

From stability to dynamics: understanding molecular mechanisms of regulatory T cells through Foxp3 transcriptional dynamics

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Summary

Studies on regulatory T cells (T_{reg}) have focused on thymic T_{reg} as a stable lineage of immunosuppressive T cells, the differentiation of which is controlled by the transcription factor forkhead box protein 3 (Foxp3). This lineage perspective, however, may constrain hypotheses regarding the role of Foxp3 and T_{reg} *in vivo*, particularly in clinical settings and immunotherapy development. In this review, we synthesize a new perspective on the role of Foxp3 as a dynamically expressed gene, and thereby revisit the molecular mechanisms for the transcriptional regulation of Foxp3. In particular, we introduce a recent advancement in the study of Foxp3-mediated T cell regulation through the development of the Timer of cell kinetics and activity (Tocky) system, and show that the investigation of Foxp3 transcriptional dynamics can reveal temporal changes in the differentiation and function of T_{reg} *in vivo*. We highlight the role of Foxp3 as a gene downstream of T cell receptor (TCR) signalling and show that temporally persistent TCR signals initiate Foxp3 transcription in self-reactive thymocytes. In addition, we feature the autoregulatory transcriptional circuit for the Foxp3 gene as a mechanism for consolidating T_{reg} differentiation and activating their suppressive functions. Furthermore, we explore the potential mechanisms behind the dynamic regulation of epigenetic modifications and chromatin architecture for Foxp3 transcription. Lastly, we discuss the clinical relevance of temporal changes in the differentiation and activation of T_{reg}.

Keywords: Foxp3, Nr4a3, regulatory T cells (T_{reg}), Time of cell kinetics and activity (Tocky), transcriptional autoregulatory circuit

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Introduction

Dynamics of Foxp3 transcription as a key to understanding regulatory T cell-mediated immune regulation

It is widely considered that regulatory T cells (T_{reg}) constitute a distinct lineage of CD4⁺ T cells dedicated for immunosuppression [1]. Key evidence for the distinct lineage include: (i) T_{reg} development is controlled by the transcription factor Foxp3 [2]; and (ii) the development of T_{reg} in the thymus is delayed to after that of other T cells under physiological conditions [3]. However, accumulating evidence shows the simultaneous development of T_{reg} and other T cells [4,5] and T_{reg} plasticity is now

widely recognized, as T_{reg} can lose forkhead box protein 3 (Foxp3) expression and become effector T cells (ex-T_{reg}) during inflammation [6,7]. Thus, studies on dynamic changes in the differentiation and activation status of T_{regs} – and other T cells – *in vivo* is essential for understanding Foxp3-mediated T cell regulation. This dynamic perspective is important not only for basic research but also clinical research and immunotherapy development, which is illustrated by the catastrophic clinical trial of the superagonistic anti-CD28 antibody TGN1412 in 2006.

TGN1412 was developed as an immunosuppressive treatment after an anti-CD28 antibody was found to suppress autoimmune reactions in rodent models [8]. TGN1412 was thus designed to bind to the CD28

molecule on the surface of T_{reg} which would, in turn, theoretically suppress non- T_{reg} [9]. This trial, however, resulted in catastrophe, where all six volunteers given TGN1412 developed a 'cytokine storm' due to stimulation of a significant proportion of T cells [10]. Later, it was found that CD28 molecules in memory-phenotype T cells are down-regulated in primates – which does not occur in humans – and this species difference was deemed to be the major cause of the incident [11]. Meanwhile, Vitetta and Ghetie pointed out that T_{reg} and non- T_{reg} may not represent strictly separate lineages, and therefore the assumption of specific activation of T_{reg} may have been inappropriate [12]. In fact, basic studies later showed the plasticity of T_{reg} : T_{reg} may lose Foxp3 expression during inflammation and non- T_{reg} may acquire Foxp3 expression [13]. Summarizing, the case provides two important lessons: first, the concepts of lineage stability may constrain hypotheses, which can be detrimental in clinical settings; and secondly, it is fundamental to investigate the dynamic changes in the differentiation and activation statuses of T_{reg} and other T cells *in vivo*, which are still poorly understood.

