



Molecular mechanisms underlying Th1-like Treg generation and function

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Abstract Since their ‘re-discovery’ more than two decades ago, FOXP3⁺ regulatory T cells (Tregs) have been an important subject of investigation in the biomedical field and our understanding of the mechanisms that drive their phenotype and function in health and disease has advanced tremendously. During the past few years it has become clear that Tregs are not a terminally differentiated population but show some degree of plasticity, and can, under specific environmental conditions, acquire the phenotype of effector T cells. In particular, recent works have highlighted the acquisition of a Th1-like phenotype by Tregs in several pathological environments. In this review we give an update on the concept of Treg plasticity and the advances in defining the molecular mechanisms that underlie the generation of Th1-like Tregs during an immune response and in different disease settings.

Keywords Foxp3 · Plasticity · Th1-Tregs · AKT · Therapy

Regulatory T cells control the immune system

The immune system is armed with a variety of effector mechanisms to recognize and destroy foreign pathogens as well as with several peripheral tolerance processes to

maintain tolerance to self. In this context, cells with regulatory capacity are crucial for maintaining immune homeostasis and peripheral tolerance, and they play an essential role in controlling autoimmune responses, allergies and limiting immunopathology [1–6]. While the spectrum of regulatory cells is wide and comprises many different cell types, naturally occurring regulatory T cells (Tregs) is the best studied population of cells with suppressive capacity [6]. They are generated in the thymus at the stage of CD4 single-positive thymocytes [7] as a separate cell lineage, and are thought to be enriched in self-reactive T-cell receptors (TCR), which is essential for maintenance of self-tolerance. As with the development of other cell lineages in the thymus [8, 9], a specific requirement for TCR signaling is essential for the induction of its lineage specification transcription factor FOXP3 and Treg cell lineage commitment, and TCR specificity plays a critical role in this differentiation. Studies with TCR transgenic mice [10–13] and sequence analysis of polyclonal TCR repertoires from Tregs as compared to conventional T cells bearing a single transgene-encoded TCR β chain [14–16] have led to the conclusion that Treg cell selection is probably instructed by TCRs with affinities for self-peptide–MHC complexes that are of an intermediate affinity between those that induce positive selection of non-Treg cells and those that mediate negative selection of self-reactive T cells. Medullary thymic epithelial cells (mTECs) are the major antigen-presenting cells in the thymus, and their phenotype and function are mediated in part by the transcription factor Aire, which regulates the expression of a multitude of genes, including antigens characteristic of fully differentiated cells in peripheral tissues, shaping the array of self-peptides presented by MHC and thus, the negative selection of effector T cells [17–19] and the positive selection of Tregs [20–23]. Additional

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signals needed for Treg differentiation in the thymus include cytokines such as IL-2 [24] and to a lesser extent IL-7 and IL-15 [25], and costimulatory molecules, among which, CD28 plays an important role in promoting natural Treg differentiation. Mice studies have shown that mice deficient in CD28 or its ligands CD80 and CD86 have significantly impaired Tregs [26, 27], whereas CTLA-4 ablation results in a higher frequency of natural Tregs [28]. Both TCR and CD28 signaling trigger a myriad of intracellular signaling events that lead to the activation of transcription factors, including NF κ B, which has been shown to be essential for natural Treg generation [29–31]. Lineage specificity is imprinted in early phases during development by the expression of FOXP3 [32] and by the induction of stable epigenetic changes [33–35].

Tregs are characterized by the expression of the master transcription factor FOXP3 [36, 37], the IL-2 receptor alpha chain, CD25 [6, 38], and the low expression of the IL-7 receptor alpha chain, CD127 [39]. Many other markers have been described to be expressed in subpopulations of Tregs, arguing for heterogeneity within this population. IL-2 is an essential cytokine for Treg homeostasis and function. Tregs constitutively express high levels of CD25, and IL-2 is essential to preserve tolerance by influencing Treg homeostasis and activation [40, 41]. In mice, knocking out or blocking IL-2 or CD25 results in Treg deficiency, impaired Treg development and reduced Treg function [42]. Furthermore, IL-2-driven signals through the JAK/STAT signaling pathway directly stabilize FOXP3 expression through STAT5 activation, subsequently driving their suppressive function [43, 44]. Due to the absence of IL-2R signaling, IL-2 and CD25 knock out mice exhibit lethal autoimmunity caused by uncontrolled CD4⁺ T cell activation and proliferation [45, 46]. Treg numbers are reduced in these mice [47] and co-transfer of functional Tregs can prevent autoimmunity [48], highlighting the importance of IL-2 signaling on Tregs for their role in controlling immune responses.

In the setting of an immune response, CD4⁺ naïve T cells produce massive amounts of IL-2 and upregulate CD25, resulting in a self-enhancing loop that favors augmented CD4⁺ T cell activation, proliferation and polarization into T helper effector CD4⁺ T cells [49]. To counteract increased effector T cell activation, Tregs respond to IL-2 and activate mechanisms to regulate effector T cells to prevent uncontrolled pro-inflammatory and potentially harmful responses.

