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REVIEW

Molecular mechanisms underlying the regulation and functional plasticity of FOXP3$^+$ regulatory T cells

Y Gao[1], F Lin[1], J Su[1,2], Z Gao[1,3], Y Li[1], J Yang[1], Z Deng[1], B Liu[2], A Tsun[1] and B Li[1]
[1]*Unit of Molecular Immunology, Key Laboratory of Molecular Virology and Immunology, Institut Pasteur of Shanghai, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China;* [2]*Department of Surgery, Shanghai Public Health Center, Fudan University, Shanghai, China and* [3]*School of Life Sciences, Fudan University, Shanghai, China*

CD4$^+$ CD25$^+$ regulatory T (Treg) cells engage in the maintenance of immunological self-tolerance and homeostasis by limiting aberrant or excessive inflammation. The transcription factor forkhead box P3 (FOXP3) is critical for the development and function of Treg cells. The differentiation of the Treg cell lineage is not terminal, as developmental and functional plasticity occur through the sensing of inflammatory signals in the periphery. Here, we review the recent progress in our understanding of the molecular mechanisms underlying the regulation and functional plasticity of CD4$^+$ CD25$^+$ FOXP3$^+$ Treg cells, through the perturbation of FOXP3 and its complex at a transcriptional, translational and post-translational level.
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Forkhead box P3$^+$ (FOXP3 in human, Foxp3 in mice; hereafter referred to as FOXP3 unless specified) regulatory T (Treg) cells have an important role during immune homeostasis through the maintenance of immune tolerance and prevention of inflammatory disease.[1] The transcription factor FOXP3 is essential for the development and function of Treg cells.[2] The loss of expression and mutations of FOXP3 lead to the development of chronic autoimmunity and is presented as the scurfy phenotype in mice[3] and X-linked autoimmunity–allergic dysregulation and immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome in humans.[4] Although Treg cells are essential for the tolerance of commensal microbiota in the gut,[5] an excessive Treg cell response may facilitate tumor growth and chronic infection by limiting anti-tumor or anti-pathogenic immune responses, respectively.[6,7] Thus, Treg cell function must be tightly controlled to heighten or dampen inflammation according to the desired response. Recent studies have also identified FOXP3 expression in epithelial cells, multipotent mesenchymal stromal cells, invariant natural killer T cells and macrophages,[8–10] but CD4$^+$ CD25$^+$ T lymphocytes remain the most characterized cell type.

CD4$^+$ helper T (Th) cells are classified into several subsets according to the expression of various lineage-specific transcription factors and cytokines, such as T box expressed in T cells (T-bet) and interferon (IFN)-γ in Th1 cells; GATA-binding protein-3 (GATA-3) and interleukin (IL)-4 in Th2 cells; retinoic acid (RA) receptor-related orphan receptor-γt (RORγt) and IL-17 in Th17 cells; and FOXP3 and IL-10 in Treg cells.[11] There are two major subtypes of Treg cells according to their differentiation origin; natural Treg (nTreg) and induced Treg (iTreg) cells differentiate and adopt FOXP3 expression in the thymus and periphery, respectively. Naïve T cells differentiate into iTreg cells in the presence of IL-2, transforming growth factor (TGF)-β and stimulation through the T-cell receptor (TcR).[12–15] TGF-β is also required for IL-6-dependent expression of the transcription factor RORγt and inhibition of FOXP3 expression during the generation of Th17 cells from naïve T cells.[16–20] However, the expression of FOXP3 and RORγt are not always mutually exclusive, as Foxp3$^+$ T cells in the gut have been shown to co-express high levels of RORγt and concurrently secrete IL-17.[21–23] Human CD4$^+$ CD25$^-$ T cells can also transiently express FOXP3 after TcR stimulation without the requirement of other extrinsic signals, but whether or not they are suppressive during this period is still under debate.[24–30]

The instability and plasticity of Treg cells

A number of recent studies have shown that Treg cells harbor the propensity to reprogram into proinflammatory cells.[31–39] For instance, Th1-like FOXP3$^+$ Treg cells have been identified in human subjects with multiple sclerosis.[40] The evolutionary advantage of this process could be explained during inflammation, whereby the suppressive nature of Treg cells should be limited for protective and proinflammatory responses to ensue.

Correspondence: Dr A Tsun or Dr B Li, Unit of Molecular Immunology, Key Laboratory of Molecular Virology and Immunology, Institut Pasteur of Shanghai, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 37 Jianguo Middle Road, Shanghai, 200025, China.
E-mail: andy@sibs.ac.cn or binli@sibs.ac.cn
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As Foxp3 expression is required for the maintenance of Treg cell identity and function,[41] recent investigations have looked into the *in vivo* role of Foxp3 expression for the reprogramming of Treg cells.[32,33] Rudensky and colleagues[32] used GFP–Cre knock-in mice driven off the Foxp3 promoter, crossed with R26-YFP mice to fluorescently track all the cells that express or had previously expressed Foxp3 *in vivo*. This system utilized a GFP–Cre mutated estrogen receptor fusion protein so that the Foxp3 driven Cre could be induced for its translocation into the nucleus after treatment with tamoxifen. Here, they found that Foxp3 expression in Treg cells was rather stable (only ~4% cells were YFP$^+$ and GFP$^-$) when incubated under a long-term physiological setting in mice, with or without viral challenge. This observation should interest those who are investigating the use of *ex vivo* expanded nTreg and iTreg cells for the treatment of autoimmune disease.[42–44] These studies suggest that the use of *ex vivo* expanded and infused Treg cells can provide long-term protective effects in humans. However, other studies have indicated that under a physiological setting Treg cells may not be terminally differentiated, and that the instability of Treg cell function is driven by the local cytokine milieu and the loss of Foxp3 expression in Treg cells.

