

Regulatory T Cells and Immune Tolerance in the Intestine

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A fundamental role of the mammalian immune system is to eradicate pathogens while minimizing immunopathology. Instigating and maintaining immunological tolerance within the intestine represents a unique challenge to the mucosal immune system. Regulatory T cells are critical for continued immune tolerance in the intestine through active control of innate and adaptive immune responses. Dynamic adaptation of regulatory T-cell populations to the intestinal tissue microenvironment is key in this process. Here, we discuss specialization of regulatory T-cell responses in the intestine, and how a breakdown in these processes can lead to chronic intestinal inflammation.

The mammalian host harbors a vast and diverse commensal microbiota. The gastrointestinal tract is a site of preferential colonization by commensal organisms, consisting of fungal, viral, and bacterial species. Initial microbial colonization of the host occurs during birth and continues until a stable commensal microbiota is established during childhood (Tannock 2007). Colonization of the gastrointestinal tract is a vital triggering stimuli for maturation of the mucosal immune system, and the presence of a commensal microbiota further benefits the host by providing resistance to invading pathogens and metabolism of dietary components (Macpherson et al. 2005; Hooper et al. 2012). A dynamic molecular dialogue between microbiota and host ensures this colonization occurs as a state of mutualism, the breakdown of which can result in chronic pathologies of the gastrointes-

tinal tract, such as inflammatory bowel diseases (IBD) (Kaser et al. 2010; Maloy and Powrie 2011). Complex interactions between the microbiota, mucosal immune system, and intestinal tissue cells provide multiple layers of regulation that control intestinal immunity. Here, we focus on the role of regulatory T cells as key components of intestinal homeostasis and discuss how tissue-specific adaptations contribute to their function when patrolling this challenging frontier.

THE GASTROINTESTINAL TRACT

The intestine constitutes a vast network of nonlymphoid and secondary lymphoid tissues, and as such, is home to numerous populations of leukocytes. Reflecting the unique challenge of maintaining intestinal immune tolerance,

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many of these cellular populations are found to be enriched in, or exclusive to, the intestine. In addition to the array of hematopoietic cell populations critical for immune tolerance within the intestine, the intestinal mucosa is anatomically specialized to promote homeostasis (Hill and Artis 2010; Harrison and Maloy 2011). The intestinal epithelium consists of a single layer of specialized epithelial cell subsets derived from multipotent and highly proliferative Lgr5⁺ stem cells located within the intestinal crypts. Although the cellular composition of the intestinal epithelium varies with anatomical location, both the colon and small intestine possess populations of absorptive and secretory cells, including enterocytes, colonocytes, goblet, endocrine, and Paneth cells (Wright 2000; Simons and Clevers 2011). Goblet-cell secretion of membrane tethered and soluble mucus components creates not only a viscous protective barrier, but a matrix loaded with secretory IgA and Paneth cell-derived antimicrobial peptides (AMP) to forge a layer impermeable to the majority of intestinal bacteria (Hill and Artis 2010). Pattern-recognition receptors (PRR), including Toll-like receptors (TLR) and Nod-like receptors (NLR), are germ-line-encoded sensors of microbial and host-derived danger signals (Schroder and Tschopp 2010). Tonic PRR-signaling within intestinal epithelial cells (IECs) drives cell-intrinsic proliferation, survival, AMP production and fortification of intercellular tight junctions, limiting bacterial translocation to the lamina propria (Maloy and Powrie 2011). Furthermore, homeostatic PRR-signaling within the intestinal epithelium serves to regulate not only localization but also composition of the microbiota. For example, deficiency in the cytosolic PRR, NLRP6, results in an altered intestinal microbiota with elevated abundance of the bacterial phyla Bacteroidetes (Prevotellaceae) and TM7 (Elinav et al. 2011). NLRP6-deficient mice have increased susceptibility to experimental DSS (dextran sodium sulphate) colitis; elegant cross-fostering and cohousing experiments revealed the colitogenic microbiota to be both vertically and horizontally transmissible. Thus, the intestinal epithelium uses multiple pathways to influence the localization and

composition of the microbiota to promote intestinal tolerance. Dysbiosis, an imbalance in the composition of microbial communities, occurs in patients with IBD, although whether this is cause or a consequence of the inflammatory environment remains unclear (Kaser et al. 2010).

ANTIGEN-PRESENTING CELLS PROMOTE INTESTINAL TOLERANCE

In line with important roles in host defense and tolerance, the intestine contains abundant and heterogeneous populations of myeloid antigen-presenting cells (APC). Subsets from distinct developmental origins vary in anatomical localization, phenotype, and function (Coombes and Powrie 2008; Varol et al. 2010). Recent studies of intestinal APC have used the differential expression of CD103 (α E integrin) and CX₃CR1 (receptor for Fractalkine [CX₃CL1]) to identify two major tissue resident populations, both of which appear to contribute to intestinal tolerance in distinct ways (Varol et al. 2010). Thus, CD103⁺ dendritic cells (DC) can take up intestinal antigens and following CCR7-dependent migration to the mesenteric lymph node (MLN), initiate T-cell responses with an intestinal tropism through induction of intestinal homing receptors CCR9 and α 4 β 7 (Iwata et al. 2004; Johansson-Lindbom et al. 2005; Jaensson et al. 2008). Under homeostatic conditions, CD103⁺ DC preferentially promote regulatory T-cell responses (Coombes et al. 2007; Sun et al. 2007; Schulz et al. 2009). However, this function is not hardwired, and as discussed below, is controlled by the intestinal microenvironment providing a mechanism through which the balance between tolerance and immunity can be controlled (Laffont et al. 2010). In addition to effects on T-cell-mediated immunity, CD103⁺ DC also promote T-cell-independent IgA class switch recombination by intestinal B cells contributing to host defense and intestinal barrier function (Uematsu et al. 2008). In contrast, intestinal CX₃CR1⁺ APC (consisting of dendritic cells and macrophages) are thought to be nonmigratory and poor primers of naïve T cells (Schulz et al. 2009), although this has recently been chal-



