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Review

Regulatory T Cells Reinforce Intestinal Homeostasis

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Regulatory T cells help maintain intestinal homeostasis by preventing inappropriate innate and adaptive immune responses. $CD4^+$ T cells that express Foxp3 and Tr1-like cells that produce IL-10 comprise the major regulatory populations in the intestine. $CD4^+Foxp3^+$ T cells play an important functional role in promoting tolerance of the flora and dietary proteins. Tr1-like cells can be generated in conditions that also promote effector T cell responses and may serve a similar function. In this review, we discuss the signals specific to the gastrointestinal tract that support both regulatory cell types and their distinct modes of action in the mesenteric lymph nodes and intestinal tissues. Dysregulation of intestinal immune homeostasis occurs in inflammatory bowel disease and can also be observed in graft-versus-host disease, tumor immunotherapy regimens, and acute HIV infection.

Introduction

The benefit derived from harboring symbiotic organisms is a force that has shaped evolution (Dale and Moran, 2006) and, in mammals, nowhere is this more apparent than in the gastrointestinal (GI) tract. The intestinal flora, which is largely composed of resident bacteria that most densely populate the colon, benefits the host by extracting dietary nutrients and preventing colonization by opportunistic pathogens (Duerkop et al., 2009, in this issue of *Immunity*). Tolerance of the endogenous flora can be advantageous, but must be achieved while minimizing the risk of systemic infection. The GI tract forms the largest mammalian epithelial surface, so this constitutes a substantial challenge. Pathogenic and commensal bacteria are diverse and derive from intermingled phylogenies, making it difficult, if not impossible, for the host to distinguish between them at a molecular level. Instead, intestinal physiology has evolved to sequester most of the flora in the lumen, in a layer of mucus and immunoglobulin A (IgA), and to reinforce the gut with multiple layers of defense, consisting of barrier, innate, and adaptive components that limit flora-driven inflammation.

Multiple Mechanisms Enforce Intestinal Homeostasis

The epithelial layer of the GI tract largely consists of intestinal epithelial cells (IECs) connected by tight junctions, as well as mucus-secreting goblet cells and antimicrobial-peptide-producing Paneth cells (Artis, 2008). Interspersed throughout the epithelium are gut-associated lymphoid tissues (GALT), including Peyer's patches in the small intestine and isolated lymphoid follicles in the colon, which contain IgA-secreting plasma cells. Together, these varied cell populations support a mucus layer, containing IgA and antimicrobial peptides, which dramatically reduces the bacterial load at the barrier between the epithelium and lumen.

Although relatively devoid of live bacteria, the intestinal epithelium may be constantly exposed to immunostimulatory molecules, such as lipopolysaccharide (LPS), that diffuse through the mucus layer. To prevent continual immune activation, IECs exclude Toll-like receptor 5 (TLR5), which senses bacterial flagellin, from the luminal interface (Gewirtz et al., 2001), and IECs, intestinal macrophages, and dendritic cells (DCs) in the intestinal draining lymph are hyporesponsive to LPS (Cerovic et al., 2009; Lotz et al., 2006). Innate immune recognition of invasive pathogens at the intestinal epithelial interface may instead rely on intracellular sensors, such as endosomal TLRs, cytoplasmic nucleic acid sensors, and Nod-like receptors. Upon detection of pathogens, inflammatory cytokines are secreted that recruit DCs, monocytes, and neutrophils to the intestine. In the absence of infection, intestinal DCs remain quiescent and promote tolerance by migrating to the GALT where they present luminal-derived antigens to lymphocytes (Coombes and Powrie, 2008).

T cells provide a third layer of intestinal defense that limits infections by pathogens that enter through the GI tract. Innate-like lymphocytes, principally intraepithelial $\gamma\delta$ T cells, provide signals that enhance barrier function (Chen et al., 2002). Conventional $CD4^+$ and $CD8^+$ T cells are also found in the intestine; however, these cells present the inherent risk of reacting toward dietary or flora antigens. Indeed, antigen-experienced T cells in the mesenteric lymph node (mLN) of healthy mice are capable of inducing intestinal inflammation upon adoptive transfer into immunodeficient recipients (Asseman et al., 2003).

Inappropriate innate and adaptive immune responses in the GI tract are normally restrained by regulatory lymphocytes (Maloy et al., 2003; Read et al., 2000). Although regulatory activity has been ascribed to several types of intestinal lymphocytes, compelling genetic and functional evidence suggests that $CD4^+Foxp3^+$ regulatory T (Treg) cells and IL-10-producing T cells carry out nonredundant functions. Notably, mice lacking Foxp3 develop fatal multiorgan inflammation that can be suppressed by adoptively transferred $Foxp3^+$ Treg cells (Fontenot et al., 2003). Mice engineered to lack the expression of specific regulatory cytokines in T cells, such as IL-10 or TGF-β, succumb to wasting disease and colitis when disease-triggering bacteria are present in the intestinal flora (Li et al., 2007; Roers et al., 2004; Rubtsov et al., 2008). In humans, intestinal inflammation often occurs in immune dysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome (Powell et al., 1982), which is caused by germline mutations in *FOXP3* (Ziegler,

2006). This review will focus on the flora- and innate immune-dependent signals that modulate intestinal Treg cell activity, and the actions of Treg cells that reinforce barrier and innate immune functions in the intestine.

Characteristics of Foxp3$^+$ Treg Cells that Develop in the Thymus

Most CD4$^+$Foxp3$^+$ Treg cells probably originate in the thymus, where their development requires signals that activate NF-κB via the PKCθ-Carma-1-IKK2 axis and limit activation of the PI3-kinase-Akt-mTOR pathway (Feuerer et al., 2009). The T cell receptor (TCR) is a dominant source of these signals. The expression of a TCR repertoire in Treg cells that is largely distinct from conventional T cells has been interpreted to indicate that thymic Treg cells bind self-peptides with moderate affinity (Feuerer et al., 2009; Josefowicz and Rudensky, 2009). After TCR stimulation, Treg cell precursors must receive costimulatory signals through CD28 and encounter the common-γ chain cytokines IL-2 or IL-15 in order to activate Stat5 and induce Foxp3 expression (Burchill et al., 2008; Lio and Hsieh, 2008). IL-2, as well as TGF-β, are also important in the periphery where they help maintain Foxp3$^+$ Treg cells.