In contrast to the above, Bluestone and colleagues[127,131–133] used a bacterial artificial chromosome Foxp3–GFP–Cre system with R26-YFP, in which they observed the downregulation of Foxp3 expression in a significant proportion of YFP$^+$ T cells (~15% cells were YFP$^+$ and GFP$^-$); these T cells exhibited an activated-memory T-cell phenotype, produced inflammatory cytokines and were dubbed 'ex-Foxp3' T cells. When these 'ex-Foxp3' T cells were transferred into non-obese diabetic Rag2$^{-/-}$ mice, they rapidly developed diabetes. Moreover, only a small percentage of YFP$^+$ cells (0.5%) were observed after the transfer of YFP$^-$ T cells into recipient mice, which suggests that the 'ex-Foxp3' T-cell population was largely derived from nTreg cells.[33] Therefore, the depletion of Foxp3 in Treg cells may have resulted in the conversion of Treg cells into autoaggressive lymphocytes ('ex-Treg' cells), a trait that was not observed in the ~4% of the 'ex-Foxp3' T cells in the study using tamoxifen-treated mice.[32] It should be noted that the loss of Foxp3 in this system could be attributed to the transient expressers of Foxp3 rather than the committed immunoregulators. In the bacterial artificial chromosomes system, Cre is driven from birth and induces YFP expression in all the cells that have expressed Foxp3 at some point, whereas the tamoxifen study could have labeled a high Foxp3-expressing and more stable proportion of the overall Foxp3$^+$ population. This supports the notion that a proportion of Foxp3$^+$ Treg cells can convert into 'ex-Treg' cells due to their natural heterogeneity, as not all FOXP3$^+$ Treg cells are fully committed to the Treg cell lineage.[45] In any case, the populations that were monitored in these two reports were intrinsically different, and may account for the differences in their findings. Another study that focused on characterizing the progenitors of CD4$^+$ follicular B helper T cells in the Peyer's patches has demonstrated that CD4$^+$ Foxp3$^+$ T cells can lose Foxp3 expression to convert into CD4$^+$ T-follicular helper cells because of the surrounding environmental signals;[36] the loss of Foxp3 expression in Treg cells may also lead to the expression of IL-4 to potentiate the differentiation of naïve T cells into the Th2 lineage.[46] Although the controversy surrounding the functional relevance of 'ex-Foxp3' or 'ex-Treg' cells still remains, it is clear that some degree of Foxp3 depletion in Foxp3$^+$ Treg cells can occur.

Foxp3$^+$ Treg cells may lose Foxp3 expression after they are transferred into lymphopenic- or lymphocyte-null mice,[34,35,45] and *in vitro* studies have shown that Treg cells can readily lose Foxp3 expression in culture.[47,48] Although the use of rather artificial experimental conditions may not be fully translatable to the physiological occurrence of these 'ex-Treg' cells, it is abundantly clear that Foxp3$^+$ Treg cells can lose the expression of Foxp3, which in turn leads to the subsequent disruption in their suppressive ability. IL-2 can induce the upregulation of FOXP3 expression in Treg cells,[49] but what are the signals that cause the initial loss of Foxp3 expression during the reprogramming of Treg cells? Proinflammatory cytokines, such as IL-1(ref. 50) and IL-6 (below), have been suggested to be the signals that are required for Treg cell conversion into non-suppressor cells, with IL-6 as one center of focus because of its shared function across the Treg–Th17 differentiation axis. An early study by Pasare and Medzhitov[51] showed that IL-6 expression, induced by Toll-like receptor stimulation, led to the loss of Treg cell suppressive function. It was shown more recently that IL-6 can convert Foxp3$^+$ Tregs into Th17-like cells to secrete IL-17.[52] But does IL-6 affect both nTreg and iTreg cells, and how does this relate to Foxp3 expression? Zheng *et al*.[53] found that IL-6 signals affected Foxp3 expression and function in nTreg but not iTreg cells due to the downregulation of the IL-6 receptor (IL-6R) after IL-2 and TGF-β treatment (used to convert naive T cells into iTreg cells). However, the treatment of nTreg cells with IL-2 and TGF-β, but not IL-2 alone, also induces the downregulation of the IL-6R in nTreg cells to render them insensitive to IL-6 stimulation. More recently, an active derivation of vitamin A, all-trans RA, has also been shown to protect nTreg cells from their conversion into Th17 cells[54,55] due to the downregulation of the IL-6R.[55,56] However, Dong and colleagues[57] found that iTreg cells were susceptible to IL-6-induced Foxp3 depletion in Treg cells even after TGF-β treatment. It is unclear as to why these two reports differ in their characterization of how iTreg cells convert into Th17 cells—it may be due to the different systems used, but what seems clear is that the expression of IL-6R on Treg cells has a role in their sensitivity to reprogramming via IL-6R signaling. We have recently developed a system to test the direct signals required for IL-6-mediated downregulation of FOXP3 expression at the protein level. Here, we found that when FOXP3 is overexpressed in FOXP3$^-$ cells, FOXP3 could be directed for degradation via IL-6 signals (unpublished data). Thus, the mechanism of 'ex-Foxp3' T-cell generation via proinflammatory cytokines, such as IL-6, may not only occur at the transcriptional level (see below) but also at the protein level.

The heterogeneity of Treg function could also be attributed to the transcriptional changes mediated by transcription factors, found downstream of the signals provided by cytokines. Signal transducer and activator of transcription 3 (Stat3) is a transcription factor required for Th17 differentiation. Various reports have observed that Treg cells can co-express Foxp3 and Th markers to contribute toward proinflammatory[31,58] and anti-inflam-

matory responses (see below). Phosphorylated Stat3 interacts with Foxp3, and the expression of Stat3 in Treg cells is essential for the suppression of fatal colitis.[59] Stat3-deficient Treg cells can inhibit T-cell proliferation *in vitro*, but fail to suppress Th17 cell differentiation and Th17-induced colitis;[59] this mechanism requires intact IL-10 signaling in Treg cells.[60] Thus, Stat3 expression in Treg cells is essential for Treg cell-mediated suppression of Th17 cell-mediated inflammation, and this occurs through the simultaneous expression of classical Treg and Th markers. Others have shown that IL-6-, IL-1- and IL-23-mediated negative regulation of Foxp3 expression is Stat3 dependent.[57,61] How Stat3 may also control 'ex-Treg' cell conversion to produce IL-17, while also facilitating Th17-directed suppression, remains to be investigated; however, it may be possible that this switch from a regulatory to inflammatory cell type is attributed to the synergistic signals provided by other proinflammatory cytokines.

