cytometry may occur primarily to immunostimulatory bacteria strong enough to induce Tfh responses⁹⁷. For example, IgA responses to bacteria obtained from co-housing with inflammasome-deficient (*Asc^{-/-}*) mice occurred only in T cell-sufficient mice¹⁰³ and were specific to *Prevotellaceae*, *Helicobacter* sp. *flexspira*, and SFB—all potential drivers of intestinal inflammation¹⁰⁴⁻¹⁰⁶. In other studies, two groups observed that SFB¹⁰⁷ and *Mucispirillum*¹⁰⁸, both bacteria that tightly adhere to the epithelium, induce TD IgA^{102, 103}. In studies of fecal bacteria from IBD patients, species that were preferentially IgA⁺, and not IgA⁻, facilitated DSS-mediated

colitis in mice¹⁰³. Similarly, a study of human undernourished children with enteropathy revealed IgA-responses to *Enterobacteriaceae*, whose pathogenicity was confirmed in mice¹⁰⁹. Finally, pathogenic secreted bacterial factors like cholera toxin specifically require Th17 cells for IgA induction¹¹⁰. Thus, bacteria with increased immunostimulatory potential such as pathobionts may be speculated to induce TD IgA responses.

In addition to these analyses, TCR Tg studies have supported a role for anti-bacterial TD IgA. CBir1 Tg Treg cells induce flagellin-specific IgA molecules when transferred into T-cell deficient mice¹¹¹. Similarly, we have observed that DP1 transgenic T cells, which recognize *Bacteroidaceae* species *in vitro*³⁶, can also induce IgA responses against those species when adoptively transferred into T cell-deficient mice (unpublished). One caveat of these studies is that they are done in lymphopenic mice, which may show altered T cell development as well as intestinal permeability to bacteria (Fig. 2). Nonetheless, these data directly demonstrate that antigen-specific T cell responses can induce bacteria-specific IgA. In summary, there has been significant progress in understanding the specificity of intestinal IgA. However, much remains to be learned about the characteristics of the bacteria that result in T and B cell responses during homeostasis and the T helper subset responses they elicit.

Role for Treg cells in Induction or Suppression of IgA

With the exception of SFB, there is a relative dearth of studies that integrate both T and B cell reactivity to intestinal bacteria at homeostasis. For example, *Clostridium* spp. induce Treg cell responses²², but the IgA responses are not characterized in normal hosts. Several *Lactobacillus* species increase the percentage of Treg cells in the colon and spleen^{104, 112}, but *Lactobacillus*-specific IgA appears to be TI^{102, 103}. In contrast, *Mucispirillum* species induce TD IgA^{102, 103}, but the T cell response to *Mucispirillum* during homeostasis has not been clearly established. One example in which data are available for both T and B cell responses is *Bacteroides thetaiotamicron*, which can induce IgA¹¹³ and expansion of colonic ROR γ t⁺ pTreg cells⁷⁷ when monocolonized in germ-free hosts. By combining the results of these two different studies, it could be hypothesized that Treg cell responses drive IgA induction during homeostasis.

A role for Treg cells in IgA induction has been proposed. In a T cell transfer model into lymphopenic mice, co-transfer of Foxp3⁺ Treg cells is necessary to limit colitis, maintain intestinal microbial diversity, and induce IgA¹¹⁴. This process appeared to result from loss of Foxp3 in a portion of the transferred Treg cells, which then upregulate Tfh markers such as Bcl6, IL-21, CD40L, and CXCR5¹¹⁵. Another group used the CBir1 TCR Tg line to show that transfer of Treg cells into TCR-deficient mice preferentially induced CBir1-specific IgA¹¹¹. In addition, depletion of Treg cells using anti-CD25 led to a marked drop in anti-CBir1 IgA, suggesting that Treg cells may directly induce IgA via TGF β . However, the use of lymphopenic models may result in loss of Treg cell stability¹¹⁶, leading to the trans-differentiation of Treg into Foxp3⁻ Tfh cells, which may not occur in normal hosts. In addition, the use of anti-CD25 antibodies may kill activated T cells or lead to gut inflammation, which may alter the range of antigens presented to the immune system⁶⁶. Thus, while there is intriguing data suggesting that gut Treg cells induce IgA, either directly

or serving as precursors for Foxp3⁻ Tfh cells, this Treg cell function during normal homeostasis requires further investigation.