The key evidence of Foxp3 as the lineage-specification transcription factor is that mutations in the *Foxp3/FOXP3* gene can lead to autoimmune disease in both mice [14] and humans [15]. However, this does not preclude the dynamic induction of Foxp3 as a negative regulator in response to T cell activation. In fact, Foxp3 expression can be induced solely by T cell receptor (TCR) signals in human T cells [16] and, although less efficiently, also in mice [17], and the induction is enhanced by transforming growth factor (TGF)- β and interleukin (IL)-2 [18]. TGF- β is produced by activated antigen-presenting cells such as dendritic cells [19] and macrophages [20], while IL-2 is produced mainly by activated T cells, particularly CD4⁺ T cells [21]. As the immunosuppressive T_{reg} population is commonly identified by the expression of Foxp3 (as Foxp3⁺ T cells in mice [2] and Foxp3^{high}CD45RA⁺ [22,23] or Foxp3⁺CD127⁻CD25^{high} T cells [24,25] in humans), the investigation of *Foxp3* dynamics *in vivo*, especially during immune responses, will be key for understanding the *in-vivo* dynamics of T_{reg} and T cell regulation. To this end, we have recently developed a new technology, the Timer of cell kinetics and activity (Tocky) system, which allows the investigation of *in vivo* dynamics of Foxp3 and T_{reg} during physiological immune responses [26,27].

In this paper, we will aim to introduce a dynamic perspective to the molecular mechanisms that account for the transcriptional and epigenetic control of the *Foxp3* gene, and thereby to improve the understanding of Foxp3-mediated T cell regulation *in vivo*.

Development of Tocky for investigating *in-vivo* dynamics of T_{reg} differentiation

The current understanding of T_{reg} differentiation and function is based significantly on evidence obtained by *Foxp3* fluorescent protein (FP) reporters such as enhanced green fluorescent protein (EGFP) [28,29] and fate mapping systems for the *Foxp3* gene (e.g. *Foxp3*^{CreGFP}:*Rosa26*^{RFP} [17] and *Foxp3*^{ERT2CreGFP}:*Rosa26*^{YFP} [30]). Notably, all these systems rely upon stable FPs such as GFP, the half-life of which is longer than 56 h. Therefore, temporal changes in *Foxp3* transcription shorter than 2–3 days cannot be investigated by these reporter systems.

In order to understand the *in-vivo* dynamics of those molecular mechanisms underlying the differentiation and function of T_{reg} , we have recently developed the Tocky system using Fluorescent Timer protein (Timer). Timer proteins exhibit a short-lived blue fluorescent form, before maturation to the stable red state [27,31]. The half-life of blue fluorescence is ~ 4 h [26,27] and that of the mature red fluorescence is ~ 5 days [26]. Thus, blue and red fluorescence (blue and red) provide a measurement of both the 'real-time' activity and the history of gene transcription [26]. Tocky uses this information to analyse dynamic changes quantitatively in transcriptional activities during cellular activation and differentiation [27]. Importantly, we have identified three characteristic dynamics of transcription in the Tocky system: blue⁺red⁻ cells are those that have just initiated transcription (new); blue⁺red⁺ cells along the diagonal line between blue and red axes are those with sustained transcription, accumulating both blue and red form proteins (persistent); and blue⁻red⁺ cells are those that have recently down-regulated gene expression under the detection threshold of flow cytometry and are inactive in transcription of the gene (arrested or inactive) [27] (Fig. 1).