The redifferentiation of Treg cells into Th-like cells, other than Th17 cells, has also been reported. In response to IFN-γ, Foxp3$^+$ Treg cells can upregulate the expression of T-bet, which promotes the expression of the chemokine receptor CXCR3 and the accumulation of T-bet$^+$ Treg cells at the sites of Th1 cell-mediated inflammation.[62] Therefore, the expression of T-bet was found to be required for the homeostasis and function of Treg cells during type 1 inflammation.[62] IFN regulatory factor 4 is a transcription factor responsible for the expression of IL-4 in Th2 cells. The co-expression of Foxp3 and IFN regulatory factor 4 in Treg cells endows them with the ability to suppress Th2 responses.[63] The overlapping expression of T-bet and IFN regulatory factor 4 with Foxp3 may be necessary for the rapid response of Treg cells to suppress excessive inflammation by Th cells. Finally, Bcl6, a transcription factor required for the generation of T-follicular helper cells and the expression of CXCR5, has been found to be responsible for the generation of CXCR5$^+$ Treg cells, which can inhibit germinal center reactions to regulate humoral immunity.[64,65] Considering the above, it would seem inappropriate that these reprogrammed cells be named 'ex-Treg' cells, as their further differentiation determines the target for suppression, but this name may be fitting for those Treg cells that have lost their ability to suppress immune responses.

The expression or loss of expression of Foxp3 in Foxp3$^+$ Treg cells may coincide with the expression of Th-specific transcription factors or cytokines, but it remains unclear as to how these populations differ. A recent investigation has shed some light on this dichotomy by demonstrating how Foxp3$^+$ IFN-γ$^+$ T cells retain regulatory functions, but are able to differentiate further into single positive IFN-γ$^+$ T cells;[66] this mechanism could be attributed to the increased activity of Stat1, which was observed in miR-146a knockout Treg cells (see below).[67] Therefore, there may be a threshold of expression and/or activation of Th-specific transcription factors in Foxp3$^+$ Treg cells that allow them to convert from a Th-specific suppressor to a contributor of proinflammatory responses. However, the exact mechanisms and co-stimulatory signals that are required for this conversion have yet to be characterized. Moreover, it may be possible that Foxp3$^+$ T cells that co-express Th markers or are fully converted into Th cells may arise from specific pools of Treg cells that are more or less committed toward the Treg lineage, respectively.[45] Further research must be undertaken to resolve the contribution of 'ex-Foxp3' or 'ex-Treg' cells as physiological regulators of immune homeostasis. The animal strain, type/magnitude of stimuli, genetic system used to track Foxp3 expression and tissue localization may all have a role in determining whether Treg cells reprogram in such a manner and the functional phenotype into which these Foxp3$^+$ Treg cells convert.

The remainder of this review aims to summarize what is known about the mechanisms that control FOXP3 expression and function in the context of Treg cell plasticity and instability, by describing how FOXP3, and its complex, are regulated at a genomic, transcriptional and post-translational level (Figure 1). How is FOXP3 expression controlled downstream of proinflammatory signals? Do any posttranslational modifications of FOXP3 affect FOXP3 or Treg cell stability and function? Understanding these mechanisms in the context of FOXP3-dependent Treg cell plasticity and instability allows for the direct targeting of the molecules at the heart of immune tolerance.

Epigenetic and transcriptional regulation of FOXP3

The *FOXP3* gene is comprised of 11 exons, and its promoter is located −6221 bp upstream of the translation start site. The 5′-untranslated region is interrupted by a 6000-bp intron, which contains a splice donor site at the 5′-end and splice acceptor site at the 3′-end, 22 bp upstream of the translation start site. The *FOXP3* gene promoter is highly conserved between humans, mice and rats.[68] Mantel *et al*.[68] used a reporter assay to analyze the human *FOXP3* functional promoter and found that the activation responsive element of the *FOXP3* promoter is located within the −511 to −307 region. The basal promoter contains six nuclear factors of activated T cells (NFAT) and activator protein-1 (AP-1) binding sites, which positively regulates the transactivation of the *FOXP3* promoter after the triggering of the TcR.[68] The regulatory regions of *Foxp3* also contains three conserved noncoding DNA sequence elements (CNS1, 2 and 3),[69] also known as enhancers.[70,71] Using a mouse knockout strategy deficient in these CNS regions, it was found that CNS1 controls peripheral, but not thymic, induction of *Foxp3* expression; CNS2 controls the heritable maintenance of *Foxp3* expression; and c-Rel binds to CNS3 and facilitates *Foxp3* induction during thymic and peripheral Treg cell differentiation.[69]

The epigenetic state of the *Foxp3* locus determines the transcriptional activity of the *Foxp3* gene. Huehn and colleagues[72,73] found that an enhancer region (CNS2) of the *Foxp3* gene contained CpG motifs (also called the 'Treg-specific demethylation region' (TSDR)), which were fully demethylated in nTreg cells, partially demethylated in TGF-β-polarized iTreg cells and methylated in naïve and Th cells. These findings suggest that the epigenetic modification of the *Foxp3* gene is critical for the stable expression and suppressive function of Treg cells. These observations have also been confirmed in cord blood Treg cells[74] and peripheral Treg cells[75] in humans, where the latter transient expressers of FOXP3 bear a partially methylated TSDR.[75] Consistent with this