The affinity of TCR binding to MHC molecules alone does not determine whether a thymocyte will express Foxp3. Interestingly, a study with transgenic mice expressing TCRs common to Treg cells demonstrates that a very small thymic niche exists for each clone with a certain specificity (Bautista et al., 2009). Limiting the thymic Treg cell niche by the availability of self-antigens would allow many clones with diverse specificities to develop. Both thymic DCs and epithelial cells are capable of presenting antigens that induce Foxp3 expression (Wirnsberger et al., 2009). Intriguingly, presentation of the full spectrum of CD4$^+$ T cell-selecting ligands requires nonhematopoietic thymic stromal cells to be able to undergo autophagy (Nedjic et al., 2008). Autophagy-deficient *Atg5*$^{-/-}$ thymi aberrantly select CD4$^+$ T cells, indicating that part of the normal T cell repertoire might recognize antigens that depend upon autophagy for their processing and presentation. When *Atg5*$^{-/-}$ thymi are transplanted into autophagy-sufficient athymic nude mice, recipient mice develop colitis despite the presence of normal Treg cell numbers in the thymus and intestine. This finding suggests the possibility that the intestine is enriched in autophagy-dependent antigens, which normally activate protective Treg cells. In recipients of *Atg5*$^{-/-}$ thymi, such Treg cells would be absent and autophagy-dependent antigens might instead activate colitogenic T cells that escape deletion in the thymus.

After exiting the thymus, some Treg cells migrate to the GI tract where they can recognize intestinal antigens and prevent inappropriate immune responses. Such a mechanism has been demonstrated via the T-cell-transfer model of colitis in which adoptively transferred naive CD4$^+$ T cells cause intestinal inflammation in SCID recipients (Powrie et al., 1993). The intestinal flora is required to drive inflammation, as indicated by the fact that colitis is attenuated in germ-free SCID mice receiving naive T cells (Aranda et al., 1997). Several factors may underlie this phenomenon, including the absence of flora-derived antigens and the impaired formation of the GALT in germ-free mice. In mice housed in specific-pathogen-free (SPF) conditions, co-transferred Treg cells prevent colitis (Read et al., 2000). By some reports, Treg cells isolated from germ-free mice are also able to prevent intestinal inflammation (Annacker et al., 2000; Singh et al., 2001), although another study has produced conflicting results (Strauch et al., 2005). These differences may reflect the adaptation of Treg cells to both self- and exogenous antigens presented in the intestine. For example, certain intestinal bacteria, such as *Helicobacter hepaticus*, promote enhanced suppressive capacity of Treg cells (Kullberg et al., 2002). Indeed, in a setting where germ-free Treg cells function, we have found that Treg cells isolated from SPF-housed mice are better suppressors than those from germ-free mice (Singh et al., 2001). Thus, additional flora-dependent shaping of the Treg cell pool can occur in the peripheral lymphoid organs that allows for efficient regulation of the GI tract.

The Intestinal Treg Cell Niche: Foxp3$^+$ Treg Cells

Generating additional Treg cells extrathymically is one way that regulation could be enhanced in the intestine. Naive CD4$^+$ T cells can differentiate into "induced" Foxp3$^+$ Treg (iTreg) cells when activated by transient TCR stimulation (Sauer et al., 2008) or TCR stimulation in the presence of TGF-β and IL-2 (Chen et al., 2003) and the absence of inflammatory cytokines that promote effector T cell differentiation (Maynard and Weaver, 2009, in this issue of *Immunity*). In vivo, transfusion of antigen under non-immunogenic conditions, homeostatic proliferation, or chronic inflammation can generate iTreg cells (Curotto de Lafaille and Lafaille, 2009). Although thymic Treg and iTreg cells may express different gene transcripts and epigenetic markers (Hill et al., 2007; Wei et al., 2009), they are currently difficult to distinguish on a single-cell basis. Therefore, it is only possible to approximate the contribution of iTreg cells to the total intestinal Treg cell pool. One approach involves adoptively transferring naive CD4$^+$CD45RB^{hi}Foxp3$^-$ T cells into *Rag2*$^{-/-}$ recipients and observing when and where cells begin to express Foxp3. Normally, naive T cell transfer into *Rag2*$^{-/-}$ recipients gives rise to very few Foxp3$^+$ Treg cells and favors the accumulation of colitogenic Th1 and Th17 effector cells (Leppkes et al., 2009; Powrie et al., 1994). However, when naive T cells are transferred into *Rag2*$^{-/-}$ recipient mice lacking IL-23 (IL-12p19), which do not develop intestinal inflammation, preferential induction of Foxp3 expression occurs among T cells that migrate to the colon lamina propria (LP) and mLN (Izcue et al., 2008). These iTreg cells function and contribute to the prevention of colitis, as indicated by the fact that intestinal inflammation occurs when Foxp3-deficient naive T cells, which cannot give rise to iTreg cells, are used as donors. Interestingly, naive T cells transferred into *Rag2*$^{-/-}$ recipients that are treated with IL-6R blocking antibody also generate a higher frequency of Foxp3$^+$ Treg cells in both the GI tract and spleen, demonstrating a general role for inflammatory Stat3-activating cytokines in limiting Foxp3 induction (Izcue et al., 2008). Lymphopenia-induced homeostatic proliferation is physiologically relevant, because it occurs in the neonatal setting in the absence of intestinal inflammation (Min et al., 2003) and could contribute to an early Treg cell population in the developing immune system.