Foxp3 transcription is controlled mainly by 5' upstream sequences and conserved non-coding sequences (CNS) 1–3 in intronic regions 7,32–34. Importantly, while TCR signals (together with TGF- β and IL-2 signals) induce Foxp3 expression in any T cells *in vitro* [18], naturally arising Foxp3 expression is found mainly in self-reactive T cells in non-inflammatory conditions [1]. Thus, we will classify the mechanisms for *Foxp3* transcription into two groups, as follows:

1. Mechanisms for the activation of *Foxp3* transcription: these are used during thymic T_{reg} selection and peripheral T_{reg} differentiation and are potentially involved in the mechanism for tonic TCR signal-mediated activation of *Foxp3* transcription.
2. Mechanisms for the consolidation and tuning of *Foxp3* transcription: these are used for sustaining *Foxp3* transcription over time, which induces effector T_{reg}

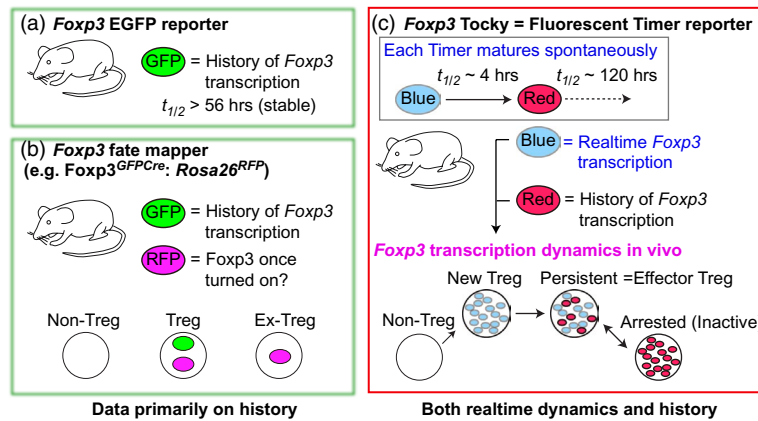


Fig. 1. Comparison of tools to investigate forkhead box protein 3 (*Foxp3*)-expressing T cells *in vivo*. (a) Most *Foxp3* reporter mice use stable fluorescent proteins (FP), such as enhanced green fluorescent protein (EGFP), the half-life of which is > 56 h. (b) *Foxp3* fate mappers such as *Foxp3*^{GFPcre}.*Rosa26*^{RFP} allow the identification of regulatory T cells (T_{regs}) with *Foxp3* expression and ex- T_{regs} that lost *Foxp3* expression. Notably, both GFP and red fluorescent protein (RFP) are stable FPs. (c) *Foxp3*-Tocky uses fluorescent timer, the emission spectrum of which changes spontaneously and irreversibly from blue to red fluorescence. The half-life of blue fluorescence is ~4 h, and thus reports the 'real-time' activity of *Foxp3* transcription. In contrast, the half-life of red fluorescence is ~120 h and thus reports the history of *Foxp3* transcription. The Tocky system combines blue and red fluorescence data and identifies characteristic transcriptional dynamics including new, persistent and arrested (inactive).

differentiation and the dynamic regulation of epigenetic modifications, such as demethylation of CpG islands in enhancer regions (Fig. 2).

Mechanisms for the activation of *Foxp3* transcription

***Foxp3* as a TCR signal downstream gene.** The differentiation and function of T_{regs} is under the control of TCR signals [35–38]. In the thymus, the recognition of cognate antigen induces not only negative selection but also the differentiation of CD25⁺*Foxp3*⁺ T_{regs} from CD4-SP cells using transgenic TCR systems [39–41]. Conversely, TCR transgenic mice in the recombination activating gene (*Rag*)-deficient backgrounds lack *Foxp3*⁺ T cells due to the absence of self-antigen recognition [42,43]. The analysis of TCR signals using reporter mice have provided insights into the mechanism for TCR-mediated T_{reg} differentiation. The Hogquist group showed that T_{regs} receive strong TCR signals in the thymus and the periphery when analysed using a Nur77(Nr4a1)-GFP transgenic reporter [44]. Using *Nr4a3*-Tocky, we have shown that *Foxp3* expression in the thymus occurs in T cells that have received temporally persistent TCR signals [27]. Furthermore, using *Foxp3*-Tocky we showed that *Foxp3* transcription is initiated in non- T_{reg} cells during inflammation in the periphery [26]. In humans, activation-induced *Foxp3* in conventional T cells suppresses their proliferation and cytokine production in a cell-intrinsic manner [45]. In addition, activated conventional T cells can express both *Foxp3* and cytotoxic T lymphocyte antigen-4 (CTLA-4), and thereby acquire the suppressive function that is dependent upon CTLA-4 [46]. These suggest that

