



## 2016 Inflammatory Bowel Disease: Global view

# Disrupted regulatory T cell homeostasis in inflammatory bowel diseases

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## Abstract

In the gut, where billions of non-self-antigens from the food and the microbiota are present, the immune response must be tightly regulated to ensure both host protection against pathogenic microorganisms and the absence of immune-related pathologies. It has been well documented that regulatory T cells (Tregs) play a pivotal role in this context. Indeed, Tregs are able to prevent excessive inflammation, which can lead to the rupture of intestinal homeostasis observed in inflammatory bowel diseases (IBDs). Both the worldwide incidence and prevalence of such diseases have increased throughout the latter part of the 20<sup>th</sup> century. Therefore, it is crucial to understand how Tregs suppress the colitogenic immune cells to establish new treatments for patients suffering from IBDs. In this review, we will first summarize the results obtained in animal model studies that highlight the importance of Tregs in maintaining intestinal homeostasis and describe the specific suppressive mechanisms involved. Next, our current knowledge about Tregs contribution to human IBDs will be reviewed, as well as the current therapeutic perspective on using Tregs for clinical IBD treatment and the challenges that remain to be resolved to ensure both the safety and effectiveness of these therapies in targeting this critical immune-regulatory

cell population.

**Key words:** Regulatory T cells; Foxp3; Gut; Inflammatory bowel disease; Colitis

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**Core tip:** The mucosal immune system must be very well-controlled to avoid immune responses against both microbial and innocuous food antigens. Regulatory T cells (Tregs) constitute a key mechanism to ensure gut homeostasis. In this review, both Treg biology and the suppressive mechanisms that have been identified using animal models of intestinal inflammation are first summarized. Then, we discuss the current knowledge concerning their contribution to human inflammatory bowel diseases (IBDs). Interestingly, these relatively recent advances have led to new therapeutic perspectives for IBD treatment by targeting this potent immunosuppressive cell population.

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## INTRODUCTION

Immune responses in the digestive tract are tightly regulated to ensure host protective immunity and prevent the development of immune-mediated pathology. The host immune system must efficiently respond to invading pathogenic microorganisms, while simultaneously and specifically blocking these mechanisms in response to antigens derived from the abundant commensal gut flora and the alimentation. The integrity of the intestinal mucosa is crucial and acts as a physical barrier, but additional tolerance mechanisms are required to prevent the excessive inflammation that can lead to the rupture of intestinal homeostasis observed in inflammatory bowel diseases (IBDs). IBDs are a group of chronic intestinal inflammatory disorders that include Crohn's disease (CD) and ulcerative colitis (UC). CD generally affects the entire gastrointestinal tract, whereas UC mainly affects the colon and the rectum. IBDs are complex diseases whose aetiology is currently incompletely understood. The current and most accepted hypothesis is that IBDs occur as a result of the complex interactions between genetic and environmental factors, leading to a breakdown in intestinal homeostasis and the development of aberrant inflammatory responses to the intestinal flora. Mouse models of IBDs that mimic features of the human pathology have been useful to better

understand and dissect the immuno-pathophysiology of IBDs. They indicate that chronic inflammation may be the result of excessive inflammatory responses or deficiencies in key negative regulatory pathways. In particular, regulatory T cells (Tregs) have been pinpointed as a key immunosuppressive population that is critically involved in the maintenance of the intestinal homeostasis. Here, we will first review the key features of Tregs before exploring their contribution to mouse models of IBDs. Then, we will focus on the implications of Tregs in human IBDs and discuss the current therapeutic perspectives based on the use of this immunosuppressive cell population.

## GENERAL FEATURES OF REGULATORY T CELLS

### *Phenotypic characterization of regulatory T cells*

The idea that a specific T cell population with regulatory capacities was able to control the immune system homeostasis and ensure tolerance emerged in the early seventies from experiments showing that neonatal thymectomy leads to the development of autoimmune manifestations<sup>[1]</sup> that can be prevented by adoptive transfer of T cells<sup>[2,3]</sup>. Since then, many studies have been performed to precisely define specific phenotypic markers of these suppressor CD4 T cells to analyze their key role in immune tolerance. The differential expression of CD5<sup>[3]</sup>, CD45RB<sup>[4,5]</sup> and CD25<sup>[6]</sup> has been successively used to define this potent regulatory population. It is now well-documented that several Treg subsets regulate the immune system, but the best-characterized Treg population is the CD4<sup>+</sup> CD25<sup>+</sup> phenotype. This subset expresses the transcription factor Foxp3 and plays a central role in preventing pathological immune responses, including autoimmunity, intestinal inflammation and allergy. Thus, Tregs ensure dominant tolerance to self and innocuous environmental antigens. The first and most convincing observation that Tregs play a major role in immune homeostasis was the dramatic autoimmune phenotype observed in the Scurfy mice that bear a mutation in the *Foxp3* gene<sup>[7,8]</sup> and the analogous fatal immune dysregulation, polyendocrinopathy, enteropathy and X-linked inheritance (IPEX) seen in humans with mutations in the *Foxp3* gene<sup>[9]</sup>. The mutations in Foxp3 in Scurfy mice and IPEX patients result in the specific absence of functional CD4<sup>+</sup> CD25<sup>+</sup> Tregs. Following these seminal observations, the use of genetically modified mice that allow to visualize or ablate Tregs *in vivo* have rejuvenated the field of T cell-mediated suppression and formally demonstrated that Foxp3 acts in Treg lineage specification<sup>[10]</sup>.

Functional studies require the isolation of a pure Treg population. Tregs are currently defined by the constitutive expression of CD25, but this molecule is also up-regulated by activated effector T cells (Teff). Additionally, although Foxp3 remains the best Treg

marker in mice, its intracellular location precludes the use of this marker for the isolation of live human cells. Furthermore, Foxp3 can be expressed by activated human Teff<sup>[11,12]</sup>. Tregs also constitutively express CTLA-4<sup>[13,14]</sup> and GITR<sup>[15]</sup>, but those markers are also impacted by T cell activation and do not provide more specificity than CD25. The lack of Treg-specific surface markers can be overcome by the use of Foxp3-reporter mice, but the identification of highly specific markers to distinguish Tregs from activated Teff remains a critical hurdle to studies in humans. The CD127 and CD27 markers have been proposed to increase the specificity of Treg identification. The level of CD127 expression is lower in CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs than in Teffs<sup>[16]</sup>. However, CD127 expression is also downregulated following Teff activation<sup>[17]</sup> and, therefore, is only useful to identify Tregs in non-inflammatory conditions. However, most of the current studies rely on Treg identification through the CD25<sup>+</sup> CD127<sup>low</sup> phenotype. The CD27 expression level in Tregs is higher than that in Teffs and identifies human Tregs under certain inflammatory conditions<sup>[18,19]</sup>.

#### **Thymic and peripheral regulatory T cell subpopulations**

Foxp3<sup>+</sup> Tregs can be divided into two main subsets: thymus-derived Tregs (tTregs), which are generated in the thymus, and peripherally-induced Tregs (pTregs), which can be induced from naive CD4 T cells in the periphery. We will briefly review the similarities and differences between these populations and discuss the relative contribution of tTregs and pTregs to intestinal homeostasis maintenance.

Tregs are generated in the thymus and represent less than 5% of the CD4<sup>+</sup> T cell population. Interestingly, tTregs develop from precursors expressing TCRs with high affinity for self-antigens. As a consequence, the TCR affinity of tTregs for self-antigens is higher than that of Teffs. Thus, although a partial overlap exists, the Treg and Teff TCR repertoires are distinct<sup>[20]</sup>. The actual model of tTreg differentiation consists of 2 steps<sup>[21,22]</sup>. A strong TCR signal associated with the engagement of costimulatory molecules leads to the upregulation of CD25 at the CD4 single positive stage. Then, signals through CD25, also known as the IL-2 receptor, lead to the expression of Foxp3. Indeed, the transcription factor STAT-5, which is activated downstream of the IL-2 receptor, binds a regulatory sequence in the *Foxp3* gene and thus promotes its expression. Several mouse models of IL-2 deficiency demonstrate that IL-2 is a key cytokine for the development and the peripheral maintenance of tTregs<sup>[23-26]</sup>. Interestingly, the lack of IL-2 in mice promotes colitis<sup>[27]</sup>.