lenged (Diehl et al. 2013). CX₃CR1⁺ APC accumulate within the lamina propria following microbial colonization where they promote intestinal tolerance through a variety of mechanisms (Niess and Adler 2010). CX₃CR1⁺ APC lie adjacent to the epithelial barrier, and project trans-epithelial processes into the intestine to sample luminal antigens (Rescigno et al. 2001; Niess et al. 2005). Highly phagocytic in nature, colonic macrophages aid intestinal tolerance through clearance of apoptotic cells and debris, while also acting to expand populations of regulatory T cells (Murai et al. 2009; Hadis et al. 2011). Together the data indicate that lymphoid and tissue dwelling populations of APC collaborate to instigate and maintain a tolerogenic intestinal microenvironment.

Foxp3⁺ Treg IN INTESTINAL IMMUNE TOLERANCE

The intestine contains a high frequency of regulatory T-cell populations and early studies revealed their importance in preventing intestinal inflammation (Izcue et al. 2009). More recent studies have emphasized the importance of Foxp3 (Forkhead box P3)-expressing populations in particular. Foxp3 is a transcription factor vital for the induction and maintenance of a regulatory T-cell phenotype (Josefowicz et al. 2012a). Mutations in the X-linked gene encoding Foxp3 illustrate the essential role for Foxp3⁺ Treg cells in immunological tolerance in both mice and humans (Ziegler 2006). The majority of patients with IPEX (immune dysregulation polyendocrinopathy enteropathy X-linked) syndrome bear loss-of-function mutations within the FOXP3 gene and suffer an early onset fatal autoimmunity (Chatila et al. 2000; Bennett et al. 2001; Wildin et al. 2001). Although IPEX syndrome affects many organs and tissues, the intestine is most frequently affected, emphasizing the key role of Foxp3⁺ Treg cells in establishing and maintaining tolerance within the intestine (Gambineri et al. 2003).

Induction of Foxp3 expression and acquisition of the regulatory T-cell program occurs at both thymic and extrathymic locations. Thymus-derived Foxp3⁺ Treg cells (tTreg) differen-

tiate in response to high-affinity TCR (T-cell antigen receptor) interactions with MHC (major histocompatibility complex) class II presented self-antigens (Stritesky et al. 2012; Xing and Hogquist 2012). Extrathymic acquisition of Treg function and Foxp3 expression is also dependent on TCR-signaling but also strongly influenced by environmental factors (Bilate and Lafaille 2012). Indeed, there is convincing evidence that the intestinal microenvironment is a preferential site of antigen-induced Treg (iTreg) development, most likely reflecting high immune challenge and the continuous need for active immune suppression to maintain intestinal homeostasis.

Indeed, results in several mouse models of colitis have shown that disruption of regulatory T-cell networks can promote chronic intestinal inflammation in the presence of a triggering intestinal microbiota (Izcue et al. 2009). We have further dissected control of colitis by regulatory T cells using a T-cell transfer model. In this model, adoptive transfer of naïve CD4⁺ CD45RB^{Hi} T cells to a lymphopenic host results in cachexia and severe intestinal inflammation (Powrie et al. 1993). Transfer of Foxp3⁺ Treg, or originally, populations enriched in regulatory potential (CD45RB^{Lo} and then CD45RB^{Lo} CD25⁺) are sufficient to prevent the onset of, or “cure” ongoing, intestinal inflammation in this and other models (Powrie et al. 1993, 1994; Maloy et al. 2003). Development of colitis is dependent on the cytokine IL-23, which functions to promote Th1 and Th17 cell-mediated pathogenic effector responses as well as restrain iTreg differentiation in the intestine (Hue et al. 2006; Izcue et al. 2008; Ahern et al. 2010).

The presence of tTreg and iTreg populations in the intestine raises the question of the functional roles of these populations (Fig. 1). This has been difficult to decipher, as there are few markers that distinguish between these two populations. In vitro activation of mature naïve CD4⁺ T cells in the presence of TGF-β1 and IL-2 is now a commonly used method of Foxp3⁺ iTreg generation. Although these cells show some facets of Treg function, as assessed in vitro, the genetic composition of these cells is vastly different to those generated in different tissue

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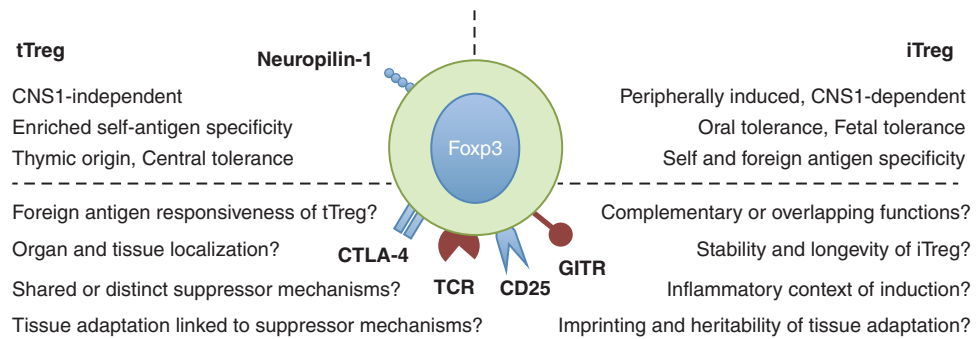


Figure 1. Properties of Foxp3⁺ Treg from thymic and extrathymic origins.



contexts in vivo (Haribhai et al. 2009; Feuerer et al. 2010). Helios, a putative tTreg marker and Ikaros transcription factor family member, is encoded by *Ikzf2*, a gene enriched in the Treg cell signature (Hill et al. 2007; Thornton et al. 2010). However, it is now apparent that a substantial proportion of experimentally induced Foxp3⁺ iTreg can express Helios, and as such, its use as a marker of Treg origin should be employed with caution (Verhagen and Wraith 2010; Gottschalk et al. 2012). In the absence of inflammation, the use of neuropilin-1 (Nrp-1) as a selective marker of tTreg appears promising; mucosa-derived iTreg are Nrp-1^{lo} compared with their tTreg Nrp-1^{Hi} counterparts (Weiss et al. 2012; Yadav et al. 2012). However, the robustness of neuropilin-1 as a marker of tTreg and its function in this context will require further experimental investigation.