Foxp3 has a role in negative feedback regulation of T cell activation in co-operation with other immunoregulatory molecules, including CTLA-4. *Foxp3* transcription, therefore, is thus under the control of TCR signals in both the thymus and the periphery. In addition, in normal homeostasis, T_{regs} and naturally arising memory-phenotype T cells are self-reactive and receive 'tonic' TCR signals in the periphery [27,44]. Considering this evidence, the biological meaning of TCR signal-induced *Foxp3* transcription in *Foxp3*⁻ cells (conventional T cells; non- T_{regs}) in the thymus and the periphery; and (ii) the effects of tonic TCR signals in *Foxp3*⁺ T_{regs} .

In line with the evidence of *Foxp3* expression upon TCR stimulation, the gene regulatory regions of the *Foxp3* gene are bound by transcription factors downstream of major branches of the TCR signalling pathway, including nuclear factor of activated T cells (NFAT) and activator protein 1 (AP1) [47], the nuclear factor kappa B (NF- κ B) components c-Rel and p65 [32,48–50], cyclic AMP response element-binding protein (CREB) [51] and Nr4a proteins [52] (Fig. 2).

Nr4a proteins (Nr4a1, Nr4a2 and Nr4a3) bind to their target sequences as homodimers or heterodimers and regulate transcription [53,54]. *Foxp3*⁺ T_{reg} differentiation is abolished in Nr4a1/2/3 triple knock-out (KO) mice and Nr4a1/3 double KO, and these mice develop fatal autoimmune disease [52]. Nr4a proteins bind to the *Foxp3* promoter upon anti-CD3 stimulation [52] and retroviral gene transduction of *Nr4a2* or *Nr4a3* induces *Foxp3* transcription [55]. Importantly, however, Nr4a triple KO lack

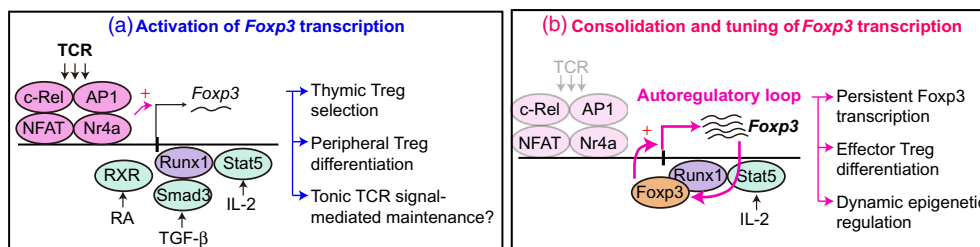


Fig. 2. Activation *versus* consolidation and tuning of *Foxp3* transcription. We propose to classify *Foxp3* transcriptional regulation into two major mechanisms. (a) Activation of *Foxp3* transcription is regulated mainly by T cell receptor (TCR) signals and enhanced by interleukin (IL)-2, transforming growth factor (TGF)- β and retinoic acid (RA). This may lead to thymic regulatory T cell (T_{reg}) selection and peripheral T_{reg} differentiation. In addition, tonic TCR signals through self-reactive TCRs may use this mechanism to regulate homeostatic *Foxp3* transcription. (b) Consolidation and tuning of *Foxp3* transcription. The maintenance of *Foxp3* transcription requires CNS2 of the *Foxp3* gene, which may provide a platform for Foxp3–Runx1/CBF- β complex to form the autoregulatory transcriptional circuit (autoregulatory loop) for the *Foxp3* gene. The activity of this loop can be affected by IL-2 signalling via phosphorylated signal transducer and activator of transcription-5 (STAT)-5. This mechanism may lead to temporally persistent *Foxp3* transcription, which promotes effector T_{reg} differentiation, and the dynamic regulation of epigenetic modifications during T_{reg} differentiation.