It is assumed that most of the Foxp3<sup>+</sup> Tregs recirculating in the lymphoid organs of healthy mice originate from the thymus. However, a large proportion of pTregs derived from conventional T cells (Tconv) are present in the gut (particularly in

the lamina propria and the gut-associated lymphoid tissues), where tolerogenic conditions are combined. Indeed, in addition to the relatively high concentration of active TGF- $\beta$ 1, the continuous presence of antigens derived from the commensal microbiota and aliments, together with the presence of tolerogenic CD103<sup>+</sup> dendritic cells (DCs), is a favorable environment for pTreg differentiation<sup>[28,29]</sup>. This conversion of Tconv to Tregs is also mediated in part by retinoic acid (RA)<sup>[29,30]</sup>. Finally, DCs are able to generate the active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25(OH)2D3), a potent immunomodulatory molecule. Indeed, stimulation of Tconv with both 1,25(OH)2D3 and IL-2 induces both CTLA-4 and Foxp3 expression, conferring immunoregulatory functions to these cells<sup>[31]</sup>. In addition, the literature describes the importance of the PD-1/PD-L1 signaling pathway for the generation, maintenance and plasticity of Foxp3<sup>+</sup> pTreg<sup>[32-34]</sup>. It has been suggested that the exhausted colitogenic Teffs cells can lose their effector functions and acquire regulatory properties. These suppressor cells are characterized by the expression of PD-1, low expression of CD25<sup>[35,36]</sup> and constitutive expression of Foxp3 and CTLA-4. This subpopulation of pTregs is able to suppress the proliferation of CD4<sup>+</sup> CD25<sup>-</sup> PD-1<sup>-</sup> Tconv *in vitro* and inhibits the development of intestinal inflammation in the transfer model of colitis. These data suggest that the PD-1 and PD-L1 pathway could be one way to generate pTregs cells during IBDs. The butyrate produced by the commensal microorganisms during starch fermentation has been shown to induce pTreg differentiation in the intestinal mucosa and to reduce the disease severity in the T cell transfer model of colitis<sup>[37]</sup>. In agreement with these data, another study revealed that, in addition to butyrate, propionate, another metabolite generated by the microbiota, exhibits the same function<sup>[38]</sup>. The composition of the microbiota is crucial for Treg accumulation<sup>[39]</sup>. Indeed, oral administration of a mix of 17 different gut bacteria strains is sufficient to promote Treg accumulation in the colon of germ-free (GF) mice. Further studies are required to investigate whether these Tregs derive from pre-existing Tregs that have migrated and/or proliferated or from the peripheral differentiation of naive Tconv. Strikingly, this treatment with the bacteria mixture protects the GF mice from developing disease in the T cell transfer model of colitis and in an allergic diarrhea model. In addition, modification of the gut microbiota by oral administration of intestinal *Clostridium* bacteria strains improves Treg function, indicating that the microbial environment in the colonic mucosa influences Treg function<sup>[39,40]</sup>. The proposed mechanism is that DCs, and likely intestinal epithelial cells, would lead to the accumulation of IL-10<sup>+</sup> CTLA4<sup>high</sup> pTregs through their production of TGF- $\beta$  and other Treg-inducing factors. Moreover, the oral administration of *Clostridium* early in life results in resistance to colitis and systemic

immunoglobulin E responses in adult mice. In addition, IBD patients exhibit some shifts in the composition of their intestinal microbiota<sup>[41,42]</sup>. This observation suggests that the equilibrium of the gut flora is important to prevent IBD development and opens new therapeutic windows. Together, these data strongly imply that the intestinal bacteria promote tolerance by producing molecules that improve Treg-mediated immunosuppression, thus allowing symbiosis between the intestinal flora and the host.

In addition to these two Treg subsets, a third subset of Tregs, referred to as type 1 regulatory T (Tr1) cells, expresses only low and transient levels of Foxp3 and produces high levels of IL-10<sup>[43-45]</sup>. Tr1 cells have been described as particularly important for maintaining intestinal tolerance. Indeed, the transfer of Tr1 cells can prevent IBDs induced by pathogenic CD4<sup>+</sup> CD45RB<sup>high</sup> T cells<sup>[43]</sup>. The coexpression of CD49b and LAG-3 enables the isolation of a highly suppressive Tr1 population<sup>[46]</sup>. However, the biology of this Treg population is still poorly understood.

The TCR repertoires of tTregs and pTregs are different. Indeed, tTregs are enriched in autospecific cells compared to the pTregs that are generated from naive T cells that have not been negatively selected in the thymus<sup>[20,47]</sup>. Therefore, pTregs are thought to be mainly responsible for tolerance to non-self-antigens, such as allergens, diet and fetal antigens<sup>[48]</sup>, whereas tTregs would be preferentially involved in the control of autospecific responses<sup>[49]</sup>.

Because both pTregs and tTregs express CD25 and the key Treg lineage transcription factor Foxp3, the distinction between these 2 populations in the peripheral organs remains challenging. Currently, Nrp-1 and Helios are two candidates for this discrimination. However, these markers do not permit a strict distinction between Teffs, pTregs and tTregs. Higher expression of the transcription factor Helios on tTregs has been proposed to distinguish them from pTregs<sup>[50]</sup>; however, recent studies have revealed that Helios could also be expressed by pTregs *in vivo*<sup>[51]</sup>. Furthermore, a fraction of human tTregs does not express Helios<sup>[52]</sup>. Nrp-1 expression by tTregs and its absence in pTregs distinguishes these 2 populations under non-inflammatory conditions<sup>[53,54]</sup>. However, in humans, Nrp-1 expression can be induced in activated Teffs<sup>[55]</sup>. Thus, the lack of clear markers limits our understanding of the relative contribution of tTregs and pTregs to the disease pathophysiology, particularly in humans.

In the context of IBDs, the relative contribution of tTregs and pTregs is difficult to assess for the reasons described above. The inhibition of pTreg differentiation (by preventing TGF- $\beta$ -dependent induction of Foxp3) demonstrated that pTregs are necessary to prevent Th2 type pathologies at mucosal sites<sup>[56]</sup>. In addition, the lack of pTregs modified the composition of the intestinal microbiota. Thus, this study suggested

that tTregs are primarily responsible for the control of autoimmune responses, whereas the main role of pTregs would be to prevent immune responses against commensal and dietary antigens. This idea is further supported by another study analyzing the TCR repertoire of colonic Tregs<sup>[57]</sup>. The comparison of the TCR repertoire of Tregs from this site with other locations revealed that gut antigens shape the Treg TCR repertoire in the intestine. Indeed, a large proportion of colonic Tregs are specific for bacterial antigens, suggesting that pTregs compose a major proportion of the gut Tregs. However, another recent study revealed that the TCR repertoires of intestinal and thymic Tregs are highly similar, suggesting that tTregs also contribute significantly to the intestinal Treg pool<sup>[58]</sup>. Therefore, both pTregs and tTregs are likely to contribute to the maintenance of intestinal homeostasis, and it remains to be elucidated which population plays a major role.

