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# The regulation and differentiation of regulatory T cells and their dysfunction in autoimmune diseases

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## Abstract

The discovery of FOXP3$^+$ regulatory T ($T_{reg}$) cells as a distinct cell lineage with a central role in regulating immune responses provided a deeper understanding of self-tolerance. The transcription factor FOXP3 serves a key role in $T_{reg}$ cell lineage determination and maintenance, but is not sufficient to enable the full potential of $T_{reg}$ cell suppression, indicating that other factors orchestrate the fine-tuning of $T_{reg}$ cell function. Moreover, FOXP3-independent mechanisms have recently been shown to contribute to $T_{reg}$ cell dysfunction. FOXP3 mutations in humans cause lethal fulminant systemic autoinflammation (IPEX syndrome). However, it remains unclear to what degree $T_{reg}$ cell dysfunction is contributing to the pathophysiology of common autoimmune diseases. In this Review, we discuss the origins of $T_{reg}$ cells in the periphery and the multilayered mechanisms by which $T_{reg}$ cells are induced, as well as the FOXP3-dependent and FOXP3-independent cellular programmes that maintain the suppressive function of $T_{reg}$ cells in humans and mice. Further, we examine evidence for $T_{reg}$ cell dysfunction in the context of common autoimmune diseases such as multiple sclerosis, inflammatory bowel disease, systemic lupus erythematosus and rheumatoid arthritis.

## Introduction

After decades of studies attempting to identify the mechanisms of T cell tolerance, the discovery of the transcription factor FOXP3 was a significant milestone that allowed the identification of regulatory T ($T_{reg}$) cells among the CD4$^+$ T cell subsets. $T_{reg}$ cells exhibit a wide spectrum of functions and contribute to peripheral tolerance by modulating the activities of diverse cell types, including CD4$^+$ T helper cells, cytotoxic CD8$^+$ T cells, B cells and dendritic cells[1]. Moreover, $T_{reg}$ cells have an important role in maintaining

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Competing interests

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tissue homeostasis and regeneration[2]. The functional versatility of FOXP3$^+$ $T_{reg}$ cells is determined by contextual cues from their microenvironment and by their stage of differentiation, spanning from naive $T_{reg}$ cells (resting state) to effector $T_{reg}$ cells[3]. Different subsets of $T_{reg}$ cells differ with regards to their expression of chemokine receptors and transcription factors and their heterogeneity mirrors the complexity observed in CD4$^+$ T helper subsets[4,5]. Moreover, the tissue microenvironment can modulate transcriptional and epigenetic profiles of $T_{reg}$ cells and determine their function[6].

The suppressive activities of $T_{reg}$ cells can be divided into contact dependent or non-contact dependent, as well as antigen specific or non-antigen specific[7-9]. These modes of action often act in concert and are in most cases complementary. Thus, the suppressive mechanisms of $T_{reg}$ cells are determined by the cells they interact with and by immunological effector molecules in their microenvironment. However, the central regulator of $T_{reg}$ cell lineage commitment and functionality is the complex gene regulatory circuit that controls FOXP3. This network is finely tuned by T cell receptor (TCR) stimulation and environmental signals that mediate epigenetic modulation (Fig. 1). FOXP3 directly controls a core set of $T_{reg}$ cell signature genes that are critical for $T_{reg}$ cell homeostasis and maintains its own transcription through an autoregulatory transcription circuit. Additionally, various transcription factors interact with FOXP3, both directly and indirectly. These interactions can activate pivotal $T_{reg}$ cell core signature genes, preserving $T_{reg}$ cell lineage commitment and suppressing genes linked to the development of effector T cells[10]. This intricate interplay bestows $T_{reg}$ cell function with a remarkable degree of adaptability and susceptibility to environmental cues. Hence, the processes by which $T_{reg}$ cells exert their suppressive functions and their potential transition to a dysfunctional state are multifactorial. In addition to a qualitative loss of $T_{reg}$ cells due to dysregulation, loss of immune tolerance can also be caused by a quantitative loss of $T_{reg}$ cells due to a defect in proliferation[11], an increased susceptibility to apoptosis[12] or a failure of thymic $T_{reg}$ ($tT_{reg}$) cell differentiation.

A significant defect of $tT_{reg}$ cell development would likely lead to lethal systemic autoimmunity (IPEX in humans or scurfy in mice), as opposed to the clinical features of more common autoimmune diseases[13,14]. Around 20 years ago, several publications demonstrated a defect in the function, but not the frequency, of $T_{reg}$ cells in patients with multiple sclerosis[15-17]. This was followed by identification of $T_{reg}$ cell dysfunction in several common autoimmune disorders including type 1 diabetes[18-20], rheumatoid arthritis[21,22], systemic lupus erythematosus (SLE)[23] and others.

Over the past decade, much research has been conducted on the biology of $tT_{reg}$ cell differentiation. Studies using $T_{reg}$ cell-specific conditional knockout (cKO) mice and fate tracing have provided insights into how FOXP3 regulates $T_{reg}$ cell development in the thymus. These studies have been summarized in several excellent review articles[1,24]. As our knowledge of $T_{reg}$ cell biology continues to evolve, it opens new avenues for targeted therapeutic strategies aimed at modulating these cells to restore immune equilibrium in autoimmune diseases. However, the mechanisms by which $T_{reg}$ cells in the periphery can become dysfunctional, particularly in the context of autoimmune disease, are less well understood.

In this Review, we first examine the factors that regulate the stability and maintenance of $T_{reg}$ cells in the extrathymic peripheral system and the mechanisms by which $T_{reg}$ cells can lose their suppressive function and become dysfunctional. We then discuss evidence for and mechanisms of $T_{reg}$ cell dysfunction in the context of autoimmune diseases such as multiple sclerosis, inflammatory bowel disease (IBD), SLE and rheumatoid arthritis.

## $T_{reg}$ cells in extrathymic tissues

$T_{reg}$ cells in the periphery can originate from the thymus ($tT_{reg}$ cells) or derive from conventional T cells (peripheral $T_{reg}$ ($pT_{reg}$)cells) in extrathymic tissues. $tT_{reg}$ cells generally have higher-affinity TCRs against autoantigens than conventional T cells and compared with $pT_{reg}$ cells. The formation of $pT_{reg}$ cells from conventional $CD4^+$ T cells can occur in response to binding of self-antigens or exogenous antigens (such as the microbiota) under TGFβ-rich conditions in both homeostatic and inflammatory conditions[25,26]. $pT_{reg}$ cells contribute to the control of peripheral tolerance at sites of inflammation, especially at the mucosal surfaces of the gut, lung and skin. However, how conventional T cells give rise to $pT_{reg}$ cells in vivo is not well understood, especially in humans. So-called induced $FOXP3^+$ $T_{reg}$-type ($iT_{reg}$) cells can be generated by stimulating mouse $CD4^+$ T cells in vitro in the presence of TGFβ and IL-2 (refs. 26,27). Although $iT_{reg}$ cells have some degree of suppressive capacity when they are transferred back into mice, their transcriptional profile differs from that of $pT_{reg}$ cells and further from that of $tT_{reg}$ cells[28]. Human conventional $CD4^+$ T cells can also be induced to express FOXP3 in vivo by stimulating their TCRs in a TGFβ and IL-2 rich environment in vitro, but these cells lack suppressive capacity[29,30]. To avoid potential confusion, here we define $pT_{reg}$ cells as $T_{reg}$ cells that are converted from conventional T cells in vivo and $iT_{reg}$ cells as $T_{reg}$ cells converted in vitro, and focus on the biology of $pT_{reg}$ cells.

In mice, $tT_{reg}$ cells and $pT_{reg}$ cells can be distinguished by the expression of the transcription factor Helios[31] and the surface expression of the immunoregulatory receptor neuropilin 1 (NRP1)[32]. However, no definitive markers that distinguish $T_{reg}$ cells of different origins have been identified in humans[33,34]. This critical discrepancy between humans and mice hinders the exploration of human $pT_{reg}$ cell biology, especially under homeostatic conditions. The $pT_{reg}$ cell and $tT_{reg}$ cell lineages in mice have similar but not identical gene expression profiles[28,35]. However, their TCR repertoires are distinct[35], highlighting the contribution of TCR affinity as one of the innate features that distinguish $tT_{reg}$ cells and $pT_{reg}$ cells. In fact, by using low-affinity and high-affinity peptides in genetically engineered mice that exclusively express specific TCRs, it was found that TCR stimulation with the low concentrations of high-affinity peptide provided optimal conditions for $pT_{reg}$ cell induction, indicating that optimal TCR avidity, which is defined by both TCR–pMHC affinity and the antigen concentration, is critical for the efficient induction of FOXP3 expression[36,37]. This is further regulated by soluble mediators such as TGFβ and retinoic acid, as well as co-stimulatory molecules such as CD28 (ref. 38), and by the strength of signalling via downstream signalling pathways (such as the NF-κB and PI3K–AKT–mTOR pathways)[39-41].