Despite these caveats, sophisticated genetic models have provided strong support for collaboration between iTreg and tTreg populations in control of intestinal inflammation. Thus, efficient prevention of T-cell transfer colitis by tTreg cells requires deviation toward an iTreg phenotype by the colitogenic transferred T cells (Haribhai et al. 2009). A nonredundant role for iTreg in maintenance of intestinal tolerance under homeostatic conditions has been shown through a series of elegant genetic manipulations of the Foxp3 locus. Within the Foxp3 locus, conserved noncoding DNA sequences (CNS) promote size, stability, and composition of the Foxp3⁺ Treg population (Zheng et al. 2010). In vivo, CNS3 deficiency leads to drasti-

cally decreased thymic output of Foxp3⁺ tTreg, and CNS2-dependent mechanisms appear responsible for heritable maintenance of Foxp3 expression in proliferating Treg. Foxp3 CNS1 contains a TGF- β /NFAT response element, and synergistic binding of Smad3 and NFAT at CNS1 are suggested to be required for Foxp3 induction (Tone et al. 2008). In contrast, CNS1-deficient mice have unaffected tTreg populations, but defective induction of Foxp3⁺ iTreg, particularly within the GALT (gut-associated lymphoid tissue). This highly selective blockade in extrathymic Foxp3⁺ Treg induction did not lead to fatal autoimmune disorders, nor exacerbated Th1 or Th17 responses in the intestine, as may be expected if there is loss of intestinal immune tolerance. Instead, complete loss of intronic Foxp3 CNS1 manifested as pronounced Th2 responses, primarily at mucosal sites (Josefowicz et al. 2012b). In line with this, aged CNS1-deficient mice develop B-cell-dependent intestinal inflammation with antibodies to food and intestinal antigens, elevated IgE and IgA, and accumulation of Gata3⁺ CD4⁺ T cells producing IL-4, IL-5, and IL-13. These data are consistent with earlier studies showing a key role for iTreg cell development in oral tolerance toward nominal antigens and control of allergic responses, and illustrate the importance of immune deviation toward a Foxp3⁺ Treg program for mucosal tolerance (Curotto de Lafaille et al. 2008). The reason for the deviation toward Th2-mediated pathology in the absence of iTreg may reflect the prevailing environmental conditions, such as, for example, the lack of particular mi-



crobial stimuli required to elicit strongly inflammatory Th1/Th17 responses. Indeed, it will be of interest to test whether infection with known colitogenic bacteria elicits exacerbated inflammation in CNS1-deficient mice. Alternatively, it is possible that deficiency in iTreg is not sufficient to drive a pathogenic Th1/Th17 response to the microbiota and that there are multiple layers of regulation, including tTreg cells and IgA among others, that contribute to this important function (Feng et al. 2010).

Together, the above results suggest important roles for both tTreg and iTreg populations in maintaining intestinal homeostasis. Whether there is shared responsibility or specific functional roles of these developmentally distinct populations is not clear. One obvious possibility is that tTreg and iTreg populations contribute different antigen specificities, increasing the frequency of potentially responsive Treg populations that can be activated. It is known that Foxp3⁺ Treg cells use a broad $\alpha\beta$ -TCR repertoire distinct from that of Foxp3⁻ effector T cells (Hsieh et al. 2006; Pacholczyk et al. 2006). Recent studies indicate that colonic Foxp3⁺ Treg use a TCR repertoire distinct from Foxp3⁺ Treg found in systemic tissues, suggesting that the local antigenic milieu shapes the colonic Foxp3⁺ Treg pool (Lathrop et al. 2011). As may be expected, microbial specificities are increased within colonic Foxp3⁺ Treg populations, and transgenic mice bearing a TCR repertoire derived from colonic Foxp3⁺ Treg could not select thymic Foxp3⁺ Treg, suggesting they are specific for exogenous antigens. Furthermore, when T cells bearing these TCR were genetically prevented from adopting a Treg cell fate, they developed colitogenic potential. These data are compatible with the notion that the intestinal microenvironment supports local differentiation of microbe-reactive iTreg as a means of preventing development of inflammatory effector responses. This local adaptation to the intestinal microenvironment provides one layer of control that may be complemented by tTreg cells, some of which may also be reactive with microbial antigens. Indeed, it would be interesting to determine whether thymectomized germ-free mice also show accumulation of Foxp3⁺ Treg on col-

onization. Further understanding of the specificity of intestinal Foxp3 expressing cell populations is required to fully understand the role of these cells in antigen-specific tolerance induction.

TOLEROGENIC MODULES USED BY INTESTINAL Foxp3⁺ Treg

Although a source of early debate, it is now widely accepted that Foxp3⁺ Treg cells use multiple regulatory mechanisms to control host immune responses. Thus, production of regulatory cytokines (TGF- β , IL-10, and IL-35), modulation of APC function (LAG-3, CTLA-4, Granzyme/Perforin), and alteration of cellular metabolism (CD25, CD39/CD73, IDO induction in DC), have all been implicated in Treg cell function in distinct inflammatory settings (Vignali et al. 2008; Shevach 2009). We focus briefly on IL-10 and TGF- β as key cytokine pathways necessary for immune tolerance within the intestine.