To assess iTreg cell generation in an adult mouse, congenically marked CD4$^+$Foxp3$^-$ T cells can be transferred into lymphocyte-replete recipients. However, in this setting, few transferred CD4$^+$Foxp3$^-$ T cells become Foxp3$^+$ and are

estimated to comprise only ~4%–7% of the normal Treg cell pool (Lathrop et al., 2008). Importantly, Foxp3$^+$ iTreg cells become twice as frequent in the mLN than the spleen or peripheral lymph nodes, suggesting that iTreg cell generation occurs more frequently in the intestine. An alternative approach with Carma-1-deficient mice, which are devoid of thymic Treg cells (Barnes et al., 2009; Medoff et al., 2009; Molinero et al., 2009) but can generate iTreg cells (Barnes et al., 2009), has yielded similar results. In these mice, Treg cells in the spleen and peripheral lymph nodes are only ~3%–4% as frequent as Treg cells in wild-type mice, but this frequency increases to ~8% and ~40% in the mLN and colon LP, respectively. Because transferred T cells have to compete with pre-existing T cells for cytokines needed to induce Foxp3 (Lathrop et al., 2008) and Carma-1-deficient CD4$^+$ T cells have a higher TCR signaling threshold for Foxp3 induction (Barnes et al., 2009), these represent conservative estimates of the normal iTreg cell population. Additionally, mice housed in SPF conditions are not exposed to persistent infections or chronic inflammation, which could further promote iTreg cell generation (Curotto de Lafaille and Lafaille, 2009). However, it is reasonable to assume that the majority of Treg cells in the GI tract of healthy mice represent Foxp3$^+$ Treg cells exported from the thymus. Taken together, these studies indicate that the noninflamed GI tract is a permissive site for the accumulation of iTreg cells, which together with thymus-derived Treg cells collaborate to reinforce intestinal homeostasis.

In order to suppress immune responses toward exogenous dietary and flora antigens, the GI tract could be enriched for reactive thymic-derived Treg cells or iTreg cells could be generated from conventional CD4$^+$ T cells. Treg cells in the mLN express TCR sequences that are distinct from those expressed by Treg cells in the peripheral lymph nodes and show little overlap with the TCRs expressed by naive or memory T cells in the mLN (Lathrop et al., 2008). This observation suggests that intestinal antigens, either self-antigens expressed by cells in the GI tract or exogenous antigens derived from the lumen, do shape the intestinal Treg cell pool. In support of the latter possibility, several studies have demonstrated that orally fed antigen can expand antigen-specific regulatory T cell populations (Chen et al., 1994). Although oral tolerance might also involve the expansion of reactive thymus-derived Treg cells, we have demonstrated, along with others, that this phenomenon involves the induction of Foxp3$^+$ iTreg cells from the conventional T cell pool and predominantly occurs in the GALT (Coombes et al., 2007; Sun et al., 2007). Orally induced Treg cells are functional and act both locally in the gut and systemically. For example, orally induced ovalbumin-specific Foxp3$^+$ Treg cells can suppress lung inflammation in a model of asthma driven by Th2 effector cells (Curotto de Lafaille et al., 2008). Different Treg cell effector mechanisms are required to prevent inappropriate immune responses to dietary- versus flora-derived antigens, with the production of IL-10 being dispensable for oral tolerance but essential in the latter situation (Fowler and Powrie, 2002). In humans with inflammatory bowel disease (IBD), oral tolerance is impaired (Kraus et al., 2004), although further research is needed to determine whether this finding represents genetic defects that predispose individuals to developing IBD (Kraus et al., 2006) or is a secondary consequence of intestinal inflammation.

The Intestinal Treg Cell Niche: IL-10-Producing T Cells

Although the generally immunosuppressive cytokine IL-10 is an important effector molecule expressed by Foxp3$^+$ Treg cells, *Il10*$^{-/-}$ mice do not develop the lymphoproliferative autoimmune disease observed in Foxp3-deficient mice. Instead, they are highly susceptible to intestinal inflammation triggered by the presence of common intestinal bacteria, such as *H. hepaticus*, in the context of a "normal" SPF-flora (Kullberg et al., 1998). Other genera of bacteria, for example segmented filamentous bacteria, can act as triggering microbes in alternate colitis models (Stepankova et al., 2007), and the flora can also contain bacteria that offset the presence of disease-triggering microorganisms by promoting IL-10 expression (Mazmanian et al., 2008). The composition and density of the intestinal flora varies greatly throughout the GI tract, so it might be fitting that the need for IL-10 varies similarly. Thus, whereas Foxp3$^+$ Treg cells represent a constitutive regulatory presence, IL-10 acts as an inducible immunoregulatory factor that can be called into action when and where inflammatory conditions demand. IL-10 acts in part by activating Stat3. The finding that mice lacking Stat3 in the myeloid compartment develop colitis similar to *Il10*$^{-/-}$ mice suggests that myeloid cells are essential targets of IL-10 signaling in the intestine (Takeda et al., 1999). Exogenous IL-10 can limit the ER stress response in an IEC cell line (Shkoda et al., 2007), so IL-10 might also contribute to the maintenance of intestinal barrier function.

Mice that coexpress Foxp3 and IL-10 reporter constructs allow IL-10 expression in Foxp3$^+$ Treg cells and other T cell subsets to be monitored at the single-cell level (Kamanaka et al., 2006; Maynard et al., 2007). Consistent with a requirement for T cell-derived IL-10 in maintaining intestinal homeostasis (Roers et al., 2004), a substantial fraction (10%–30%) of tissue-resident intestinal CD4$^+$ T cells can produce IL-10 (Maynard et al., 2007). In the colonic LP, nearly all of the IL-10-producing T cells are Foxp3$^+$ Treg cells. These cells have a nonredundant function, as shown by the fact that *Helicobacter*-infected mice with a specific deletion of IL-10 in Foxp3$^+$ cells develop typhlitis (inflammation of the caecum) and mild colitis (Rubtsov et al., 2008). However, the colitis observed in these mice is less severe than in *Il10*$^{-/-}$ mice, suggesting that other sources of IL-10 are also functionally important. Among intraepithelial lymphocytes in the small intestine, most IL-10-producing CD4$^+$ T cells are Foxp3$^-$ and do not secrete effector T cell cytokines or IL-2 (Maynard et al., 2007), reminiscent of previously described IL-10-Treg or T regulatory type 1 (Tr1) cells (Vieira et al., 2004). In the small intestine LP, both Foxp3$^+$ Treg and Tr1-like cells produce IL-10. This compartmentalization of intestinal regulatory T cell subsets suggests that the GI tract contains several distinct immunological microenvironments that differentially promote IL-10 production (Figure 1).