not only Foxp3⁺ T_{regs} but also most of the double-positive (DP) cell population [52], which suggests that the T_{reg} reduction in these KO mice is a consequence of defective regulation of positive and negative selection. Meanwhile, we have identified *Nr4a3* as the gene that is the most correlated with the effects of TCR signals in the thymus and the periphery, followed by *Nr4a1* [27]. Specifically, using canonical correspondence analysis (CCA) [56], we analysed the transcriptome data set of thymic T cell populations and that of resting and anti-CD3 stimulated peripheral T cells, and thereby identified the genes that were correlated with both thymic T cells under selection (*in-vivo* TCR signals) and peripheral T cell activation [27]. By developing *Nr4a3*–Tocky, we have shown that temporally persistent TCR signals sustain *Nr4a3* transcription and initiate *Foxp3* transcription [27]. This leads to the new model for Nr4a, that the recognition of cognate antigen conveys persistent TCR signals, which induce and accumulate Nr4a proteins and thereby control thymic selection and differentiation processes including T_{reg} differentiation.

***Foxp3* transcription-enhancing cytokine signals.** *Foxp3* transcription is activated by IL-2 signalling in the presence of TCR stimulation and TGF- β signalling [18]. However, it is unknown whether these cytokine signals can regulate *Foxp3* transcription independently from TCR signalling.

IL-2 signalling is a central cytokine for T cell activation, proliferation and differentiation [21]. The expression of CD25 (IL-2R α -chain) is induced by TCR and CD28 signals and forms the high-affinity IL-2R, together with IL-2R β -chain (CD122) and the common γ -chain (CD132) [57,58]. IL-2 binding to IL-2R triggers phosphorylation of signal transducer and activator of transcription (STAT)-4 and STAT-5 by the associated kinases Janus kinase (Jak)1 and Jak3, which promote cell cycle entry and proliferation of

TCR-stimulated T cells [59]. In addition to the role in T cell activation, CD25 is also a surface marker for T_{reg} in mice [60] and humans [61]. In fact, IL-2 signalling is functional in T_{regs} . Phosphorylated STAT-5 binds to the promoter and CNS2 and activates *Foxp3* transcription [62,63]. KO mice for the genes that are involved in IL-2 signalling (*Il2* [64], *Il2ra* [64], *Il2rb* [65], *Jak3* [66] and *Stat5a/Stat5b* [67]) have reduced Foxp3⁺ T cells in the thymus and periphery. Thus, IL-2 signalling is required for the activation of *Foxp3* transcription, most probably during both the early phase of T_{reg} differentiation as well as the maintenance of both *Foxp3* transcription and the T_{reg} population. Considering the primary role of IL-2 for the activation and proliferation of T cells [21], this suggests a role of *Foxp3* as a sensor for the IL-2 abundance in the environment surrounding individual T cells. In other words, when T cells are activated IL-2 becomes abundant, which enhances Foxp3 expression in nearby T cells. Given that IL-2R expression in T_{regs} absorbs IL-2 and suppresses IL-2-mediated T cell proliferation [68], the size of the T cell population may be self-regulated through the feedback mechanism involving IL-2, CD25 and Foxp3 [38].