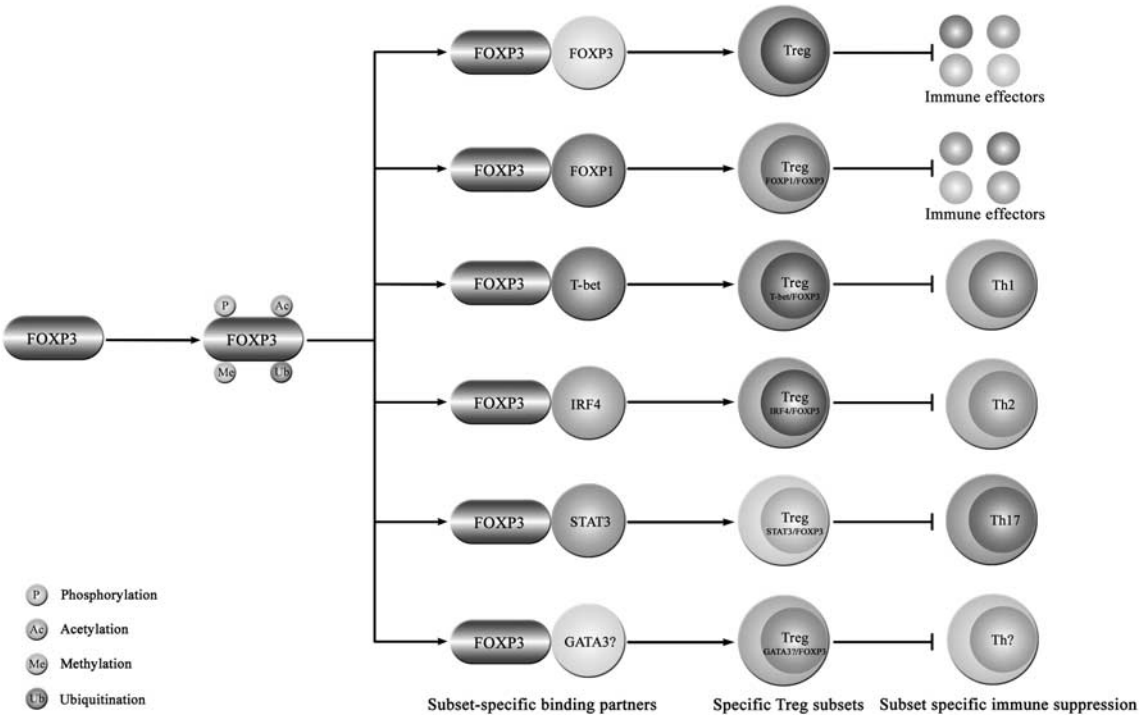

Figure 1 FOXP3 functions with other transcription factors to regulate Treg cell plasticity. FOXP3 may modulate the destiny of Treg cells by interacting with certain transcription factors that define the polarization of other Th subsets. After Treg cells are stimulated by various physiological stimuli, FOXP3 protein may undergo modifications, including phosphorylation, acetylation, methylation and/or ubiquitination, to modulate its function. FOXP3 may also bind to Th-subset-specific transcription factors to suppress specifically the corresponding T-cell lineages.

notion, a TcR response element was found in the first intron of the *Foxp3* gene that is located within this CNS region[76] and also Est-1 binding sites, which are important for positively regulating *Foxp3* transcription.[77,78] The TSDR also overlaps with the binding site for the transcription factor cyclic AMP (cAMP) response element-binding, where an increase in TSDR methylation negatively correlates with cAMP response element-binding and *Foxp3* expression.[70] Moreover, the treatment of Treg cells using 5′-azacytidine, a DNA methyltransferase inhibitor, leads to the rapid passive demethylation of DNA in this region to increase *Foxp3* expression, even in the absence of TGF-β.[70] Naïve T cells that are treated with DNA methyltransferase inhibitors and TGF-β can convert into Treg cells, with high Foxp3 expression and stable suppressive function.[79] Conversely, IL-6 can reduce Foxp3 expression and increase TSDR methylation in nTreg cells.[79] More recently, the SUMO E3 ligase PIAS1 has been shown to recruit DNA methylases and heterochromatin protein 1 to reduce *Foxp3* promoter accessibility.[80] The elucidation of how Treg cell instability relates to the known properties of epigenetic regulation of the *Foxp3* locus could therefore provide important insights into the reversibility of 'ex-Foxp3' T cells. Additionally, any future therapies involving the use of the *ex vivo* expanded and infused Treg cells in humans[42,43] may require further testing of their epigenetic state to ensure that the most stable Treg cells are utilized.

The binding of transcription factors to the promoter regions of *Foxp3* serve to augment or inhibit the transcription of this gene. Even though the epigenetic modifications detailed above offer us an indication of the basal stability and conformation of the *Foxp3* gene, the transient changes in expression and activity of transcription factors ultimately determine the outcome of gene transcription. Smad3 and NFAT can activate a *Foxp3* enhancer (CNS1).[71] The binding of both of these factors increases the acetylation of histones (such as histone 4) in both nTreg and iTreg cells stimulated by TGF-β plus TcR activation.[71] More recently, Smad3 phosphorylation has been shown to be inhibited by S1P1 signaling via the activation of the mTOR pathway to inhibit Treg cell differentiation and function.[81,82] The inhibition of the mTOR pathway is now commonly used to selectively expand Treg cells over Teff cells;[42,43] thus, this model of mTOR and Smad3 cross-talk can explain at least one aspect of how they cooperate to affect Treg cell function. Another molecule that upregulates Treg cell function is RA; RA induces the binding of the RA receptor and retinoid X receptor to a site at CNS1, which leads to local histone acetylation at the Smad3 binding site. Therefore, RA augments the TGF-β-dependent pathway and Treg cell induction by increasing the accessibility of Smad3 to the *Foxp3* locus.[83] More recently, the inhibitor of DNA binding 3 was identified as a regulator of TGF-β-dependent *Foxp3* expression. Inhibitor of DNA binding 3 can relieve the inhibition of *Foxp3* expression by reducing the binding of the transcription factor GATA-3 (see below) to the *Foxp3* promoter, to allow for the enrichment and increased binding of transcription factor 3 (E2A). Furthermore, it was shown that inhibitor of DNA binding $3^{-/-}$ T cells were more prone to differentiate into Th17 cells but not into Foxp3^{+} Treg cells.[84] TGF-β signaling may also interact with another set of transcription factors, FOXO1/FOXO3a, to positively regulate Foxp3 expression by binding to its promoter,[85–87]

but the exact pathways by which they meet remain to be characterized. Itch, an E3 ubiquitin ligase, has been found to ubiquitinate the transcription factor TGF-β-inducible early gene 1 in a TGF-β-dependent and non-proteolytic-dependent pathway at the *Foxp3* promoter.[88] These reports support the role of TGF-β as an important positive regulator of Foxp3 expression and shows how this pathway signals down to the gene level in Treg cells.