## The role of FOXP3 in peripheral $T_{reg}$ cells

FOXP3 plays an essential role in lineage commitment during the development of $tT_{reg}$ cells in the thymus, but also in maintaining the extrathymic $tT_{reg}$ cell pool in mice by sustaining '$T_{reg}$ cell signature' gene expression[42]. Genetic fate mapping or adoptive transfer of FOXP3-expressing T cells in mice further demonstrated that stable FOXP3 expression under homeostatic conditions stabilizes $tT_{reg}$ cell lineage commitment for most $T_{reg}$ cells after thymic egress. However, a noticeable fraction (10–20%) of $FOXP3^+$ $T_{reg}$ cells lose FOXP3 expression and become 'ex$T_{reg}$ cells'[43,44]. Further study of this population in cell fate tracing mice revealed that they are mostly derived from activated conventional T cells that temporally express FOXP3, and from $pT_{reg}$ cells[45]. By contrast, only a minor fraction (<3%) of $tT_{reg}$ cells appear to lose FOXP3 expression, indicating a stable lineage commitment of $tT_{reg}$ cells in mice[44,46]. Whereas the conventional role of FOXP3 in $T_{reg}$ cell lineage commitment is well established, recent evidence suggests that the $T_{reg}$ cell core gene programme and their suppressor function do not exclusively depend on FOXP3 expression[47-50]. This raises the question of whether there is a FOXP3-independent mechanism that confers extrathymic tTreg cell suppressive capacity and suggests that there are several key molecules and mechanisms that are likely to contribute to regulating both $tT_{reg}$ cell and $pT_{reg}$ cell function in the periphery, in addition to FOXP3.

Insights into $pT_{reg}$ cell development were gained from studies of mice that lack conserved non-coding sequence 1 (CNS1), a regulatory element in the *FOXP3* locus. These studies revealed that CNS1 is dispensable for the development of $tT_{reg}$ cells but is required for the development of both $pT_{reg}$ cells and $iT_{reg}$ cells[51,52]. It is important to note that loss of CNS1 does not completely deplete $pT_{reg}$ cells, indicating that CNS1 has a crucial role in $pT_{reg}$ cell differentiation but is not the sole determining factor[33,53]. Furthermore, genetic tracing in mice showed that transcriptional features of $pT_{reg}$ cells are established before FOXP3 induction and that FOXP3 is dispensable for $pT_{reg}$ cell fitness and lineage commitment in the gut after its colonization by microbiota[50]. These findings indicate that numerous transcriptional signatures in $pT_{reg}$ cells develop in a FOXP3-independent manner.

FOXP3 reporter-null $T_{reg}$ cells, which are similar to precursor $T_{reg}$ cells but lack FOXP3 expression, have been utilized to study the role of FOXP3 in the lineage commitment of $T_{reg}$ cells. These cells are referred to as 'wannabe $T_{reg}$ cells'. Notably, FOXP3 reporter-null $pT_{reg}$ cells can suppress effector T cell proliferation. However, their regulatory function is not sufficient to prevent pathogenic features in mouse models of colitis, indicating that 'wannabe $pT_{reg}$ cells' remain committed to the $T_{reg}$ cell lineage but FOXP3 is required to confer full suppressive capacity[50]. This suggests that FOXP3-independent mechanisms, such as the epigenetic regulation of the $T_{reg}$ cell core gene programme, play an important role in the commitment of T cells to the $T_{reg}$ cell lineage and in providing the foundation for the acquisition of full suppressive function. Further studies are needed to better understand these mechanisms.

## Epigenetic control of $T_{reg}$ cell stability

The development and maintenance of $T_{reg}$ cell function depends on the stable and coordinated expression of FOXP3 and other $T_{reg}$ cell signature genes. Epigenetic modifications, such as the methylation of CpG motifs and the acetylation, methylation and ubiquitination of histones, can modulate the transcriptional regulation of $T_{reg}$ cell signature genes. These modifications can be established and maintained in FOXP3-dependent and FOXP3-independent manners and their dysregulation can lead to $T_{reg}$ cell dysfunction. Recent investigations into genetic factors in autoimmune diseases revealed that causal genetic variants were enriched in non-coding regulatory elements that are accessible in immune cells, especially in $T_{reg}$ cells[54,55]. These findings, together with increases in the incidence of autoimmune diseases over the past three decades that cannot be explained by genetic factors alone, point to environmental cues that lead to epigenetic modifications as key mediators of autoimmune risk.

### Epigenetic regulation of the *FOXP3* gene locus

A comparative genomic approach of the *FOXP3* gene locus in human, rat and mouse identified three highly conserved non-coding sequences (CNS1–3) and a promoter element[56,57] (Fig. 2). All conserved non-coding sequences were highly enriched in demethylated CpG motifs, indicating that they serve as binding sites for factors involved in the control of *FOXP3* gene expression. Subsequently, CNS0, a conserved region upstream of the *FOXP3* transcription start site and outside the *FOXP3* gene locus, was discovered. The accessibility of the conserved non-coding sequence regions and other regulatory elements to transcriptional regulators is determined by their CpG methylation state and by modifications of the histones they are bound to. Importantly, these regulatory elements appear to contribute not only to $tT_{reg}$ cell development but also to $T_{reg}$ cell maintenance in the periphery[58].

In precursor $tT_{reg}$ cells, $T_{reg}$ cell-specific super-enhancers that are associated with *FOXP3* and other $T_{reg}$ cell signature genes are activated by the epigenetic modifier SATB1, which binds to CNS0 and demethylates $T_{reg}$ cell-specific demethylated regions (TSDRs). SATB1 acts as a ‘pioneering factor’, as its expression precedes FOXP3 expression in $T_{reg}$ cell precursors[59]. CNS0 is also bound by MLL4, a subunit of the methylated histone H3 Lys4 (H3K4me1) complex[60], BRD9, a non-canonical BAF chromatin-modifying complex component[61], and the signalling protein STAT5 (ref. 62). Experiments in mice with a genetic deletion of CNS0 indicated that this region is required for the induction of FOXP3 expression in response to signalling via TCR stimulation and IL-2-induced STAT5 signalling in $tT_{reg}$ cell precursors, as well as for $iT_{reg}$ cell induction[63].

CNS1 and CNS2 are *cis*-elements within the first intron of the *FOXP3* locus, whereas CNS3 is a *cis*-element within the second intron of the *FOXP3* locus. CNS1 is dispensable for $tT_{reg}$ cell differentiation but is one of the critical factors for TGFβ-induced FOXP3 expression, and therefore likely important for the development of $pT_{reg}$ cells[52,64]. CNS2 is the most studied *cis*-element because of its indispensable role in $T_{reg}$ cell lineage commitment and in stabilizing FOXP3 expression in both humans and mice[65-67]. The maintenance of elevated FOXP3 expression requires a strong TCR signal that activates the transcription factor NFAT to bind to CNS2, which facilitates the interaction between CNS2 and the

*FOXP3* promoter element[68]. Other transcription factors, including FOXP3 itself, also bind CNS2. A complex of the transcription factors RUNX1 and CBFβ has been shown to interact with demethylated CNS2 (refs. 69,70), which is critical for *FOXP3* locus activity during $T_{reg}$ cell maturation[52,71]. Moreover, when activated by IL-2 signalling, STAT5 binds CNS2, promoting $T_{reg}$ cell differentiation[72].

CNS3 is essential for the induction of thymic FOXP3 expression but not for mature $T_{reg}$ cell maintenance or function[73,74]. Of note, it has been shown that activation of FOXP3 expression via CNS3 can also broaden the $T_{reg}$ cell TCR repertoire by allowing for the development of $T_{reg}$ cells with weak-affinity TCRs, as it is bound even in response to weak signalling[74]. The CNS0 and CNS3 regions become accessible during early $tT_{reg}$ cell differentiation in response to IL-2 and TCR signalling, which is crucial for FOXP3 stability and the $T_{reg}$ cell lineage commitment[75]. Independently of FOXP3 expression but dependent on TCR engagement, $T_{reg}$ cell lineage-committed cells acquire CpG hypomethylation on *cis*-elements for $T_{reg}$ cell signature genes, which is essential for the expression of these genes and for the suppressive function of the cells[76].

Although these findings highlight the fundamental role of demethylated *cis*-regulatory elements in the *FOXP3* locus, conserved non-coding sequence-mediated *FOXP3* induction is not sufficient to convert a conventional naive $CD4^+$ T cell into a fully functional $T_{reg}$ cell in both humans and mice[77,78] or to confer the full capacity of suppressive function of $pT_{reg}$ cells (Fig. 1).