TGF- β signalling has multi-faceted effects on tissue development and regeneration, inflammation and cancer in a context-dependent manner [69]. The importance of TGF- β signalling in T cells is recognized particularly in mucosal and tumour immunity [70]. The transcriptional response of T cells to TGF- β signalling is also context-dependent, and is illustrated by the reciprocal differentiation of T helper type 17 (Th17) and T_{regs} by IL-6 and IL-2, respectively, in the presence of TGF- β [71,72]. TGF- β signal-activated Mothers Against DPP Homologue 3 (SMAD3) binds to the CNS1 of the *Foxp3* gene [32,73]. However, the genetic deletion of the SMAD-binding site does not change the frequencies of T_{regs} in the thymus

and the periphery, apart from marginal reductions of Foxp3⁺ T cells in Peyer's patches and lamina propria in aged mice [74]. This suggests that TGF- β controls *Foxp3* transcription through multiple sites in the *Foxp3* gene and/or through the induction of other factors. While IL-2 signalling is intrinsically required for T_{reg} differentiation, as discussed above, the opposing effects of IL-6 signalling seem to be reactive and inflammation-dependent, as the genetic deletion of *Stat3* does not affect T_{reg} populations, while inhibiting the differentiation of T_{regs} in the CD45RB^{high} T cell-mediated colitis model [75].

Veldhoen and Stockinger have proposed the model that TGF- β skews CD4⁺ T cell differentiation from Th1 to Th17 [76], and as such, TGF- β may shift T cells from the Th1–Th2 axis to the Th17–T_{reg} axis. In the TGF- β -rich microenvironment, such as in the intestines, tumour or damaged tissues undergoing regeneration and remodelling, the persistence of pathogen or autoantigen may activate monocytes and dendritic cells, and thereby repress *Foxp3* transcription and promote Th17 differentiation, as observed in rheumatoid arthritis patients [77]. In contrast, once the activation of innate immune cells is terminated, *Foxp3* transcription may be initiated in antigen-reactive T cells, as observed by *Foxp3*–Tocky [26], especially when adjacent T cells are proliferating and producing IL-2, inducing the resolution of inflammation.

Mechanisms for the consolidation and tuning of *Foxp3* transcription – the role of autoregulatory transcriptional circuit for the *Foxp3* gene

The maintenance of *Foxp3* transcription in T_{reg} requires conserved non-coding sequences 2 (CNS2), which includes the widely studied T_{reg}-specific demethylated region, TSDR [33]. The cytosine–phosphate–guanine (CpG) motifs in the TSDR are methylated in non-T_{reg} cells and fully demethylated in thymic T_{regs} [22,33]. The genetic deletion of CNS2 results in the reduction of Foxp3 expression in thymic T_{regs} but does not affect Foxp3 induction *in vitro* [32]. CNS2 is bound by several key transcription factors, including the Runx/Cbf- β complex [78–81], Ets-1 [82], which makes an active complex with Runx1 [83], Foxp3 protein [32] and STAT-5 [63].

Foxp3 binding to CNS2 is dependent upon Runx1/CBF- β [32]. Importantly, the expression of Foxp3 in T_{regs} is reduced in both CBF- β -deficient T_{regs} [78] and CNS2-deleted T_{regs} [34]. CNS2 is required for maintaining the number of T_{regs} in the periphery during homeostasis and is also important for sustaining Foxp3 expression during inflammation [7,34]. CNS2-deleted T_{regs} lose Foxp3 expression in the presence of proinflammatory cytokines, including IL-4 and IL-6, and become effector T cells to enhance

autoimmune inflammation in mice [7]. Furthermore, analysis of TCR repertoires in human T_{regs} also suggests the dynamic regulation of both CD25 and Foxp3 on T cells in rheumatoid arthritis [84]. These data together suggest that, although Foxp3 expression is commonly recognized to be stable, it is in fact dynamically regulated in Foxp3⁺ T_{regs} during homeostasis and during immune responses.