FOXP3 as a master regulator of the Treg lineage

FOXP3 is crucial for Treg development [37, 50], function [51, 52] and maintenance [53]. Thus, forced expression of FOXP3 in CD4⁺ T cells results in the acquisition of a

regulatory phenotype, although it does not completely recapitulate Treg gene-specific signature [54, 55], and experimental deletion of the *FOXP3* gene in Tregs results in the loss of their suppressive capabilities [32, 37, 53]. Mutations in the *FOXP3* gene leads to the human autoimmune immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), characterized by a loss of Treg function and severe autoimmunity. Patients with IPEX suffer from early-onset insulin-dependent diabetes mellitus, thyroiditis, massive lymphoproliferation, eczema, enteropathy and other autoimmune pathologies that are usually fatal during the first years of life [56, 57]. Due to its essential role in maintaining Treg function and stability, it is not surprising that *Foxp3* expression is tightly regulated. Transcription of *Foxp3* gene has been shown to be modulated at the epigenetic level [58], and FOXP3 protein expression and stability may be controlled by post-translational modifications such as phosphorylation [59–61], acetylation [62, 63] and ubiquitination [64, 65], among others. Experiments with genetically engineered mouse models have shown that the genomic region of the *Foxp3* locus has several conserved non-coding sequences (CNS1, CNS2, CNS3), which perform diverse functions in the regulation of *Foxp3* transcription. CNS1 region contains binding sites for NFAT and AP-1, being important for peripheral generation of adaptive Tregs [58, 66], while CNS3 plays a role in both natural and adaptive Treg generation and contains binding sites for transcription factors such as c-Rel [58]. Runx1-CBF β complexes bind to CNS2 region to control *Foxp3* expression and stability [67]. Moreover, epigenetic modifications of highly conserved regions within CNS in the *Foxp3* locus are involved in the transcription of *Foxp3*. Thus, CNS2 contains a conserved CpG island (TSDR region) that is highly demethylated in natural Tregs and hypermethylated in conventional CD4⁺ T cells [34, 68, 69], which determines *Foxp3* expression and the stability of the Treg lineage [33, 69, 70]. This TSDR region has been widely used to distinguish *bona fide* Tregs from T cell populations that can transiently upregulate FOXP3 upon activation [71]. Lastly, although FOXP3 is an essential transcription factor required by Tregs to maintain their phenotype and function, over the last few years several works in the literature have demonstrated that FOXP3 does not function alone but forms protein complexes with more than 300 potential partners [72]. Many of these partners are transcription factors such as, among others, NFAT, Gata-3, Smad, Runx1 and FOXO [66, 72–75]. These transcription factors have been shown to be required to define the Treg cell phenotype and to establish their unique transcriptional program [76].

Functionally, Tregs utilize cell–cell contact mechanisms and soluble factors to inhibit the activation of many different cell types. Thus, Tregs can suppress not only CD4⁺ and

CD8⁺ T cells [77] but also other immune cells such as B lymphocytes [78–81], dendritic cells [82–84], monocytes [85, 86], and NK cells [87, 88], as well as non-immune cell types such as osteoclasts [89, 90], underscoring the importance of this population to maintain immune homeostasis.

FOXP3[−]CD4⁺ T cells in the periphery can also acquire FOXP3 expression and suppressive function when they encounter their cognate antigen in the presence of TGFβ and IL-2 under certain environmental conditions. These Tregs are termed adaptive or induced Tregs (iTregs), and they show important epigenetic differences as compared to natural Tregs; however, we currently lack specific markers that distinguish both populations [91].

Finally, FOXP3 expression also defines a population of CD8⁺ T cells with regulatory capacity both in mice and humans that seems to play a role in autoimmune, infectious and transplantation settings [92, 93], although their origin and their function in the immune response in these disease scenarios is less studied than those of CD4⁺ Tregs. Interestingly, some early reports suggested that their suppressive function mainly depends on HLA-E recognition [94, 95] and is mediated by IFNγ secretion [96, 97], although the molecular mechanisms underlying this observation have not been examined in depth.

Regulatory T cell plasticity

Traditionally, Tregs have been considered as a stable cell lineage with strong suppressive capabilities and a terminally differentiated phenotype. But the idea of phenotype irreversibility has been recently challenged by a body of work demonstrating that Tregs are not a completely committed cell lineage, but can retain some degree of plasticity. This observation is not surprising in the context of an immune response, as multitude of works have clearly demonstrated that cell plasticity is an inherent property of most, if not all, immune cells that helps them adapt their phenotype and function to the changing environment [98–102]. In this regard, it is important to distinguish between functional plasticity and lineage instability. For the purpose of this review, we will consider functional plasticity as the capacity of Tregs to acquire a different phenotype due to environmental cues, anatomical location, among other factors, but maintaining either FOXP3 expression or Treg-specific epigenetic patterns. As such, three major plasticity events have been described with regards to Treg phenotype and/or function:

‘ex-FOXP3’ cells

Cell-fate reporter mice have revealed that under certain inflammatory conditions, a small number of Tregs can lose

FOXP3 expression and acquire effector-like phenotypes (‘ex-FOXP3’ cells), producing pro-inflammatory cytokines such as IL-2 and TNF and contributing to inflammation [103–106]. In some experimental conditions, these ex-FOXP3 cells appear to retain the Treg-specific epigenetic signature, potentially being able to be reconverted to FOXP3⁺ Tregs in the absence of the environmental cues that induced loss of FOXP3 expression [107]. The de-differentiation of Tregs into effector-like cells has also been observed in humans under several pathological settings [108–110].

In relation to the loss of FOXP3 expression on Tregs, other studies have shown that FOXP3 degradation favors the secretion of cytokines such as IL-2, TNF and IFNγ and the decrease in suppressive function, especially in type I pathogenic settings [64]. The E3 ubiquitin ligases Stub1 and USP21 seem to play antagonistic roles in modulating the degradation of FOXP3 with Stub1 promoting degradation while USP21 stabilizing FOXP3 expression [64, 65]. Pro-inflammatory cues such as cytokines and LPS signaling induce K48-linked polyubiquitination of FOXP3 by its interaction with Stub1, resulting in FOXP3 degradation, increased expression of IFNγ and reduced expression of characteristic Treg genes like CD25 and CTLA-4 [64] and subsequently disrupting Treg function. On the contrary, specific deletion of the ubiquitin ligase USP21 on mice Tregs induces an immune disorder characterized by increased expression of IFNγ by effector cells, and a Th1-like phenotype by Tregs. USP21 prevents FOXP3 degradation through deubiquitination, thus stabilizing Treg phenotype and antagonizing the development of Th1-like Tregs [65]. While USP21 and Stub1 directly interact with FOXP3, the E3 ubiquitin ligase VHL indirectly regulates Th1-like Treg generation by increasing the expression of HIF-1α, which binds to the *IFNG* promoter, increasing IFNγ production [111].