## Histone modifications in $T_{reg}$ cells

Chromatin accessibility is determined by the dynamic competition between histone acetyltransferases (HATs), which acetylate lysine residues of histones and thereby promote chromatin accessibility, and histone deacetylases (HDACs), which deacetylate lysine residues[79,80]. HAT and HDAC complexes can modulate FOXP3-mediated transcriptional repression[81]. A pan-HDAC inhibitor was shown to increase the acetylation of histones at the regulatory elements of *FOXP3* and of FOXP3 itself and to enhance $T_{reg}$ cell suppressive function both in vivo and in vitro[82]. However, this effect was not observed with a class I-specific HDAC inhibitor[83,84]. By using knockout mice for each class of HDACs, it was determined that the loss of HDAC6 (ref. 80), HDAC9 (ref. 83), HDAC10 (ref. 85) and sirtuin 1 (ref. 86) can improve $T_{reg}$ cell suppressive function[84], whereas the loss of HDAC3 (ref. 87), HDAC5 (ref. 88) or sirtuin 3 (ref. 89) lowered $T_{reg}$ cell suppressive function. HDAC7, which is highly expressed in $T_{reg}$ cells, has been shown to interact with FOXP3 and the HAT TIP60 (ref. 90). cKO mice with a $T_{reg}$ cell-specific deletion of *Hdac7* had no significant loss of $T_{reg}$ cell frequency or number, and there was no sign of autoimmunity in heterozygous *Hdac7*-cKO mice, indicating that partial loss of HDAC7 does not impair $T_{reg}$ cell development and maintenance[91]. Surprisingly, heterozygous *Hdac7*-cKO mice developed more severe neuroinflammation in the EAE mouse model of multiple sclerosis, suggesting an essential role of HDAC7 in maintaining $T_{reg}$ cell function in the periphery[91]. Of note, a recent genetic association study of low-frequency coding variations in patients with multiple sclerosis identified several susceptibility loci, including a protective variant that is located in the amino-terminal region of the *HDAC7* locus (rs148755202,

HDAC7.p.R166H)[92]. Mice expressing the orthologous human HDAC7 R166H variant did not show any changes in the immune cell compartments in secondary lymphoid organs or signs of autoimmunity. When EAE was induced, homozygous and heterozygous *HDAC7*$^{R166H}$ knock-in mice were protected from severe disease. Furthermore, human $T_{reg}$ cells that overexpress *HDAC7*$^{R166H}$ have an increased suppressive function compared with wild-type *HDAC7*-overexpressing $T_{reg}$ cells[91]. However, it has not yet been determined whether the R166H missense variant affects the composition of the FOXP3–HDAC7–TIP60 complex that stabilizes FOXP3 through acetylation. HDAC7-R166H is located at the amino-terminal region of its interaction domain with MEF2, a transcription factor that is known to regulate $T_{reg}$ cell suppressor function by interacting with HDAC9 and FOXP3 (refs. 93,94).

In addition to acetylation, histones can also be epigenetically modified by methylation of lysine residues. Here, polycomb repressive complex 2 (PRC2) is a key regulator for dimethylated histone H3 Lys27 (H3K27me2) and trimethylated histone H3 Lys27 (H3K27me3)[95]. The enrichment of H3K27me3, which is indicative of gene repression, on histones at FOXP3-binding sites within FOXP3-repressed genes suggested that PRC2 interacts with FOXP3 (ref. 96). Indeed, in mouse $T_{reg}$ cells, FOXP3 was found to directly interact with SUZ12, a key component of PRC2 (ref.97). In activated mouse and human $T_{reg}$ cells, FOXP3 was also shown to interact with EZH2, another PRC2 component, and bind to loci enriched in histones with H3K27me3 (refs. 98,99). $T_{reg}$ cell-specific deletion of *Ezh2* in mice leads to spontaneous autoimmunity with reduced stability of FOXP3 in $T_{reg}$ cells in non-lymphoid tissues and $T_{reg}$ cells failed to be activated by their specific antigen[99]. In a model of EAE, these mice failed to control autoimmune inflammatory responses in the brain after the initiation of the disease. $T_{reg}$ cells lacking *Ezh2* are prone to apoptosis after antigen encounter, indicating that EZH2 is necessary to shape the activation-induced epigenetic landscape that allows effector $T_{reg}$ cell differentiation and long-term survival. Interestingly, the IBD-related FOXP3 mutation cysteine 232 (FOXP3-C232) abrogates its interaction with EZH2, implicating impaired $T_{reg}$ cell differentiation and survival in IBD disease pathology[100]. Similarly, IL-6 signalling, which is known to play a pathogenic role in autoimmune diseases including IBD, can disrupt the FOXP3–EZH2 interaction, indicating that the loss of this interaction is relevant to the development of human IBD[101]. EZH2 was also reported to have a critical role during regulatory T follicular helper cell development[102]. Given that EZH2 controls T helper 1 ($T_H1$) cell/$T_H2$ cell differentiation by inducing lineage-specifying genes in terminally differentiated conventional FOXP3$^{-}$CD4$^{+}$ T cells[103], it is likely that EZH2 can also act independently of FOXP3 in $T_{reg}$ cells[99]. Recent CRISPR screening studies have further highlighted the contribution of chromatin remodelling complexes, such as the SWI/SNF and SAGA complexes[61,104], to histone modification-mediated regulation of FOXP3 expression and $T_{reg}$ cell function.

## FOXP3 modulates the 3D chromatin landscape

During thymic differentiation of $T_{reg}$ cells in mice, the $T_{reg}$ cell-specific epigenetic landscape is pre-established even before FOXP3 expression is induced. At this stage, FOXO1 acts as a precursor to FOXP3 (ref. 105). At the same time, FOXP1, another member of the forkhead transcription factor family, engages in joint binding and synergistic regulation of FOXP3 target genes. This cooperative activity helps to sustain $T_{reg}$ cell

fitness, enhances FOXP3 stability and prevents the initiation of gene programmes that could cause $T_{reg}$ cell dysfunction[106,107]. These findings highlight a complex FOXP3-centred gene regulation programme that extends beyond the simplified model of transcription factor binding to gene promoters. Gene expression depends on complex interactions of several partners that establish physical connections between regulatory elements and gene promoters, creating enhancer–promoter loops. Although it is evident that FOXP3 regulates $T_{reg}$ cell lineage development and stability by modulating $T_{reg}$ cell signature genes[108], the precise mechanisms by which FOXP3 and its cofactors orchestrate such enhancer–promoter loops between regulatory elements and $T_{reg}$ cell signature genes remain an ongoing area of investigation[97,109]. Analyses of conventional $CD4^+$ T cells and $T_{reg}$ cells by HiChIP[110], a tool to map enhancer–promoter architecture, revealed that FOXP3 controls $T_{reg}$ cell identity and stability by interacting with promoters and enhancers of core $T_{reg}$ cell signature genes and maintaining enhancer–promoter connectivity[111].

The looping of the *FOXP3* promoter to the CNS2 region was also shown to stabilize *FOXP3* expression via a 3D genome structure consisting of the transcriptional co-activator mediator and cohesin[68]. Another study showed that FOXP3 can maintain $T_{reg}$ cell identity and stability not only directly but also indirectly by regulating the expression of intermediary transcription factors such as TCF1 (ref. 112). However, although it is plausible that some $T_{reg}$ cell signature genes are indirectly regulated, the majority of the genes responsible for $T_{reg}$ cell stability and linage determination are directly FOXP3 dependent[113].

These studies highlight the importance of genome topology in controlling $T_{reg}$ cell stability and function. The genome organizers CTCC-binding factor (CTCF) and cohesin bind super-enhancers that control $T_{reg}$ cell signature genes[59] and FOXP3-bound enhancers are highly enriched in CTCF motifs[111], indicating that interactions between FOXP3 and CTCF–cohesin are important for $T_{reg}$ cells. $T_{reg}$ cell-specific deletion of *TCF1* (which interacts with CTCF) and *LEF1* in mice did not impair $tT_{reg}$ cell development or in vitro suppressor function, but these mice developed spontaneous systemic autoimmunity with enhanced humoral responses[114]. TCF1 and LEF1 were specifically required to maintain the $TCF1^+$ $T_{reg}$ cell subset that contained a pool of $T_{reg}$ cells that develop into regulatory T follicular helper cells. Interestingly, the loss of TCF1 expression by $TCF1^+$ $T_{reg}$ cells was necessary to allow their differentiation into effector $T_{reg}$ cells. To fully understand the genetic regulation of $T_{reg}$ cell identity and stability, a clear grasp of regulatory mechanisms determined by genome topology is imperative.

## The role of TCR signalling in $T_{reg}$ cells

Changes in the amplitude of TCR signal strength may affect the thymic development of $T_{reg}$ cells and $pT_{reg}$ cell function, leading to autoimmunity[115]. The requirements for TCRs in differentiated extrathymic $T_{reg}$ cells have been investigated in mice with $T_{reg}$ cell-specific TCR cKO (TCR cKO $T_{reg}$ cells). Surprisingly, TCR expression was largely dispensable for $T_{reg}$ cell lineage stability and FOXP3 expression. Although the majority of $T_{reg}$ cell signature gene expression (such as *Il2ra, Entpd1* and *Ctla4*) was intact and the $T_{reg}$ cell-specific epigenetic pattern was not affected by TCR ablation, their suppressive function was impaired and TCR cKO $T_{reg}$ cells failed to induce peripheral tolerance[116,117].

TCR-dependent genes such as *Egr2, Il1r2, Lag3, Il10, Ebi3, Irf4, Ikzf2* and *Ccr8,* and effector $T_{reg}$ cell markers such as CD38, CD44, OX40, GITR and CD69, were decreased in TCR cKO $T_{reg}$ cells[116,117]. Notably, the regulation of TCR-dependent gene expressions appeared to depend on the transcription factors EGR2, EGR3, c-REL and, importantly, IRF4 (ref. 117). These data suggest that TCR signalling is indispensable for both the induction and maintenance of mature $T_{reg}$ cells in the periphery. Other studies examining $T_{reg}$ cell localization within secondary lymphoid organs identified highly suppressive mature $T_{reg}$ cells that are strategically positioned in distinct clusters where they interact with migratory dendritic cells, which might present self-antigen to $T_{reg}$ cells, and these clusters are lost when TCRs are genetically ablated in $T_{reg}$ cells[118].