The signals that turn on IL-10 expression in T cells are distinct from those that induce Foxp3 expression. IL-10 can be expressed by both Foxp3$^+$ Treg cells and Tr1-like cells (Asseman et al., 1999; Vieira et al., 2004). Effector T cells can also coexpress IL-10 along with IFN-γ, IL-4, or IL-17 in infectious contexts (O'Garra and Vieira, 2007), and this has been shown, in vitro, to require Erk1 and Erk2 activation (Saraiva et al., 2009). The best-characterized pathway for inducing IL-10 expression occurs in response to IL-27, a member of the IL-12 family of cytokines

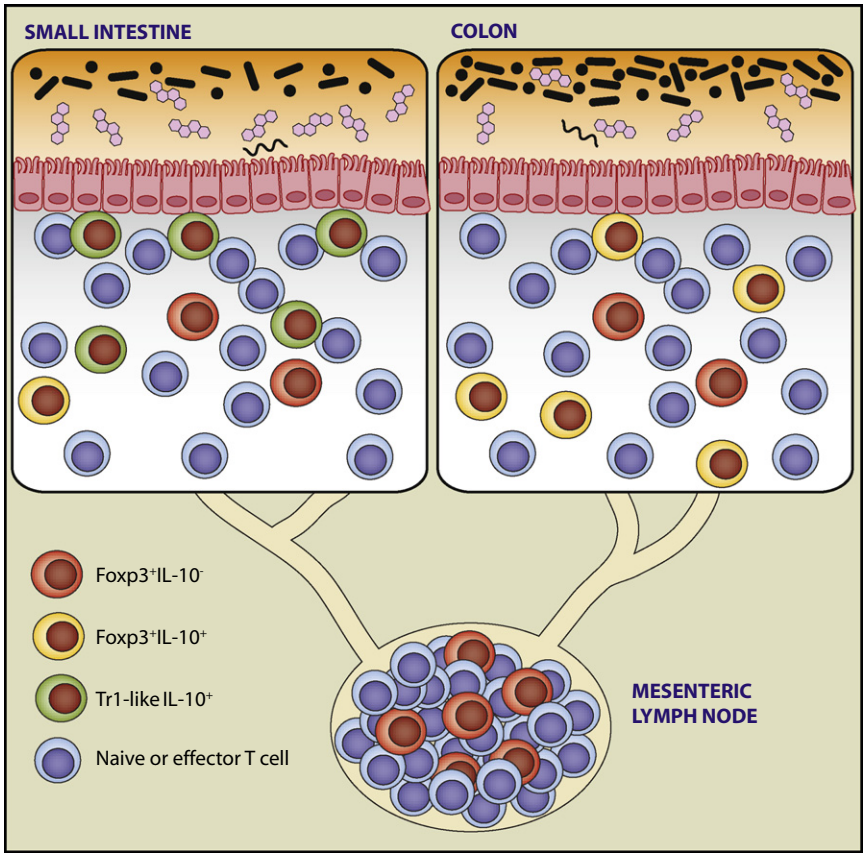

Figure 1. The Anatomy of Intestinal Regulatory T Cell Populations

$CD4^+$ T cell populations with regulatory functions in the intestine include $Foxp3^+$ Treg cells and $Foxp3^-$ Tr1-like cells. In the colon, which harbors a high bacterial load, most of the $Foxp3^+$ Treg cells in the lamina propria produce IL-10 and Tr1-like cells are less frequent. Fewer bacteria colonize the small intestine, where the bulk of dietary nutrients are absorbed. Here, a high frequency of Tr1-like cells patrol the intraepithelial layer, whereas both Tr1-like cells and $Foxp3^+$ Treg cells populate the lamina propria. In the draining mLN, fewer IL-10-producing cells are found than in the intestinal tissue and the major regulatory population is the $Foxp3^+$ Treg cell. Some intraepithelial lymphocytes are present in the colon, but the frequency of $Foxp3^+$ Treg and Tr1-like cells in this location has not been reported.

that has both effector and regulatory properties. Exposure to IL-27 can induce IL-10 expression in $CD4^+$ T cells by triggering a sequence of events that include upregulation of the transcription factor c-Maf and subsequent induction of IL-21, which acts as a growth factor for IL-10-producing T cells (Pot et al., 2009; Spolski et al., 2009). IL-27-deficient mice are not susceptible to flora-triggered colitis, like IL-10-deficient mice, in part because IL-27 also promotes effector T cell responses. However, *Il27r*$^{-/-}$ *Il10*$^{-/-}$ double-deficient mice still develop colitis, although with slower kinetics than *Il10*$^{-/-}$ mice (Villarino et al., 2008). These findings suggest that other pathways for both effector T cell responses and IL-10 induction operate in the intestine. For example, in the presence of TGF-β, IL-6 can drive IL-10 production independently of IL-21 signaling (Spolski et al., 2009). IL-6 is produced in large amounts during intestinal inflammation, but its contribution to the induction of IL-10 in the steady state is less obvious and merits investigation. Furthermore, although IL-27 and TGF-β can induce iTreg cells that express IL-10 in vitro (Stumhofer et al., 2007), the signals that regulate IL-10 expression in $Foxp3^+$ Treg cells in vivo remain elusive (Maynard et al., 2007, 2009).

Mouse models have identified several transcription factors as potential regulators of IL-10 expression, including two that have been implicated in IL-21 signaling, Blimp-1 and c-Maf. Mice lacking hematopoietic or T cell expression of Blimp-1, which is encoded by *Prdm1* and highly expressed by both activated T cells and $CD4^+CD25^+$ T cells, develop spontaneous colitis (Kallies et al., 2006; Martins et al., 2006). Colitis may occur because of excessive effector T cell responses, because Blimp-1 is needed to limit IL-2 expression and polarization toward a Th1 cell effector phenotype. Blimp-1 may also be required for regulatory T cell function. Whereas mRNA encoding Blimp-1 is not expressed in large amounts among resting $Foxp3^+$ Treg cells (Hill et al., 2007), the promoter of *Prdm1* does contain a Foxp3 binding site (Zheng et al., 2007) making it unclear whether activated T cells or perhaps activated $Foxp3^+$ Treg cells are the major source of Blimp-1 in the $CD4^+CD25^+$ T cell pool. Although Treg cells from Blimp-1-deficient mice can protect in the T-cell-transfer model of colitis (Kallies et al., 2006), both the $CD25^+$ and $CD25^-$ $CD4^+$ T cell populations show a reduced frequency of $IL\text{-}10^+$ cells compared to wild-type mice (Martins et al., 2006); however, this reduction could be a secondary effect of ongoing inflammation. Another candidate transcription factor for promoting IL-10 expression, c-Maf, can be induced by IL-27 and IL-21 (Pot et al., 2009) or signaling through the inducible costimulator (ICOS) molecule (Bauquet et al., 2009). ICOS-deficient $CD4^+$ T cells show defects in IL-10 expression (Pot et al., 2009), and many IL-10-producing $CD4^+$ T cells, including $Foxp3^+$ Treg cells, coexpress ICOS (Huehn et al., 2004; Ito et al., 2008). Intriguingly, preliminary reports suggest that ICOS-deficient Treg cells fail to control T-cell-transfer-induced colitis (Zheng et al., 2009). Transcription factors activated by TGF-β signaling are also likely to be important in generating intestinal IL-10-producing T cells (Kitani et al., 2003; Maynard et al., 2007).