Treg plasticity as a means of controlling immune responses and/or adapting to the tissue where they reside

Several studies have demonstrated that Tregs utilize the transcription factor program of the population they are suppressing. Thus, Tregs that express T-BET efficiently suppress type 1 inflammation [112], IRF4 expression on Tregs is essential for controlling Th2 responses [113], and STAT3 is utilized by Tregs to control Th17 responses [114] in mouse models of inflammation.

Recent studies in mice have also indicated that tissue-resident Tregs show a distinct gene expression pattern and TCR usage as compared to circulating Tregs [115–117]. For instance, the peroxisome proliferator-activated receptor gamma (PPARγ) was identified as the characteristic

transcription factor utilized by fat tissue-resident Tregs to maintain their unique phenotype [115]. Interestingly, PPAR γ is predominantly expressed on adipocytes, where its function is to regulate adipocyte differentiation and to mediate glucose metabolism [118]. This suggests that tissue-specific Treg phenotypes are also driven by tissue-specific transcription factors, introducing an additional type of plasticity. Similarly, other studies have identified roles for Treg cells in muscle repair [119, 120], regulation of skin homeostasis, and prevention of skin infections [121]. Both muscle- and skin-derived Tregs show a differential gene expression pattern as compared to blood- and fat-derived Tregs, indicating that Tregs might adapt their phenotype and function to the tissue they populate, a process that is probably mediated by the specific tissue microenvironment.

Th-like Tregs

Lastly, under certain inflammatory settings, some Tregs can acquire an effector T helper (Th)-like phenotype with the capacity to express pro-inflammatory cytokines, mainly IFN γ (Th1-like Tregs), IL-17 (Th17-like Tregs) and IL-13 (Th2-like Tregs), and lose suppressive capacities while maintaining FOXP3 expression. In this regard, little is known about the phenotype and function of Th17-like Tregs under inflammatory conditions. For instance, when Tregs are stimulated in vitro in the presence of dectin-1-activated dendritic cells (DC), they upregulate ROR γ t and express IL-17 [122]. Interestingly, Th17-like Tregs have been observed in vivo in humans under physiological conditions [123, 124] and in mice, preferentially located in the intestine [125]. Human Th17-like Tregs seem to maintain their suppressive capacity despite IL-17 expression in healthy individuals [123, 124], and they can be induced in vitro by stimulation of Tregs in the presence of IL-6 and IL-1 β [123]. These cells could have pathogenic potential, contributing to mucosal disease and being involved in the development of colon cancer [110, 126, 127] and inflammatory bowel disease [128]. In this respect, Saito et al. have recently observed that some tumors from patients with colorectal cancer contain an increased frequency of Foxp3^{low} Tregs with increased IL-17 secretion and decreased suppressive function as compared to peripheral blood, and this group of patients seems to have a better prognosis than those who do not display the increase in Th17-like Tregs [110]. Other recent reports have described the presence of Th17-like Tregs in the skin of psoriasis [129] as well as arthritis [130] patients.

Th2-like Tregs have been recently observed in a mouse model of food allergy, and they are increased in frequency in children with milk allergy as compared to healthy donors or children with other food allergies. These Th2-like Tregs

are characterized by an increased expression of the transcription factors GATA3 and IRF4 and increased secretion of IL-4 and IL-13 [131, 132]. The production of both cytokines by Tregs directly contributes to disease, as specific disease amelioration is observed upon Treg cell-specific deletion of IL-4 and IL-13 [132]. Moreover, viral infection with the respiratory syncytial virus (RSV), which is known to increase the risk for asthma in adults when infection occurs early in life, induces a Th2-like inflammation in the lung, which promotes a Th2-like effector phenotype in Treg cells and a loss of suppressive function [131]. In vitro, Th2-like Tregs can be polarized by the stimulation of Tregs in the presence of IL-4 and IL-13 [131, 132].

Similarly, we and others have shown that Tregs from healthy individuals stimulated in vitro in the presence of IL-12 acquire a Th1-like phenotype characterized by the secretion of IFN γ , upregulation of T-BET and other Th1-related markers, such as CXCR3 and CCR5, both in mice and humans [68, 133]. Despite the maintained expression of FOXP3, these Th1-like Tregs are defective in suppressive capacity as compared to Tregs, although they retain some degree of inhibitory function in most cases [68, 133, 134]. Several reports have described the in vivo generation of Th1-like Tregs in inflammatory environments such as *Toxoplasma gondii* infection [135], neurotropic hepatitis virus [136] and in patients with autoimmune diseases such as relapsing-remitting Multiple sclerosis [133, 137] and type 1 diabetes [134].

In this review we give an update on the molecular mechanisms responsible for Th1-like Treg generation focusing on two Treg plasticity aspects: the acquisition of a Th1-suppressing phenotype to control type 1 immune responses, and the acquisition of an effector-like phenotype characterized by their inflammatory nature. We also review recent literature on the role of Th1-like Tregs in several pathological settings such as autoimmune diseases, infections and cancer, and the potential modulation of Treg plasticity as a therapeutic strategy in human disease.

Suppressive Th1-like Tregs: a method to control specific immune responses

During the past years, it has become clear that Treg function is a finely modulated process during the extent of an immune response and is very much dependent on the anatomical location of the occurring reaction, as well as the type of immune response they are controlling. In this regard, it has been observed that Tregs acquire the expression of the master transcription factor that is similar to the effector T cell population or type of immune response that they are suppressing. Thus, Tregs co-opt for

IRF4 to inhibit Th2 responses [113] and STAT3 to control Th17 pathology [114], while T-BET expression acquisition by Tregs is necessary for the control of type 1 inflammation *in vivo* [112].