GATA-3 is a crucial transcription factor for the development and function of Th2 cells. FOXP3 expression cannot be induced in mature Th2 cells and IL-4-producing T cells by TcR stimulation and TGF-β treatment.[89] The block in Foxp3 expression may be due to Stat6-mediated repression of *Foxp3* transcription at its promoter region.[90] Luciferase reporter assays have supported the finding that GATA-3 can bind to the promoter of *Foxp3*, in order to mediate its transcriptional inhibition, and thus inhibit the differentiation of GATA-3$^+$ T cells into iTreg cells.[89] However, in nTreg cells, the low expression of Foxp3 seems to account for a degree of GATA-3 upregulation by some unclear intrinsic mechanism that favors nTreg-to-Th2 conversion.[46] A recent study by the same group analyzed the effect of GATA-3 depletion in Treg cells and found that these mice developed inflammatory disorder.[91] Here, GATA-3-deficient Treg cells expressed reduced amounts of Foxp3 and were enhanced in the ability to produce inflammatory cytokines. GATA-3 was also found to interact with the CNS2 region of the *Foxp3* gene to promote its expression. Therefore, the role of GATA-3 to inhibit or increase Foxp3 expression could be determined by its interaction with Th2- or Treg cell-specific molecules, and seem to differ between iTreg and nTreg cells.

Runt-related transcription factor 1 (RUNX1)/acute myeloid leukemia 1 and RUNX3/acute myeloid leukemia 2 are also specifically involved in the process of T-cell lineage commitment.[92] The mRNA of RUNX1 and RUNX3, as well as FOXP3, can be upregulated by the stimulation of naïve T cells using anti-CD3/CD28 antibodies plus TGF-β treatment.[93] RUNX1 and RUNX3 can bind to the *Foxp3* promoter in the presence of TGF-β to enhance the expression of *Foxp3*. The inactivation of the gene encoding for RUNX cofactor–core-binding factor-β in mice and the knockdown of RUNX1 and RUNX3 in human T cells reduces the expression of FOXP3 and the suppressive function of Treg cells. Another report also found that the RUNX–core-binding factor-β complexes can control the expression of *Foxp3*, and the evidence suggests that this complex acts at the CNS2 region.[69,94] Thus, RUNX transcription factors are required for positively regulating FOXP3 expression and the function of Treg cells.[93]

The nuclear factor (NF-κB) signaling pathway is a key regulator of Foxp3 expression;[69,95,96] c-Rel, an NF-κB family member, can bind to the *Foxp3* enhancer region (CNS3) and control the development of Treg cells by promoting the formation of a Foxp3-specific enhanceosome. c-Rel-deficient mice have up to a 90% reduction of Treg cells compared with wild-type mice, and are compromised in Treg cell differentiation.[69,95,96] More recent studies have shown how the cooperative expression of c-Rel and JunB significantly enhances Foxp3 promoter activity,[97] and the role of RelA has also been observed to be important for iTreg cell differentiation in a CD28 signal-dependent manner.[98]

IL-2 and its receptor IL-2R are crucial for the expansion and survival of Foxp3$^+$ Treg cells *in vivo*.[99–101] When mice are deficient in the *IL-2R-β* gene, the number of Treg cells reduces remarkably, indicating that the signaling pathway downstream of IL-2 affects the differentiation of Treg cells.[99] Additionally, limiting the availability of IL-2 to Treg cells increases their propensity to convert into proinflammatory cells via the signals provided by inflammatory cytokines such as IL-12.[31] IL-2 can activate Stat5, which binds to the promoter of the *Foxp3* gene to promote Treg cell differentiation by regulating the expression of *Foxp3*.[102,103] Conversely, IL-6 stimulation leads to the binding of Stat3 to the CNS2 region to inhibit transcription, thus controlling Treg/Th17 polarization.[49,104] The combination of IL-6 and TGF-β also induces the expression of the Th17 transcription factor RORγt, which can bind to the *Foxp3* promoter to inhibit its transcription.[105]

Other pathways which are involved in the direct regulation of the *Foxp3* gene include the aryl hydrocarbon receptor, which can induce Foxp3 expression in Treg cells by binding to the conserved aryl hydrocarbon receptor-binding sites in the *Foxp3* promoter.[106] The notch pathway has also been implicated in negatively controlling *Foxp3* expression[107] through Hes1, which can interact with the promoter region of the *Foxp3* gene[108] and also by signaling through the protein kinase C and canonical NF-kB pathways.[109] Finally, Bcl11b has also been shown to increase the suppressive nature of Treg cells, and was found to regulate the genes that encode Foxp3 and IL-10.[110]

Histone modifications allow for the control of gene accessibility and may also regulate the transcription of *Foxp3*. The trimethylation of histone H3 lysine 4 (H3K4me3) is a permissive mark that facilitates the transcription of target genes, whereas the trimethylation of histone H3 lysine 27 (H3K27me3) inactivates them. Wei *et al*.[111] generated genome-wide H3K4me3 and H3K27me3 maps in naive, Th1, Th2, Th17, iTreg and nTreg cells, and found that the plasticity of Th cells was more flexible than that previously envisioned. For example, H3K27me3 was detected at the *Il4* gene in naïve, Th1 and Th17 cells, whereas nTreg cells had little or no repressive marks in the *Il4* gene. This indicates that Treg cells have a higher propensity for their induction into IL-4-secreting cells and, as mentioned above, have the increased plasticity to convert into Th2 cells.[46,111] More importantly, the authors found no evidence of significant H3K27me3 marks in association with the *Foxp3* promoter in all of the tested T-cell populations, which suggests that Foxp3 may be expressed more widely and transiently than previously thought.