These studies highlight the importance of TCRs in p$T_{reg}$ cell maturation and in the establishment of a tolerant environment. However, a recent study suggested that TCR signalling may not be necessary for sustaining the function of terminally differentiated effector $T_{reg}$ cells within the mouse colon[119]. This raises the question of at which stage of effector $T_{reg}$ cell differentiation TCR signalling is needed, and whether this phenomenon also applies to human $T_{reg}$ cells. Moreover, it is not clear how TCR-independent 'innate-like' features are acquired in peripheral tissues. Further studies are needed to explore the role of TCR in terminally differentiated effector $T_{reg}$ cells in peripheral tissues.

## Adaptability of $T_{reg}$ cells to inflammation

$T_{reg}$ cells can develop effector functions that resemble the context-dependent effector gene expression signatures of conventional T cells. If the differentiation of conventional effector T cells and $T_{reg}$ cells is not coordinated, the immune response can become aberrantly activated. For example, in scenarios where a $T_H1$-type response is required, such as during viral infections, conventional T cells differentiate into $T_H1$ cells under the regulation of the transcription factor T-bet. Although $T_{reg}$ cells have the ability to induce T-bet expression and require T-bet expression to suppress $T_H1$-type inflammation (such cells are known as T-bet$^+$ $T_{reg}$ cells or $T_H1$-type $T_{reg}$ cells), it is important to note that T-bet expression alone is not adequate to confer the suppressive characteristics of $T_{reg}$ cells within a $T_H1$ cell-skewed microenvironment[120]. It was shown that mouse $T_{reg}$ cells that successfully adapt their phenotype under $T_H1$ cell skewing conditions have a unique TCR repertoire compared with that of other $T_{reg}$ cells, indicating that $T_{reg}$ cells harbouring specific TCRs can be 'licensed' to control effector T cell activation under conditions of $T_H1$-type inflammation or cancer[120,121]. These findings suggest that particular $T_{reg}$ cell functions are predetermined by their TCR repertoire, thus highlighting the fundamental role of the TCR repertoire in the development of their adaptive effector differentiation programme[120]. Another aspect of T-bet$^+$ $T_{reg}$ cells, which applies in particular to the subset that also has elevated expression of the checkpoint receptor TIGIT, is their ability to produce the anti-inflammatory cytokine IL-10 and skew dendritic cells towards a tolerogenic phenotype[122,123]. This concept of $T_{reg}$ cell adaptability depending on the tissue microenvironment can be extended to other types of T cell responses. For example, RORγt and STAT3 expression in $T_{reg}$ cells are necessary to regulate $T_H17$ cell-mediated responses[124-127], and IRF4 and STAT6 expression in $T_{reg}$ cells is critical to control $T_H2$ cell-mediated responses[128,129]. Although GATA3 is known to control $T_H2$-type T cell differentiation, its role in $T_{reg}$ cells goes beyond controlling

$T_H2$-type T cell-mediated inflammation because GATA3 can also directly bind to and modulate the activity of FOXP3 (refs. 97,130-132) (Fig. 3). $T_{reg}$ cells owe their ability to adapt to a microenvironment that is associated with various T helper cell lineages to their plasticity and heterogeneity. However, when factors that maintain $T_{reg}$ cell function, such as IL-2, are missing or only present at insufficient levels under inflammatory conditions, $T_{reg}$ cells can become unstable. In these cases, 'T helper cell signature transcription factors' can disturb gene regulation circuits in $T_{reg}$ cells, leading to $T_{reg}$ cell dysfunction in mice[127,133,134] and humans[16]. In addition to T helper cell signature transcription factors, there are several other transcription factors that regulate effector $T_{reg}$ cell differentiation and function in the periphery (Box 1). Overall, intrinsic mechanisms of $T_{reg}$ cell maintenance, extrinsic environmental factors and inflammatory contexts that alter the $T_{reg}$ cell suppressive programme are important for controlling immune responses and establishing peripheral tolerance.

## $T_{reg}$ cells in human autoimmune diseases

Under certain conditions, $T_{reg}$ cells can acquire conventional effector T cell functions and secrete inflammatory cytokines. In vitro experiments with human $T_{reg}$ cells have shown that a combination of IL-1β and IL-6 can induce IL-17 secretion, and IL-12 can induce IFNγ secretion[16,135,136]. More importantly, IFNγ secretion was associated with an in vitro loss of $T_{reg}$ cell suppressor function. Indeed, $T_{reg}$ cells that can lose their suppressive function and exert effector T cell functions have been detected in patients with multiple sclerosis, IBD, SLE and rheumatoid arthritis. Nevertheless, the precise underlying mechanisms remain incompletely elucidated. Below, we present evidence of functionally altered $T_{reg}$ cell characteristics across a spectrum of distinct autoimmune diseases and discuss the potential mechanisms responsible for the impairment of $T_{reg}$ cell functionality within each specific disease context.

### Multiple sclerosis

Circulating $CD4^+CD45RA^-CD25^{hi}CD127^{low}$ $T_{reg}$ cells (corresponding to Fr. II $T_{reg}$ cells)[3] from patients with multiple sclerosis (MS $T_{reg}$ cells) were shown to contain a significantly higher proportion of cells that produced IFNγ compared with those from healthy controls[16,137]. $IFN\gamma^+FOXP3^+$ $T_{reg}$ cells maintained a similar TSDR demethylation pattern to $IFN\gamma^-FOXP3^+$ $T_{reg}$ cells from both patients with multiple sclerosis and healthy controls and expressed comparable levels of FOXP3 (ref. 16). These data indicate that the FOXP3 autoregulatory circuit remained intact, yet the $T_{reg}$ cell transcriptional programme, independent of FOXP3, might have become dysfunctional, allowing for aberrant effector cytokine production. Moreover, the impaired suppressive activity of MS $T_{reg}$ cells was recovered in the presence of neutralizing antibodies to IFNγ, whereas $T_{reg}$ cells from healthy individuals remained unaffected. This implies that IFNγ secretion could be a hallmark of $T_{reg}$ cell dysfunction in patients with multiple sclerosis[12]. A similar observation was made with $T_{reg}$ cells from patients with type 1 diabetes. However, it should be noted that TSDR demethylation appeared to be lost in $IFN\gamma^+FOXP3^+$ $T_{reg}$ cells in patients with type 1 diabetes (ref. 138), a discrepancy that might be explained by a different gating strategy. This dysfunctional feature of $T_{reg}$ cells in human autoimmunity shares a significant

similarity to the $T_{reg}$ cell fragility observed in the mouse tumour microenvironment[139,140]. These examples from autoimmunity and tumour immunity complement each other and highlight that a IFNγ signature in $T_{reg}$ cells is a critical hallmark of $T_{reg}$ cell dysfunction and/or fragility.

A genome-wide gene expression approach and pathway analysis that examined IFNγ$^+$ versus IFNγ$^-$ $T_{reg}$ cells identified the PI3K–AKT–FOXO1/3 signalling cascade as the major pathway involved in IFNγ secretion by human $T_{reg}$ cells[141]. Ex vivo experiments demonstrated a critical role for specific AKT isoforms in the generation of $T_H$1-type IFNγ$^+$FOXP3$^+$ $T_{reg}$ cells[142]. A key finding was that blockade of the AKT pathway in MS $T_{reg}$ cells inhibited IFNγ secretion and restored suppressive function in vitro[143]. Similarly, recent experiments with MS $T_{reg}$ cells showed that binding of CD155 to TIGIT, which leads to a suppression of the PI3K–AKT pathway, can inhibit IFNγ secretion and restore the suppressive function of $T_{reg}$ cells[144]. Inhibition of FOXO1 activity has been shown to contribute to $T_{reg}$ cell dysfunction, which is associated with mTOR pathway-mediated control of $T_{reg}$ cell function (Box 2).

Further investigation of pathways that are specifically activated in IFNγ$^+$ $T_{reg}$ cells compared with IL-10$^+$ $T_{reg}$ cells in humans identified β-catenin signalling as a suppressor of FOXO activity[137]. In a high salt environment, the unphosphorylated stabilized (‘activated’) form of β-catenin, together with the serine/threonine kinase SGK1, can potentiate signalling via the AKT pathway and promote the differentiation of $T_H$1-type $T_{reg}$ cells, and stabilized β-catenin was found to be increased in MS $T_{reg}$ cells[137].