In mice, Koch et al. demonstrated that a Treg subpopulation expressing CXCR3 (a surface marker preferentially expressed on Th1 CD4⁺ T cells) is present in the circulation of wild-type animals [112]. CXCR3 expression is regulated by the transcription factor T-BET [138]. T-BET expression in turn is further induced in Tregs during type-I inflammation, subsequently resulting in an enrichment of T-BET⁺ Tregs that is necessary to control T cell responses during *Mycobacterium* sp. infection [112]. Although T-BET is classically defined as a Th1-driving transcription factor controlling the expression of IFN γ , T-BET⁺ Tregs do not seem to express IFN γ in this model; this is likely to be due to the absence of IL-12 receptor beta 2 subunit (*IL12RB2*) expression and the lack of IL-12-induced signaling pathways [139]. The differentiation of Tregs into Th1-suppressing Tregs is likely to be tightly controlled by cytokines in the microenvironment. In the case of a type 1 immune response, antigen presenting cells (APCs) and other cells from the innate immune system, such as NK cells and macrophages, release Th1-associated cytokines, IL-12, TNF α and IFN γ , which induce a Th1 phenotype on CD4⁺ effector T cells. These cytokines also shape the phenotype and function of Tregs.

TNF α can trigger pro- and anti-inflammatory pathways in Tregs. A subset of both murine and human Tregs express the TNF receptor 2 (TNFR2) [140, 141]. TNF α stimulation induces, synergistically with IL-2, the expression of other members of the TNFR superfamily such as 4-1BB and OX40 [142]. Interestingly, OX40 (CD134; TNFRSF4) has recently been shown to induce Treg activation and suppressive function [143]. Furthermore, *in vitro* experiments have demonstrated the induction of Treg proliferation and promotion of Treg stability upon OX40 engagement via APC-mediated activation and stimulation with OX40 ligand (OX40L). TNF α stimulation increases OX40 expression and promotes Treg suppressive function [143, 144]. During type I inflammation, Th1 effector T cells as well as macrophages release a large amount of TNF α . TNF α in turn is essential to stabilize Treg function in a mouse model of colitis [145] as well as murine autoimmune diabetes [146]. Mediating the accumulation of suppressive Tregs in a TNF α -dependent manner poses a potential feedback mechanism to regulate strong type I inflammatory responses. On the other hand, TNF α can inflict negative effects on Treg stability and function. For instance, Valencia et al. demonstrated *in vitro* that the addition of TNF α in concentrations of 50 ng/ml inhibits Treg suppression and it is accompanied by decreased FOXP3 expression in a TNFR2-dependent manner [147].

In rheumatoid arthritis, TNF α negatively modulates Treg suppressive function [147] and dephosphorylates FOXP3 [61]. Additionally, anti-TNF α therapy induced Tregs in rheumatoid arthritis patients [148, 149]. However, Zhong et al. observed inhibitory effects of TNF α mainly on Helios^{low} Tregs [150], suggesting that the observed differential effects of TNF α might be highly dependent on the specific Treg subpopulation and/or the inflammatory milieu.

IFN γ is mainly released by NK cells and Th1 effector cells in the context of type-I inflammation. In Tregs, IFN γ increases *TBET* expression in a STAT1-dependent manner resulting in induction of *IL12RB2* expression [139]. The impact of IFN γ on Tregs is thus dichotomic as the upregulation of *IL12RB2* renders Tregs susceptible to IL-12 signaling and subsequent polarization into Th1-like Tregs. However, during type-I immune responses, IFN γ fosters the polarization of Th1-suppressing Tregs [112]. As IFN γ signaling induces *TBET* expression, IFN γ released by Th1 effector CD4⁺ T cells can act as a feedback mechanism to control type-I immune responses through expansion of Th1-suppressing Tregs and maintenance of high levels of T-BET expression. In regards to IL-12 signaling on Tregs, some reports in the literature suggested that in steady state conditions, Tregs expressed much lower levels of the *IL12RB2* subunit mRNA as compared to FOXP3⁻ T cells [139], suggesting that these cells would not be responsive to IL-12 *ex vivo*. IFN γ induces *IL12RB2* in a STAT4-dependent manner in both murine and human Tregs *in vitro* [139, 143]. Subsequent exposure of IFN γ -primed Tregs to IL-12 renders Tregs dysfunctional and favors the polarization of Th1-like IFN γ -producing Tregs leading to a disrupted regulation of type-I inflammatory responses [151]. However, the upregulation of IFN γ at RNA and protein levels by human and mice Tregs upon *ex vivo* stimulation in the presence of IL-12 has been widely demonstrated [68, 133–135]. IL-12 induces IFN γ -producing Th1-like Tregs that in addition show inhibited proliferative capacities, reduced suppressive function and expression of CD25 [113, 133, 134]. In agreement with these results, recent data suggest that human Tregs do express *IL12RB2* at the RNA level in the steady state [152], which would explain the rapid increase in *IFNG* expression upon IL-12 stimulation. Thus, the balance between differentiation into Th1-suppressing or Th1-like Tregs might be somewhat different between mice and humans and might not be controlled by the expression of *IL12RB2* and sensitivity to IL-12, but rather by the amount and availability of IL-12 in the microenvironment and/or the duration of IL-12 signaling (Fig. 1). In this regard, a subpopulation of Tregs expressing OX40, which can be induced by TNF α , has been found to differentiate into Th1-suppressing Tregs and OX40 to compete for IL-12 [143], unraveling another

possible mechanism that drives Tregs into different Th1-associated phenotype and functions.