As the current evidence shows, there are many transcription factors that can function positively or negatively at the *Foxp3* gene to regulate Foxp3 expression. However, an extra level of regulation is exerted upon the *Foxp3* gene through epigenetic means at the TSDR. Similar to Foxp3, transcription factors may confer either upregulatory or inhibitory effects on gene transcription dependent on the timing of their recruitment and the accumulation of other transcription modulators. In this sense, there is still a long way before we will fully understand the kinetics of how these transcription factors interact *in vivo* as multiple signals are received by Treg cells at any given time. The expression and

degree of activation of Th-specific lineage markers may also determine whether Treg cells further differentiate into lineage-specific suppressor cells or reprogram into proinflammatory/non-suppressive 'ex-Treg' cells.

Regulation of microRNA expression in FOXP3+ Treg cells

The discovery and recent drive for the identification of microRNAs (miRNAs)[112–117] that are involved in gene expression regulation has added a whole new dimension to our understanding of biological regulatory systems. miRNAs are small (~22 nucleotides) noncoding RNA molecules that can target partially complementary sequences primarily at the 3′-untranslated region of mRNAs, leading to their degradation or the prevention of translation.[118,119] This ultimately results in the downregulation of protein expression. So far, more than 700 miRNAs have been identified in the human genome, whereby each miRNA has the ability to target and downregulate multiple mRNAs to create an overwhelming complex gene regulatory network.[118,119]

Unsurprisingly, miRNAs were found to be expressed in the hematopoietic system,[120] where they have been shown to have a broad role in regulating immunity—extending to both the innate and adaptive arms of the immune system.[121–124] In lymphocytes, miRNAs have been shown to determine their differentiation and function.[125] In this respect, the disruption of the RNaseIII Dicer—an endonuclease that facilitates miRNA maturation—in T and B cells causes a dramatic reduction in the numbers of thymocytes due to the increase in cell apoptosis.[126–129] However, although Dicer is involved in the maturation of thymocytes, it does not affect CD4/CD8 lineage commitment.[126]

The disruption of Dicer specifically in the CD4 lineage using CD4–Cre Dicer$^{fl/fl}$ mice results in normal thymocyte numbers, a general reduction in T-cell numbers in the periphery, the induction of a Th1 bias[127,130] and a substantial reduction of Treg cell numbers during their differentiation in the thymus.[127] IL-2 production is also highly dampened.[130] These peripheral conventional T cells are less able to express Foxp3 during *in vitro* stimulation in the presence of TGF-β, and the instability of Treg cells in this system leads to a late onset of colitis.[127] A more recent investigation into the disruption of Drosha (another RNAseIII enzyme related to miRNA biogenesis) in the CD4 T cell population has revealed a similar phenotype to the Dicer model described above.[131]

Subsequent studies that utilized Foxp3–Cre mice to eliminate miRNA specifically in Treg cells via Dicer[131–133] and Drosha[131] have shown no disruption in their development but a reduction in their suppressive ability, as indicated by the presentation of fatal early-onset lymphoproliferative autoimmune syndrome.[131–133] Moreover, these mice display a similar phenotype to those that lack Foxp3 and/or are depleted of Treg cells. A milder and later onset of disease progression seen in the CD4–Cre-based disruption of miRNA[127,130] may be attributed to the concurrent disruption of T-effector cell function. In the periphery, Rudensky and colleagues[132] observed a reduced number of Treg cells under non-inflammatory conditions; however, Treg cells could be activated to proliferate under inflammatory conditions but to the loss of suppressive capacity by the downregulation of Treg cell-specific effector molecules, such as cytotoxic T-lymphocyte antigen-4 (CTLA-4) and IL-10. Bluestone and colleagues[127,131–133] found that peripheral Treg cells deficient in miRNA may adopt Th1-, Th2- and Treg cell like effector profiles, with a reduction of Foxp3 levels in Treg cells to confer this instability. These studies show a defined requirement of miRNAs for the differentiation and suppressive function of Treg cells, with the possibility that miRNA expression profiles may be indicative of Treg cell instability.

The Treg miRNA signature has been explored in mice[127] and humans[134] to reveal Treg-specific miRNA candidates for further functional analysis. In human Treg cells, miR-31 has been shown to bind to the 3′-untranslated region of *FOXP3* mRNA for its downregulation, and miR-21 can positively—albeit indirectly—regulate FOXP3 expression.[134] The miR-31-low and miR-21-high signatures have also been shown in valproate-treated T cells in which the inhibition of histone deacetylases can induce FOXP3 expression through the induction of the transcription factor Ets-1;[77,135] however, this miRNA signature was shown not to occur as a consequence of FOXP3 expression.[135] T-cell responses in miR-155-deficient mice are biased toward Th2 differentiation but not Th17 and Th1 effector responses,[124,136,137] and are protected against EAE, thus reflecting a role for miR-155 in the immune system. Rudensky and colleagues[132] have recently shown that miR-155-deficient Treg cells have impaired proliferation, and along with other independent investigators found miR-155 to be highly expressed in Foxp3+ T cells and that Foxp3 can directly upregulate the expression of miR-155.[127,138–140] Vigorito and colleagues[141] have also investigated the role of miR-155 in Treg cells and found that mice deficient in miR-155 have reduced numbers of Treg cells in the spleen and thymus, but their suppressive capacity, the expression of Treg markers and peripheral survival rate remained intact. Suppressor of cytokine signaling-1 protein expression is regulated by miR-155, which determines Treg cell responsiveness to IL-2 signaling.[140] The repression of suppressor of cytokine signaling-1 allows for Treg cell competitiveness in environments that are limited in the availability of IL-2.[140] A recent study using a Treg cell line HOZOT identified FOXO3a as a target for miR-155.[142] As Foxp3 is proposed to upregulate miR-155 expression by inducing its resident gene, and FOXO3a is a positive regulator of Foxp3 expression, this could provide a negative feedback of Foxp3 expression through miRNAs. Other studies have found that miR-142-3p can regulate adenyl cyclase 9 mRNA and the subsequent production of cAMP in Treg cells.[143] cAMP is a determinant of Treg cell suppressor function.[144,145] Foxp3 mediates the downregulation of miR-142-3p expression, which has yet to be characterized as a direct or indirect process, to disrupt the maintenance of cAMP production.[143] Lu *et al*.[67] recently identified that mir-146a expression is crucial for Treg cell function, where miR-146a-mediated downregulation of Stat1 is required for Treg-mediated suppression of the Th1 response.[67] In this model, the expression level of Stat1 is crucial for Treg function, such that low Stat1 expression in Treg cells render them unable to mitigate Th1 responses, but excessive Stat1

activation due to the loss of mir-146a expression results in Treg cell instability and their reprogramming into Th1-like IFNγ-secreting cells.[67] Furthermore, a recent analysis of miR-146a has revealed its major role in the dampening of inflammation and anti-tumor responses in mice.[146]