### **Heterogeneity of regulatory T cells**

Effector CD4<sup>+</sup> T helper (Th) cells are heterogeneous and differentiate into at least 4 subsets (Th1, Th2, Th17 and Tfh) that differ in their cytokine production and functions. For instance, Th1 cells are defined as producing IFN- $\gamma$  and expressing the transcription factor T-bet. Th1 cells can enhance the killing potential of macrophages and CD8 T cells. Th2 cells produce IL-4, IL-5 and IL-13, express the transcription factor GATA3 and contribute to eosinophilic inflammation and allergic reactions. Th17 cells release IL-17A, IL-21 and IL-22, express the transcription-factor ROR $\gamma$ t and are involved in the activation and recruitment of neutrophils. Follicular helper T cells (Tfh) are characterized by the expression of CXCR5 and the transcription factor Bcl-6. This subpopulation plays a key role in humoral responses by promoting germinal center reactions and providing critical signals that shape the B cell responses. There is now a large body of evidence indicating that Foxp3<sup>+</sup> Tregs are also heterogeneous and could be further subdivided into highly specialized subpopulations with different functions. The expression of distinct effector molecules, chemokine receptors and transcription factors varies within Foxp3<sup>+</sup> Tregs isolated from distinct anatomical sites<sup>[59]</sup>. This differential expression of specific molecules endows Tregs with the ability to specifically control the various types of immune responses in different anatomical locations. The mechanisms involved in Treg specialization to control the Th1/Th2/Th17/Tfh responses have been recently elucidated. Expression of the Th1 transcription factor T-bet by a subtype of Tregs controls the expression of CXCR3 and allows Tregs to accumulate at Th1 inflammation sites<sup>[60]</sup>. The acquisition of T-bet expression by Tregs depends on STAT1 activation. Both IFN- $\gamma$  and IL-27 are involved in this differentiation by acting in different anatomical sites, and these 2 cytokines also induce



distinct gene expression profiles<sup>[60-62]</sup>. Treg expression of miR-146a, which is involved in the control of STAT1 expression, is also critical for their ability to control the Th1 responses<sup>[63]</sup>. Treg expression of the Th2 transcription factor IRF4 allows Tregs, in cooperation with Foxp3, to specifically control the Th2 responses. Indeed, specific deletion of IRF4 in Foxp3<sup>+</sup> T cells leads to the spontaneous development of a deregulated Th2 response, lymphoproliferation associated with a selective increase in IL-4/IL-5-producing CD4<sup>+</sup> T cells, the aberrant production of IgE and tissue lesions<sup>[64]</sup>. IRF4 physically interacts with Foxp3. Moreover, expression of the protein kinase CK2 is also involved in the Treg-mediated control of the Th2 responses. A Treg-specific CK2 deficiency induces the expression of the ILT3 receptor by a discrete population of Foxp3<sup>+</sup> Tregs. These ILT3<sup>+</sup> Tregs retain the ability to control the Th1 responses but favor the emergence of Th2-promoting PD-L2<sup>+</sup> IRF4<sup>+</sup> DCs, leading to a Th2 lymphoproliferative disease<sup>[65]</sup>. Tregs ability to suppress Th17 responses relies on their expression of STAT3, a key Th17 differentiation factor. Treg-specific ablation of STAT3 induces specific Th17 activation and an intestinal pathology<sup>[66]</sup>. STAT3 expression by Tregs is involved in the expression of the chemokine receptor CCR6, thus impacting their intestinal location. STAT3 also controls IL-1R and IL-6R expression. Expression of these cytokine receptors is likely to impact Teff polarization by depriving them of the cytokines important for Th17 differentiation. However, this phenomenon has only been demonstrated *in vitro*. Finally, the expression of the Tfh differentiation factor BCL6 by Tregs induces CXCR5 expression, promoting Treg migration into the germinal center (GC), where they become T follicular regulatory T cells (Tfr). The absence of BCL6 or CXCR5 expression by Tregs leads to increased GC activity<sup>[67]</sup>. Finally, Tregs can also be separated into subsets of naive and antigen-primed/memory Tregs. These populations are defined by the expression of CD45RA and CD45RO, respectively, and are both suppressive but functionally distinct<sup>[68-72]</sup>. Naive Tregs have the ability to recirculate between lymphoid tissues and are believed to function at the priming site, where their activation endows them with the ability to migrate into inflamed sites.

### Stability of regulatory T cells

The fine-tuning of gene expression is critical for Treg specialization and optimal control of various immune responses, and it has been suggested that Tregs can lose Foxp3 expression and their suppressive function to be reprogrammed into a phenotype resembling effector cells, particularly under inflammatory conditions where these ex-Tregs could contribute to disease development<sup>[73-77]</sup>. Other studies revealed that these "ex-Tregs", which are initially found at a high frequency, even in homeostatic conditions<sup>[73]</sup>, are mainly generated in the thymus where transient Foxp3

expression can occur without generating tTregs<sup>[78]</sup> or by transient expression of Foxp3 in non-Treg cells<sup>[79]</sup>. Thus, these results support the view that the differentiated Tregs are ultimately a plastic, but stable lineage. Foxp3 controls the activation or repression of genes and plays a key role in the development and suppressive function of Tregs<sup>[80]</sup>. However, the expression of Foxp3 alone is not sufficient to establish a complete and stable regulatory T cell phenotype. Indeed, ectopic expression of Foxp3 in Teffs induced only one-third of the Treg genetic signature<sup>[80-82]</sup>. Retroviral transduction of Foxp3 does not induce the activation of the endogenous Foxp3 locus, indicating that Foxp3 cannot activate its own transcription<sup>[81,83]</sup>. Furthermore, an analysis of the genetic signature of Tregs in Foxp3-GFP-KO mice, where the *Foxp3* gene is deleted and replaced by GFP under control of the same promoter, shows that the GFP<sup>+</sup> Foxp3<sup>-</sup> cells express some genes of the Treg signature<sup>[83]</sup>, revealing that if Foxp3 is essential for Treg development, it is not the only factor involved in the acquisition of a complete regulatory T cell phenotype.

The key role of epigenetic modifications in the regulation of cellular differentiation processes and lineage stabilization has been recently revealed in many cell types<sup>[84-86]</sup>. These epigenetic modifications regulate transcriptional activity and are transmitted during cell division<sup>[86]</sup>. A specific methylation profile of Tregs has been established. Compared to Teffs, Tregs exhibit hypomethylation of genes coding for key proteins involved in their functions, such as Foxp3, CTLA-4, and GITR<sup>[87-89]</sup>. In parallel, the Teff-specific genes that are repressed in Tregs are hypermethylated. These epigenetic modifications are initiated in Tregs during tTreg development in the thymus. A partial demethylation profile is indeed found in thymic Foxp3<sup>+</sup> cells<sup>[87,88]</sup>. The establishment of the hypomethylation profile is independent of Foxp3 expression<sup>[88,90]</sup>. Epigenetic modifications affect the DNA structure and increase the accessibility to Foxp3 binding sites in specific gene promoters involved in Treg functions, thus modifying the transcriptional activity of these genes in a Foxp3-dependent manner<sup>[90]</sup>. The epigenetic modifications differ between the thymus-derived tTregs and the induced Tregs (iTregs) *in vitro*<sup>[87,88]</sup>. The incomplete methylation profile of iTregs compared to tTregs explains the reduced stability of this population, which loses its Foxp3 expression and suppressive functions when restimulated in the absence of TGF- $\beta$ <sup>[87]</sup>. Furthermore, iTregs do not express all of the genes of the Treg-specific signature and show different capacities to control the effector responses *in vivo*<sup>[81]</sup>. Therefore, the Treg instability and loss of Foxp3 expression observed in inflammatory settings is thought to occur through pTreg conversion rather than through the tTreg population.

In addition to the epigenetic modifications that control Treg stability, numerous other mechanisms are

involved, such as the repression of SATB1 transcription factor expression by Foxp3<sup>[91]</sup>, which is essential for maintaining Treg function and preventing the expression of effector cytokines, or the expression of the transcription factors BACH2 and Eos, which ensure the repression of the effector program<sup>[92,93]</sup>. The miRNAs expressed by Tregs also play a key role in preserving the stability of the Treg program<sup>[94,95]</sup>. Likewise, the expression of the phosphatase PTEN is critical for maintaining Treg stability, given that PTEN-deficient Tregs can lose Foxp3 expression and up-regulate molecules of the Th1 and Tfh lineages<sup>[96,97]</sup>.

In the context of IBDs, the inflammatory environment might influence Treg stability. Indeed, in mouse models of autoimmunity, Treg functions may be directly influenced by multiple pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-6 and IL-21<sup>[98-101]</sup>. Of these cytokines, TNF- $\alpha$  is the best example because it has a negative effect on Treg functions in the context of rheumatoid arthritis<sup>[102]</sup>. It is worth noting that many researchers believe that anti-TNF antibody treatment in patients suffering from autoimmune diseases is so successful because it promotes the recovery of Treg suppressive functions. By contrast, the IL-33 cytokine has been described as positively modulating Treg biology during gut inflammation<sup>[103]</sup>. Indeed, IL-33 signaling can both potentiate TGF- $\beta$ 1 signaling to favor pTreg differentiation and improve Treg functions *in vivo* during intestinal inflammation. In the T cell transfer model of colitis, signals through the receptors for the C3a and C5a complement molecules also decrease Treg suppressive functions<sup>[104]</sup>. The specific suppressive functions of Tregs that are impaired have not been studied, although decreased expression of Foxp3 and CTLA-4 is documented.