Th1-like Treg signaling

In humans, *in vitro* stimulation of Tregs with IL-12 induces a Th1-like Treg phenotype with upregulation of T-BET, CXCR3, CCR5 and IFN γ expression [133, 151]. In contrast to mice studies [139], human Treg stimulation in the presence of IFN γ does not induce a Th1-like phenotype, with IL-12 efficiently inducing *IFNG* gene expression as soon as 24 h after stimulation *in vitro* ([133, 153] and unpublished data). While FOXP3 expression and the methylation status of the TSDR locus remain unaffected by IL-12 exposure, Th1-like Tregs diminish their suppressive function in *in vitro* co-cultures with Treg-depleted CD4⁺ T cells. In relation to the signaling pathways that drive the generation of Th1-like Tregs in humans, we recently defined the PI3K/AKT/FOXO pathway as a major axis involved in IFN γ production by Tregs [153]. PI3K/AKT is a critical signaling node in all eukaryotic cells and the AKT family of proteins is one of the most versatile and important kinases in human physiology and disease. The AKT family of proteins comprises 3 mammalian isoforms (AKT1, AKT2 and AKT3), encoded by separate genes [154]. AKT is activated downstream of PI3K, which can be triggered by multiple stimuli such as T cell receptor (TCR) engagement [155], costimulatory molecules such as CD28 [156], cytokine receptors [153, 157], G protein-coupled receptors [158] and insulin [159], among others. AKT is fully activated by phosphorylation at both Ser 473 by mTORC2 [160], and at Thr 308 by PI3K-activated PDK1 [161]. Although phosphorylation at Ser 473 is necessary for full activation, once triggered by growth factors, phosphorylation at this residue by mTORC2 targets AKT for degradation by the ubiquitin system [162]. Once activated, AKT is able to phosphorylate and activate a myriad of downstream substrates, influencing diverse cellular and physiological processes such as cell cycle progression, cell growth, cell differentiation, cell survival, metabolism, angiogenesis and motility [163–165]. One of these multiple AKT targets is the FOXO family of transcription factors, which has been widely involved in Treg development and function in mice studies [73, 166, 167]. The PI3K/AKT/FOXO axis is tightly regulated by the function of several phosphatases that act at different levels of the pathway, among which, PTEN inhibits PI3K by dephosphorylating PI(3,4,5)P₃ to PI(4,5)P₂ and PHLPP phosphatases dephosphorylate AKT kinases [168].

IFN γ ⁺ Tregs isolated from healthy individuals show an increased expression of AKT1 and decreased expression of FOXO3, AKT3 and PTEN. Interestingly, using IL-12 as an

in vitro model for Th1-like Treg generation, it is observed that IL-12 directly induces Th1 polarization by activation of the PI3K/AKT/FOXO1/3 pathway as measured by phosphorylation of AKT at residue Thr 308 and FOXO1/3 at Ser 319 [133, 153]. Despite the well known involvement of AKT in cell survival, *in vitro* experiments did not show increased Th1-like Treg survival as compared to control Tregs [153], although the *in vivo* role of this pathway in promoting Th1-like Treg cell survival remains to be elucidated. Previous studies had highlighted the importance of the PI3K/AKT axis in Treg development [169, 170] and function [171], demonstrating that diminished AKT activation is necessary for human Tregs to foster suppressive capacities [171]. The importance of the PI3K/AKT/FOXO1/3 pathway is further highlighted by a series of *in vitro* experiments where interference with the pathway by either pharmacological activation of PI3K or AKT1, or inhibition of PTEN or FOXO1/3 resulted in increased expression of IFN γ and T-BET as well as reduced suppressive function [153]. These data are in agreement with some recent works demonstrating that PI3K and PTEN are essential for Treg stability *in vivo* in mouse Tregs [172, 173]. PTEN stabilizes the metabolic balance between glycolysis and mitochondrial fitness, and PTEN-deficient Tregs show increased phosphorylation of AKT and higher expression of activation markers. In these mice, activated memory-effector T cells produce high amounts of IFN γ and upregulate CXCR3, underscoring the importance of the PTEN/AKT axis to stabilize Treg phenotype and modulate Treg-mediated control of type 1 immune responses [172]. Tregs deficient in PTEN show increased PI3K activity and downregulate the expression of CD25 and FOXP3 which leads to reduced suppressive capacity further demonstrating the relevance of the PI3K/AKT/FOXO1/3 pathway for Treg homeostasis and function [173]. FOXO1 has also been involved in the stability and function of Tregs, as FOXO^{-/-} Tregs display an increase in IFN γ expression and a pro-inflammatory phenotype, and mice with Treg-specific deletion of FOXO1 succumb to a fatal autoimmune disorder similar in severity to that observed in FOXP3 deficient mice [73] (Fig. 2).

Interestingly, AKT isoforms exhibit differential functions on the context of Th1-like Treg polarization [153]. While AKT1 is upregulated in IFN γ -producing Th1-like Tregs and AKT1 blockade can prevent Th1-like Treg polarization [153, 171], AKT3 silencing was sufficient to induce IFN γ production by human Tregs [153]. Other studies have suggested non-overlapping functions of AKT isoforms in the context of cancer biology and vascular disease [154, 165, 174]. Moreover, in the context of autoimmunity, a recent study has attributed AKT3 a protective role in EAE development in mice [175].

Besides the PI3K/AKT/FOXO pathway, gene expression analysis of human IFN γ ⁺ and IFN γ ⁻ Tregs has shown

Fig. 1 Functional differentiation of Treg cells into Th1-suppressing and Th1-like Tregs. FOXP3⁺ Tregs upregulate T-BET expression upon type I inflammatory stimuli such as IFN γ in a STAT1-dependent manner. T-BET⁺FOXP3⁺ Tregs retain their suppressive function and contribute to resolution of type I inflammation. Additional or subsequent exposure to IL-12 drives Tregs to express T-BET and release IFN γ . IFN γ -producing Tregs either contribute to resolution of type I inflammation (Th1-suppressing) or lose suppressive function and fail to efficiently control autoimmune responses (Th1-like Tregs)