Our recent investigations using *Foxp3*–Tocky have shown that, intriguingly, resting T_{regs} have intermittent *Foxp3* transcription, while activated effector T_{regs} with high expression of immunoregulatory molecules (including CTLA-4 and IL-10) have more sustained *Foxp3* transcription throughout time [26]. The phenotype of these effector T_{regs} with temporally persistent *Foxp3* transcription is in fact very similar to those of the effector T_{regs} that are dependent upon *Myb* [85] and the CD44^{high}CD62L^{low} activated T_{regs} that are dependent upon TCR signals [35], which supports the model that TCR signals induce temporally persistent *Foxp3* transcription and thereby enhance the suppressive phenotype of T_{regs}. Furthermore, by analysing female mice with heterozygosity for a hypomorphic Foxp3 mutant (namely, *Scurfy* mutation), Foxp3 protein sustains the temporally persistent *Foxp3* transcriptional dynamics that promote effector T_{reg} functions [26]. In the thymus, the active demethylation of the TSDR occurs only after the initiation of *Foxp3* transcription and when *Foxp3* transcription is highly sustained over time [27]. These indicate that Foxp3 protein and the *Foxp3* gene form an autoregulatory loop that consolidates the T_{reg}-type TSDR demethylation during thymic differentiation [27], and tunes *Foxp3* transcriptional activities and thereby activates their suppressive activity during inflammation [26]. Given the critical roles of the Runx1/Cbf- β complex in the maintenance of Foxp3 expression and the Foxp3–Runx1 interaction in T_{reg} differentiation and function, it is plausible that this autoregulatory transcriptional circuit is formed via the binding of Foxp3–Runx1/Cbf- β complex [32] to CNS2 of the *Foxp3* gene (Fig. 2).

Dynamic regulation of epigenetic modifications and chromatin architecture of the *Foxp3* gene

TCR-induced *Foxp3* transcriptional activities can be opposed by epigenetic mechanisms for silencing *Foxp3* transcription. The SUMO E3 ligase Pias3 binds to the *Foxp3* promoter, and *Pias1* KO mice have increased frequencies of Foxp3⁺ cells in CD4⁺ T cells and reduced methylation of histone H3 at Lys9 (H3K9), which is a hallmark of repressed genes [86]. The DNA methyltransferase DNA (cytosine-5)-methyltransferases (Dnmt1) and the high mobility group transcription factors Tcf1 and Lef1 constitutively repress *Foxp3* transcription in CD8+

T cells, as *Dnmt1*^{-/-} or *Tcf1*^{-/-} *Lef1*^{-/-} double KO permits the differentiation of Foxp3⁺CD8⁺ T cells, which are rarely found in normal mice [87,88]. In addition, the induction of Foxp3 expression in *Dnmt1*^{-/-} T does not require TGF-β [87], suggesting that TGF-β probably modulates epigenetic mechanisms in normal mice. Strong TCR signalling *in vitro* causes the accumulation of Dnmt1 at the *Foxp3* promoter, which can lead to increased CpG methylation and inhibition of *Foxp3* transcription [89]. Thus, TGF-β may be important for tuning Dnmt1 expression during T cell activation.