### TCR signaling defects impact regulatory T cell functions

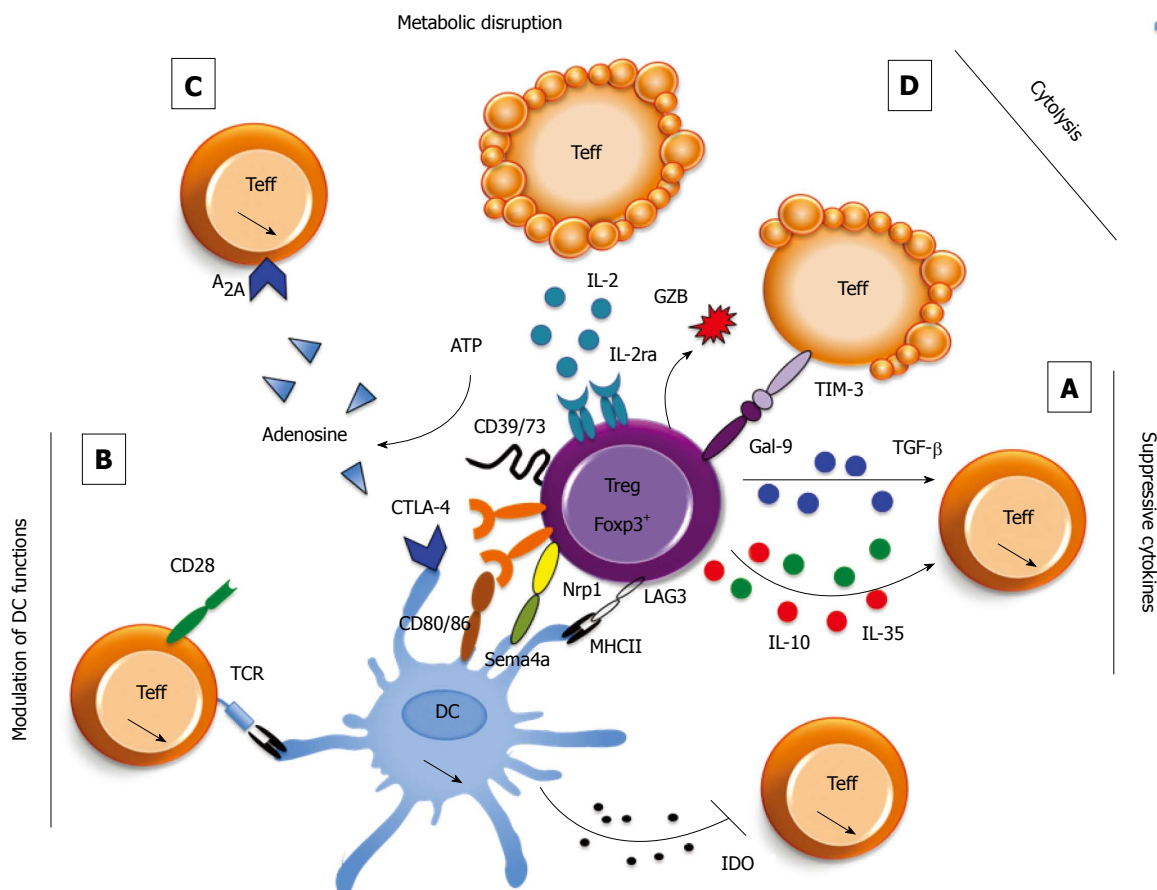
TCR signals are absolutely required for thymic development and the suppressive function of Tregs<sup>[105-107]</sup>. In mice, Ly-6C expression is inversely correlated with the strength of the TCR signals received and divides the Tregs into two subpopulations<sup>[108]</sup>. Ly-6C<sup>+</sup> Tregs exhibit better survival and suppressive functions *in vitro* and in the T cell transfer model of colitis *in vivo*. These data highlight the requirement for a minimal TCR signal for Treg survival and their suppressive functions, which is consistent with the fact that they recognize self-ligands during their recirculation in the secondary lymphoid organs.

T cell-specific defects in genes of the proximal TCR signaling cascade (such as PLC- $\gamma$ 1 and LAT) lead to a partial block in thymic T cell development<sup>[109,110]</sup>. As a consequence, the number of both peripheral Tconv and Tregs is reduced and is associated with the development of autoimmune manifestations, particularly those affecting the gut. The deletion of LAT in T cells reduces the expression of Foxp3, CTLA-4, and CD25 in Tregs and impairs their function<sup>[110]</sup>. This data

has been further confirmed in a study that restricted this defect in TCR signaling to the Treg population<sup>[111]</sup>. Similarly, the LAT-PLC- $\gamma$ 1 interaction in Tregs is necessary for their suppressive functions *in vivo*, as disruption of this interaction leads to the development of a lymphoproliferative syndrome. Specific mutations resulting in an impaired scaffolding function of ZAP-70 also result in a lymphopenia, a loss of the Treg suppressive functions and the development of rheumatoid factor autoantibodies, but it is not known whether those mice develop intestinal lesions<sup>[112]</sup>. Interestingly, mutations affecting the catalytic domain of ZAP-70 have no impact on Treg functions<sup>[113]</sup>. Lck inactivation in Tregs also compromised their homeostatic proliferation *in vivo* and their regulatory functions *in vitro*, but it remains to be determined whether this defect in TCR signaling impairs the Treg functions in a colitis model<sup>[114]</sup>. Moreover, rats deficient in Themis1, a molecule involved in the TCR proximal signaling hub, exhibit CD4<sup>+</sup> T cell lymphopenia, which affects both the Tconv and Treg cell numbers. This phenotype has been associated with a defect in Treg functions and the spontaneous development of intestinal inflammation<sup>[115]</sup>. Furthermore, we reported that an epistatic interaction between *Themis1* and *Vav1* controls regulatory T cell function and IBD development in Themis1-deficient BN rats by modulating TCR signaling strength<sup>[116]</sup>. It would be interesting to analyze the consequences of a conditional deletion of Themis1 in Treg cells to investigate its role in Treg biology and disease development independently of the defect in thymic T cell development and subsequent lymphopenia.

### Suppressive mechanisms of regulatory T cells

Tregs are equipped with several different suppressor mechanisms that allow them to control both the innate and adaptive immune cells in various pathophysiological contexts. Tregs can modulate the activation of diverse cellular populations, including CD4<sup>+</sup> and CD8<sup>+</sup> T cells<sup>[117]</sup>, NK cells<sup>[118]</sup>, NK-T cells<sup>[119]</sup>, B cells<sup>[120]</sup> and macrophages<sup>[121,122]</sup>. Tregs can exert their functions by directly acting on effector populations or through indirect mechanisms that modulate the antigen presenting cell (APC) phenotype<sup>[123]</sup>. Tregs suppressive activities can be divided into two main categories, contact-dependent or contact-independent mechanisms, through the secretion of soluble mediators or metabolic disruption (Figure 1). Briefly, Tregs can modulate APC functions by controlling their expression of major histocompatibility complex (MHC) and costimulatory molecules, thereby limiting the APC-Teff contacts and inducing the production of the immunosuppressive molecule IDO. The expression of CTLA-4, Nrp-1 and LAG-3 in Tregs has been shown to mediate this contact-dependent APC modulation. Tregs are also able to produce immunosuppressive molecules, such as IL-10, IL-35 and TGF- $\beta$ , that can



**Figure 1** Regulatory T cells control immune responses through a large panel of suppressive mechanisms. In order to control Teff functions, regulatory T cells (Tregs) can use diverse immunosuppressive strategies: A: Treg suppression can be mediated through the inhibitory cytokines IL-10, IL-35, and TGF- $\beta$ ; B: Tregs can modulate DC maturation or function through the interaction of CTLA-4, LAG-3 and Nrp1 expressed by Tregs and the CD80/86 costimulatory molecules, MHC class II and Sema4a expressed by DC, respectively leading to IDO production and preventing DC maturation and Teff activation; C: Tregs can disrupt metabolic functions through the expression of the ectoenzymes CD39/73 allowing adenosine generation, or by IL-2 deprivation; D: Tregs can also induce direct killing of Teff through the production of granzyme B (GZB) or via the interaction between Gal-9 expressed by Tregs and TIM-3 expressed by Teff.

act directly on Teffs to inhibit their activation or *via* APCs. The expression of the CD39/CD73 ectoenzymes by Tregs can mediate the conversion of extracellular ATP to adenosine, a metabolite that modulates the functions of Teffs and innate immune cells. Tregs are even able to directly kill their target cells. There is some redundancy among those mechanisms because a deficiency in only one does not recapitulate the dramatic phenotype of the Foxp3-deficient mice. The mechanisms that are employed in specific situations and pathologies constitute an active area of research.