SGK1, which interacts with the mTOR–AKT and FOXO pathways, has been implicated as playing a role in the development of multiple sclerosis and EAE. Although initially known for its role in maintaining the salt balance by inducing the production of aldosterone in renal tubule epithelial cells[145], the role of SGK1 in the differentiation of CD4$^+$ T helper cells became evident when examining mouse $T_H$17 cells. Here, SGK1 emerged as a pivotal driver of IL-23R expression, thereby contributing to a phenotype skewed towards $T_H$17 cells rather than $T_{reg}$ cells with potential for pathogenesis[146]. This effect requires p38 MAPK and SGK1 signalling, is enhanced under elevated NaCl conditions and has also been demonstrated in in vitro experiments with human $T_{reg}$ cells[147]. Here, high salt exposure impaired $T_{reg}$ cell function without altering TSDR methylation or FOXP3 expression, favouring a shift towards $T_H$1 cell differentiation which was marked by elevated T-bet expression and IFNγ production[148]. Inhibition of SGK1, or deletion or silencing of *SKG1,* causes retention of unphosphorylated FOXO1 in the nucleus, which leads to an upregulation of FOXP3, CTLA4, ICOS and CD25, and restores function under high salt conditions[148-150]. Interestingly, the phenotype of $T_{reg}$ cells from patients with multiple sclerosis mirrors that of $T_{reg}$ cells cultured under high salt conditions, with elevated β-catenin activation, phosphorylated FOXO1 and IFNγ expression[137]. Furthermore, it has been shown that brain lesions in patients with multiple sclerosis have an increased sodium concentration[151], suggesting that salt-sensing via SGK1 plays an important role in $T_{reg}$ cell function. A recent study indicated the SGK1–FOXO axis as a key pathway implicated in the dysfunctional $T_{reg}$ cell programme observed in MS $T_{reg}$ cells[152]. However, a previous study had also documented its involvement in the regulation of $T_H$1 cell versus $T_H$2 cell

differentiation within conventional T cells[153], thus suggesting context-dependent functions for SGK1 in T cell biology (Fig. 4).

## Inflammatory bowel disease

The importance of β-catenin in $T_{reg}$ cell biology was first shown in a study where aberrant activation of β-catenin in $T_{reg}$ cells caused exaggerated colonic inflammation in a mouse model of colitis and in colon tissues from patients with IBD[154]. $T_{reg}$ cells with stabilized β-catenin maintained FOXP3 expression but had a competitive fitness disadvantage and produced higher levels of IFNγ, IL-17 and TNF. TCF1 is considered to be a transcriptional repressor in the absence of β-catenin stabilization; however, it can act as an activator upon β-catenin binding[155]. Another study investigated the epigenetic landscape of $T_{reg}$ cells from mice in which β-catenin was stabilized in $T_{reg}$ cells and showed that TCF1 and FOXP3 bound cooperatively to accessible chromatin sites that are associated with T cell activation and $T_H17$ cell differentiation[137]. Moreover, chromatin regions bound by both TCF1 and FOXP3 became accessible, suggesting that activated β-catenin can switch TCF1 from a repressor into an activator, and thereby allow for T cell activation and $T_H17$ cell-associated effector function. This is supported by the observation that β-catenin is activated by TCR stimulation, especially when IL-12 is present[137]. These findings provide additional complexity to the TCF1–FOXP3 interaction; both factors exert dual functions as repressors and activators, depending on the co-binders that are dynamically changed by the amplitude of TCR stimulation and external environmental cues. Despite this complexity, it appears that the β-catenin–TCF1 axis could be one of the pathways driving $T_{reg}$ cell dysfunction in the context of multiple sclerosis and IBD. Although it is challenging to obtain the requisite tissue samples, further studies are needed to explore the characteristics of $T_{reg}$ cells from sites of inflammation, especially in the brain tissue of patients with active multiple sclerosis.

## Systemic lupus erythematosus

The number and function of $T_{reg}$ cells in patients with SLE are controversial and the definitive role of $T_{reg}$ cells in SLE remains unclear. A preclinical study using lupus-prone NZW mice indicated that a decreased sensitivity to trophic cytokines, such as IL-2 and IL-33, resulted in impaired $T_{reg}$ cell competitive fitness and FOXP3 destabilization[156]. The transcriptomic signature of NZW $T_{reg}$ cells showed an upregulation of type I interferon response genes, which resembles the signature of peripheral blood immune cells, including that of T cells in patients with SLE[157,158]. A recent study using single-cell RNA sequencing demonstrated that peripheral blood $T_{reg}$ cells in patients with SLE are increased in frequency and expressed higher levels of co-inhibitory receptors such as PD1, TIGIT, LAG3 and CTLA4, with stronger TCR activation and type I interferon signatures and impaired in vitro suppressor function[159]. The authors described these $T_{reg}$ cell signatures as ‘exhaustion-like’ signatures (Box 3); however, given that type I interferon can induce co-inhibitory receptor expression[160], it can alternatively be explained by stronger type I interferon signalling in SLE $T_{reg}$ cells. $T_{reg}$ cell-specific IFNα/β receptor-deficient mice are susceptible to chronic viral infection and tumour development. $T_{reg}$ cells from these mice also displayed transcriptomic signatures that indicated more activated effector $T_{reg}$ cells with enhanced suppressor function, suggesting that type I interferon signalling downregulates $T_{reg}$ cell suppressor function[161]. Although other studies provided conflicting results[162,163], this study

provided direct evidence for endogenous IFNα/β receptor signalling specifically in $T_{reg}$ cells. Nevertheless, the mechanisms of $T_{reg}$ cell function in localized tissue sites with SLE-associated inflammation, such as in the skin and kidney, are vitally important but remain poorly understood due to the limited number of $T_{reg}$ cells that can be isolated from tissues and the relatively lower frequency of $T_{reg}$ cells among infiltrating immune cells.

## Rheumatoid arthritis

The relatively easy isolation of $T_{reg}$ cells from the inflamed synovial tissue of patients with rheumatoid arthritis is a great advantage for the study of $T_{reg}$ cell function in this disease. Although the quantitative and qualitative features of circulating blood $T_{reg}$ cells in patients with rheumatoid arthritis are still controversial, accumulating evidence suggests that $T_{reg}$ cell frequency is increased in their synovial fluid[164]. Moreover, synovial fluid $T_{reg}$ ($sfT_{reg}$) cells are potently suppressive in vitro[165-168], which may be due to their higher expression of CTLA4, GITR, OX40 and FOXP3. However, they appear to be impaired with regards to proliferation in response to TCR stimulation[167]. There are mixed results regarding the production of $T_H1$-type or $T_H17$-type cytokines by $sfT_{reg}$ cells and whether their dampened proliferative response in vitro is indicative of a highly differentiated state[169,170].

Interestingly, the TCR repertoire of $T_{reg}$ cells in patients with juvenile idiopathic arthritis, the most common paediatric rheumatic disorder, is restricted and clonally expanded $T_{reg}$ cells are present both in peripheral blood and in synovial fluid[171]. Recent advances in single-cell RNA sequencing technology, together with TCR repertoire analysis, have exposed novel aspects of $sfT_{reg}$ cells and led to the identification of four different $sfT_{reg}$ cell clusters in juvenile idiopathic arthritis: one naive $T_{reg}$ cell cluster and three effector $T_{reg}$ cell clusters that were defined as suppressive, cytotoxic and $CXCL13^+$ clusters[168,172]. The $sfT_{reg}$ cells in the $CXCL13^+$ cluster also expressed *LAG3, PDCD1, GPR56, ID2, HAVCR2* and *IFNG* and displayed some overlap with the gene signature of peripheral T helper cells[173]. Given the possible suppressive features of these cells in synovial inflammation, $CXCL13^+$ $sfT_{reg}$ cells could be considered as peripheral helper $T_{reg}$ cells as they have the potential to counteract peripheral T helper cells, thereby preventing synovial inflammation. Although the suppressive function of $sfT_{reg}$ cells is not impaired in vitro, traditional co-culture-based assays of conventional T cells and $T_{reg}$ cells may not be the correct context to test suppression capacity. Given that the key function of peripheral T helper cells is to facilitate the activation of B cells[173,174], the suppression of this activity by $sfT_{reg}$ cells can only be assessed in vivo in the context of B cell activation. As shown by the loss of regulatory T follicular helper cell maintenance in $T_{reg}$ cell-specific *TCF1* and *LEF1*-double knockout mice[114], impairment of the $sfT_{reg}$ cell pool and/or function might lead to aberrant humoral immunity. Moreover, single-cell TCR repertoire analysis demonstrated that the $CXCL13^+$ $sfT_{reg}$ cell cluster displayed a relatively unique TCR repertoire as compared with the other two effector $T_{reg}$ cell clusters. Of note, there was a small overlap of the TCR repertoires of $T_{reg}$ cell and non-$T_{reg}$ cell clones in synovial fluid $CD4^+$ T cells. Although mouse $T_{reg}$ cells are known to lose their identity and to become $exT_{reg}$ cells that drive pathogenic inflammation in the mouse rheumatoid arthritis model[175], the human data indicate that there is no direct evidence of conversion of $T_{reg}$ cells into $exT_{reg}$ cells at local sites of inflammation. Taken together, in rheumatoid arthritis synovial fluid, $T_{reg}$ cells maintain

in vitro suppressor function and display a differentiated effector $T_{reg}$ cell phenotype with evidence of clonal expansion in the inflamed tissue.