Foxp3-Tocky has shed light on the dynamics of *Foxp3* epigenetic regulation following the initiation of *Foxp3* transcription. Importantly, *Foxp3* transcription precedes the demethylation of TSDR in the thymus. Both thymic new *Foxp3* expressors, which are identified by Tocky [27], and immature CD24^{high}FoxP3⁺CD45P by *Foxp3*-EGFP mice [90] have fully methylated TSDR. The active process for TSDR demethylation occurs only after *Foxp3* transcription is sustained over time and the Foxp3 autoregulatory loop is formed [26]. Collectively, the interactions between Foxp3-inducing and inhibiting factors occur during the early phase of T_{reg} differentiation when the *Foxp3* gene is still 'silenced', and we would therefore hypothesize that Foxp3 protein may also have roles in dynamically regulating the epigenetic modifications of the *Foxp3* gene. Future studies could therefore address the role of Foxp3 in the dynamic regulation of chromatin architecture, which can be investigated by chromatin conformation capture (3C) and derivative methods (e.g. Hi-C). For example, the Zheng group showed that, using 3C, NFAT activation induces the interaction of the TSDR-containing CNS2 with the *Foxp3* promoter, which facilitates enhanced *Foxp3* transcription [34]. Using Hi-C and clustered regularly interspaced short palindromic repeats (CRISPR)-mediated mutation, the Zhao group showed that the mixed lineage leukaemia (MLL) family methyltransferase MLL4 binds to -8.5k upstream enhancer of the *Foxp3* gene, and makes a chromatin loop to promote the monomethylation of histone H3 at Lys4 (H3K4me1) in the promoter and CNS3, which activates *Foxp3* transcription [91]. The chromatin organizing factor special AT-rich sequence binding protein 1 (SATB1) is also involved in activating *Foxp3* transcription in the thymus, as the genetic deletion of SATB1 results in the marked reduction of Foxp3⁺ T_{regs} and the accumulation of thymic CD25⁺Foxp3⁻ T_{reg} precursors with reduced enhancer activity [which are identified by acetylation of histone H3 at Lys27 (H3K27ac)] [92]. Thus, it is likely that chromatin remodelling of the *Foxp3* gene underlies the temporally dynamic *Foxp3* autoregulatory loop, suggesting that the former is also induced dynamically through the interactions between Foxp3

protein and key chromatin organizers and epigenetic regulators. In addition, as those chromatin organizers and epigenetic regulators control not only the *Foxp3* gene but also other genes, the chromatin remodelling of Foxp3-target genes may be also induced dynamically in activated T_{regs} and differentiating T_{regs}. Future studies, therefore, should investigate the role of Foxp3 protein and its co-factors in the temporally dynamic regulation of chromatin structure within and outside the *Foxp3* gene region.

Dynamic *Foxp3* expression *in vivo*: perspectives for basic immunology and clinical relevance

After the emergence of single-cell technologies and the Tocky tool, studies on T cell regulation are shifting from the stability and plasticity of T_{reg} to the investigation of temporal changes in Foxp3-mediated mechanisms *in vivo*. Our analysis of T_{regs} in peripheral immune compartments show that, in non-inflammatory conditions, *Foxp3* transcription is most probably modelled by intermittent gene activity [26]. This intermittent transcription may offer an explanation for the low frequency of T_{reg} cells with detectable *Foxp3* transcripts in T_{reg} cells analysed by single-cell RNA-seq [93,94], although these data sets have limitations due to shallow sequencing depths. Given that the temporal changes in *Foxp3* transcription control T_{reg} function and effector T_{reg} differentiation, future work will investigate the molecular mechanisms that control the real-time transcribing of the *Foxp3* gene, which can be analysed by the Tocky system. In addition, in line with the temporally dynamic regulation of *Foxp3* transcription *in vivo*, the significance of thymic and peripheral T_{reg} markers needs to be readdressed. Our investigation using *Foxp3*-Tocky has confirmed that the expression of Neuropilin 1 [95] and Helios [96] are dynamically regulated in T_{regs} according to *Foxp3* transcription dynamics [26], and therefore are not faithful markers of thymic T_{regs}, as has been noted previously in the literature [97].

Importantly, clinical studies and immunotherapy development may be benefited by the endorsement of the dynamic perspective. Whether targeting T_{regs} or not, immunotherapy may dynamically change *Foxp3* transcription. If these dynamic responses are clarified, immunotherapy targeting T cells may be better designed with a more tailored strategy, as we recently showed by manipulating *Foxp3* transcriptional dynamics through targeting inflammation-reactive effector T_{regs} by OX40 (CD134) and tumour necrosis factor receptor II (Tnfrsf1b which are expressed specifically in T_{regs} with temporally persistent *Foxp3* transcription [26]. We therefore envisage that the investigation of dynamic changes in molecular mechanisms during T cell responses *in vivo* will improve the predictability of

preclinical studies and thereby contribute to the development of new immunotherapies for autoimmune and cancer patients.

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Disclosures

The authors declare that no conflicts of interest exist in relation to this paper.

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