## REGULATORY T CELLS IN ANIMAL MODELS OF IBDs

Murine models of IBDs are useful tools for the study of the pathogenesis and regulation of intestinal inflammation. They can be divided into 4 relatively distinct categories: chemically induced barrier disruption models, spontaneous models (due to genetic mutations), intestinal pathogen-induced models and the model of naive T cell transfer in

immune-deficient mice, which is the most widely used experimental model of IBD. In the last, colitis is induced by an injection of naive ( $CD4^+ CD45RB^{high}$ ) T cells into syngeneic immune-deficient (*e.g.*, SCID or RAG-deficient) mice<sup>[5]</sup>. As a consequence, these animals develop Th1/Th17-mediated colitis that shares some features with human IBDs<sup>[124-127]</sup>. This intestinal pathology is characterized by severe mononuclear cell infiltration into the colon, which is associated with epithelial hyperplasia and goblet cell depletion<sup>[5,128]</sup>. In this model, gut inflammation would be driven by the resident commensal bacteria, given that the immune-deficient recipients that have been transferred with naive T cells but raised under a germ-free environment or flora-restricted conditions are partially protected from colitis development. This experiment suggests that T cells specific for enteric bacteria are present in the inoculated  $CD45RB^{high} CD4^+$  T cells and are most likely responsible for the pathogenesis of the disease<sup>[129]</sup>. Interestingly, the transfer of Tregs can prevent disease development<sup>[130-132]</sup>, providing a model to discover the factors that control the accumulation

and functions of colitogenic T cells as well as Tregs *in vivo*. Importantly, from a clinical perspective, Tregs can also cure established colitis<sup>[4,133]</sup>. Thus, in contrast to the *in vitro* reductionist Treg/Teff coculture model, this mouse model of intestinal inflammation is a potent tool to dissect the Treg immunosuppressive mechanisms *in vivo*.

### Role of Treg suppressive cytokines in IBDs

**TGF- $\beta$ :** The TGF- $\beta$  family consists of three members, TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3, which all have similar roles in the regulation of immune cells. TGF- $\beta$ 1 is a pleiotropic cytokine that has been described for its broad immunoregulatory effects. This cytokine influences the biology of many different immune cells<sup>[134,135]</sup>. TGF- $\beta$ 1 can be secreted or expressed at the cell surface by different non-immune and immune cells, including Tregs. Its bioavailability is tightly regulated; to be functional, TGF- $\beta$ 1, which is secreted as an inactive precursor molecule [combined with latency-associated peptide (LAP)], needs to be cleaved by enzymes (such as furin) or modified by integrins (such as  $\alpha$ v $\beta$ 8 and  $\alpha$ v $\beta$ 6)<sup>[136,137]</sup>. DCs, which express  $\alpha$ v $\beta$ 8 integrins, and Tregs, which express furin, are able to perform this post-translational modification of TGF- $\beta$ 1, again highlighting their potent immunosuppressive function.

TGF- $\beta$ 1 is present in large quantities in the intestine. TGF- $\beta$ 1 is critical for the maintenance of gut homeostasis, because TGF- $\beta$ 1-deficient mice develop lethal multiorgan lymphoproliferative disease, which particularly affects the gut. Of note, this pathology is similar to the one observed in the Foxp3-deficient mice<sup>[138,139]</sup>. CD4<sup>+</sup> T cells are responsible for the phenotype of the TGF- $\beta$ 1-deficient mice, because their depletion improves the animal survival<sup>[140,141]</sup>. The administration of TGF- $\beta$ 1 blocking antibodies is sufficient to inhibit the Treg control of colitogenic Teffs in the T cell transfer model of colitis<sup>[13]</sup>. Activated TGF- $\beta$ 1 acts on Teffs rather than on Tregs because the expression of a dominant negative TGF- $\beta$ -receptor (TGF- $\beta$ R) type II or overexpression of an endogenous inhibitor of TGF- $\beta$ 1, Smad7, in Teffs impairs Treg-mediated suppression<sup>[142,143]</sup>. Surprisingly, TGF- $\beta$ 1-deficient Tregs are still able to prevent colitis, unless TGF- $\beta$  blocking antibodies are administered along with the colitogenic CD45RB<sup>high</sup> CD4<sup>+</sup> T lymphocytes<sup>[142]</sup>. This data highlights the relevance of TGF- $\beta$  production by cells other than Tregs and even raises the question of the relevance of TGF- $\beta$  secretion by Tregs. TGF- $\beta$ 1 signaling seems to be important for Treg survival<sup>[144]</sup>, for extra-thymic induction in pTregs<sup>[145]</sup> and for the inhibition of Th17 cell differentiation<sup>[146]</sup>. Therefore, these data suggest that TGF- $\beta$ 1 would inhibit Teff functions in the context of IBD development, but would also drive the conversion of Teffs into pTregs. Therefore, further investigation is required to determine whether either of these effects is a

dominant immunoregulatory mechanism. In addition, the ability of Tregs to activate TGF- $\beta$  is crucial in the regulation of TGF- $\beta$ 1 production, particularly in the gut. Indeed, furin-deficient Tregs exhibit impaired regulatory function in the T cell transfer model of colitis<sup>[137]</sup>. Likewise, myeloid cells (particularly DCs) participate in intestinal homeostasis by promoting pTreg differentiation, in part, by activating TGF- $\beta$ 1 in the intestinal mucosa through the expression of the  $\alpha$ v integrin<sup>[147,148]</sup>.

**IL-10:** IL-10 is a potent anti-inflammatory cytokine that is produced by both immune and non-immune cells. This cytokine exhibits a broad range of target cell types. IL-10 signaling inhibits T cell proliferation and cytokine secretion<sup>[149-152]</sup>. A Treg-specific IL-10-deficient mouse demonstrated the key role of this cytokine in mucosal homeostasis<sup>[153]</sup>, and its neutralization abolished the Tregs impact on fetal-maternal tolerance<sup>[154]</sup>. The first evidence that IL-10 is a major immunosuppressive cytokine in the gut comes from a study using IL-10-deficient mice<sup>[155]</sup>. These mice spontaneously develop chronic enterocolitis if they are not housed in a specific pathogen-free environment. This data highlights the importance of IL-10 in maintaining the symbiosis between the gut microbiota and the organism. Additional studies confirmed the critical role of IL-10 in intestinal homeostasis by showing that the administration of recombinant IL-10 or intestinal bacteria able to produce IL-10 can dampen an established intestinal inflammation in mice<sup>[124,156]</sup>. IL-10 secretion is one of the key Treg suppressive mechanisms responsible for immunological tolerance to self and environmental antigens at environmental interfaces, particularly in the colon, lung and skin<sup>[153]</sup>. Indeed, Foxp3<sup>+</sup> Treg-specific IL-10-deficient mice spontaneously exhibit signs of inflammation in the colon, lung and skin. In the colon, most of the IL-10-producing Tregs express Foxp3<sup>[157]</sup>. According to these data, in this model, Foxp3<sup>+</sup> Tregs, rather than Tr1 cells, would constitute the main IL-10 source, preventing colitis. Moreover, the secretion of IL-10 by Tregs and other cells also controls the activation of proinflammatory macrophages in an IBD mouse model<sup>[121,122]</sup>. IL-10 can directly suppress the Th1 and Th1+Th17 colitogenic T cells<sup>[158]</sup>, and, interestingly, IL-10 signaling in Tregs is necessary for colitogenic Th17 Teff control<sup>[159]</sup>. Thus, IL-10 secretion by Tregs might amplify the negative regulatory mechanisms, because IL-10 secretion by Tregs directly inhibits colitogenic cell activation, and IL-10 signaling in Tregs is required for Treg suppressive functions. Taken together, these data demonstrate a crucial role for IL-10 in Treg activation and suppressive functions.