Moreover, it has been argued that Treg cells actually inhibit B cell germinal center responses via the T follicular regulatory (Tfr) cell subset. In the spleen, Tfr cells have been shown to develop from Foxp3⁺ tTreg cells, maintain expression of Foxp3, and upregulate expression of Tfh markers such as PD-1, CXCR5, and Bcl6¹¹⁷. However, Tfr cells do not express CD40L or the Tfh cytokines IL-21 and IL-4, and function to limit the number of Tfh cells in germinal centers as well as inhibit the non-antigen-specific selection of B cells to prevent autoantibody formation¹¹⁷⁻¹¹⁹. Consistent with these observations in non-mucosal sites, Peyer's patch (PP) Foxp3⁺ germinal center Tfr cells were necessary to produce more specific, affinity-matured IgA¹¹⁴. Defective Tfr function resulted in a larger percentage of fecal bacteria that stained IgA⁺, suggesting that Tfr responses are more important in the gut for limiting IgA induction, rather than directly inducing IgA responses themselves.

Interestingly, CNS1^{-/-} Foxp3 mice, which show decreased induction of pTreg cells, develop plasmacytic enteritis characterized by increased serum antibodies against small intestine, large intestine, and chow antigens³⁴, which could hint at the necessity of antigen-specific Tfr cells to maintain intestinal homeostasis. However, it can be difficult to dissect in this model whether this is due to Treg cells controlling effector T cell activation versus Tfh cells in the germinal center reaction. Thus, future studies are required to determine whether gut Treg cells promote and/or inhibit IgA responses.

In brief, the role of Treg cells in TD IgA remains unclear. A straightforward hypothesis is that bacteria induce Treg cells, which, via expression of TGFβ, facilitate IgA class switch. However, it may be possible that Treg cells do not induce TD IgA. In this case, it may be possible that bacteria could induce a small fraction of effector T cells that stimulate IgA production, which may be subsequently regulated by Treg cell-derived Tfr cells. Thus, new studies are required to understand the concomitant T and B cell response to intestinal bacteria at homeostasis.

Conclusions

In summary, there has been considerable progress recently in our understanding of adaptive immune responses to colonic bacteria during homeostasis. First, it is clear that intestinal bacteria play a crucial role in educating the colonic immune system, with distinct effects on the TCR repertoire. TD IgA responses are also induced, although it has not been established whether this occurs in the colon or elsewhere. Second, the T cell response to colonic bacteria can result in naïve T cell differentiation into pTreg cells. Commensal bacteria appear to facilitate this process via provision of antigens and generation of metabolites such as SCFA or inducing TGFβ production by epithelial cells. Third, pTreg cell responses can be the dominant outcome of naïve T cell differentiation *in vivo*, making it a bone fide peripheral T helper subset. Finally, bacteria-induced pTreg cells are necessary *in vivo* to prevent colitis.

However, these data also raise important issues. For example, what are the bacterial specificities of colonic Treg cells during homeostasis in the context of a normal microbiota

(Fig. 1)? If IgA specificity is a guide, we might predict that there will be relatively few bacterial species that induce pTreg cell responses in a normal host. This would be consistent with the observation that CBir1 TCR Tg cells do not see their antigen under homeostatic conditions⁶⁶, even though it is clear that the antigen is relatively abundant in the lumen. We speculate that this means that the mucous/mucosa layer functions as a “wall” to prevent many/most bacterial antigens access to immune cells (Fig. 2). This contrasts with the small intestine, where oral administration of ovalbumin results in antigen presentation to T cells and Treg cell induction^{120, 121}. This is further supported by the recent observation that the majority of small intestinal pTreg cells are induced by dietary antigens¹²². Yet, it is evident that commensal bacteria have a tremendous impact on the colon TCR repertoire, suggesting that those bacteria that do get through are immunodominant over self-antigen presentation. One possible explanation is that OMVs¹²³ or soluble antigens¹²⁴ may have easier access to APCs than bacterial-associated antigens such as flagellin. Nonetheless, we predict that only a small fraction of intestinal bacterial antigens is presented to the adaptive immune system in the colon.

Another issue is the use of lymphopenic mice for the study of intestinal tolerance (Fig. 2). While it has been the classic model by which to study the role of Treg cells in the colon¹²⁵, our study suggests that pTreg cell differentiation is markedly perturbed in this environment³³. This may be due to abnormal barrier function, which results in bacterial translocation into the lamina propria, mesenteric lymph nodes, and spleen¹¹. This may facilitate increased proliferation¹²⁶ and induce an unbalanced cytokine environment that favors effector T cell differentiation of bacteria-specific T cells. Nonetheless, it may be possible in the future to establish conditions that “normalize” the lymphopenic model by adding in sub-therapeutic amounts of polyclonal Treg cells³⁹ or other manipulations such as anti-cytokine antibody, thereby permitting the study of commensal-specific pTreg cell differentiation and function in a setting where the antigen-specificities can be experimentally controlled.

