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**Promises and paradoxes of regulatory T cells in inflammatory bowel disease**

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**Abstract**

Since their discovery two decades ago, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs) have become the subject of intense investigation by immunologists. Unlike other T cells, which promote an immune response, Tregs

actively inhibit inflammation when activated by their cognate antigen, thus raising hope that these cells could be engineered into a highly targeted, antigen-specific, immunosuppressant therapy. Although Tregs represent less than 10% of circulating CD4<sup>+</sup>T cells, they have been shown to play an essential role in preventing or limiting inflammation in a variety of animal models and human diseases. In particular, spontaneous intestinal inflammation has been shown to occur in the absence of Tregs, suggesting that there may be a Treg defect central to the pathogenesis of human inflammatory bowel disease (IBD). However, over the past decade, multiple groups have reported no qualitative or quantitative deficits in Tregs from the intestines and blood of IBD patients to explain why these cells fail to regulate inflammation in Crohn's disease and ulcerative colitis. In this review, we will discuss the history of Tregs, what is known about them in IBD, and what progress and obstacles have been seen with efforts to employ them for therapeutic benefit.

**Key words:** Foxp3; Regulatory T cells; Crohn's disease; Th17; Ulcerative colitis; Inflammatory bowel disease

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**Core tip:** Regulatory T cells (Tregs) have received much interest in animal models of inflammatory bowel disease (IBD), but have yet to demonstrate a clear defect in human Crohn's disease or ulcerative colitis. This review will detail our current knowledge about this important regulatory arm of the immune system in human IBD, and discuss the potential role for Tregs as immunotherapy.

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## INTRODUCTION

In the summer of 1995, Shimon Sakaguchi published the first report of what later came to be recognized as regulatory T cells (Tregs) by demonstrating that mice depleted of CD4<sup>+</sup>CD25<sup>+</sup> T cells spontaneously developed multiorgan autoimmunity, including gastrointestinal (GI) inflammation<sup>[1]</sup>. More importantly, such autoimmunity could be prevented by administration of these CD4<sup>+</sup>CD25<sup>+</sup> Tregs, suggesting that they might someday represent a potent cellular therapy for autoimmune and chronic inflammatory conditions. In the twenty years since this initial report, well over 10000 original manuscripts have been published concerning Tregs, making them one of the most intensely studied T cell populations of the 21<sup>st</sup> century.

Interest in Tregs took a quantum leap forward shortly after the turn of the millennium, when it was discovered that the gene *FOXP3* was central to Treg development and function, and could serve as an excellent marker for these relatively rare cells. A genetic defect in the *FOXP3* gene which precluded Treg development was found to be the cause of a mouse multiorgan inflammatory condition called scurfy<sup>[2]</sup>. At roughly the same time, a similar human condition called immune polyendocrinopathy enteropathy X-linked (IPEX) was reported to result from mutations in the human *FOXP3* gene resulting in humans with no Tregs<sup>[3,4]</sup>. As the name implies, an inflammatory enteropathy, resembling severe pan-intestinal Crohn's disease, is a central feature of IPEX, and generally causes fatal malnutrition in the absence of a hematopoietic cell transplant (HCT). This condition made it clear that the Tregs which had been receiving increasing attention in murine models were also critical for intestinal immune homeostasis in humans.

## TREG MECHANISMS OF ACTION

We now know that FOXP3<sup>+</sup> Tregs reside within the intestinal lamina propria and represent up to 10% of circulating CD4<sup>+</sup> T cells in humans<sup>[5-8]</sup>. Tregs recognize specific MHC-II-bound peptide antigens through a clonally unique T cell receptor (TCR), just like any other CD4<sup>+</sup> T cells<sup>[7,9]</sup>. However, while other T cells will deliver pro-inflammatory signals upon TCR ligation, Tregs do the opposite. They inhibit the activation of bystander T cells in a contact-dependent manner<sup>[10]</sup>. While no single molecular mechanism for this inhibition has been elucidated, several regulatory signals appear to be important (Figure 1), augmentation of which would represent an attractive opportunity for IBD therapy.