IL-10, a regulatory cytokine, is produced by multiple leukocyte populations and protects the host by limiting inflammatory responses to microbial challenge (Saraiva and O'Garra 2010). IL-10 signals via a cytokine receptor complex consisting of IL-10R α and IL-10R β subunits, promoting STAT3 activation via Jak1 and Tyk2 (Glocker et al. 2011). In contrast with the fatal consequences of Foxp3 deficiency, genetic ablation of IL-10 or IL-10R leads to colitis in the presence of a triggering microbiota (Kuhn et al. 1993; Davidson et al. 1996; Sellon et al. 1998). Mutations in genes encoding IL-10 or IL-10R subunits are found in patients with very severe early-onset IBD (eo-IBD) and are associated with immune hyperactivation within the intestine (Glocker et al. 2009). Many of these patients are unresponsive to immunosuppressive therapies and require bone marrow transplantation. Recent studies suggest up to 15% of eo-IBD cases are attributable to mutations in the IL-10/IL-10R axis, clearly illustrating the important role of this pathway in intestinal homeostasis (Kotlarz et al. 2012). Single nucleotide polymorphisms in *IL10* have also been associated with susceptibility to adult ulcerative colitis

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and Crohn's disease, although in this setting the genetic contribution to disease is modest (Franke et al. 2008, 2010; Jostins et al. 2012).

IL-10 production is enriched within mucosal tissues and CD4⁺ T cells at these sites are an abundant source. Furthermore, both Foxp3⁺ and Foxp3⁻ populations contribute to the production of IL-10 within the gastrointestinal tract. However, strikingly there is compartmentalization of this response, with high levels of IL-10 production in the colon primarily restricted to Foxp3⁺ cells and the reverse in the small intestine where Foxp3⁻ cells represent the major source (Uhlir et al. 2006; Maynard et al. 2007). Functional studies support the expression data as the adoptive transfer of IL-10^{-/-} CD4⁺ T cells to lymphopenic hosts results in severe intestinal inflammation even in the face of host derived IL-10 production, and development of colitis ensues following specific deletion of IL-10 from CD4⁺ T cells (Davidson et al. 1996; Asseman et al. 1999; Roers et al. 2004). Early studies showed that IL-10-deficient Treg cells could prevent, but not cure, colitis in a T-cell transfer model (Asseman et al. 1999; Uhlir et al. 2006). In a lymphocyte-replete model, deletion of IL-10 from Foxp3⁺ Treg also leads to intestinal inflammation, albeit with less severity than deletion in total CD4⁺ T cells, implicating CD4⁺ Foxp3⁻ cells as a functional source of IL-10 that contributes to intestinal homeostasis (Rubtsov et al. 2008).

HOW DOES IL-10 ACT TO PROMOTE INTESTINAL TOLERANCE?

The IL-10 receptor complex is expressed on innate and adaptive immune cell populations within the intestine and acts to control intestinal CD4⁺ T cells through direct and indirect mechanisms. Myeloid-specific ablation of STAT3 leads to spontaneous colitis driven by dysregulated CD4⁺ T-cell responses. In this setting, defective IL-10R signaling in colonic macrophages results in elevated levels of costimulatory molecules CD80/86 and hyperactivation of colitogenic CD4⁺ T cells (Takeda et al. 1999; Kayama et al. 2012). Direct IL-10R signaling into CD4⁺ T cells also serves to limit colitogenic

responses within the intestine. Intestinal Th17 and Foxp3⁺ Treg express IL-10R α , and CD4-specific expression of a dominant-negative IL-10R α , results in selective expansion of Th17 and IFN- γ /IL-17A coproducing populations during intestinal inflammation (Huber et al. 2011). Under these circumstances, IL-10 production from both Foxp3⁺ and Foxp3⁻ CD4⁺ T cells is required to control colitis.

In addition to effects on effector T cells, there is also accumulating evidence that IL-10R signaling into Foxp3⁺ cells is important for their function. Thus, IL-10R β ^{-/-} Foxp3⁺ Treg cells fail to protect mice from intestinal inflammation following naïve T-cell transfer (Murai et al. 2009). In that study, IL-10R β ^{-/-} Treg cells lost Foxp3 expression and regulatory function following transfer into a lymphopenia-induced disease setting. In a genetic model, specific deletion of IL-10R α in Foxp3⁺ Treg cells resulted in spontaneous intestinal inflammation characterized by selective dysregulation of Th17 cells (Chaudhry et al. 2011). However, in that and other lymphocyte replete settings, Foxp3⁺ Treg cells show remarkable stability and are not observed to lose expression of Foxp3 (Rubtsov et al. 2010; Chaudhry et al. 2011). Whether the loss of Foxp3 expression in lymphopenia-driven colitis represents a failure to maintain the Treg program or is a specific consequence of the cell transfer system requires further study, especially given the interest in Treg cell therapy in the clinical setting. Finally, it should not be overlooked that many other cell types produce IL-10, including B cells and macrophages, and there is evidence that this plays a functional role in intestinal homeostasis (Mizoguchi et al. 2002; Murai et al. 2009; Hadis et al. 2011).

TRANSFORMING GROWTH FACTOR (TGF)- β

The transforming growth factor (TGF)- β family of cytokines have developmental and physiological roles throughout the mammalian lifespan. Encoded by individual genes, the TGF- β family consists of three isoforms, TGF- β 1, TGF- β 2, and TGF- β 3, which signal via the TGF- β R complex (TGF- β R1 and TGF- β R2). TGF-