While most of this review has focused on Treg cells, the role of effector T cells during homeostasis is not well characterized. As noted above, the observed bacterial translocation from the intestinal lumen to secondary immune organs in lymphopenic mice suggests that T cells are required for full barrier function. However, other than Th17 cells reactive to SFB, identification of effector cells reactive to colonic bacteria during homeostasis has not been successful³⁶. One possibility is that Treg cells, perhaps via ROR γ t, are capable of generating the signals required for epithelial homeostasis. Another is that commensal bacteria may induce, along with pTreg cells, some effector T cells that acts to improve barrier function and limit bacterial invasion. The small fraction of bacteria-specific effector T cells would be constrained by Treg cells. Like self-reactive effector T cells, their presence may only be recognized when Treg cells are depleted¹²⁷. A combined effector:Treg cell response to commensal antigens would be analogous to concomitant immunity such as that observed with Treg cells in *Leishmania* infection¹²⁸. Future studies will be required to determine the role of effector T cells for intestinal homeostasis to commensal bacteria.

Finally, the type and extent of antigen-specific T cell responses involved in IgA induction is poorly characterized during homeostasis. It is unknown whether induction of IgA to

commensal bacteria even occurs in the colon, as it may occur in more proximal parts of the intestine which have a thinner mucous layer and more defined antigen uptake mechanisms. Another question is whether Treg, Th17, or another subset is involved in IgA induction to commensal bacteria. Thus, many questions remain regarding gut bacterial interactions with the adaptive immune system.

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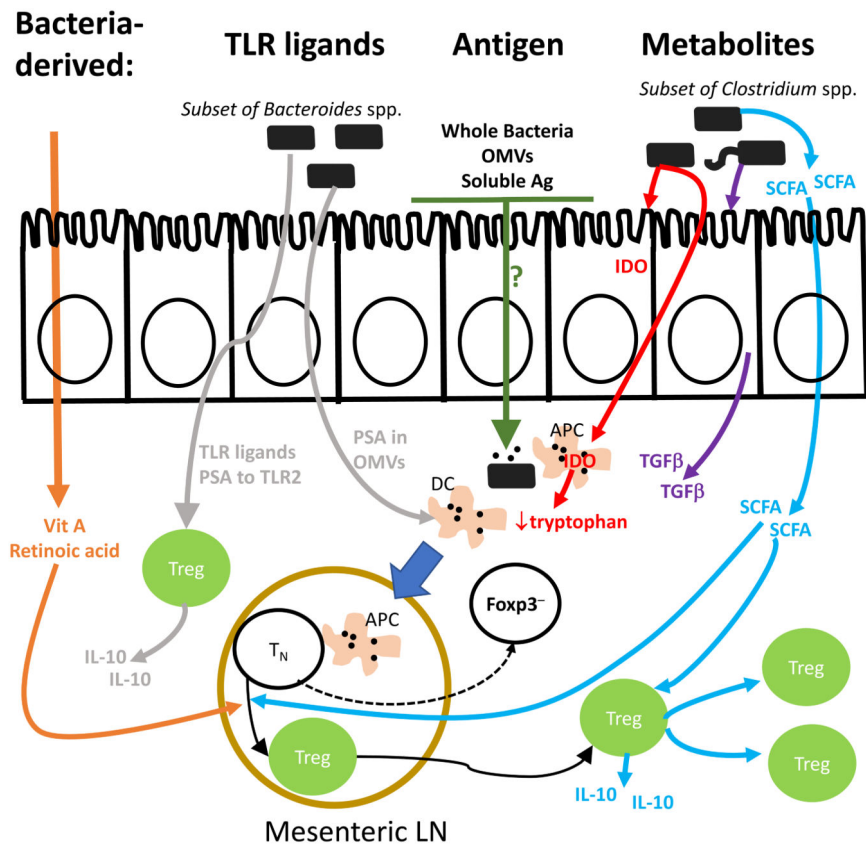


Figure 1. Antigen Specific and Non-Specific Mechanisms of Bacteria-Induced Treg Cell Generation and Function