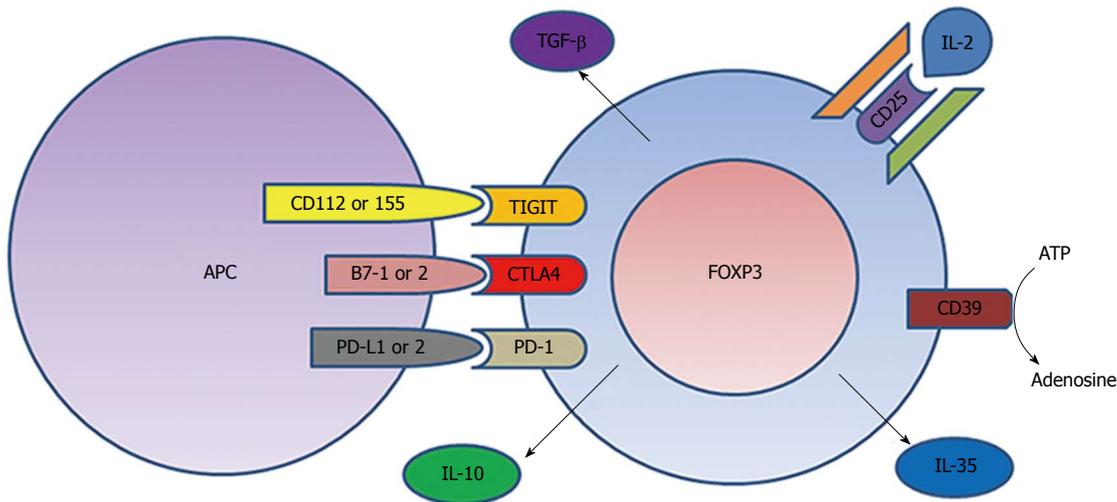
By definition, Tregs express more CD25 than any

other T cells<sup>[1]</sup>, and because CD25 is an essential component of the high-affinity IL-2 receptor, Tregs may absorb local IL-2, depriving nearby T cells of this T cell growth and survival factor when its concentration is limiting. However, IL-2 is evidently not essential for pro-inflammatory T cell growth and survival because mice genetically engineered to lack CD25<sup>[11]</sup> or the beta chain of the IL-2 receptor (CD122)<sup>[12]</sup> do not develop immunodeficiency, but rather a lymphoproliferative disorder including spontaneous autoimmunity and IBD. This was evidently due to a lack of Tregs<sup>[13]</sup>, as the latter are uniquely dependent upon IL-2. Thus, depriving other T cells of IL-2 is certainly not central to the inhibitory effect of Tregs *in vivo*.

Tregs also constitutively express more of the immunoregulatory CTLA4 molecule (CD152) than other T cells<sup>[8,14,15]</sup>, and this molecule appears to be necessary for Treg inhibitory function<sup>[15,16]</sup>. CTLA4 can bind up B7-1 (CD80) and B7-2 (CD86) costimulatory molecules on the surface of antigen presenting cells (APC), preventing them from costimulating CD28 receptors on other T cells<sup>[17]</sup>. Mice lacking the CTLA4 gene develop multiorgan autoimmunity<sup>[18]</sup> not unlike mice lacking Tregs. Similarly, patients who receive the CTLA4-blocking antibody ipilimumab as a cancer immunotherapy can develop spontaneous autoimmunity, including enterocolitis in over 20% of recipients<sup>[19,20]</sup>, thus demonstrating the importance of this molecule in maintaining intestinal immune homeostasis. However, whether CTLA4's role is primarily mediated through Tregs is unclear, as ipilimumab also limits CTLA4 engagement on activated T cells.

TIGIT, a molecule analogous to CTLA4, is also enriched on a subset of Tregs<sup>[21,22]</sup>, and likewise binds costimulatory molecules (CD112, CD155) on APC, preventing them from ligating a costimulatory receptor (CD226) on effector T cells, and thereby inhibiting the latter<sup>[23]</sup>. TIGIT<sup>+</sup> Tregs have been reported to selectively inhibit Th1 and Th17 cells, the CD4<sup>+</sup> T cell populations commonly associated with autoimmune and inflammatory conditions like IBD<sup>[24]</sup>. Tregs also express PD-1 (CD279)<sup>[25]</sup>, an inhibitory receptor that interacts with PD-L1 (CD274) and PD-L2 (B7-DC, CD273) on APCs and has, like CTLA4, recently become a target for cancer immunotherapy<sup>[26-29]</sup>. Like CTLA4 blockade, PD-1 blockade has caused spontaneous intestinal inflammation in clinical trials, albeit at a lower rate, affecting < 10% of recipients<sup>[30,31]</sup>.