**IL-35:** IL-35, a member of the heterodimeric IL-12 cytokine family, has been described as a suppressive cytokine produced by Tregs. In T cells, IL-35 signals



through the homodimers of the IL-12R $\beta$ 2 or gp130 molecules or through IL-12R $\beta$ 2-gp130 heterodimers, leading to STAT1 and STAT4 phosphorylation<sup>[160,161]</sup>. IL-35 is critical for Treg-mediated control of the inflammatory responses in the gut. Indeed, in the T cell transfer model of colitis, mice receiving Tregs from animals deficient in one subunit of the IL-35 receptor were less protected than mice transferred with wild-type (WT) Tregs<sup>[160]</sup>. It has been proposed that IL-35 exerts its regulatory effects by inducing the conversion of Tconv to pTregs. Indeed, CD4<sup>+</sup> T cell activation, which is induced by both IL-35 and TCR signals, promotes the generation of a stable pTreg population<sup>[162]</sup>. Interestingly, these Foxp3<sup>+</sup> pTregs are very efficient in controlling inflammation, including colitis, *in vivo* in an IL-10- and TGF- $\beta$ 1-independent manner.

### **Regulatory T cell-mediated metabolic disruption in IBDs**

Tregs highly express the ectoenzymes CD39 and CD73. These two molecules can metabolize extracellular ATP to ADP, 5'-AMP, and eventually adenosine<sup>[163,164]</sup>. Thus, Tregs are able to generate adenosine, which is an immunomodulatory molecule. Adenosine nucleosides signal through four different receptor subtypes (A<sub>2A</sub>), which are expressed by diverse cells, particularly immune cells (APC, Teff and Tregs)<sup>[165,166]</sup>. Thus, Tregs can also moderate inflammatory responses by consuming ATP, a well-described inflammatory molecule, or by generating adenosine, which positively impacts Treg activation but also decreases Teff functions<sup>[167-169]</sup>. Adenosine can not only inhibit the *in vivo* development of both Th1 and Th17 CD4<sup>+</sup> T responses but may also act on DCs, neutrophils and macrophages<sup>[170]</sup>. In addition, adenosine signaling can induce the generation of LAG-3<sup>+</sup> pTregs<sup>[171]</sup>, allowing Tregs to inhibit DC maturation after interactions with their MHC class II molecules<sup>[172]</sup>. Because adenosine is widely expressed by diverse cell types, the specific role of the Treg-generated adenosine remains to be determined. In the context of IBDs, the administration of the highly selective A<sub>2A</sub> adenosine receptor agonist ATL-146e decreases the disease severity in both acute and chronic colitis models<sup>[173]</sup>. Moreover, the expression of A<sub>2A</sub> adenosine receptor by colitogenic Teffs is crucial because A<sub>2A</sub> adenosine receptor-deficient Teffs are resistant to Treg-mediated suppression<sup>[174]</sup>. Interestingly, it has been shown that CD39 deficiency exacerbates murine intestinal inflammation<sup>[175]</sup>, but to date, it has not been directly shown that CD39 expression by Tregs is absolutely required for them to exert their suppressive functions in this inflammatory context. In contrast, CD73 expression by Tregs plays a nonredundant role in their regulatory effects because CD73-deficient Tregs are unable to control WT colitogenic CD4<sup>+</sup> CD45RB<sup>high</sup> Teffs in the T cell transfer model of colitis<sup>[176]</sup>.

### **Induction of effector T cell apoptosis by regulatory T cells in IBDs**

In addition to immunoregulatory cytokine production, Tregs may suppress immune responses by killing effector T cells. Tregs are able to induce Teff apoptosis using the granzyme machinery. Indeed, granzyme B is expressed by activated Tregs and promotes immunosuppression by directly killing effector cells or APCs<sup>[177,178]</sup>. This suppressive mechanism might be implicated in transplantation<sup>[179]</sup> and tumor<sup>[180]</sup> models *in vivo*, but its role in IBDs is still unknown. The constitutive expression of CD25 by Tregs not only promotes their peripheral maintenance but can also permit them to preferentially bind IL-2 and deprive the effector cells of this critical cytokine for T cell proliferation, inducing apoptosis<sup>[181]</sup>. This mechanism of Teff control has been demonstrated *in vitro*, but the direct proof that Tregs increase Teff apoptosis through cytokine deprivation in the T cell transfer model of colitis *in vivo* is lacking and difficult to obtain. Finally, Gal-9 might also be involved in this mechanism. Indeed, Gal-9 is highly expressed in pTregs but not tTregs<sup>[182]</sup>. Gal-9 deficiency impairs pTreg regulatory functions both *in vitro* and *in vivo*; this deficiency also impairs pTreg stability, a feature associated with decreased Foxp3 expression and Smad3 activation<sup>[182]</sup>. Interestingly, Gal-9-deficient pTregs are unable to control intestinal inflammation in the T cell transfer model of colitis. Gal-9 may function *in vivo* by regulating Teff apoptosis or exhaustion<sup>[183,184]</sup>.

### **Modulation of antigen presenting cell biology by regulatory T cells in IBDs**

The maturation of APCs, particularly DCs, is necessary to fully activate Teffs in both physiological and pathological conditions. A key mechanism used by Tregs to suppress immune responses is interference with the DC maturation process. Indeed, *in vivo* two-photon real-time experiments nicely showed that Teffs and Tregs directly interact with DCs in different contexts<sup>[185-189]</sup>. Tregs are able to modulate DC maturation and promote the immunosuppressive function of APCs through diverse mechanisms.

The CTLA-4 and CD28 glycoproteins share the CD80 and CD86 ligands expressed by activated APCs (such as DCs and B cells). The CD80 and CD86 molecules are recognized by CTLA-4 with a higher affinity than CD28, allowing CTLA-4 to limit CD28-dependent costimulation and consequently limit T cell activation<sup>[190]</sup>. Therefore, CTLA-4 is a potent negative regulator of T cell activation. Indeed, the disruption of the CTLA-4 gene in mice results in fatal multiorgan inflammation very early in life, which particularly affects the gut<sup>[191,192]</sup>. The constitutive expression of CTLA-4 by Tregs plays a key role in their immunosuppressive function. Treg-specific CTLA-4-deficient mice develop a fatal multiorgan pathology

characterized by an exacerbated Th1/Th2 response and autoantibody production<sup>[193]</sup>. CTLA4 expression by Tregs confers at least two main functions to Tregs: the modulation of APC maturation and the induction of indoleamine 2,3-dioxygenase (IDO) in APCs. The engagement of CTLA-4 has been shown to deplete CD80 and CD86 expression on DCs by trans-endocytosis<sup>[194]</sup>. Given that CTLA-4 and CD28 share the costimulatory CD80 and CD86 ligands expressed by APCs, CTLA-4 engagement reduces the activation potential of DCs by inhibiting costimulation of Tregs through CD28 engagement<sup>[193,194]</sup>. In addition, CTLA-4 engagement with its ligands expressed on DCs also represents a major regulatory mechanism through the induction of IDO production<sup>[195]</sup>. The IDO produced by DCs inhibits Treg proliferation by depriving them of IDO-catabolized tryptophan. Furthermore, the metabolites generated during tryptophan catabolism can also induce T cell apoptosis<sup>[195]</sup>. In the T cell transfer model of colitis, the administration of CTLA-4 blocking antibodies is sufficient to inhibit the control of colitogenic Tregs by Tregs<sup>[13]</sup>. Interestingly, CTLA-4-deficient Tregs exhibit an impaired ability to inhibit both Treg proliferation and activation *in vitro* and *in vivo* in models of gastrointestinal inflammation<sup>[193,196]</sup>. Moreover, Treg-specific CTLA-4-deficient mice develop systemic lymphoproliferation and fatal T cell-mediated autoimmune disease, pathologies similar to those developed by CTLA-4-deficient mice that also affect the intestine. Together, these data identify the major role of CTLA-4 in Treg function and, more importantly, in the maintenance of immune homeostasis. Of note, a recent study showed that CTLA-4 expression promotes pTreg production and Treg accumulation in the intestine<sup>[197]</sup>.