The Intestinal Treg Cell Niche: TGF-β

TGF-β is a pleiotrophic cytokine, important for the maintenance and effector function of both $Foxp3^+$ Treg and Tr1-like cells in the intestine (Chen et al., 1994; Li et al., 2006; Marie et al., 2005, 2006; Maynard et al., 2007; Powrie et al., 1996). Unlike the susceptibility to colitis observed in *Il10*$^{-/-}$ mice, *Tgfb1*$^{-/-}$ mice succumb to a T cell-dependent lymphoproliferative autoimmune disease by several weeks of age (Diebold et al., 1995). This difference has made studying the role of TGF-β in the GI tract

especially challenging, necessitating the use of both adoptive transfer and conditional gene-targeting approaches. In the absence of signaling through TGF-βRII, T cells become partially activated and prone to differentiate into autoreactive effector T cells (Gorelik and Flavell, 2000; Li et al., 2006; Marie et al., 2006). With the T-cell-transfer model of colitis, we have shown that transferred naive T cells expressing a dominant-negative TGF-βRII molecule cause intestinal inflammation that cannot be suppressed by Treg cells (Fahlén et al., 2005). Furthermore, $CD4^+$ T cells from mice that overexpress Smad7, an endogenous inhibitor of TGF-β signaling through Smad2 and Smad3, are similarly resistant to suppression (Fantini et al., 2009). Therefore, TGF-β signaling into T cells is essential to limit colitogenic effector T cell responses.

Regulatory T cells are one source of intestinal TGF-β. We found, along with others, that Treg cells from *DO11.10.Tgfb1*$^{-/-}$ mice (Fahlén et al., 2005) or *Tgfb1*$^{-/-}$ neonates (Kullberg et al., 2005) function in the T-cell-transfer model of colitis. However, another study using the same model reported that TGF-β1-deficient Treg cells do not limit weight loss or intestinal inflammation (Li et al., 2007). Differences in the intestinal flora, genetic background, Treg cell TCR repertoire, or ongoing inflammation in the donor mice might underlie these conflicting results. Interestingly, TGF-β1-deficient Treg cells cotransferred with TGF-β1-deficient naive T cells cause worse disease than TGF-β1-deficient Treg cells cotransferred with wild-type naive T cells (Li et al., 2007). Furthermore, Tr1-like cells can suppress T cell proliferation in vitro by producing TGF-β and IL-10 (Maynard et al., 2007). These findings are consistent with the existence of Treg cell-dependent and -independent sources of TGF-β in the $CD4^+$ T cell pool that contribute to intestinal homeostasis.

Although the intestine harbors a high concentration of TGF-β, the majority is thought to exist in an inactive form. Integrins have a key role in activating TGF-β, including $\alpha_v\beta_6$ integrin expressed by IECs and $\alpha_v\beta_8$ integrin expressed by DCs (Lacy-Hulbert et al., 2007; Munger et al., 1999; Travis et al., 2007). *Helicobacter*-infected mice lacking β_8 integrin expression in $CD11c^+$ cells develop spontaneous colitis, demonstrating an essential role for DCs expressing TGF-β-activating integrins in the intestine (Travis et al., 2007). In addition to being a source of TGF-β, T cells might also express molecules important for TGF-β activation, thereby promoting both Treg cell induction and maintenance (Andersson et al., 2008). Thrombospondin is one such molecule (Crawford et al., 1998), and we note that a population of latency-associated peptide-expressing $CD4^+CD25^-CD45RB^{lo}$ T cells are enriched for thrombospondin expression and can prevent intestinal inflammation in the T-cell-transfer colitis model (Oida et al., 2003). Another important molecule is the proprotein convertase Furin, which can activate TGF-β along with a number of other substrates. Mice engineered to lack Furin expression in T cells have normal to elevated numbers of $Foxp3^+$ Treg cells, but show impaired TGF-β-dependent processes in the GI tract and develop colitis by 6 months of age (Pesu et al., 2008). In summary, $CD4^+$ T cells, DCs, and IECs all collaborate to activate TGF-β and maintain T cell tolerance in the intestine.

The Intestinal Treg Cell Niche: Conditioning Factors

TGF-β is one of many factors that control the size and composition of the intestinal Treg cell niche. The frequency of peripheral Treg cells is reduced in the absence of TGF-βRII signaling (Li et al., 2006; Marie et al., 2006) but remains normal in mice with a T cell-specific deletion of TGF-β1 (Li et al., 2007). Although it is possible that other TGF-β isoforms compensate for the absence of TGF-β1, non-T cell sources of TGF-β likely contribute to the maintenance of the intestinal Treg cell compartment. Apoptotic cells represent an important potential source of bioactive TGF-β (Chen et al., 2001), capable of enhancing TGF-β production from immature DCs and macrophages, and, in turn, generating iTreg cells (Perruche et al., 2008). Because epithelial cells have a high rate of turnover, apoptosis of IECs may be an important process in establishing the TGF-β-rich intestinal environment that supports a high frequency of Treg cells. In support of this idea, mice lacking expression of α_v integrins in hematopoietic cells show defects in the phagocytosis of apoptotic cells and develop spontaneous colitis (Lacy-Hulbert et al., 2007). Like β_8 integrin-deficient mice (Travis et al., 2007), these mice have a specific reduction of $Foxp3^+$ Treg cells in the colon LP, but not the spleen or lymph nodes (Lacy-Hulbert et al., 2007). $\alpha_v\beta_8$ integrin can activate TGF-β, so it is not yet clear whether the intestinal inflammation and Treg cell deficiency in α_v integrin-deficient mice reflects an absence of bioactive TGF-β or impaired apoptotic cell uptake. It is also possible that these processes are intertwined (Perruche et al., 2008). Apart from sustaining Treg cells, apoptotic cell-derived TGF-β can instead support effector T cell responses during inflammatory conditions. For example, during *Citrobacter rodentium* infection, the apoptosis of IECs accelerates, resulting in the production of large amounts of TGF-β in an inflammatory milieu that contributes to the Th17 cell response in this acute model of intestinal inflammation (Torchinsky et al., 2009).