In addition to their contact-dependent immunomodulatory mechanisms, Tregs may control inflammation through soluble factors. CD39 is an ectonucleotidase preferentially expressed by Tregs, which hydrolyzes ATP and ADP to AMP, and ultimately adenosine<sup>[32,33]</sup>. ATP has been reported to enhance pro-inflammatory Th17 cells<sup>[34,35]</sup>, while adenosine may inhibit effector T cells through the A<sub>2A</sub> receptor<sup>[36-39]</sup>, so this surface receptor may change the local environment of the Tregs to regulate inflammation.



**Figure 1** FOXP3<sup>+</sup> Tregs may mediate their inhibitory function through multiple soluble and cell-surface factors. CTLA4, TIGIT and PD-1 interact with costimulatory molecules on antigen presenting cells (APC). CD25 binds the T cell growth factor IL-2. CD39 converts local ATP to adenosine. The cytokines IL-10, IL-35 and TGF- $\beta$  have suppressive functions on nearby immune cells.

Reduced Treg expression of CD39 has been described in lupus<sup>[40]</sup> and multiple sclerosis<sup>[32,32,41]</sup>, but has not yet been described in IBD.

Tregs have also been reported to control inflammation through cytokines. TGF- $\beta$  is expressed by Tregs, and has immunomodulatory properties, although it may function as a cell-surface protein on Tregs<sup>[42]</sup>, and may not be necessary for Treg inhibitory function<sup>[43]</sup>. IL-10 is likewise an immunomodulatory cytokine made by Tregs<sup>[42]</sup>, and is essential for preventing spontaneous bowel inflammation in mice<sup>[44]</sup> and humans<sup>[45]</sup>. However, the immunoregulatory roles of IL-10 and TGF- $\beta$  may be more appropriately ascribed to other "regulatory T cell" populations that do not express FOXP3, namely Tr1<sup>[46,47]</sup> and Th3 cells<sup>[48]</sup>, which are beyond the scope of this review. More recently, FOXP3<sup>+</sup> Tregs have been shown to mediate their inhibitory function through the cytokine IL-35<sup>[49,50]</sup>.

## TREGS IN IBD

A number of clinical observations and experiments in animal models<sup>[51,52]</sup> have suggested that Tregs or their inhibitory mechanisms are critical for preventing spontaneous intestinal inflammation, and thus suggested that a defect in Tregs may be central to the pathogenesis of UC and/or Crohn's disease. Out of 38 distinct animal models of IBD reviewed in 2003, nine involved Tregs or their inhibitory mechanisms<sup>[51,53]</sup>. As an iatrogenic inflammatory bowel disease, human gastrointestinal graft vs host disease (GVHD) following HCT has been associated with evidence of decreased Tregs in the blood<sup>[54]</sup> and intestinal mucosa<sup>[55]</sup>.

Despite this wealth of data implicating Tregs in intestinal immune homeostasis, direct evaluation of Tregs in the intestines of IBD patients has not identified obvious defects. The first report of CD4<sup>+</sup>CD25<sup>+</sup> Tregs

isolated from the intestinal lamina propria (LP) of IBD patients, published more than a decade ago, demonstrated that these cells are present, express CTLA4, and show *in vitro* suppressive activity against other T cells which is no different from those of controls<sup>[56]</sup>. This and subsequent reports found that these Tregs paradoxically represent a greater fraction of LP CD4<sup>+</sup> T cells in the intestines of IBD patients than healthy control subjects<sup>[5]</sup> and are no less common in bowel affected by IBD than in bowel inflamed for other reasons, such as infection<sup>[51]</sup>. Paradoxically, Tregs are even more common in actively inflamed than uninfamed IBD mucosa<sup>[5,57-59]</sup>, with a reciprocal drop in circulating Treg frequency in the peripheral blood of symptomatic IBD patients likely reflecting sequestration of these cells to the site of inflammation. Thus, the mucosal inflammation of IBD appears to be different from that of IPEX in that it does not result from any local dearth of FOXP3<sup>+</sup> cells.