## Common genetic risk factors

Genome-wide association studies can identify genes that are potentially causal to disease pathophysiology[176,177]. Most of the common allelic variants are found in non-coding regions, where they are enriched in active enhancer or promoter regions that are unique to immune cell types, including $T_{reg}$ cells[54,178,179]. Accumulating evidence from genetic studies supports the idea that these variants contribute to the regulation of gene expression rather than directly disrupting the function of proteins. A common approach to elucidate the link between genetic variation and phenotype is to examine the effects of variants on downstream gene expression, called expression quantitative trait locus (eQTL) mapping[180]. As these variants have small effect sizes with complex interactions that are highly cell type and cell state dependent, and given their rarity, it is challenging to decipher how these genetic variations affect $T_{reg}$ cell biology. Recent eQTL mapping efforts to decode immune cell types have been conducted, such as DICE[181] and ImmuNexUT[158]. Of note, ImmuNexUT provides a rich resource of transcriptomic data for three different $T_{reg}$ cell subpopulations – namely, naive $T_{reg}$ (Fr. $IT_{reg}$) cells, effector/memory $T_{reg}$ (Fr. II $T_{reg}$) cells and activated conventional T (Fr. III) cells – at a population scale, which enables the data mining of subpopulation-specific eQTL effects. Another study assessed the enrichment of autoimmune disease-associated variants that are also associated with the $T_{reg}$ cell-specific DNA CpG hypomethylation status[55]. Common variants that are associated with autoimmunity were found to be enriched in $T_{reg}$ cell-specific CpG hypomethylated regions as opposed to CpG hypomethylated regions associated with conventional T cell activation. DNA CpG hypomethylation was also enriched in $T_{reg}$ cell-specific super-enhancer regions that are known to be associated with FOXP3 and other $T_{reg}$ cell signature genes[59]. Moreover, compared with common variants associated with non-autoimmune diseases or traits, those that were commonly associated with autoimmune diseases were more selectively enriched in Fr. I $T_{reg}$ cell-specific CpG hypomethylated regions[55]. These findings highlight the contribution of the $T_{reg}$ cell-specific DNA hypomethylation status and the super-enhancer region as regulatory components of genetic susceptibility in autoimmune diseases, which is consistent with previous studies demonstrating highly enriched common autoimmune variants in epigenetically active $T_{reg}$ cell-specific super-enhancer regions[54,143,182]. These studies also provided evidence that variants associated with susceptibility to autoimmune diseases are likely to affect $T_{reg}$ cell function through gene regulation; however, the specific genes or pathways linking genetic variants and gene expression had not been clarified. A recent study mapped the regulatory variants controlling the gene expression and chromatin accessibility in $T_{reg}$ cells in a cohort of 124 individuals and identified 133 unique immune disease loci that showed functional relevance in $T_{reg}$ cells[183]. Of note, a risk allele in the CD28 gene locus that is associated with multiple sclerosis exhibited a positive eQTL effect on CD28 expression in $T_{reg}$ cells. Conversely, a risk allele linked to coeliac disease demonstrated an opposing effect, suggesting that eQTL effects are specific to each disease. Although this requires further investigation, this

study highlights the candidate genes and variants that are potentially causal to $T_{reg}$ cell dysfunction in association with each autoimmune disease[183].

Although it is challenging to apply functional genomics tools to human primary $T_{reg}$ cells, recent advances in functional genomics using CRISPR–Cas9 technology have allowed us to interrogate the functions of non-coding regions both in vitro[184] and in vivo[185]. The identification of the functional impact of regulatory variants will advance our understanding of the causal role of $T_{reg}$ cells in the pathophysiology of autoimmune diseases.

## Conclusion and future directions

The identification of $T_{reg}$ cells as mediators of peripheral tolerance has revolutionized our understanding of the potential destruction that can be caused by the immune system. Attention has focused on the quantitative and qualitative loss of $T_{reg}$ cells as the key drivers of autoimmune diseases. Although the investigation of the detailed mechanisms by which $T_{reg}$ cells become dysfunctional is complicated by the plasticity and multifunctional nature of $T_{reg}$ cells, recent findings have extended our understanding of the complex mechanisms by which FOXP3 confers suppressive function and lineage identity to $T_{reg}$ cells in both direct and indirect manners. Here, the key components involved in the fine-tuning of $T_{reg}$ cell function include TCR signalling and the factors that regulate FOXP3 expression, which are regulated by environmental cues that alter the epigenetic landscape. The disruption or ‘rewiring’ of this FOXP3-centred regulatory circuit likely promotes $T_{reg}$ cell dysfunction; thus, it is essential to emphasize the importance of obtaining detailed epigenetic and genome topology information to better understand the factors contributing to $T_{reg}$ cell dysfunction. Additionally, important questions in the context of human autoimmune disease concern the $T_{reg}$ cell TCR repertoire and genetic variants associated with susceptibility to autoimmune disease $T_{reg}$ cell dysfunction. Moreover, the distinction between $tT_{reg}$ cells and $pT_{reg}$ cells in peripheral tissue remains uncertain due to a lack of established markers and a genetic system that allows fate tracing in mice and the perturbation of $pT_{reg}$ cell function in peripheral tissues. By acquiring a better understanding of this complex and plastic system via the integration of genetic tools in mice, the interrogation of disease-relevant genetics in humans and immunological tools, we may be able to develop therapeutic options that restore $T_{reg}$ cell-mediated immune tolerance. These may not only be applicable to autoimmune diseases but may also be of use to enhance tolerance to transplantation, as well as chronic inflammation.

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**Box 1**

## Key transcription factors that regulate effector $T_{reg}$ cell differentiation and function in the periphery

Upon T cell receptor (TCR) stimulation, naive regulatory T ($T_{reg}$) cells differentiate into effector $T_{reg}$ cells that can exert strong suppressive function. Effector $T_{reg}$ cells adapt to the microenvironment and acquire effector features that counter-regulate effector functions of T helper cell lineages. The differentiation into T helper 1-type ($T_H1$-type), $T_H2$-type and $T_H17$-type $T_{reg}$ cells is initiated by transcription factors. Here, the expression of T-bet promotes the differentiation of $T_H1$-type $T_{reg}$ cells, RORγt and STAT3 promote the differentiation of $T_H1$-type $T_{reg}$ cells, and IRF4, GATA3 and STAT6 promote the differentiation of $T_H2$-type $T_{reg}$ cells. GATA3 is necessary for preventing the conversion of $T_{reg}$ cells into $T_H17$-type FOXP3$^+$ $T_{reg}$ cells that have lost FOXP3 expression (‘ex$T_{reg}$ cells’) by limiting the expression of RORγt and IL-17 (ref. 130), whereas BATF[186] and IRF4 (ref. 128) block the acquisition of a $T_H2$ cell phenotype. Another T helper cell lineage-associated $T_{reg}$ cell is the regulatory T follicular helper cell, which requires the transcription factors TCF1, LEF1 and BCL-6 as well as MAF for its differentiation[141]. General effector $T_{reg}$ cell maturation and non-lymphoid tissue resident programmes are regulated by BATF, IRF4 and GATA3 (refs. 187-189). The loss of BATF in $T_{reg}$ cells results in reduced expression of activation markers and inhibitory receptors, such as GITR, PD1, LAG3 and TIGIT[189], and decreased trafficking to various tissues, including the visceral adipose tissue, lung, colon and bone marrow. These alterations are associated with tissue-specific inflammation and destabilized immune cellularity[188,190]. BATF cooperates with IRF4 and plays a key role in the development and maintenance of visceral adipose tissue $T_{reg}$ cells by promoting the expression of PPARγ and ST2 (ref. 191). Along with BATF and IRF4, GATA3 has also been identified as a key transcription factor in the trafficking of ST2$^+$ $T_{reg}$ cells into tissues and the maintenance of tissue residency[192]. In the context of cancer, BATF and IRF4 have been implicated as partners in the establishment of highly immunosuppressive $T_{reg}$ cells and together control programmes of activation, proliferation and differentiation[193]. Independent of BATF, IRF4 controls IL-10 signalling pathways[193] via the induction of BLIMP1 (ref. 194). Moreover, IRF4 and BLIMP1 are required for IL-10 production as both participate in chromatin remodelling at the *Il10* locus[194]. BACH2 functions as a transcriptional repressor of IRF4, BLIMP1 and GATA3, playing a crucial role in controlling effector $T_{reg}$ cell programmes by suppressing pro-inflammatory cytokine expression[195] and maintaining the resting $T_{reg}$ cell pool[196]. BACH2 inhibits peripheral activation of effector $T_{reg}$ cells by modulating TCR responsiveness and obstructing IRF4 binding to its target sequence[197]. TCF1, another regulator that is suppressed by TCR stimulation, cooperates with FOXP3 to suppress the expression of $T_{reg}$ cell signature genes and effector function[198]. Similar to BACH2 (ref. 196), the transcriptional regulators FOXP3 and BATF downregulate the TCF1 response to T cell activation[112,189].