Neuropilin 1 (Nrp-1) is involved in the physical interaction between Tregs and DCs. Nrp-1 inhibition decreases the interaction time between Tregs and immature DCs, whereas Nrp-1 overexpression in Tregs increases their interactions with APCs<sup>[198]</sup>. Therefore, Treg-specific Nrp-1 expression enhances their capacities to respond to the low antigen quantities presented in the absence of inflammatory signals and potentially modulates their functions. Nrp-1 expression by Tregs promotes their inhibition of anti-tumor responses<sup>[199,200]</sup>. Likewise, the interaction between neuropilin expressed by Tregs with semaphorin-4a expressed by the DCs allows the Tregs to modulate DC activation<sup>[200]</sup>. This mechanism is particularly important for the Treg suppressive functions in the T cell transfer model of colitis because neuropilin-deficient Tregs are unable to cure an established colitis. Tregs can also exert their suppressive function by controlling DC homeostasis, a model of particular interest in the context of IBDs<sup>[201]</sup>.

### **Regulatory T cell migration defects and IBDs**

It seems evident that highly coordinated Treg tra-

fficking is crucial for Tregs to be in the right place at the right time, but several studies also argue that this well-regulated Treg trafficking is necessary for the generation of a tailored phenotype to efficiently address the situation. This trafficking has been extensively studied in the intestine. Both  $\beta 7$  integrin and CCR9 expression play a role in T cell trafficking in the gut<sup>[202-204]</sup>. The expression of these molecules can be induced by RA secretion by intestinal DCs and non-hematopoietic stromal cells<sup>[205-207]</sup>. In the intestinal context of oral tolerance, RA is also involved in the conversion of Tconv cells to pTreg cells<sup>[208]</sup>. *Ex vivo* pretreatment of Tregs with RA enhances Treg suppressive functions in a model of acute but not chronic colitis<sup>[209]</sup>. Thus, RA has critical roles in regulating T cell migration towards intestinal inflammatory sites and in promoting pTreg production in the gut. Interestingly,  $\beta 7$  integrin-deficient Tregs retain their ability to suppress colitis, even if they may not populate the intestine, suggesting that, in this model, migration to the mesenteric draining lymph nodes is sufficient to control intestinal inflammation<sup>[210]</sup>. In agreement with these data, in the T cell transfer model of colitis, the transferred CCR4- or CCR7-deficient Tregs are unable to reach the mesenteric lymph nodes, leading to impaired control of colitogenic Tregs and the development of colitis<sup>[211,212]</sup>. Taken together, these data indicate that Tregs must enter the mesenteric lymph nodes to exert their suppressive function and prevent IBD development but do not necessarily need to migrate into the intestinal mucosa. However, one study contradicts this hypothesis. Indeed, in the T cell transfer model of colitis, the transferred CCR6-deficient Tregs are less efficient in their migration to the colonic mucosa but not to the mesenteric lymph nodes, and they are also unable to control colitis development compared to WT Tregs<sup>[213]</sup>. CCR6 is the receptor for the chemokine CCL20. Because CCL20 is highly expressed in the inflamed colon of humans and mice suffering from IBDs, CCR6 expression by Tregs may be mandatory for their migration into the inflamed gut. Thus, it is still an open question whether Tregs need to be present at the inflammatory sites in the context of IBDs.

## **REGULATORY T CELLS IN HUMAN IBDs**

Experimental models have clearly shown that Tregs can not only prevent the development of intestinal inflammation<sup>[5]</sup>, but they can also control an established IBD<sup>[133]</sup>. In humans, Tregs also play a key role in controlling intestinal homeostasis and therefore represent an attractive therapeutic target for clinical treatments. The most striking evidence of Treg involvement in the control of intestinal homeostasis is that intestinal inflammation is the most prominent feature of human immune dysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX)

syndrome, which is caused by mutations of the *Foxp3* gene<sup>[214]</sup>.

Numerous studies investigating Tregs in human IBDs show that patients exhibit decreased Treg numbers in the blood and increased Treg numbers in the inflamed intestinal mucosa compared to the uninfamed tissues<sup>[215-218]</sup>. The disease activity has a differential impact on the circulating and mucosal Treg numbers<sup>[215,218]</sup>. The circulating Treg percentages inversely correlate with disease activity<sup>[215,219]</sup>, and a concomitant increase in the mucosal Treg numbers is associated with a reduction of the circulating Treg population in active disease<sup>[220]</sup>. The increased Treg localization in the inflamed mucosa could be due to an active recruitment and expansion of this population in inflamed areas in an attempt to regulate the inflammation. Mucosal Treg recruitment could be responsible for the reduction of circulating Tregs. However, Treg accumulation in the inflamed intestines is also observed in other inflammatory conditions, such as diverticulitis or CMV-induced colitis<sup>[215,221]</sup>, where it does not correlate with a reduction in the number of circulatory Tregs. The Treg increase in the inflamed bowel mucosa is not as strong in IBD patients as in other intestinal inflammatory diseases<sup>[215]</sup>. This suggests that although they are recruited to the inflamed areas, Tregs may still be present in insufficient numbers in IBD lesions to efficiently control the inflammation, or that they exert their immunoregulatory functions in a different location, such as the mesenteric lymph nodes. This question remains unresolved due to obvious limitations in studying the cells from patient and healthy control mesenteric lymph nodes. Increased caspase activation and PARP cleavage is observed in blood Tregs as well as mucosal Tregs in UC and CD patients<sup>[222]</sup>, suggesting that their anti-inflammatory effect might be limited due to increased apoptosis. A more recent study in patients with Crohn's disease corroborated these findings and also revealed that the apoptosis of circulating Tregs was higher in female than in male patients<sup>[223]</sup>. Although no causal link can be established from the current knowledge, this increased Treg apoptosis is correlated with the increased prevalence and severity of the disease in females. Retrospective studies examining the evolution of the circulating Treg counts before disease onset and during the disease would be of great interest to determine whether a decreased Treg count precedes the disease occurrence or is secondary to the appearance of symptoms.

In many studies, the Tregs from IBD patients were functional in *in vitro* suppression assays. This has been shown for both the peripheral blood<sup>[215]</sup> and mucosal Tregs<sup>[217,218,220,224]</sup> of CD and UC patients. A potential reason for the development of a deleterious immune response, despite the presence of functional Tregs in the inflamed mucosa, could be the resistance of Tregs to the immunosuppressive effects of TGF- $\beta$ . The

upregulation of SMAD7 in the intestinal mucosa of IBD patients renders the cells resistant to Treg inhibition<sup>[225]</sup>. However, other studies have reported different results, showing that IBDs can be associated with functional defects of Tregs<sup>[226,227]</sup>. It is impossible to determine whether these defects are initially responsible for the disease development in those patients or is secondary to the excessive inflammation triggered by other mechanisms. A whole exome sequencing study in a genetic familial case of IBDs identified a mutation in the *Foxp3* gene linked to impaired Treg suppressive activity *in vitro*<sup>[227]</sup>, suggesting that a Treg defect can be a key cofactor for disease development. In addition, human genetic studies have pinpointed that mutations in the IL-10R genes that abrogate IL-10 signaling can lead to inflammatory disease development<sup>[228]</sup>, demonstrating the relevance of this key immunosuppressive cytokine to human pathology. Because IL-10 production by Tregs is critical in mouse colitis models, IL-10 production in human Tregs may also represent a central mechanism for intestinal homeostasis maintenance. Interestingly, disruption of the *Themis1* gene in rats leads to a functional Treg defect that is associated with spontaneous IBD development<sup>[115]</sup>, and a *Themis1* polymorphism has been linked to Crohn's disease in humans<sup>[229]</sup>. It is not known whether any qualitative or quantitative Treg defects caused by *Themis1* polymorphisms contribute to disease development in those patients. IBDs are complex, multifactorial diseases that depend on an intricate interplay between genetic and environmental factors. Although genetic Treg defects may not occur in the majority of cases as the primary cause of IBD, the association of Treg defects with other genetic or environmental variables is likely to contribute to disease development and/or severity.