## ACTIVATION INDUCED FOXP3 EXPRESSION

Confounding these analyses was the discovery that FOXP3 expression could be induced *de novo* in human T cells that were originally FOXP3 negative by TCR activation in the presence of TGF- $\beta$ <sup>[60,61]</sup>. Thus the seemingly paradoxical excess of FOXP3<sup>+</sup> cells in the inflamed mucosa of an IBD patient could simply be locally activated T cells. Complicating matters, by some accounts, T cells induced to express FOXP3 by activation are nonetheless effective regulators of other immune cells *in vitro*<sup>[62,63]</sup>. Whether these "induced Tregs" (iTregs) have all the same suppressive function *in vivo* as constitutively FOXP3<sup>+</sup> "natural" Tregs (nTregs) has been debated<sup>[64]</sup>, and is difficult to establish experimentally in humans. One significant difference

between iTregs and nTregs concerns their ability to make cytokines. Classical nTregs do not make pro-inflammatory cytokines, such as IL-2 or IFN- $\gamma$ , and additionally show demethylation of CpG sites in the FOXP3 promoter<sup>[6]</sup>. In contrast, iTregs generated from effector T cells retain their ability to produce these cytokines<sup>[64]</sup>, and do not demethylate their FOXP3 promoter<sup>[65]</sup>, although they do up-regulate CD25 and CTLA4 to resemble nTregs<sup>[64]</sup>, making it difficult to discern the two Treg populations by surface markers. Adding to the complexity, it has become clear that the "nTregs" that constitutively express FOXP3 *in vivo* are actually a mix of Tregs that either acquired FOXP3 expression in the thymus (tTregs) or periphery (pTregs), thus reflecting their antigen specificity and perhaps phenotype<sup>[66]</sup>.

The nuclear protein Helios has been shown to be constitutively expressed by thymically-derived tTregs, but not *in vitro*-generated iTregs<sup>[67]</sup>, making this a potentially unique marker with which to distinguish at least these two populations. The fraction of FOXP3<sup>+</sup> LP T cells that express Helios is no lower in IBD patients than controls<sup>[68]</sup>, suggesting that the paradoxically increased FOXP3<sup>+</sup> T cells in IBD are not exclusively iTregs. However, there is evidence that activation-induced FOXP3<sup>+</sup> T cells may acquire Helios expression<sup>[69]</sup>, thus compromising the reliability of Helios as a marker for distinguishing iTregs from nTregs.

The TCR gene is uniquely rearranged in each nascent T cell, making it a stable genetic marker with which to identify T cells from a common clonal origin. By comparing the TCR V $\beta$  hypervariable domain repertoires of FOXP3<sup>+</sup> and FOXP3<sup>-</sup> T cell populations from the colon LP, it has been shown that these are predominantly distinct populations, even in IBD<sup>[68]</sup>. Indeed, LP Helios<sup>-</sup> Tregs show no more similarity in their TCR repertoire to effector T cells than they do to Helios<sup>+</sup> Tregs<sup>[68]</sup>. Thus, the paradoxically increased mucosal FOXP3<sup>+</sup> cells in IBD cannot be explained solely by activation-induced FOXP3 expression among effector T cells.

## TREG VS TH17 CELLS

Several groups have noted that an unusually high fraction of mucosal Tregs from IBD patients are able to produce IL-17A<sup>[70-72]</sup>. IL-17A is a potent pro-inflammatory cytokine associated with neutrophil recruitment<sup>[73]</sup>, and hence thought to play a central role in anti-bacterial immune responses. It is made by a subset of effector T cells, called Th17 cells, which can be identified by CCR6<sup>[74]</sup> and CD161 expression<sup>[75]</sup>, and have been implicated in multiple autoimmune conditions<sup>[76]</sup>. Thus, by sharing characteristics with a potentially pathogenic class of T cells, the copious intestinal FOXP3<sup>+</sup> Tregs present in IBD could paradoxically promote rather than suppress intestinal inflammation.

Like iTregs, Th17 cells require TGF- $\beta$  for their development, but additionally require IL-6, which in turn suppresses the formation of FOXP3<sup>+</sup> Tregs<sup>[77,78]</sup>. The differentiation of Th17 cells is governed by the transcription factor ROR $\gamma$ t<sup>[74,79]</sup> instead of FOXP3. In cells that express both transcription factors, FOXP3 physically interacts with ROR $\gamma$ t in the nucleus to prevent the latter from promoting IL-17A expression<sup>[80]</sup>. This interaction requires a region of the FOXP3 protein encoded by exon 2 of the FOXP3 mRNA<sup>[80]</sup>, which is deleted in a splice variant ( $\Delta$ exon 2) that represents approximately half the FOXP3 transcripts expressed by humans<sup>[81]</sup>. This would suggest that IL-17-producing FOXP3<sup>+</sup> T cells, as seen in IBD, could be exclusively expressing the  $\Delta$ exon 2 variant of FOXP3. However, no predominance of  $\Delta$ exon 2 relative to full-length FOXP3 expression is seen in IBD, nor are there cells which exclusively express  $\Delta$ exon 2, even among IL-17-expressing FOXP3<sup>+</sup> T cells<sup>[57]</sup>. Thus, how Th17-like FOXP3<sup>+</sup> T cells arise in IBD remains a mystery, but could be due to an increased responsiveness to IL-6, as has been seen in T cells from multiple sclerosis patients<sup>[82]</sup>.