β R activation triggers intrinsic kinase activity and phosphorylation of downstream signaling components, of which transcription factors Smad2/3 are an example (Derynck and Zhang 2003). Inhibitory Smads, for example, Smad7, act to negatively regulate TGF- β R signaling, making this a finely tuned system. Further control occurs at the posttranscriptional level as TGF- β is secreted to the cell surface in a latent complex, requiring further posttranslational modification to become biologically active. Activation of the latent TGF- β 1 complex requires enzymatic cleavage of latency-associated peptides, or integrin-mediated conformational change. The dominant role for TGF- β 1 in immunological tolerance is highlighted by the T-cell-dependent, early onset, fatal autoimmune disease of TGF- β 1^{-/-} mice that occurs within weeks of birth (Shull et al. 1992; Diebold et al. 1995; Letterio et al. 1996; Kobayashi et al. 1999). Similar to Foxp3 deficiency, complete ablation of TGF- β 1 production, or TGF- β R expression in T cells, leads to catastrophic autoimmune disease early after birth. In contrast with complete loss of this pathway, quantitative reductions in mice with impaired but not abolished TGF- β regulatory pathways leads to intestinal inflammation, indicating the intestine is particularly sensitive to TGF- β signaling. Genetic perturbation of Smad signaling, through loss of Smad3/4, or forced expression of Smad7 results in intestinal inflammation, implicating active TGF- β in intestinal tolerance. Likewise, inability to activate latent TGF- β in either T cell or myeloid cells results in loss of intestinal homeostasis. Mice with APC-specific ablation of integrin subunits, α v or β 8, or T-cell-specific deletion of convertase furin are prone to development of intestinal inflammation (Lacy-Hulbert et al. 2007; Travis et al. 2007; Yang et al. 2007; Pesu et al. 2008). In addition to the importance of T-cell-produced TGF- β 1, it is also crucial that T cells can respond to this cytokine (Li et al. 2006). Naïve T cells unable to respond to TGF- β cannot be controlled by Treg cells (Fahlen et al. 2005); however, whether TGF- β mediates suppression by inhibition of proliferation, acquisition of colitogenic potential, or generation of iTreg cell populations warrants

further investigation. Furthermore, the distinct signaling pathways initiated by different TGF- β isoforms remains to be examined in this and multiple other contexts.

Foxp3⁺ Treg-CELL SPECIALIZATION IN THE INTESTINE

An emerging concept in T-cell biology is that of adaptation to the tissue environment and context-dependent specialization of the adaptive immune response is well documented (Littman and Rudensky 2010). Differentiation of CD4⁺ T cells to distinct effector populations is dependent on appropriate TCR signaling, costimulation, and surrounding cytokine milieu. Expression of lineage-specifying transcription factors T-bet, Gata3, and ROR(γ)t is required for Th1, Th2, and Th17 responses, respectively (Zheng and Flavell 1997; Szabo et al. 2000; Ivanov et al. 2006). However, expression of these differentiation programs is not mutually exclusive and it is increasingly appreciated that interplay between these factors contributes to the fate and flexibility of effector T-cell responses (Oestreich and Weinmann 2012). As has been described for effector T-cell populations, it is now evident that Foxp3⁺ Treg can acquire expression of transcription factor modules that allow context-dependent immune suppression (Barnes and Powrie 2009). For example, in response to IFN- γ or IL-27, Foxp3⁺ Treg cells up-regulate expression of the Th1 cell transcription factor, T-bet (Koch et al. 2009; Hall et al. 2012). T-bet expression in Foxp3⁺ Treg promotes CXCR3 expression and migration to Th1-inflamed sites facilitating control of the Th1 response (Koch et al. 2009). Equally, the reliance of Th17 cells on STAT3-dependent cytokines for their differentiation is mirrored by the STAT3-dependency of Foxp3⁺ Treg-mediated control. In that case, targeted deletion of STAT3 (or IL-10R α , which signals via STAT3) in Foxp3⁺ Treg lead to severe intestinal inflammation dominated by accumulation of Th17 cells (Chaudhry et al. 2009, 2011).

In contrast with the Th1-context-dependent expression of T-bet in Foxp3⁺ Treg, the majority of peripheral Foxp3⁺ Treg express transcription factors IRF4 and/or Gata3, suggesting these

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transcription factors may play more general roles in Treg cell function (Zheng et al. 2009; Wang et al. 2011; Wohlfert et al. 2011; Rudra et al. 2012). Indeed, the specific loss of IRF4 in Foxp3⁺ Treg results in an autoimmune syndrome characterized by mucosal Th2 responses, similar in reported phenotype to mice deficient in Foxp3 CNS1 (Zheng et al. 2009). IRF4 seems to be an important pioneer transcription factor involved in multiple T-cell effector phenotypes including Th2, Th17, and Follicular Helper T-cell (T_{FH}) differentiation (Lohoff et al. 2002; Rengarajan et al. 2002; Huber et al. 2008; Bollig et al. 2012; Glasmacher et al. 2012). Based on these findings, IRF4 may play multiple roles in Foxp3⁺ Treg cells that promote specific modules of suppression. In support of this, Blimp-1 is a regulator of IL-10 production and its expression in Treg is dependent on IRF4 (Cretney et al. 2011). Furthermore, IRF4-deficient Foxp3⁺ Treg cells are unable to control Th1 and Th17 responses in a T-cell transfer model of colitis (Zheng et al. 2009). Further understanding of the various molecular mechanisms through which IRF4 expression in Foxp3⁺ Treg cells contributes to mucosal immune tolerance is required.

Beyond its essential role in embryogenesis, within the hematopoietic system, the transcription factor Gata3 is required for thymic T-cell development and mature CD4⁺ T-cell-differentiation to a Th2 lineage (Zheng and Flavell 1997). In addition, this lineage-specifying factor promotes immune tolerance through transcriptional enhancer activity at Foxp3 CNS2 to maintain Foxp3 expression in regulatory T cells (Wang et al. 2011). Genetic ablation of Gata3 in Foxp3⁺ Treg results in a systemic autoimmune syndrome, dermatitis, and intestinal inflammation (dependent on experimental system) due to reduced expression of Foxp3 mRNA and protein on a per-cell basis and associated decrease in regulatory capability (Wang et al. 2011; Wohlfert et al. 2011; Rudra et al. 2012). Alongside decreased Foxp3 expression, Foxp3⁺ Treg-specific ablation of Gata3 results in uncontrolled intestinal Th2 responses, with increased frequencies of Gata3⁺, IL-4, 5, and 13 producing populations in the intestinal lamina propria (Rudra et al. 2012) (Fig. 2A).