**Box 2**

## The role of the mTOR–FOXO pathway in $T_{reg}$ cell function

The mTOR pathway plays a key role in the negative regulation of regulatory T ($T_{reg}$) cell development, and the mTOR inhibitor rapamycin can promote the generation of $FOXP3^+$ $T_{reg}$ cells in the periphery[199]. Independent of FOXP3 expression, the modulation of mTOR components mTORC1 and mTORC2 can impact $T_{reg}$ cell function during $T_{reg}$ cell activation[200]. However, $T_{reg}$ cell-specific deletion of *Raptor,* which encodes the regulatory unit of mTORC1, results in a scurfy-like phenotype without affecting FOXP3, IL-10 or TGFβ expression; conversely, CTLA4 and ICOS expression are abrogated[201]. mTORC1 signalling was found to be enriched in activated as opposed to resting $T_{reg}$ cells and inhibiting mTOR in activated $T_{reg}$ cells resulted in a reduced suppressive capacity, downregulation of CTLA4 and limited IRF4 induction. These findings indicate a key role of mTORC1 in establishing and maintaining the effector $T_{reg}$ cell population[202]. Enhanced mTORC2 activity has also been implicated in $T_{reg}$ cell dysfunction as the loss of FOXP3 is associated with the downregulation of *Phlpp1,* which encodes a phosphatase that negatively regulates the mTORC2–AKT pathway, thereby affecting the phosphorylation and cytosolic retention of FOXO1 (ref. 203). The translocation of FOXO1 from nucleus to cytosol leads to impaired $T_{reg}$ cell development and reduced CTLA4 and CD25 expression, and skews $T_{reg}$ cells towards a T helper 1 ($T_H1$) cell phenotype[204]. Deletion of *Rictor,* the regulatory unit of mTORC2, in FOXP3-sufficient $T_{reg}$ cells has been shown to have little impact on $T_{reg}$ cell function and identity. By contrast, the deletion of *Rictor* in FOXP3-deficient $T_{reg}$ cells can reverse the aberrant T cell effector and metabolic programme, thereby rescuing the dysfunctional phenotype[205]. That said, deletion of *Raptor* led to mTORC2 overactivity and downstream FOXO1 phosphorylation, and thereby impaired $T_{reg}$ cell function due to the loss of mTORC1 regulation of mTORC2 (ref. 201).

FOXO1 retention in the nucleus maintains $T_{reg}$ cell identity and function by suppressing the expression of IFNγ[203] and balances the activation state of $T_{reg}$ cells by promoting the expression of lymphoid organ homing genes in resting $T_{reg}$ cells as well as repressing migratory programmes[206]. In turn, activation of $T_{reg}$ cells through the T cell receptor (TCR) resulted in the upregulation of FOXO1-repressed genes that encode factors involved in migration. In mice with $T_{reg}$ cell-specific constitutively active FOXO1, $T_{reg}$ cells were adequately suppressive and had normal expression of *Ctla4, Lag3* and *Gitr.* Yet mice displayed increased immune infiltrates in the liver and colon as well as immunopathology driven by increased number of $CD8^+$ T cells. A lack of peripheral tolerance was attributed to sustained expression of molecules involved in lymphoid homing in $T_{reg}$ cells, such as CCR7, which prevented the migration of activated $T_{reg}$ cells to peripheral tissues[206]. This phenotype is also observed in *Raptor*-deficient $T_{reg}$ cells, further supporting the proposed complicated crosstalk of mTORC1 and mTORC2 in $T_{reg}$ cell function.

**Box 3**

## Exhaustion-like features of $T_{reg}$ cells

T cell exhaustion typically occurs in the context of chronic viral infections, cancer or prolonged antigen exposure, especially in $CD8^+$ T cells. The exhaustion of T cells is primarily attributed to continuous exposure to persistent antigen stimulation and sustained activation of inhibitory receptors, such as PD1, CTLA4, LAG3 and TIM3. Based on those signatures, $CD4^+$ T cell exhaustion has been detected in the context of cancer and chronic inflammation. Recently, not only $CD4^+$ conventional T cells but also regulatory T ($T_{reg}$) cells were shown to display T cell signatures of exhaustion, such as expression of the co-inhibitory receptors PD1, TIM3 and CTLA4, indicating that an exhausted-like state may occur in $T_{reg}$ cells under conditions of chronic inflammation or after prolonged immune activation[207]. Although a definitive definition of exhaustion-like $T_{reg}$ cells has not yet been established, there is a current consensus that $T_{reg}$ cells displaying functional defects, such as impaired suppressor function, and expressing T cell exhaustion markers can be considered as exhausted. Previous studies have demonstrated that $T_{reg}$ cell-specific *Pd1*-conditional knockout (cKO) mice exhibit stronger $T_{reg}$ cell suppressive capacity in the context of the EAE model of multiple sclerosis[208,209]. Furthermore, $T_{reg}$ cell-specific *Lkb1*-cKO mice express a high level of PD1 on $T_{reg}$ cells and lose the ability to control T helper 2 ($T_H2$) cell-mediated inflammation. This phenomenon is reversible with anti-PD1 mAb treatment, highlighting the role of LKB1 in $T_{reg}$ cell metabolomic and functional fitness[210]. This anti-PD1-mediated invigoration of $T_{reg}$ cell function is involved in the resistance to anti-PD1 checkpoint inhibition in some tumour types[211,212]. In tumour-infiltrating $T_{reg}$ cells, lactic acid (LA) binding to monocarboxylate transporter 1 (MCT1) promotes the expression of PD1 and thus contributes to resistance to PD1-targeted therapy[213]. In patients with glioblastoma multiforme, tumour-infiltrating $PD1^{hi}$ $T_{reg}$ cells were shown to display a gene expression signature indicative of exhaustion and were impaired in their suppressive function. Moreover, they were found to produce IFNγ[214]. PD1 expression was also increased in dysfunctional $T_{reg}$ cells from patients with multiple sclerosis[215]. These findings suggest that enhanced PD1 signalling in $T_{reg}$ cells may promote $T_{reg}$ cell dysfunction and an exhaustion-like state. Finally, a recent study addressed the question of whether human $T_{reg}$ cells acquire an exhausted state in response to repetitive T cell co-stimulation or in response to tonic signalling though chimeric antigen receptors[216]. In both cases, stimulation induced $T_{reg}$ cell dysfunction with a high expression of co-inhibitory receptors, such as PD1 and TIM3, together with the exhaustion-associated transcription factors TOX and BLIMP1. Although the definition of $T_{reg}$ cell exhaustion is not fully established, there is accumulating evidence that $T_{reg}$ cells can become exhausted and lose their suppressive function. However, the high expression of co-inhibitory receptors is not sufficient to mark exhausted dysfunctional $T_{reg}$ cells and their induction seems to be context dependent. Further studies are required to understand underlying molecular mechanisms by which $T_{reg}$ cell exhaustion is induced in different contexts, including autoimmunity and cancer.

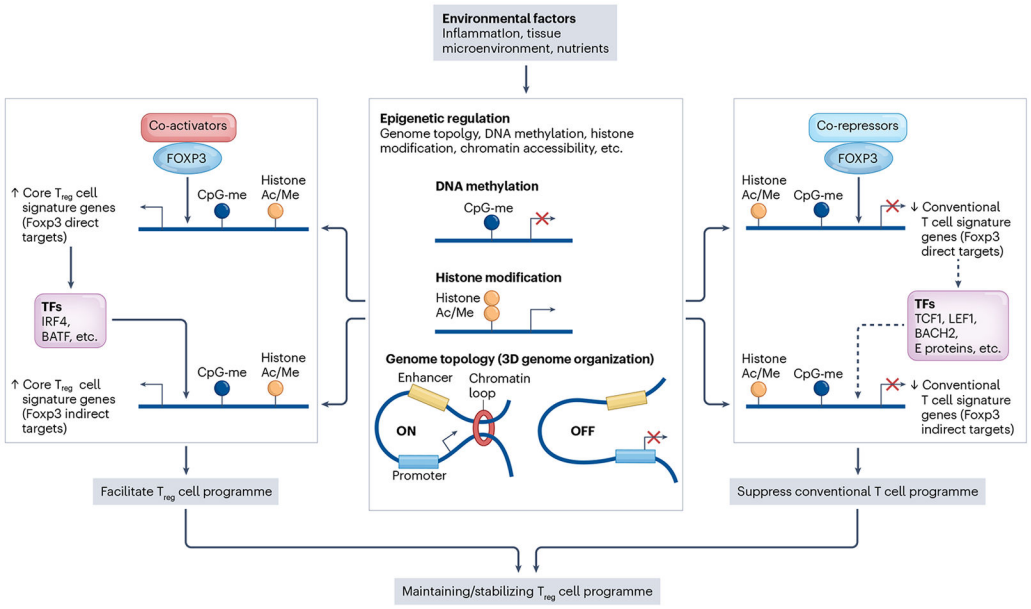


**Fig. 1 |. FOXP3-centred gene regulatory network: epigenetic modulation of $T_{reg}$ cell function and stability.**

FOXP3 plays a central role in governing the regulatory T ($T_{reg}$) cell gene regulatory network through both direct and indirect manners. Depending on interacting cofactors, FOXP3 can act as an activator or a repressor. Environmental factors, such as inflammation or nutrient availability, affect the epigenetic regulation of genes (DNA methylation, histone modifications and 3D genomic conformational changes) and can thereby directly and indirectly affect the expression of FOXP3 target genes. The FOXP3-centred gene regulatory network reinforces the expression of core $T_{reg}$ cell signature genes, ensuring their stability. FOXP3 can also suppress the differentiation of conventional T cells by downregulating transcription factors (TFs) that promote the differentiation of these cells. This dual activity of FOXP3 synergistically maintains $T_{reg}$ cell lineage stability and function. This multilayered FOXP3-centred gene regulatory network is indispensable for maintaining $T_{reg}$ cell functionality and stability. ac, acetylation; me, methylation.