## TREG AND THE INTESTINAL FLORA

With the recent advent of inexpensive, high-throughput nucleic acid sequencing techniques, the bacterial flora, or "microbiome", of the GI tract has recently come under intense scrutiny. Differences between the intestinal microbiomes of people with and without IBD have been described by many independent researchers<sup>[83-86]</sup>, although it is difficult to determine whether such differences are a cause or effect of IBD once sufficient inflammation has occurred in the GI tract to diagnose an individual with IBD. Nonetheless, a leading hypothesis about the pathogenesis of IBD dictates that the immune system is losing tolerance to intestinal commensal flora, suggesting a dominant role for the microbiome.

Studies in germ-free mice have demonstrated that the gut microbiome is important for development of the normal intestinal immune system, as reviewed elsewhere<sup>[87]</sup>. This includes IL-10-producing, peripherally-induced FOXP3<sup>+</sup> Tregs, whose development can be driven by specific intestinal microbiota in animal models<sup>[88,89]</sup>. While some intestinal Treg development may simply be due to exposure to luminal peptide antigens, non-peptide bacterial products, such as short-chain fatty acids<sup>[90]</sup> or specific polysaccharides<sup>[88]</sup>, are important for Treg induction in the gut. Likewise, ingested micronutrients, such as retinoic acid, have been shown to contribute to the peripheral generation of FOXP3<sup>+</sup> Tregs in the gut<sup>[91]</sup>. Thus, exposure of the intestinal mucosa to the fecal stream may be an important means by which the mucosal immune system develops tolerance, or perhaps fails to do so in IBD.

## TREG IN IBD THERAPY

Contemporaneous with the growth of research on Tregs in the early 21<sup>st</sup> century was the use of biopharmaceutical therapy for IBD and other inflammatory conditions involving TNF- $\alpha$  blockade. Perhaps as a consequence, a number of groups analyzed the effect of anti-TNF agents, particularly infliximab, on circulating FOXP3<sup>+</sup> Tregs, and found that the latter were enriched in the peripheral blood of patients demonstrating a good clinical response to therapy<sup>[92-95]</sup>. This suggests that the blockade of TNF- $\alpha$  *in vivo* may enhance Treg development, expansion, or viability if this cytokine normally inhibits Tregs in the setting of inflammation. Alternatively, because anti-TNF drugs can cause apoptosis of TNF-producing cells, and Tregs do not make TNF- $\alpha$ , it is possible this effect reflects a selective “pruning” of the FOXP3-effector T cell population rather than expansion of FOXP3<sup>+</sup> Tregs. However, caution should be taken in drawing conclusions about IBD from peripheral blood analyses, as the intestinal lamina propria houses more lymphocytes than the circulation. Thus selective sequestration or release of cell populations to or from the gut can actually cause the blood to reflect the opposite of what is actually happening at the site of inflammation in IBD. Indeed, the effect of anti-TNF agents on intramucosal Tregs has been less clear, with some researchers reporting a drop in FOXP3<sup>+</sup> cells on therapy<sup>[94]</sup>, and others reporting an increase<sup>[95]</sup>. Further confounding these analyses is the observation that histological IBD activity correlates inversely with Treg frequency in tissue sections<sup>[5,57-59]</sup>, such that a drop in tissue Tregs in the setting of effective therapy could obscure any local enrichment, and if mediated by a release of Tregs into circulation, produce the observed increase in blood Tregs.

The effect of other immunosuppressive therapies on Tregs has been less intensely studied in IBD, but data exists from other conditions for which these drugs are used. In liver transplant recipients, use of the immunosuppressive drug azathioprine has been paradoxically associated with decreased colonic FOXP3<sup>+</sup> cells, although only as cotherapy with prednisone and calcineurin inhibitors<sup>[96]</sup>. Likewise, in autoimmune hepatitis, azathioprine use, again in conjunction with prednisone, resulted in decreased intrahepatic Tregs, although a higher ratio of these Tregs to other lymphocytes correlated with biochemical remission<sup>[97]</sup>. Although these effects could be attributed to cotherapy with prednisone, studies in asthmatics have shown no effect of oral glucocorticoids on circulating Treg frequency<sup>[97]</sup>. Furthermore, as with anti-TNF agents, it is difficult to demonstrate that changes in Tregs associated with a given therapy represent a cause or effect of changes in inflammatory activity. Whether the newer anti-integrin biopharmaceutical vedolizumab will have an effect on intramucosal Tregs has yet to be seen, but a similar agent, natalizumab, did not

alter the ratio of Tregs to other T cells in the intestinal mucosa of Crohn's patients receiving it<sup>[98]</sup>.