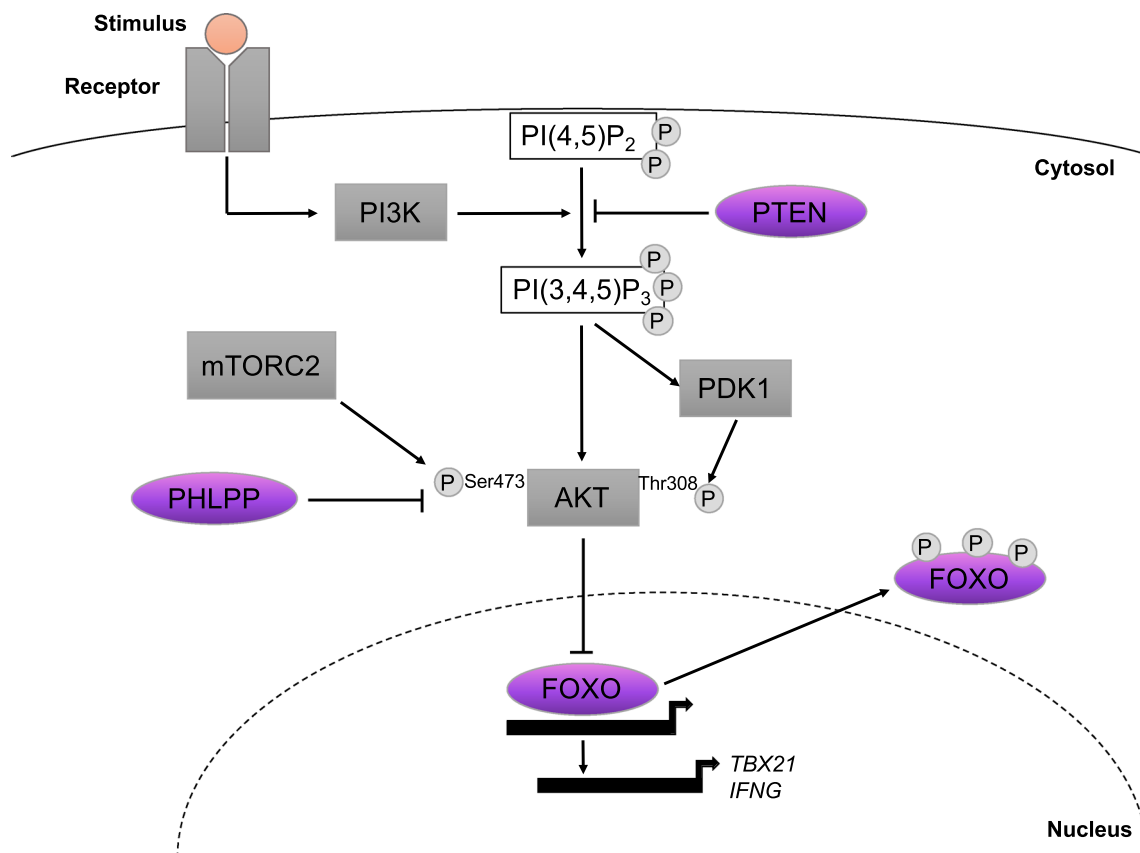
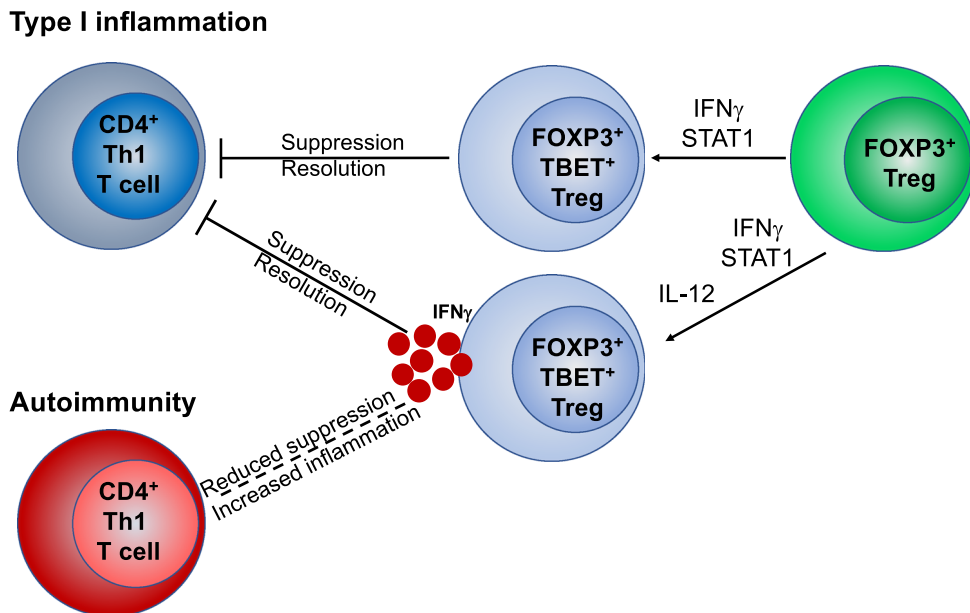


Fig. 2 The PI3K/AKT/FOXO pathway is a key regulator of Th1-like Treg differentiation. Upon T cell activation via the T cell receptor or cytokine receptors, the phosphatidylinositol 3-kinase (PI3K) phosphorylates PI(4,5)P₂ to PI(3,4,5)P₃ which in turn recruits PDK1. PTEN counteracts PI3K activity by dephosphorylating PI(3,4,5)P₃ to PI(4,5)P₂. PDK1 phosphorylates AKT at Thr 308. Additional phosphorylation of AKT at Ser 473 by mTORC2, activates AKT to

phosphorylate FOXO transcription factors, which promotes their nuclear export. FOXO1/3 are essential to stabilize FOXP3 expression in Tregs. In the case of Th1-like Tregs, AKT phosphorylation is enhanced resulting in increased FOXO1/3 nuclear export, which enables other signaling events to trigger *TBET* and *IFNG* gene expression

that other signaling pathways are differentially expressed in Th1-like Tregs, suggesting that they could also be involved in Th1-like Treg generation, although their functional relevance remains to be determined [153].