An increase in the number of circulating T cells co-expressing the Treg transcription factor *Foxp3* and the Th17 cytokine IL-17 is observed in CD and UC patients<sup>[226]</sup>. These *Foxp3*<sup>+</sup> IL-17<sup>+</sup> T cells also express the Th17 transcription factor ROR $\gamma$ t, revealing an intermediate or transitional phenotype between the Treg and Th17 subsets. *Foxp3*<sup>+</sup> IL-17<sup>+</sup> T cells can be found in the mucosa of IBD patients. One study identified *Foxp3*<sup>+</sup> IL-17<sup>+</sup> double positive T cells in the intestinal mucosa of CD but not UC patients<sup>[230]</sup>, whereas another study reported their presence in UC patients<sup>[231]</sup>. The *Foxp3*<sup>+</sup> IL-17<sup>+</sup> T cells are likely to represent a transitional stage between Tregs and Th17 cells. They would be induced by the inflammatory microenvironment or would come from incompletely differentiated Tregs. Whether these cells retain their suppressive activity remains controversial. A significant decrease in the Tregs ability to suppress autologous T-cell proliferation *in vitro* was associated with an increased proportion of IL-17<sup>+</sup> T cells among the *Foxp3*<sup>+</sup> Tregs<sup>[226]</sup>, while another study concluded that the mucosal *Foxp3*<sup>+</sup> IL-17<sup>+</sup> T cells from UC patients

showed suppressor capacities *in vitro*<sup>[231]</sup>. Interestingly, additional investigations of the mucosal Foxp3<sup>+</sup> IL-17<sup>+</sup> T cells functional capacities in the latter study revealed that they increased IL-1 and IL-6 inflammatory cytokine production by colonic tissue cultures in an IL-17-dependent manner<sup>[231]</sup>. Thus, the acquisition of pro-inflammatory capacities by Tregs, concomitant with the loss of suppressive activity is likely to contribute to the uncontrolled inflammation *in vivo*.

One major caveat for understanding Treg function in IBD pathogenesis is the lack of reliable markers for identifying Tregs. The classical CD25 and Foxp3 markers, which are used in many studies, can also be expressed by activated Teffs<sup>[232]</sup>. Therefore, drawing firm conclusions will require revisiting those studies using a more refined phenotype for Treg identification. Currently, the identification of human Tregs under non-inflammatory conditions is based on their high CD25 expression and low CD127 expression. However, in rheumatoid arthritis patients, CD127 is also downregulated on activated effector T cells under inflammatory conditions<sup>[17]</sup>.

## REGULATORY T CELLS AS THERAPEUTIC TOOLS FOR IBD TREATMENT

Some treatments that were not designed to specifically target Tregs exert beneficial effects on the disease and a concomitant impact on Tregs. Lymphocytapheresis and *Granulocyte/monocyte apheresis (GMA)*, therapies that deplete the circulating immune cells in patients with active UC so they cannot reach the target organ and promote inflammation, have showed positive clinical results. The clear clinical efficacy of GMA in UC patients with severe disease who are refractory to conventional treatments is associated with a significant increase in the circulating Treg numbers<sup>[233]</sup>. Furthermore, this increase was specifically observed in responder patients<sup>[234,235]</sup>. The numbers of mucosal Tregs were concomitantly decreased in the responders, likely because of the reduced inflammation. Together with depletion of the pathogenic populations, this increased circulating Treg ratio is likely to exert a beneficial impact. An anti-TNF treatment associated with clinical benefits also increases the circulating Treg numbers and decreases the mucosal Treg infiltrates of IBD patients, particularly the responders<sup>[219,236,237]</sup>. The anti-TNF treatment can reverse the increased Treg apoptosis observed in UC patients<sup>[222]</sup>, increase their suppressive functions *in vitro*<sup>[237]</sup> and increase the seric levels of TGF- $\beta$  and IL-10 in responder CD patients<sup>[238]</sup>. Combined with the neutralization of this important proinflammatory cytokine, the impact of the anti-TNF treatment on Tregs is highly likely to also contribute to its clinical benefits. A low dose IL-2 treatment that selectively targets Treg expansion, likely because of their high expression of IL2R $\alpha$  (CD25)<sup>[239]</sup>, has shown

promising results in patients with type-1 diabetes<sup>[240]</sup>. A clinical trial investigating the impact of low dose IL-2 treatment in several autoimmune and inflammatory diseases including UC and CD is currently ongoing (TRANSREG, NCT01988506).

Treg transfer can cure intestinal pathology in mice<sup>[133]</sup>. This prompted the scientific community to use Tregs as a therapeutic modality for IBD treatment. The generation of new protocols allowing the large-scale expansion of Tregs from a small initial population has made possible the use of autologous Treg therapy to treat the disease in humans. This strategy has already been successfully applied to graft-versus-host disease<sup>[241]</sup> and type-1 diabetes<sup>[242]</sup> and has shown promising clinical efficiency.

In a first escalating dose phase I and II a clinical trial, autologous ovalbumin-specific Tregs (OVA-Tregs) were expanded *in vitro* and injected back into 20 patients with refractory CD<sup>[243]</sup>. Although ovalbumin is not implicated in intestinal inflammation in animal models or in patients with CD, this common diet antigen was chosen to ensure OVA-Treg antigen-specific activation in the gut. A reduction of CD disease activity was observed in 40% of the patients. This promising study has led to the development of a larger, ongoing, placebo-controlled clinical trial to evaluate the impact of autologous Treg cell therapy in patients with active Crohn's disease who are refractory to conventional treatments (NCT02327221 Ovasafe).

Treg-based therapies present the risk of adverse effects, such as increased sensibility to infections or cancer development caused by immunosuppression. These off-target effects are likely to be reduced by controlling the antigen specificity of the Tregs. Additionally, the phenotype of the starting population and the culture conditions are critical to obtain the maximal purity of the therapeutic Tregs and ensure phenotypic stability. In this regard, a recent study suggested that CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>low</sup> CD45RA<sup>+</sup> Tregs might be the most appropriate population from which to expand Tregs for autologous Treg therapy for CD to reduce the potential deleterious effects of Th17 conversion<sup>[244]</sup>.

## CONCLUSION

There is little doubt that Tregs represent one of the major research areas in immunology. Their critical role in intestinal homeostasis maintenance is now well established because defects affecting Treg development or suppressive functions have clearly been associated with IBD development, and Tregs can be used to cure an established IBD. This has led to an increasing interest in the possibility of using Tregs as targets for therapy to preserve intestinal integrity. However, although substantial progress in understanding Treg biology has been achieved in the past 10 years, currently, no phenotypic marker can



be used to isolate pure Tregs for cellular therapy. The identification of such markers, together with developing a better understanding of the extrinsic factors that impact Treg stability and function, represents a great challenge. The recent discovery of the critical role of epigenetic modifications that affect Treg stability constitute an important advance in our understanding of Treg lineage specification. The identification of factors that control these modifications and their maintenance in peripheral Tregs will hopefully pave the way for the design of new strategies aimed at improving Treg stability and functions in the context of Treg transfer therapies.

In addition, when developing new strategies to improve Treg function, we also need to consider the fact that excessive Treg activity could lead to adverse effects by coincidentally impairing protective immunity towards pathogens and tumors. Therefore, investigations of how Tregs function, how they are activated/inactivated, and the effector mechanisms that are required for the control of various immune responses are critical to specifically target some disease-specific mechanisms and limit the potential negative side effects.

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