The intestinal flora also influences the balance of intestinal Treg and Th17 cells by generating immunomodulatory metabolites. A comparison of serum from germ-free and conventionalized mice by mass spectrometry found that the flora influences the concentration of ~10% of common circulating metabolites (Wikoff et al., 2009). Affected molecules include the aromatic amino acids—phenylalanine, tryptophan, and tyrosine—and their derivatives, including the signaling molecule serotonin. Interestingly, these classes of molecules directly influence the differentiation of Th17 cells in vitro (Veldhoen et al., 2009). In the intestinal lumen, resident bacteria secrete adenosine 5′-triphosphate (ATP), which similarly favors the expansion of Th17 cells (Atarashi et al., 2008). Accumulation of Th17 cells can come at the expense of the maintenance of $Foxp3^+$ Treg cells. For example, mice colonized with an intestinal flora that supports high frequencies of Th17 cells in the small intestine LP have correspondingly lower frequencies of $Foxp3^+$ Treg cells among $CD4^+$ T cells (Ivanov et al., 2008). In the presence of an intestinal flora that favors Th17 cell accumulation, the induction of Tr1-like cells might represent a backup or alternative regulatory system (Figure 2).

The flora also contains bacterial molecules that promote regulation. Polysaccharide A (PSA), a carbohydrate expressed by the human commensal bacterium *Bacteroides fragilis*, is sufficient to ameliorate T cell-driven colitis in an IL-10-dependent manner (Mazmanian et al., 2008). Previous studies demonstrated that PSA can be presented to $CD4^+$ T cells by MHC class II molecules and favors Th1 cell effector responses, which are normally

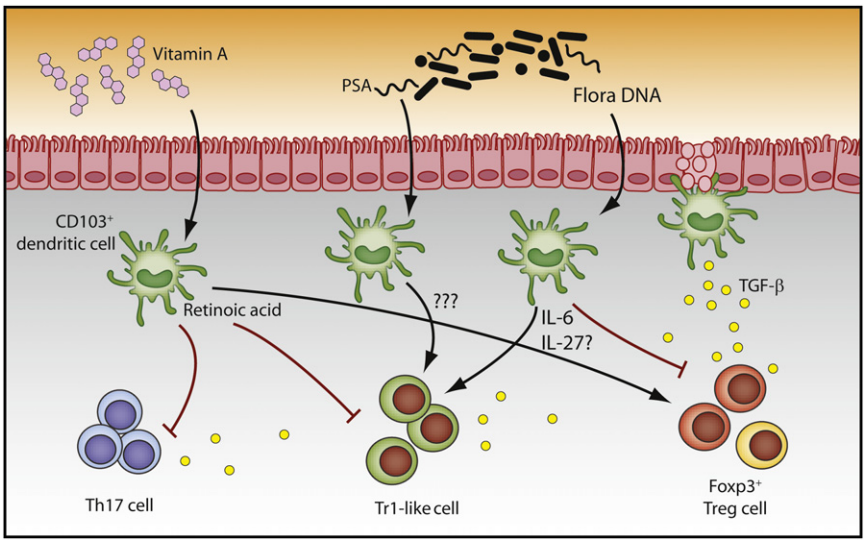

Figure 2. Distinct Stimuli Promote Intestinal Th17, Tr1-like, and Foxp3$^+$ Treg Cells

Of the many factors unique to the intestinal environment, several stimuli that affect the differentiation of T cell subsets have been identified. Retinoic acid, a metabolite of dietary vitamin A, can promote iTreg cell generation and inhibit Th17 and Tr1-like cell responses in part by reducing IL-21 transcription and IL-6Rα expression in T cells. PSA, a molecule expressed by the bacterium *Bacteroides fragilis*, can ameliorate intestinal inflammation by an IL-10-dependent mechanism, which might involve the expansion of Tr1-like cells. Bacterial DNA from the flora can activate TLR9, thereby limiting Treg cell accumulation and potentially favoring Tr1-like cell responses via the production of IL-6 and perhaps IL-27, which can induce the IL-10-promoting cytokine IL-21. All three T cell subsets utilize TGF-β for their maintenance. Apoptotic IECs are one potential source of TGF-β, which might be activated by both myeloid cells expressing α_v integrins and T cells expressing TGF-β-activating molecules.

associated with intestinal inflammation (Mazmanian et al., 2005). Whether PSA also induces protective Tr1-like or Treg cell responses remains an important unanswered question. Another intestinal bacterium, *Faecalibacterium prausnitzii*, has been shown to induce IL-10 expression in circulating T cells (Sokol et al., 2008). Discovering the molecules and mechanisms underlying the many "probiotic" properties of the intestinal flora is an area of great therapeutic interest.