Th1-like Tregs in autoimmunity

In regards to autoimmune pathologies, several works in the literature have defined the presence of Th1-like Tregs in patients with various diseases, such as Multiple Sclerosis (MS) [133, 153], type 1 diabetes (T1D) [134], de novo autoimmune hepatitis in patients with liver transplant [176] and in inflammatory bowel disease [177–179] (IBD). We described some years ago that untreated relapsing-remitting (RR) MS patients display an increased frequency of Th1-like Tregs in peripheral blood. These Tregs express increased levels of T-BET, CXCR3, CCR5, and IFN γ and decreased levels of TGF β , and CTLA-4, being defective in function [133]. Interestingly, IFN γ is involved in the decreased suppressive capacity observed in Tregs from RRMS patients, as IFN γ blockade in ex vivo co-cultures of Tregs and Treg-depleted CD4⁺ T cells from patients with MS, significantly increased their suppressive function. Moreover, the elevated frequency of Th1-like Tregs in RRMS patients is due, at least in part, to the in vivo activated status of the AKT/FOXO pathway as compared to Tregs from healthy individuals, with increased expression of phosphorylated AKT (Thr 308) and FOXO1/3 (Ser 319) [153]. Therefore, blockade of PI3K activation in ex vivo stimulated Tregs from MS patients decreased the frequency of IFN γ -producing Tregs and increased their suppressive capacity. The presence of Th1-like Tregs has also been observed in Tregs infiltrating the CNS in vivo, during EAE development, utilizing a FOXP3 knock-in mouse model. In this model, antigen-specific Tregs and effector T cells were traced by MOG-specific tetramers at different points after EAE induction [137]. Upon EAE induction, MOG-specific Tregs were not capable of suppressing CNS-infiltrating MOG-specific T cells in vivo and in vitro and prevent disease onset, and secreted IFN γ at the onset and peak of the disease, decreasing the amount during the recovery phase and increasing IL-10 secretion [137].

Similarly, patients with T1D display an increased frequency of Th1-like Tregs as compared to healthy individuals [134] that can be observed in in vitro expanded or in ex vivo isolated Tregs. Similar to the observation made with MS patients, Th1-like Tregs in T1D patients contained both Helios⁺ and Helios⁻ Tregs. Although initially thought to be a marker of thymus-derived Tregs [180], contradictory works have occluded the use of this marker to differentiate natural versus adaptive Tregs [181–184]. In vivo, a recent work has demonstrated that in

the pre-diabetes phase in the NOD mouse model there is an increased frequency of Th1-like Tregs in the draining lymph nodes, characterized by the expression of T-BET, CXCR3, ICOS and IFN γ . These CXCR3⁺ICOS⁺ Th1-like Tregs, however, retain some degree of suppressive capacity in vivo in adoptive transfer experiments [185].

Tregs are increased in frequency in inflamed intestinal tissue in animal models and patients with IBD [178, 179] and most of them express IFN γ and IL-17, potentially enhancing inflammation or inhibiting regulation. But contradictory results have been found in in vivo models of colitis with regards to the protective or pathogenic role of Th1-like Tregs in this setting [177–179]. While antigen-specific IFN γ ⁺ Tregs were able to prevent colitis in an adoptive transfer model with flagellin-specific Tregs [178], FOXO^{-/-} Tregs, which show a Th1-like phenotype, were unable to prevent diseases in a colitis model, with IFN γ being involved on the Treg defect in function, as FOXO^{-/-}IFN γ ^{-/-} double knock-out mice were able to partially recover from the wasting syndrome [73].

Th1-like Tregs in infections and tumor environments

Both Th1-suppressing and Th1-like Tregs have been described in several models of infection and tumor environments, with each of the two populations contributing to disease manifestations and outcome. Th1-suppressing Tregs, characterized by elevated levels of T-BET and CXCR3 but not IFN γ , are induced in a model of type I inflammation with *Leishmania major* and *Mycobacterium tuberculosis* infections in mice [112]. T-BET-expressing Tregs accumulated at the site of infection, balanced Th1 inflammatory responses and maintained their homeostasis and function. In other infection models, Th1-like Tregs gained the ability to express IFN γ resulting in differential clinical outcomes of the infection models. Thus, during *T. gondii* infection, T-BET and IFN γ expression were triggered in Tregs while FOXP3 expression declined coinciding with strong immunopathology and subsequent lethal disease progression [135]. Although IFN γ is considered to be a highly pro-inflammatory cytokine, Hall et al. reported the rise of IFN γ ⁺ Tregs that were able to suppress Th1 effector T cells and limit effector T cell responses to *T. gondii* infection [186]. Along this line, IFN γ ⁺ Tregs in a colitis model retained suppressive function in vitro and inhibited the induction of colitis by microbiota antigen-specific T cells in vivo [113]. IFN γ ⁺ Th1-suppressing Tregs were also observed in a chronic corona-virus-induced encephalomyelitis model during both acute and chronic phases of infection [136]. Again, Th1-suppressing Tregs produced IFN γ and retained their

function in this model, being more likely to contribute to diminishing immunopathology. Koenecke et al., further demonstrated the protective role of IFN γ -producing Tregs as these cells appear in both *Listeria monocytogenes* infection and in acute graft-versus-host disease (GVHD). Ablation of IFN γ in Tregs resulted in the development of lethal GVHD undermining the importance of IFN γ -production by Tregs to prevent GVHD [187].