## TREGS AS IBD THERAPY

Shortly after their discovery, Tregs were proposed as a potential therapy for autoimmune or inflammatory disease in more reviews and editorials than can be listed here. Indeed, in many animal models, adoptive transfer of Tregs proved effective for the prevention or treatment of inflammatory conditions, including IBD<sup>[99]</sup>. However, more than a decade later, the application of Tregs to human disease has been surprisingly limited. Given their rarity in peripheral blood, a major obstacle to therapeutic application of Tregs has been simply having enough Tregs to administer, so much work went into expanding or generating Tregs *in vitro* into a large, stable population with stable suppressive function. The earliest and most extensive efforts applying Tregs as anti-inflammatory therapy have been directed at GVHD complicating HCT<sup>[100-102]</sup>, a condition which, like IBD, commonly involves deregulated intestinal inflammation. As an alternative to adoptive transfer of *in vitro* expanded Tregs, *in vivo* expansion of Tregs post HCT through the use of low-dose IL-2 has demonstrated efficacy against GVHD<sup>[103-105]</sup>. Low dose IL-2 also expanded Tregs in type- I diabetes<sup>[106,107]</sup>, but it paradoxically accelerated autoimmunity, even when given with the immunosuppressant rapamycin, perhaps because it also expanded eosinophils and NK cells<sup>[107]</sup>. However, some efficacy has been seen with adoptive transfer of Tregs in type- I diabetes<sup>[108,109]</sup>.

The first trial of adoptive transfer of Tregs as a therapy for IBD was recently published as an 8-wk, open-label, dose-ranging study involving 20 Crohn's patients<sup>[110]</sup>. In contrast to the aforementioned trials in GVHD and diabetes, the transferred Tregs were selected and cloned to be specific for a dietary antigen (chicken egg ovalbumin) so that antigen-specific activation of the transferred cells could be stimulated in the gastrointestinal tract through an egg-intensive diet (meringue cake). 40% of recipients demonstrated clinical improvement, although the most improvement was paradoxically seen in recipients of the smallest number of Tregs (10<sup>6</sup>), and only minimal improvement was observed by objective measures of inflammation, such as C reactive protein and fecal calprotectin. Thus, the efficacy of Tregs as IBD therapy was neither straightforward nor overwhelming, suggesting that other factors, such as Treg antigen specificity or inhibitory function, may be more important than Treg numbers. Curiously, the number of circulating FOXP3<sup>+</sup> T cells decreased in responders, while rising in non-responders. However, the frequency of Tregs in the intestines was not evaluated, so this dichotomy could reflect mucosal Treg sequestration if such a phenomenon was associated with therapeutic response.

## CONCLUSION

Despite extensive interest in Tregs as central mediators of intestinal immune homeostasis, there is surprisingly little evidence that a defect in Tregs is associated with either form of human IBD. The fact that inflammation persists in Crohn's and UC despite an excess of Tregs in the mucosa relative to healthy bowel indicates that the inflammation of IBD is resistant to their presence. Whether the mucosal Tregs of IBD patients are intrinsically defective in their ability to regulate mucosal inflammation *in vivo* is unknown, but *in vitro* assays have shown no such functional defect<sup>[56,58,59]</sup>. Alternatively, Treg-extrinsic factors could undermine the immunoregulatory function of Tregs. Other immune cells, such as FOXP3-negative effector T cells, could be resistant to the inhibitory function of Tregs in IBD, as has been described in multiple sclerosis and diabetes<sup>[82,111]</sup>. Mucosal dendritic and other antigen presenting cells with which Tregs and other T cells interact could deliver signals which undermine Treg-mediated inhibition. Finally, the mucosal micro-environment in general, including soluble factors and components of the extracellular matrix, such as hyaluronic acid<sup>[112]</sup>, could be actively detrimental to, or passively unsupportive of, the inhibitory function of Tregs in IBD. A better understanding of the factors that undermine Treg function in IBD will be necessary before the promise of Tregs as an IBD therapy can ultimately be realized.

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