Although it is clear that Foxp3 and Gata3 cooperate to promote intestinal tolerance, the precise mechanisms are not known. Foxp3 mediates its effects at a molecular level through multimeric protein complexes, and directly regulates the expression of many of its interacting partners, including Gata3 (Rudra et al. 2012). Cooperation between Foxp3 and Gata3 promotes the Treg transcriptional program and subset stability. In the case of CNS1-deficient mice, it is possible that reduced Foxp3 expression during iTreg induction leads to sole binding of Gata3 at classically Foxp3/Gata3 co-bound sites, resulting in an aberrant Gata3 transcriptional response that leads to mucosal Th2 pathologies. As such, the mucosal Th2 response observed in CNS1-deficient mice may represent a cell intrinsic failure of effector T cells to divert their fate to a Foxp3⁺ iTreg phenotype, rather than specific requirement for Foxp3⁺ iTreg cells to control aberrant Th2 responses to the local antigenic milieu (Fig. 2B).

It is important to note that expression levels of effector T-cell transcription factors by Foxp3⁺ Treg cells are low relative to their Foxp3⁻ counterparts, suggesting that Foxp3⁺ Treg cells may use this strategy to adopt some, but not all aspects of distinct effector programs. For example, the acquisition of homing receptors, CXCR3 in the example of T-bet, without production of proinflammatory cytokine IFN- γ , renders Foxp3⁺ Treg cells able to migrate, but not contribute, to Th1-driven pathologies. In the instance of T-bet⁺ Foxp3⁺ Treg, incomplete commitment to an effector phenotype is regulated by suppression of IL-12R β 2 expression required for STAT4-mediated acquisition of effector function (Koch et al. 2012). The mechanisms by which IFN- γ /STAT1 signals in effector T cells, but not Foxp3⁺ Treg, lead to IL-12R β 2 expression remain to be elucidated. As yet, whether the role of effector T-cell-associated transcription factors in Foxp3⁺ cells is similar for tTreg and iTreg populations remains to be examined. Interestingly, Gata3 has been shown to inhibit the induction of Foxp3 in TGF- β -induced iTreg (Mantel et al. 2007), in comparison to the requirement for maintenance of Foxp3 in mature Treg.

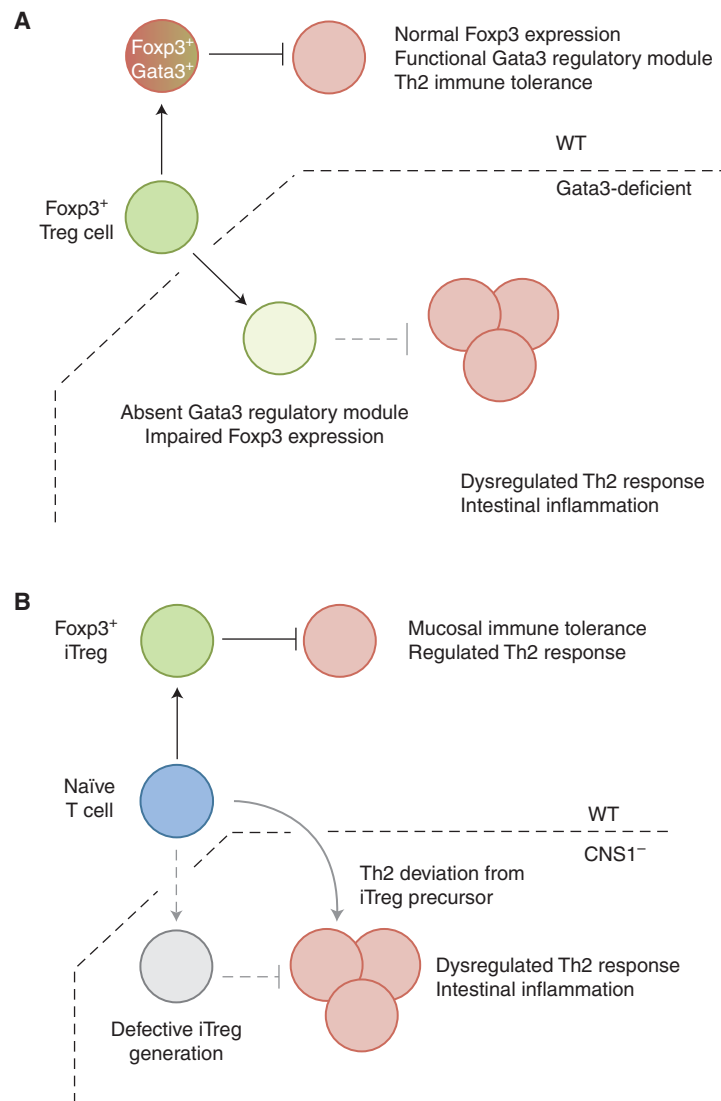


Figure 2. Foxp3⁺ Treg specialization on microenvironment adaptation maintains mucosal immune tolerance. (A) Gata3-dependent mechanisms promote Foxp3 expression and regulation of mucosal Th2 responses. Whether these mechanisms are distinct is not known. (B) CNS1 deficiency and loss of Foxp3⁺ iTreg-mediated regulation of mucosal Th2 responses may occur through cell-extrinsic (absolute requirement for Foxp3⁺ iTreg in control of Th2 responses) or cell-intrinsic (naïve T-cell precursors to Foxp3⁺ iTreg deviate to Th2 phenotype in absence of CNS1) mechanisms.