A recent study identified differential surface expression of OX40 to coincide with IFN γ -expression and Treg function in Tregs isolated from liver tissue from human hepatocellular carcinoma and cirrhosis patients [143]. These data suggest that OX40 expression efficiently separates Th1-like (OX40⁻) Tregs with IFN γ expression and reduced suppressive function from Th1-suppressing (OX40⁺) Tregs. In this study, cirrhosis tissue and tumor microenvironments favor the accumulation of OX40⁺ Th1-suppressing Tregs, whereas OX40⁻ Th1-like Tregs preferentially accumulate in non-cirrhotic chronic HCV-associated liver tissue. OX40 stimulation can abolish Treg function and thus it has been investigated as a potential antitumor target [188]. Furthermore, recent data in patients with colorectal cancer have shown that a small percentage of Tregs from either peripheral blood or infiltrating the tumor express IFN γ at similar levels, but the study lacks healthy individuals for absolute comparison [110].

Taken together, these data demonstrate that Th1-suppressive and Th1-like regulatory T cells appear in a variety of infections and tumor environments. Whereas the expression of the transcription factor T-BET seems to be a characteristic feature in all models, additional differentiation and expression of IFN γ can either be connected with protective or pro-inflammatory function in these disease settings.

Treg plasticity as a potential therapeutic strategy

Tregs represent a major barrier to effective immune responses in antitumor immunity, as well as in chronic viral infections. The identification of pathways that maintain Treg cell stability or that induce Th-like effector functions on Tregs could potentially present important novel therapeutic approaches to undermine intratumoral Tregs or Tregs in chronic infections and to enhance disease clearance.

Modulation of Treg differentiation might be a potential therapeutic target but also harbors some pitfalls. Engagement of certain surface molecules and activation of subsequent signaling pathways that might drive Tregs into either Th1-like or Th1-suppressing subtypes can dictate the beneficial outcome in settings of infection or tumor development. Several clinical studies have been undertaken to exploit antibody therapy against CTLA-4 and PD-1/PD-L1 thus dampening intratumoral Treg responses and enabling

tumor clearance by effector T cells [189, 190]. However, systemic administration of antibodies or other reagents that interfere with Treg signaling pathways harbor the danger of shifting the subpopulation balance in disfavor when considering the performance of other immune responses.

Furthermore, the underlying pathways that drive Treg plasticity into either direction seem to be critically dependent on the microenvironment. MS patients display an increased number of IFN γ ⁺ Th1-like Tregs that lost suppressive function in peripheral blood and are thus believed to be a crucial factor as to why autoimmune reactions in these patients are not controlled efficiently. The PI3K/AKT/FOXO1/3 pathway has been identified as one of the key pathways involved in Th1-like Treg generation and is an interesting prospective target for immunomodulation [153]. However, IFN γ ⁺ Th1-like Tregs in other disease settings do not lose their suppressive function which might indicate the involvement of different signaling pathways ultimately resulting in similar but yet different phenotypic outcomes.

Clinically, Tregs have recently been beneficially used for therapy against graft versus host disease following allogeneic bone marrow or stem cell transplantation [191, 192] and type 1 diabetes patients [193]. In these settings, autologous polyclonal Tregs are expanded in vitro and re-transferred into the patient. The use of in vitro Treg expansion protocols gives the opportunity to analyze Treg phenotype and function as well as specifically control cell numbers necessary to re-transfer for a beneficial outcome. Several clinical trial studies have reported successful application of Treg transfer therapy in T1D and GVHD [193, 194]. As antigen-specific Tregs are more efficient at regulating disease-specific immunological processes, the generation of antigen-specific Tregs using chimeric antigen receptors (CAR) has been explored as well, as an improvement to Treg therapy. CAR Tregs have been shown to prevent GVHD in a humanized mouse model highlighting their improved therapeutic potential [195].

Combination of adoptive transfer of antigen-specific Tregs with modulation of Treg plasticity proposes strong therapeutic potential. In vitro expansion of Tregs provides the opportunity to also include cytokine treatments to reprogram Tregs into phenotypic subtypes most beneficial for the respective disease. Furthermore, defective Treg phenotypes could be corrected by either interfering with involved pathways by using pharmacological agents or by introducing recent gene editing technologies such as the CRISPR/Cas9 system.

Conclusions and future perspectives

During the past few years it has become clear that Tregs possess some degree of plasticity and can adapt their phenotype to the microenvironment where they exert their

functions. Furthermore, aberrant reprogramming of Tregs into Th1-like Tregs has been observed in several human autoimmune diseases, suggesting their contribution to disease. The relative importance of Th1-like Tregs in the pathophysiology of the diseases where they have been observed, their role in promoting or protecting from inflammation-derived damage and their influence in disease outcome are fundamental open questions that remain to be answered and that will undoubtedly provide valuable information for the potential manipulation of Treg plasticity with therapeutic purposes for these diseases. In order to design better therapeutic options targeted to Tregs in cancer, infectious and autoimmune diseases, it is imperative to understand the signaling pathways that govern the acquisition of specific effector characteristics by Tregs in different disease settings. In this regard, most of the current literature on Th1-like Tregs defines $\text{IFN}\gamma$ and IL-12 as the major inducers of Th1- reprogramming in Treg cells. However, the discovery of the PI3K/AKT axis as a major signaling pathway that regulates Th1-like Treg generation, and the variety of upstream ligands/receptors that can activate it, makes the generation of Th1-like Tregs a plausible event in many disease settings, and it strongly suggests that there are likely other environmental cues apart from Th1 cytokines that induce the generation of dysfunctional Th1-Tregs or Th1-suppressing Tregs. Moreover, there are likely many other signaling pathways involved in Th1-like Treg generation in specific disease settings yet to be defined [93], that will further improve our knowledge on the molecular mechanisms that regulate human Treg plasticity with potential therapeutic applications.

In conclusion, it is crucial to fully understand the underlying pathways and mechanisms that regulate Treg plasticity and the environmental cues that induce such phenotypes in specific disease settings in order to be able to take advantage of Treg plasticity for therapeutic purposes. Furthermore, it will be important to perform investigations that focus on understanding the differences between Th1-suppressive Th1-Tregs and dysfunctional Th1-Tregs observed in autoimmune disease settings and acute phases of infection as well as how to transition from one state to another, with potential important applications in therapy.

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