In addition to affecting the ratio of Treg cells to effector T cells, dietary- and flora-derived molecules influence the frequency of intestinal Foxp3$^+$ Treg and Tr1-like cells. DNA in the intestinal lumen, presumably released from dying bacteria, contains TLR9-activating motifs (Hall et al., 2008). In vitro, stimulation of DC and T cell cocultures with TLR9 ligands induces inflammatory cytokines, including IFN-γ, IL-4, and IL-6, which inhibit the generation of iTreg cells. The increased frequency of Foxp3$^+$ Treg cells in the small intestine LP of *Tlr9*$^{-/-}$ mice suggests that such a mechanism may operate in vivo. TLR9-mediated activation of the IL-6 and IL-21 signaling pathways also induces IL-10 expression among Foxp3$^-$ CD4$^+$ T cells in vitro (Maynard et al., 2009). Whether TLR9 signaling exerts similar effects in vivo, promoting Tr1-like cell responses, remains to be determined. Retinoic acid (RA), a vitamin A metabolite, can act as an opposing influence, favoring the generation of Foxp3$^+$ iTreg cells (Coombes et al., 2007; Mucida et al., 2007; Sun et al., 2007) and limiting the induction of IL-10 expression (Maynard et al., 2009). RA might inhibit IL-10 expression by reducing the T cell surface expression of IL-6Rα and transcription of IL-21 in activated T cells (Hill et al., 2008). Mice raised on a vitamin A-deficient diet show an abundance of IL-10-producing Tr1-like cells throughout the intestine, yet retain a relatively normal frequency of IL-10-producing Foxp3$^+$ Treg cells (Maynard et al., 2009). Therefore, both TLR9 ligands and RA reciprocally influence the accumulation of Tr1-like and Foxp3$^+$ Treg cells in the intestine (Figure 2), but other factors appear to regulate IL-10 expression in Foxp3$^+$ Treg cells.

Identifying cells that deliver IL-10- or Foxp3-inducing signals is an area of intense investigation. Intestinal DCs expressing $\alpha_E\beta_7$ integrin (CD103) and CD11b$^+$ intestinal macrophages are enriched in their capacity to store and produce RA and are thus potent generators of iTreg cells (Coombes et al., 2007; Denning et al., 2007; Sun et al., 2007). CD103$^+$ DCs comprise about 30%–50% of intestinal DCs and are rather homogenously distributed throughout the colon LP, small intestine LP, and mLN of healthy mice (Annacker et al., 2005; Sun et al., 2007). Yet, the composition of Foxp3$^+$IL-10$^-$, Foxp3$^+$IL-10$^+$, and Tr1-like cells varies greatly between intestinal compartments (Figure 1; Maynard et al., 2007). Currently available data cannot explain the heterogeneous distribution of Treg and Tr1-like cells in the intestine. However, we note that these populations in the small intestine LP are particularly sensitive to changes in the flora induced by vancomycin treatment, TLR9 deficiency, or RA depletion (Hall et al., 2008; Ivanov et al., 2008; Maynard et al., 2009). The colon LP contains a large population of IL-10-producing Foxp3$^+$ Treg cells and comparatively fewer Tr1-like cells that are less sensitive to such perturbations. It may be that RA and flora-derived DNA are dominant forces that shape the differentiation of CD4$^+$ T cells in the small intestine. Because the colon harbors the highest concentration of resident bacteria in the GI tract, additional, partially overlapping pathways may be needed to maintain intestinal homeostasis.

Modes of Regulation in the Lymph Nodes and Tissues

Of the many mouse models of colitis, the T-cell-transfer model is unique in its ability to probe the regulatory mechanisms utilized by Treg and Tr1-like cells (Strober et al., 2002). Although the effector functions of Treg cells have been recently reviewed (Shevach, 2009), their mode of action in the GI tract is distinct. Somewhat surprisingly, Treg cells lacking β_7 integrin, an important molecule for trafficking to the intestinal LP and intraepithelial tissues, function in the T-cell-transfer colitis model (Denning et al., 2005). By contrast, CCR4- and CCR7-deficient Treg cells are impaired in their ability to repopulate the mLN and fail to prevent colitis (Schneider et al., 2007; Yuan et al., 2007). Together, these findings suggest that Treg cells can prevent colitogenic T cell responses via actions in the mLN. One potential mechanism involves limiting the duration of contact between T cells and DCs, perhaps because of the high expression of

CTLA-4 on Treg cells, thus reducing the likelihood that a naive T cell would become activated (Tadokoro et al., 2006). Treg cells may accomplish this by forming aggregates around DCs, preventing access by other T cells (Onishi et al., 2008). In this context, an intestinal Treg cell population with a TCR repertoire able to recognize dominant intestinal antigens would be able to outcompete naive or effector T cells with similar antigen specificities for access to DCs, thereby preventing the priming or proliferation of colitogenic T cells.

Large numbers of adoptively transferred Treg cells can also cure established colitis induced by the transfer of naive T cells into *Rag2*$^{-/-}$ recipients (Mottet et al., 2003). In this setting, Treg cells migrate into the inflamed LP and secrete IL-10 (Uhlig et al., 2006). The requirement for IL-10 in the cure setting, but not classic T-cell-transfer model (Asseman et al., 2003), is of particular note. In colitic mice, we have observed changes in the flora, including an increase in segmented filamentous bacteria in close proximity to the epithelium (Stepankova et al., 2007). It is also worth noting that the prevention of T-cell-transfer colitis requires Treg cell-derived IL-10 when recipient mice are infected with *H. hepaticus* (Kullberg et al., 2002). Among common microbes in the murine intestinal flora, *H. hepaticus* is somewhat unique in that it can partially penetrate the mucus layer and approach the epithelium in the caecal crypts (Chan et al., 2005). Through a flora-centric lens, these requirements for IL-10 might reflect the need to temper exuberant innate immune responses toward bacteria that become associated with the epithelium, either as a result of mucus-secreting goblet cell depletion during the course of colitis or penetration of the mucus layer by certain microbes. IL-35 is another Treg cell effector cytokine important for resolving established inflammation (Collison et al., 2007), although it acts through indirect effects on other T cells in vitro (Collison et al., 2009) and demands further mechanistic study in vivo.

An emerging concept in Treg cell biology is that cytokines and signals that promote the expression of transcription factors classically associated with effector T cell subsets can also induce expression of the same transcription factors in subsets of Treg cells, allowing their proliferation and acquisition of particular regulatory functions (Koch et al., 2009; Zheng et al., 2009). Such a strategy could tailor Treg cells to best respond to different types of inflammation. This paradigm also appears to apply to intestinal Treg cells, because deletion of Stat3 in Foxp3^{+} Treg cells leads to Th17 cell-dependent intestinal inflammation (Chaudhry et al., 2009). Further studies are required to identify how Stat3 orchestrates the function of Treg cells in the intestine.