Clostridium spp. establish an environment favorable for pTreg cell generation via the induction of TGFβ secretion by intestinal epithelial cells (IECs) (purple arrow), IDO in IECs and APCs (red arrows), and the production of SCFAs from dietary components (blue). *Bacteroides* spp. such as *B. fragilis* have been described to increase Treg cell function through PSA activation of TLR2 ligation on T cells and DCs (grey arrows). PSA, as well as SCFAs (blue arrow), induce IL-10 secretion from LP Treg cells. SCFAs also act directly on T cells to induce Treg differentiation/expansion. Dietary vitamin A from the intestinal lumen (orange arrows) can enhance pTreg cell differentiation through its metabolite retinoic acid, which can affect pTreg cell selection by inducing transcription factor binding to a Foxp3 enhancer element in CNS¹⁴⁸⁻⁵⁰, or via blockade of effector cytokine production by effector T cells¹²⁹. Bacterial antigens (green arrow), may gain access to immune cells by a variety of mechanisms that are currently unclear, including via goblet-associated passages, transcytosis through IECs, extension of APC processes into the intestinal lumen, or direct invasion of whole bacteria^{130, 131}. Naïve T cells have been reported to encounter commensal antigens in the mesenteric lymph node and undergo pTreg cell selection³³. The Peyer's patches and isolated lymphoid follicles may also be important sites of pTreg cell selection, but this requires further study.

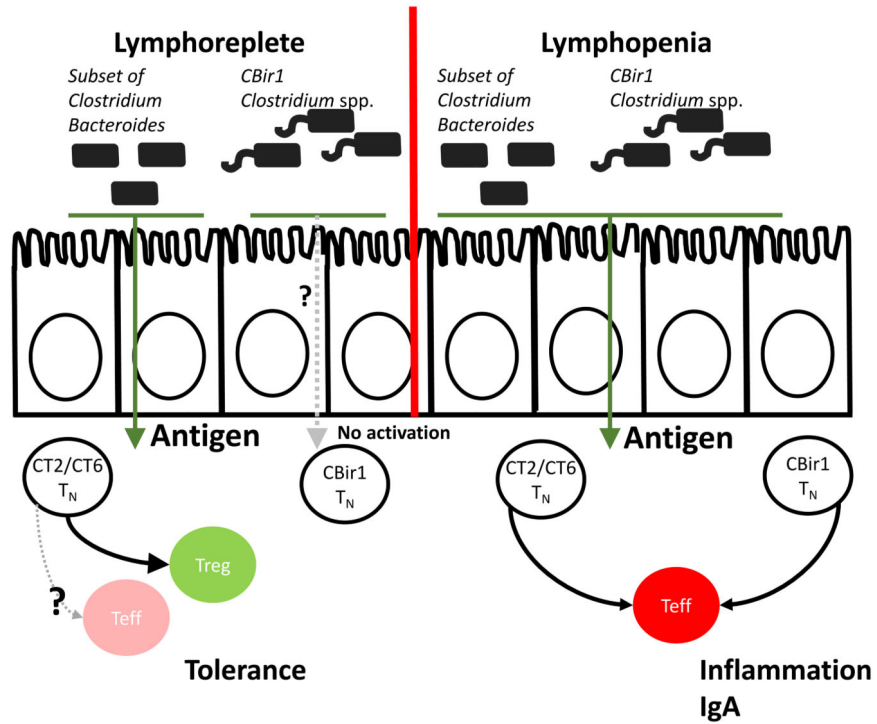


Figure 2. Alternative Regulation of Intestinal Bacterial Antigen Presentation and T Cell Fate in Lymphoreplete vs. Lymphopenic Mice

In lymphoreplete mice, commensal spp. induce naïve colonic TCR transgenic CT2/CT6 cells into Treg cells. Based on *in vitro* and *in vivo* studies, it is predicted that select *Bacteroides* and *Clostridium spp.* induce antigen-specific Treg cells in lymphoreplete mice. However, naïve CT2/CT6 T cells develop into effector T cells in *Rag1*^{-/-} lymphopenic mice. In contrast to the behavior of CT2/CT6, CBir1 antigen, a component of flagella present in many gut bacteria species including those in *Clostridium* subphylum XIVa, does not activate T cells or induce differentiation of Treg cells in lymphoreplete mice. However, these antigens are presented in lymphopenic mice leading to T cell activation and IgA production. Mucosal injury or infection will also lead to antigen presentation to CBir1 reactive T cells. In these environments, CBir1 reactive T cells become effector, and not Treg, cells.