Treg cells present a miRNA signature that is unique compared with conventional T cells; however, T cells with an induced overexpression of Foxp3 (and during their early activation) may also upregulate the expression Treg cell-specific miRNAs.[127] During autoimmune disease, an alteration in the miRNA signature of Treg cells has been identified between healthy and diseased patients. In multiple sclerosis, 23 miRNAs were found to be differentially expressed between diseased and healthy human subjects by array studies, where miR-106b, miR-19b and miR-25 were upregulated in $CD4^+CD25^{hi}$ $CD127^{low}$ Treg cells of multiple sclerosis patients.[147] During diabetes, an increased miR-510 and decreased miR-342 and miR-191 expressions were found in Treg cells of diseased patients compared with healthy controls.[148] In a mouse model of systemic lupus erythematosus, Divekar *et al*.[149] had an unexpected observation of a decrease in Dicer but an increase in miR-155 expression in Treg cells from diseased mice prone to systemic lupus erythematosus, which suggests the existence of a Dicer-independent miR-155 processing mechanism that precedes the onset of disease. Here, they found that 12 miRNAs increased and 54 reduced in Treg cells from diseased patients compared with healthy donor controls.[149] Our recent miRNA microarray studies have also identified similar patterns of miRNA expression in a Treg cell line as the studies above (unpublished data). These investigations bring a correlative expression of miRNA in diseased conditions, but whether or not miRNA profile changes arise as a contributor or as a consequence of inflammation require further investigation.

The discovery of Dicer/Drosha-independent miRNA processing by Argonaute 2 adds further complexity to the global role of miRNA in the immune system; this has yet to be investigated in Treg cells.[150–152] Combined with the invention of next-generation sequencing methods for the analysis of miRNA expression in the immune system,[153,154] there remains a lot to be discovered before we can fully understand the role of different miRNAs in Treg cell function. Future studies of Treg cell miRNA signatures under different settings, such as cancer, infection and autoimmune disease, may provide clues into the intrinsic stability of Treg cells that lead to the development of disease. Other miRNAs that are important for Th cell function may also have a role in Treg cells when subjected to proinflammatory differentiation cues. Recent efforts have pursued to therapeutically target miRNAs in the immune system. Thus, the targeting of miRNAs specifically in Treg cells may also allow for the treatment of human diseases.

Dynamic regulation of the FOXP3 complex

FOXP3 is a key transcription factor required for the suppressive function of Treg cells. Upon the induction of FOXP3 expression in Treg cells, a number of inflammatory cytokines, such as IL-2 and IFN-γ, are downregulated, whereas IL-10, CTLA-4, glucocorticoid-induced tumor necrosis factor receptor (TNFR)-related protein (GITR) and CD25 are upregulated.[99] Recent studies have suggested that FOXP3 does not function as a single molecule, but by forming a large supramolecular complex.[155,156] Li *et al*.[155] detected an endogenous FOXP3 complex of more than 500 kD in size in human T cells, and found that FOXP3 may exist as homodimers or homotetramers. We propose that FOXP3 may determine the function and plasticity of Treg cells by interacting with different binding partners, and that the regulation of the FOXP3 complex is highly dynamic (Figure 1).

FOXP3 protein contains a proline-rich region, zinc-finger domain, leucine-zipper domain and a forkhead domain. In humans, FOXP3 is expressed as two isoforms; one is the full-length form representing an ortholog to murine Foxp3 and the other is a smaller form lacking exon 2 (amino acids 72–106 of the full-length form), which only exists in humans. Human T cells over-expressing FOXP3Δexon2 have an intermediate proliferative response to TcR stimulation and produce marginally more IL-2 than cells expressing only the full-length protein.[2] However, the distinct physiological function of FOXP3Δexon2 remains unclear. Ziegler and colleagues[157] have revealed that the RA receptor-RORα can interact with exon 2 of FOXP3 for the inhibition of RORα-mediated transcriptional activation. Moreover, FOXP3 may bind to the AF2 domain of RORα to downregulate the expression of IL-17, IL-22 and CXCR3, which shows that the high expression of FOXP3 in T cells can inhibit the expression of proinflammatory cytokines and subject them to differentiate into Treg cells.[157] However, the reverse effects of RORα on FOXP3 requires further investigation at the protein level. Additionally, Foxp3 can to be processed by convertases into a shorter form by its cleavage at the N and/or C terminals, which are functionally distinct from one another.[158,159]

NFAT binds cooperatively to composite DNA with AP-1 to regulate the expression of *IL2*, *IL4* and *IFN-γ*. NFAT is activated by calcium and calcineurin, whereas AP-1 is induced by the protein kinase C/Ras signal pathways.[160,161] Wu *et al*.[162] found that the FKH domain of FOXP3 could bind to NFAT at the same DNA region of the NFAT–AP-1 complex. They then proposed that FOXP3 could compete with the NFAT–AP-1 complex to repress the transcription of NFAT-AP-1 target genes.[162] In light of these findings, NFAT therefore displays bifunctional-like properties by binding to various transcription factors, and may affect the plasticity of T cells by acting like a switch in response to different stimuli. Recently, the structure of the NFAT1–FOXP3–DNA complex was solved.[163] These investigators found that the FKH domain of FOXP3 can form stable domain-swapped dimers in solution in the presence and absence of DNA. The interface of the domain-swapped dimer is important for the suppressive function of FOXP3, whereby the mutations at this interface eliminate FOXP3-mediated suppressive function.[163] As FOXP3 was earlier shown to form dimers via its leucine-zipper domain,[155,164,165] its dimerization therefore occurs at both the leucine-zipper and FKH domain. However, the question remains as to how the FKH domain is regulated by its interaction partners to allow for FOXP3-mediated suppression or induction of its target genes.