Adaptation of Foxp3⁺ Treg to the tissue microenvironment and the ability to use multiple regulatory mechanisms is key to instigating and maintaining active immunological tolerance. Distinct antigen specificities, enrichment in regulatory cytokine production and utilization of effector transcription factor networks show

that adaptation of Foxp3⁺ Treg to the tissue microenvironment is critical in maintaining intestinal immune tolerance. The role of the host immune system in continuous tolerance to the microbiota is evident; yet, the commensal microbiota itself is intimately involved in the dynamic molecular dialogue required for im-

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immune tolerance at both mucosal and peripheral sites.

MICROBIOTA AND DIETARY METABOLITES AFFECT INTESTINAL CD4⁺ T-CELL SUBSETS

Colonization of the host by the commensal microbiota and the presence of multiple dietary components is key to proper mucosal immune cell function (Fig. 3). Indeed, individual bacterial species promote differentiation of distinct intestinal T-cell populations. Reared in sterile surrounds, and on a diet free of live microbes, germ-free (GF) mice provide a useful experimental tool in understanding the relationship between host immune system and commensal microbiota. Controlled colonization of germ-

free mice allows analysis of the role for individual or multiple bacterial species in mutualistic interactions with the host immune system.

Segmented filamentous bacteria (SFB) are Gram-positive members of the *Clostridiales* genus able to penetrate the small intestinal mucus layer and interact intimately with the epithelium to colonize the host. Colonization with SFB results in accumulation of effector T cells within the small intestine, primarily Th17, and to a lesser extent, Th1 cells (Gaboriau-Routhiau et al. 2009; Ivanov et al. 2009). The mechanisms by which SFB promotes effector T-cell polarization and accumulation within the intestine remain largely unknown. Whether direct interactions between SFB and the intestinal epithelium, host immune cells, or production of

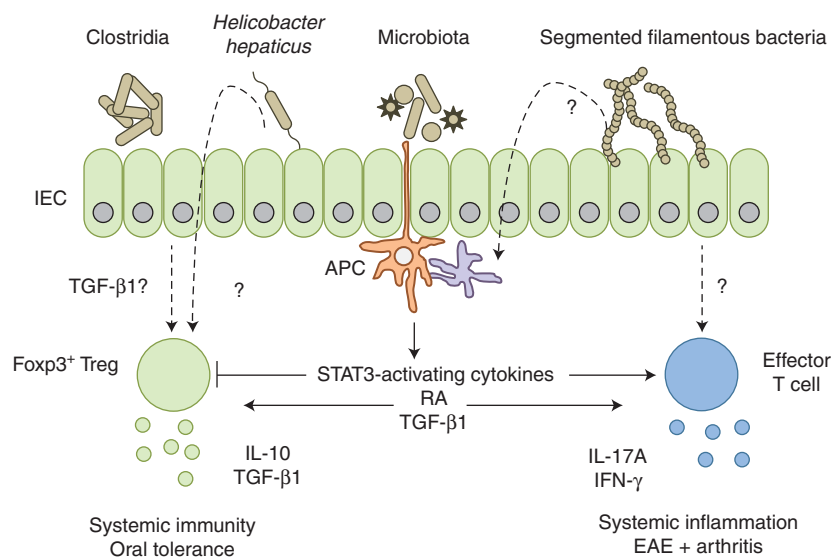


Figure 3. Commensal microbiota and dietary metabolites influence intestinal T-cell adaptation to direct mucosal and peripheral immune responses. The intestinal mucosa is constantly exposed to a diverse array of foreign antigens, including dietary antigens, metabolites, and components of the commensal microbiota. The presence of distinct microbial species promotes specialized host immune responses through differentiation of appropriate effector and regulatory T-cell populations. Segmented filamentous bacteria promote effector T-cell accumulation, particularly of Th17 cells, within the intestine. Accumulation of Foxp3⁺ regulatory T-cell populations and induction of IL-10 production arise in response to colonization of germ-free mice with a restricted flora (*Clostridiales* IV and XIVa, or Altered Schaedler Flora), or conventionally housed mice with *Helicobacter hepaticus*. In both cases, the mechanism of induction and antigen-specificity of the response require further investigation. Antigen-presenting cell production of soluble mediators heavily influences the balance between effector and regulatory T-cell responses. Production of STAT3 activating cytokines (IL-6, IL-23, IL-21) favors effector T-cell differentiation, whereas retinoic acid (RA) and TGF-β1 production modulate the balance between effector and regulatory responses, depending on the inflammatory microenvironment and associated cytokine production.



unique metabolic modulators is required for induction of effector T cells is not understood. Equally, whether the immune response to SFB colonization is antigen-specific remains to be established. Identifying the genetic attributes of SFB that drive this highly immunostimulatory response will be crucial to understanding how distinct bacterial species modulate the mucosal immune system.

Regulatory T-cell populations are also affected by microbial colonization, which can lead to accumulation of intestinal Foxp3⁺ Treg populations and induction of IL-10 production (Honda and Littman 2012). For example, *Bacteroides fragilis* is a human commensal able to induce IL-10 production from intestinal T cells and limit Th17 responses during intestinal inflammation (Round and Mazmanian 2010). Colonization of germ-free mice with a cocktail of 46 spore-forming murine Clostridia strains promotes accumulation of colonic Foxp3⁺ Treg (Atarashi et al. 2011). The nature of the exact strains, microbial attributes, and associated host molecular responses to this complex gnotobiotic mixture are as yet unknown. However, accumulation of Foxp3⁺ Treg and induction of IL-10 production are associated with increased colonic levels of active TGF- β 1 in these gnotobiotic mice. As discussed earlier, although the reliability of Helios expression to determine iTreg from iTreg is still controversial, *Clostridium*-colonized gnotobiotic mice showed a shift from Helios⁺ Foxp3⁺ to Helios⁻ Foxp3⁺ Treg following colonization, indicative of iTreg generation. The antigen-specificity of Clostridia-induced colonic Foxp3⁺ Treg was not assessed, but in the context of TCR repertoire of colonic Foxp3⁺ Treg, it is possible to hypothesize that these iTreg would bear TCR-specificity for Clostridia-derived epitopes. Furthermore, analysis of TCR repertoire in this setting may aid identification of individual Clostridia strains responsible for Foxp3⁺ Treg accumulation and/or IL-10 induction. In addition, colonization with a benign altered Schaedler flora (ASF) promoted accumulation of colonic Helios⁻ Foxp3⁺ Treg that served to promote intestinal tolerance during experimental DSS colitis (Geuking et al. 2011). Similarly, infection with the mouse