Gaining Therapeutic Insights from Murine Colitis Models

Despite its utility in dissecting intestinal effector and regulatory pathways, one limitation of the T-cell-transfer model is the introduction of homeostatic proliferation as an additional variable predisposing mice to autoimmunity (reviewed in Coombes et al., 2005). Despite this complication, a strong correlation exists between genes involved in T cell-transfer colitis and those linked to human IBD-susceptibility alleles by genome-wide association studies, such as *ICOSLG*, *IL10*, *IL12B*, *IL23R*, *STAT3*, and multiple autophagy-related genes (Barrett et al., 2008; Franke et al., 2008). Therefore, the T-cell-transfer model seems to have general utility for discovering factors involved in IBD pathogenesis, including environmental factors or genes that association studies might not identify because of low frequency risk alleles in human populations. One potential insight offered by the T-cell-transfer model is that the GI tract might support sustained proliferation and competition between effector and regulatory T cells. Costimulatory molecules have a central role in controlling Treg cell proliferation and accumulation that has been reviewed extensively elsewhere (Bour-Jordan and Bluestone, 2009). Intriguingly, we have found that Treg cells require expression of the costimulatory molecule OX40 in order to efficiently repopulate the peripheral lymphoid organs and colon of *Rag2*$^{-/-}$ recipients. However, in mixed OX40-deficient and wild-type bone marrow chimeras, accumulation of OX40-deficient Treg cells was normal in the secondary lymphoid organs, but selectively reduced in the colon LP (T. Griseri and F.P., unpublished observations). Thus, genes required for homeostatic proliferation may also be particularly important for the maintenance of Treg cells in the intestine. We note that the role of costimulation in the generation of Tr1-like cells remains largely unexplored.

Enhancing Tr1-like or Treg cell function represents a potential therapeutic strategy for treating human IBD, which most often presents in patients as patchy, recurring inflammation involving the ileum and colon (Crohn's disease) or continuous inflammation along the length of the colon (ulcerative colitis). In both diseases, increased numbers and frequencies of Foxp3^{+} Treg cells are observed in the intestine (Uhlig et al., 2006). Treg cells in human IBD retain some functionality as they secrete IL-10 in the colon LP, although it is possible that inflammatory conditions dampen IL-10-independent functions of Treg cells. Importantly, effector T cells in human IBD express large amounts of the inhibitor of TGF-β signaling, SMAD7, rendering them resistant to Treg cell-mediated suppression (Fantini et al., 2009; Monteleone et al., 2001). In order for Treg cells to restore intestinal homeostasis, innate immune activation might first need to be controlled in order to remove stimuli that render effector T cells unresponsive to TGF-β signaling. Although it is suspected that bacteria in the flora trigger innate immune activation, the human flora can contain viruses, fungi, protozoan parasites, and worms that could also be involved in IBD pathogenesis (Artis, 2008).

The most effective IBD therapies currently used in the clinic might have underappreciated roles in enhancing regulatory activity. Steroid regimens serve as an initial therapeutic option for IBD and have systemic anti-inflammatory properties. In vitro, combinations of the steroid dexamethasone and vitamin D_3 potently induce Tr1-like cells (Vieira et al., 2004), and it is possible that similar effects occur in the GI tract after treatments with steroid regimens. TNF-α neutralization represents an alternative therapeutic strategy that, in addition to reducing innate immune activation, may enhance the function of Treg cells (Valencia et al., 2006). Blockade of IL-12p40, a component of IL-12 and IL-23, reduces innate immune activation and effector T cell responses, but might also result in the induction of iTreg cells in the GI tract (Izcue et al., 2008). Designing therapeutic strategies that target innate immune activity or intestinal barrier function, but also leave behind an enhanced population of Tr1-like or Treg cells, might improve the prospects for long-term

remission of IBD symptoms and restore the symbiotic relationship of the host with the intestinal flora. Indeed, one unique therapeutic approach confers regulatory function on the flora itself by expressing an IL-10 transgene in the intestinal bacterium *Lactococcus lactis* (Braat et al., 2006).

Finally, insights into intestinal immune regulation gained from murine colitis models have potential applications beyond understanding the etiology of IBD. In tumor immunotherapy, one promising approach involves CTLA-4 blockade, which acts in part by reducing Treg cell function in order to enhance antitumor immune responses (Peggs et al., 2006). As predicted from mouse models (Read et al., 2000), colitis is a problematic, sometimes lethal side effect of this strategy. In bone marrow transplantation, a procedure utilized to treat a number of hematological conditions, MHC incompatibility can result in graft-versus-host disease (GVHD). Colitis is a common manifestation of GVHD and is associated with impaired accumulation of $Foxp3^+$ Treg cells in the intestine (Rieger et al., 2006). During acute HIV infection, rapid depletion of intestinal $CD4^+$ T cells that express the HIV coreceptor CCR5 occurs in the GI tract (Brenchley and Douek, 2008). In the SIV macaque model of HIV infection, this phenomenon includes the loss of intestinal $Foxp3^+$ Treg cells and the absence of regulatory function among total intestinal $CD4^+$ T cells by 14 days after infection (Chase et al., 2007). Consequently, the remaining intestinal $CD4^+$ T cells proliferate and acquire effector function without restraint, resulting in intestinal inflammation that likely contributes to further viral dissemination. Incorporating targeted therapies that reduce immune activation and reinforce immune regulation in the intestine, such as blockade of the IL-23 signaling pathway, into the treatment of these devastating conditions is worth considering.

Future Perspectives

Here, we have discussed the immunological niches, cell types, and molecules unique to the intestine that promote immune regulation. In humans, intestinal inflammation can be initiated at a specific location in the GI tract, but then spread in the absence of effective intervention. Although this spreading suggests a degree of communition between different niches in the gut, less is known about how events in one part of the GI tract affect another. For example, interesting studies have implicated the liver in inducing tolerogenic T cells (Crispe, 2009) that could potentially play an underappreciated role in maintaining intestinal homeostasis. Apart from anatomical differences, the availability of dietary- and flora-derived molecules also varies throughout the GI tract. In the light of emerging evidence that regulatory T cells are both affected by and influence organ-specific metabolism (Cobbold et al., 2009; Lumeng et al., 2009), understanding the cellular targets and pathways activated by these molecules in the GI tract promises to be a fertile area for further research.

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