The transcription factor RUNX1 can upregulate the expression of IL-2 and downregulate cell surface molecules, such as CD25, CTLA-4 and in particular GITR.[166] RUNX1 is expressed in both Teff and Treg cells, and in Foxp3+ Treg cells RUNX1 can interact physically with Foxp3. Foxp3-mediated upregulation of Treg cell-associated molecules, such as CD25, CTLA-4 and GITR, relies on its interaction with RUNX1.[167] Therefore, RUNX1 functions differently in Teff and Treg cells. It is therefore conceivable that FOXP3 could determine the differential fate of T cells by binding to a number of key transcription factors during different inflammatory settings.

Eos is a zinc-finger transcription factor that belongs to the Ikaros family, and a recently identified functional component of the Foxp3 complex that acts specifically to promote Foxp3-mediated target gene repression.[168] Eos is highly expressed in Treg cells, especially in activated Treg cells ($CD4^+$ $CD25^{hi}$ $CD62L^{lo}$) and can bind to the proline-rich domain of Foxp3. The knockdown of Eos reverses Foxp3-mediated suppression of IL-2 expression, but has little effect on CD25, CTLA-4 and GITR expression.[168] Therefore, Eos is necessary for gene silencing but not for the expression of Foxp3-activated genes. The mechanism by which Eos is involved in Foxp3-dependent gene silencing may be through its co-repressors such as C-terminal binding protein 1, which affects histone modification and promoter methylation involved in selective gene silencing.[168]

Finally, FOXP3 has also been found to directly interact with c-Rel through its N-terminal region to repress the NF-κB pathway in mature Treg cells.[169] It is highly likely that FOXP3 has many other binding partners. We are currently investigating the role of various interaction partners of FOXP3 that were elucidated via the purification of the FOXP3 complex and by high-affinity *in vitro* protein–protein binding studies. Dissecting the interaction motifs of FOXP3 with its binding partners could allow for the targeting of these protein–protein interaction interfaces to block particular FOXP3-mediated pathways.

Post-translational modification and stability of FOXP3

Post-translational modifications are essential for the expression, location, stability and function of many functional proteins, including histones, transcription factors and other cellular proteins.[170–172] The different modes of protein post-translational modifications include acetylation, methylation, phosphorylation, ubiquitination, neddylation and sumoylation. These modifications may cross-talk and regulate one another synergistically;[173] currently, data is lacking as to the exact modifications that Foxp3 could undergo. The upstream signals that may lead to these modifications and the resultant downstream functions must also be dissected to fully understand these mechanisms in the context of Treg cell instability and plasticity. In addition, the post-translational modification of FOXP3 may affect a plethora of pathways, including the dynamic regulation of the FOXP3 complex and FOXP3 stability.

FOXP3 was recently identified as an acetylated protein in human primary $CD4^+$ $CD25^+$ Treg cells.[174] Both the histone acetyltransferase TIP60 and histone deacetylase HDAC7 can be recruited to the proline-rich domain of FOXP3, and are required for FOXP3-mediated suppression of IL-2 expression.[174] HDAC9 may also interact with FOXP3 in resting Treg cells, which can be disrupted by TcR stimulation and reversed by the pretreatment of Treg cells using the protein deacetylation inhibitor trichostatin A.[175] This suggests that the interactions between the components of the FOXP3 complex are highly dynamic and dependent on the circumstantial stimuli. The histone acetyltransferase p300 was recently identified for its ability to acetylate and stabilize Foxp3 protein.[176] This process can be reversed by the histone deacetylase SIRT1, which has been shown to colocalize with Foxp3 to mediate this process.[176–178] Impaired proteasome-mediated Foxp3 degradation through the reduction of Foxp3 ubiquitination, combined with its hyperacetylation, can increase the suppressive function of Treg cells[176] and affect target gene occupancy of Foxp3;[175,179] however, this could be perturbed under the influence of Treg cell-destabilizing cytokines such as IL-6.[180] We have recently identified a stress-signal-activated E3 ubiquitin ligase, named STUB1, that can interact with FOXP3 to promote its polyubiquitination and degradation *in vitro* and *in vivo*, whereby the overexpression of STUB1 in Treg cells impairs their suppressive function (unpublished data). This process is linked to the heat-shock response of Treg cells, which may indicate the role of heat and/or the combination of proinflammatory signals to downregulate FOXP3 expression in Treg cells and allow for their conversion into proinflammatory cells (unpublished data).

Post-translational modification is a truly efficient and dynamic process. Our knowledge in this research field in the context of FOXP3 is still very limited. Modifications such as phosphorylation, sumoylation and neddylation have not been reported but are currently being investigated in our lab, and the distinct sites of acetylation and ubiquitination, regulation of different HATs and HDACs, and their corresponding upstream signaling pathways remain unclear. How these post-translational changes in FOXP3 regulate its target gene expression and its own stability would be of high interest to the Treg cell instability field as a means of finding new drug targets to therapeutically manipulate Treg cells to suit our requirements.

Conclusions

FOXP3, as a master regulatory transcription factor, has a central role in immune regulation mediated by FOXP3+ Treg cells. Its transcription, expression, modification and function all require strict and precise regulation, including extracellular stimulation, intracellular signaling, transcriptional and translational regulation, post-translational modification and its interaction with other enzymatic and non-enzymatic nuclear cofactors. All these phenomena could affect the plasticity toward the development and function of Treg cells within the local tissue microenvironment. Understanding these molecular mechanisms in the context of the flexibility and plasticity of FOXP3+ Treg cells would therefore provide us with clues on how to design new and novel tools for the therapeutic modulation of Treg cell function to treat

immune diseases, such as autoimmunity, infectious disease, allergy and cancer.

Conflict of interest

The authors declare no conflict of interest.

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