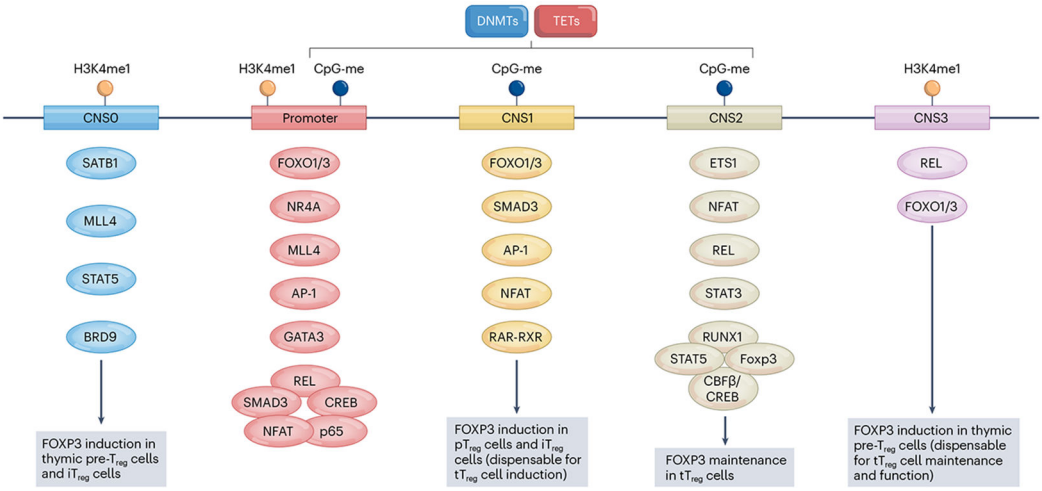


**Fig. 2 |. *cis*-Regulatory elements in the *FOXP3* locus that control $T_{reg}$ cell induction, maintenance, stability and function.**

The *FOXP3* locus contains several regulatory elements, such as the conserved non-coding sequences CNS0–3 and the FOXP3 promoter element. These regions are bound by transcription factors and complexes of transcription factors, and the binding of these factors is controlled by DNA CpG methylation (CpG-me) and methylated histone H3 Lys4 (H3K4me1), which, in turn, is determined by the balance between DNA methyl transferases (DNMTs) 1–3 and the demethylating enzymes ten–eleven translocations (TETs) 1–3. Shown are transcription factors and the complexes they form that have been reported to bind to regulatory elements. Different conserved non-coding sequence regions play a role at different stages of regulatory T ($T_{reg}$) cell differentiation. $iT_{reg}$ cell, induced $FOXP3^+$ $T_{reg}$-type cell; $pT_{reg}$ cell, peripheral $T_{reg}$ cell; $tT_{reg}$ cell, thymic $T_{reg}$ cell.

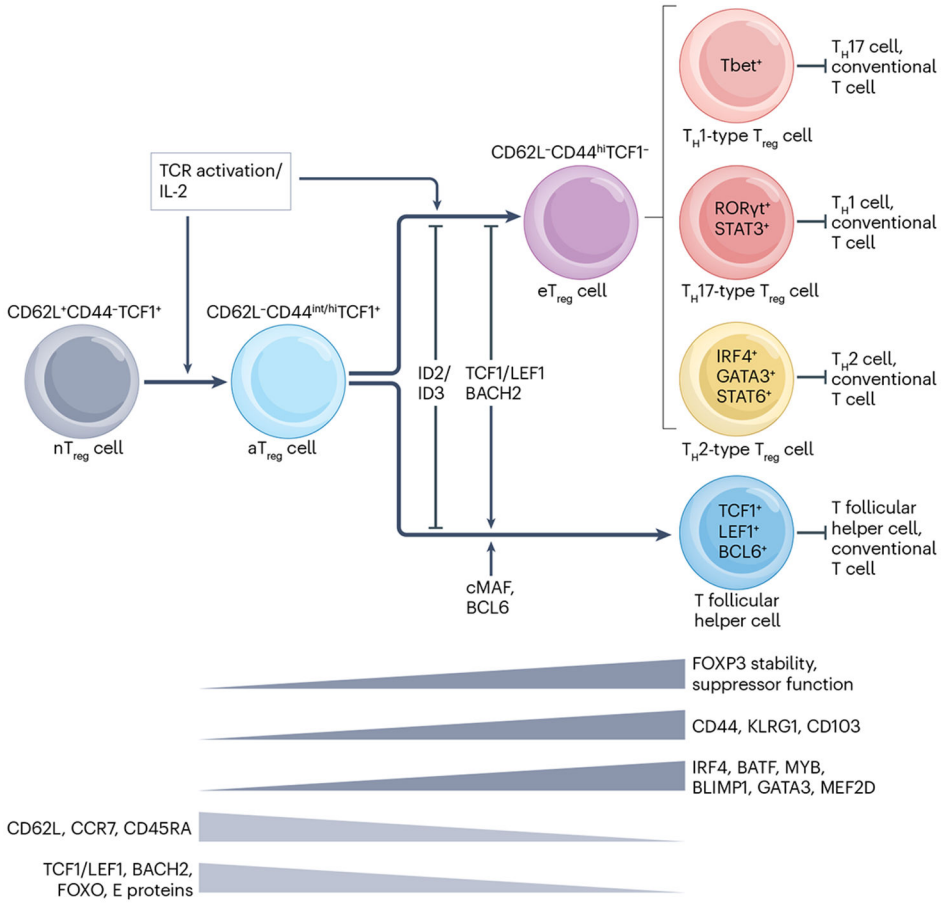


**Fig. 3 |. Transcription factors that regulate $T_{reg}$ cell differentiation and function in the periphery.**

Naive $CD62L^{+}CD44^{-}TCF1^{+}$ regulatory T ($nT_{reg}$) cells that are stimulated via T cell receptor (TCR) signalling in the presence of IL-2 become $CD62L^{-}CD44^{mid/hi}TCF1^{+}$ activated regulatory T ($aT_{reg}$) cells and then differentiate into $CD62L^{-}CD44^{hi}TCF1^{-}$ effector regulatory T ($eT_{reg}$) cells. Specific transcription factors that regulate different $eT_{reg}$ cell subsets and the differentiation steps of $eT_{reg}$ cells are shown. Cell surface markers and transcription factors that change according to differentiation state from $nT_{reg}$ cell to $eT_{reg}$ cell are also shown (bottom). $T_H1$ cell, T helper 1 cell; $T_H2$ cell, T helper 2 cell; $T_H17$ cell, T helper 17 cell.

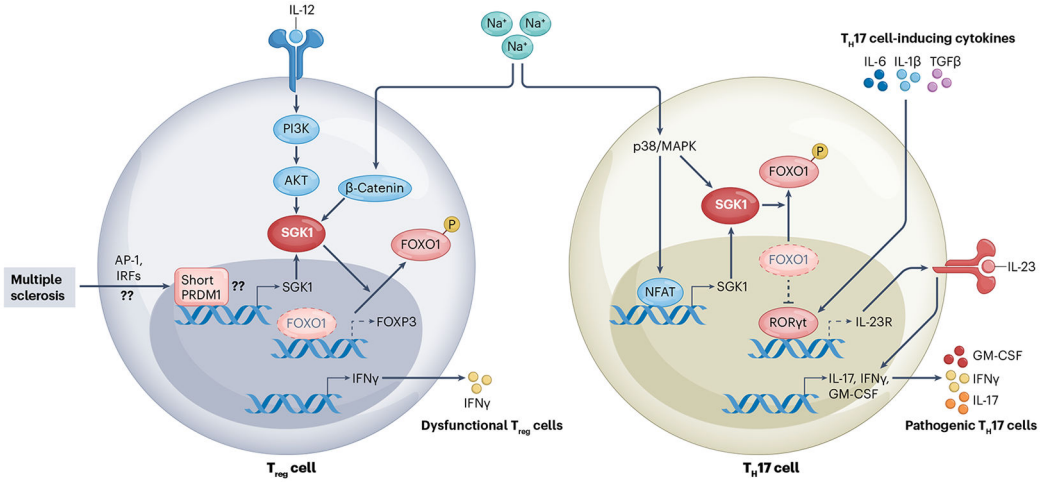


**Fig. 4 | Activation of the SGK1–FOXO1 axis is common to dysfunctional $T_{reg}$ cells and pathogenic $T_H17$ cells.**

IL-12 stimulation In the presence of a high salt environment induces regulatory T ($T_{reg}$) cell dysfunction. The upregulation of the serine/threonine kinase SGK1 due to the activation of β-catenin and/or the PI3K–AKT pathway under high salt conditions leads to the phosphorylation of FOXO1, which induces the translocation of FOXO1 from the nucleus to the cytosol where it becomes inactivated. This leads to reduced *FOXP3* induction, higher IFNγ production and loss of $T_{reg}$ cell suppressive function. $T_{reg}$ cells from patients with multiple sclerosis express higher levels of the short isoform of the transcription factor BLIMP1 compared with $T_{reg}$ cells from healthy individuals. This can upregulate SGK1 and, potentially, enhance FOXO1 inactivation. During the differentiation of T helper 17 ($T_H17$) cells, a high sodium environment activates p38 MAPK and NFAT5, which results in the activation of SGK1 and subsequent FOXO1 phosphorylation. The inactivation of phosphorylated FOXO1 allows for the derepression of the transcription factor RORγt, which, in turn, induces IL-23R expression. This promotes the pathogenic $T_H17$ cell phenotype with higher IFNγ, IL-17 and GM-CSF production. IRF, interferon regulatory factor.