pathogen *Helicobacter hepaticus* induces an IL-10-producing Treg response, which when missing leads to bacteria-driven chronic intestinal inflammation (Kullberg et al. 2002). Together, these data suggest that induction of a Treg/IL-10-producing response may be an important part of host-commensal mutualism allowing persistence of microbes in the absence of a damaging inflammatory response. It is clearly of interest to further understand the molecular pathways and metabolites that control microbe-induced Treg function, as these may provide novel immune modulators and underlie the poorly characterized properties of probiotic bacteria. Clostridia are fermenting bacteria and it is notable that fermentation products such as short-chain fatty acids promote gut health (Maslowski et al. 2009). Whether iTreg induction and IL-10 production following bacterial colonization requires constant microbial exposure remains to be examined and the use of reversible colonization systems will be a useful tool to examine this question.

Retinoic acid (RA) is a metabolite of diet-derived vitamin A, a nutrient essential for multiple physiological processes including an array of immunological functions (Hall et al. 2011b). Within the GALT, RA promotes both regulatory and effector immune responses through imprinting of intestinal homing receptors α 4 β 7 and CCR9 on activated T cells, and acts as a cofactor in the de novo generation of Foxp3⁺ iTreg by CD103⁺ DC (Coombes et al. 2007; Sun et al. 2007). Induction of Foxp3 expression by CD103⁺ DC is dependent on TGF- β 1 and is enhanced by RA, accordingly CD103⁺ DC highly express mRNA for *aldh1a1* and *aldh1a2*, the retinal metabolizing enzymes required for RA synthesis (Coombes et al. 2007). In vivo corroboration of the role for RA in iTreg induction stems from the observation that mice reared on a vitamin-A deficient diet are defective in iTreg generation on oral antigen exposure (Hall et al. 2011a). As such, during intestinal homeostasis, RA promotes regulatory responses to bolster immunological tolerance to dietary antigens. However, recent studies show context-dependent roles for this metabolite in intestinal immune tolerance. Thus, RA

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acts to promote effector responses in settings of intestinal inflammation. Oral infection with *Toxoplasma gondii* is controlled by a strong intestinal Th1 response, which is impaired in vitamin-A deficient mice (Suzuki et al. 1988; Hall et al. 2011a). Furthermore, normal tolerance to dietary antigens is disturbed in patients with Celiac disease, whereby aberrant immune responses to gluten, a component of wheat, result in intestinal inflammation. Contrary to its recognized role in oral tolerance, RA, in combination with IL-15, a cytokine highly abundant in the mucosa of patients with Celiac disease, promotes proinflammatory cytokine production from intestinal APC to drive intestinal inflammation (DePaolo et al. 2011). As such, RA acts as a proinflammatory adjuvant in settings of intestinal stress, while promoting intestinal tolerance during homeostasis.

THE INTESTINAL MICROBIOTA AFFECTS SYSTEMIC IMMUNITY

The effect of the commensal microbiota on distinct lymphocyte subsets modulates immune function and tolerance beyond the intestinal microenvironment. Many models of autoimmunity or chronic inflammation are attenuated in the absence of a commensal microbiota (Chervonsky 2010). Although Th17 cells are strongly associated with immunopathology in multiple models of autoimmunity, colonization with SFB does not result in immunopathology and instead aids in protection from intestinal infection with murine pathogen *Citrobacter rodentium* (Ivanov et al. 2009). However, colonization with SFB is required for inflammation in models of intestinal, joint, and central nervous system pathologies (Stepankova et al. 2007; Wu et al. 2010; Lee et al. 2011). Therefore, SFB colonization can promote tissue pathology in experimental settings predisposed to chronic inflammation, either in the absence of Treg (colitis) or in autoimmune prone strains (arthritis and experimental autoimmune encephalomyelitis [EAE]). However, whether SFB can be classed as a true commensal species is contentious, given its intimate interaction with the intestinal epithelium. The regulatory response to

SFB remains ill-defined. Although clearly highly immunostimulatory, SFB is not essential for tissue inflammation. Transgenic SJL/J mice that express a myelin peptide-specific TCR develop relapsing-remitting EAE under conventional, but not GF conditions, indicating a requirement for microbial colonization as a triggering factor for disease pathogenesis (Berer et al. 2011). The colonization of GF mice with commensal microbiota, but not monocolonization with SFB, results in the acquisition of relapsing-remitting disease, highlighting the requirement for microbiota-derived signals as priming stimuli to trigger tissue pathologies in settings already predisposed to chronic inflammation. In the context of intestinal inflammation, the explosion in knowledge and techniques used in detailing the intestinal microbiota will be fundamental to understanding whether dysbiosis represents a triggering factor or is a consequence of inflammation, particularly given the high frequency of IBD-susceptibility SNPs within the healthy population.

CONCLUSION

The mucosal immune system promotes intestinal tolerance by controlling localization and composition of the commensal microbiota. Equally, the commensal microbiota is a fundamental stimulus for priming and activation of both mucosal and peripheral immune responses. However, dysregulated immune responses to the commensal microbiota have profound effects on human health. The dynamic interactions between microbiota and the immune system represent the key to targeted future therapies in chronic intestinal inflammation. To use natural tolerogenic mechanisms occurring under homeostatic microbial-mammalian dialogue, we require not only greater understanding of how the immune system regulates the microbiota, but equally how the distinct microbial communities influence host immunity.

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