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

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Abbreviations: regulatory T cells (Treg); Timer of cell kinetics and activity (Tocky); T cell receptor (TCR); interleukin (IL); fluorescent protein (FP); conserved non-coding sequences (CNS); double-positive (DP); knock out (KO); Treg-specific demethylated region (TSDR); chromatin conformation capture (3C).

Summary

Studies on regulatory T cells (Treg) have focused on thymic Treg as a stable lineage of immunosuppressive T cells, the differentiation of which is controlled by the transcription factor Foxp3. This lineage perspective, however, may constrain hypotheses regarding the role of Foxp3 and Treg in vivo, particularly in clinical settings and immunotherapy development. In this review, we synthesise 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*. Particularly, 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 Treg 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 Treg 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 Treg.

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

It is widely considered that regulatory T cells (Treg) constitute a distinct lineage of CD4+ T cells dedicated for immunosuppression [1]. Key evidence for the distinct lineage include: (**i**) Treg development is controlled by the transcription factor Foxp3 [2]; and (**ii**) the development of Treg in the thymus is delayed to after that of other T cells under physiological conditions [3]. However, accumulating evidence show the simultaneous development of Treg and other T cells [4, 5] and Treg plasticity is now widely recognised as Treg can lose Foxp3 expression and become effector T cells (ex-Treg) during inflammation [6, 7]. Thus, studies on dynamic changes in the differentiation and activation status of Treg – and other T cells – in vivo is essential for understanding Foxp3-mediated T cell regulation. This dynamic perspective is important for not only 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 Treg, which would theoretically in turn suppress non-Treg [9]. This trial, however, resulted in catastrophe where all 6 volunteers given TGN1412 development 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 downregulated 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 Treg and non-Treg may not represent strictly separate lineages, and therefore the assumption of specific activation of Treg may have been inappropriate [12]. In fact, basic studies later showed the plasticity of Treg: Treg may lose Foxp3 expression during inflammation and non-Treg 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; second, it is fundamental to investigate the dynamic changes in the differentiation and activation statuses of Treg 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, in mice as well [17], and the induction is enhanced by 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 mainly produced by activated T cells, particularly CD4+ T cells [21]. Since the immunosuppressive Treg 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 Treg and T cell regulation. To this end, we have recently developed a new technology **T**imer **o**f **c**ell **k**inetics and activit**y** (Tocky) system, which allows the investigation of in vivo dynamics of Foxp3 and Treg during physiological immune responses [26, 27].

In this article, 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 Timer of cell kinetics and activity (Tocky) for investigating in vivo dynamics of Treg differentiation

The current understanding of Treg differentiation and function is significantly based on evidence obtained by Foxp3 fluorescent protein (FP) reporters (such as 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 on stable FPs such as GFP, the half-life of which is longer than 56 hours. Therefore, temporal changes in *Foxp3* transcription shorter than 2 – 3 days cannot be investigated by these reporter systems.

In order to understand in vivo dynamics of those molecular mechanisms underlying the differentiation and function of Treg, 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 hours [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 quantitatively analyse dynamic changes 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 downregulated gene expression under the detection threshold of flow cytometry and are inactive in transcription of the gene (Arrested or Inactive) [27] (**Figure 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 mostly in self-reactive T cells in non-inflammatory conditions [1]. Thus, we will classify the mechanisms for *Foxp3* transcription into two groups:

(i) Mechanisms for the activation of *Foxp3* transcription: these are used during thymic Treg selection and peripheral Treg differentiation and are potentially involved in the mechanism for tonic TCR signal-mediated activation of *Foxp3* transcription.

(ii) Mechanisms for the consolidation and tuning of *Foxp3* transcription: these are used for sustaining *Foxp3* transcription over time, which induces effector Treg differentiation and the dynamic regulation of epigenetic modifications, such as demethylation of CpG islands in enhancer regions (**Figure 2**).

Mechanisms for the activation of *Foxp3* transcription

Foxp3 as a TCR signal downstream gene

The differentiation and function of Treg 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 $CD25^{+}Foxp3^{+}$ Treg from CD4-SP cells using transgenic TCR systems [39-41]. On the other hand, TCR transgenic mice in the Recombination activating gene (*Rag*) deficient backgrounds lack Foxp3+ T cells due to the absence of self-antigen presentation [42, 43]. The analysis of TCR signals using reporter mice have provided insights into the mechanism for TCR-mediated Treg differentiation. The Hogquist group showed that Treg 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-Treg 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 CTLA-4 and thereby acquire the suppressive function that is dependent on CTLA-4 [46]. These suggest that Foxp3 has a role in negative feedback regulation of T cell activation in cooperation 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, Treg 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 expression includes two situations: (i) antigen recognition-induced *Foxp3* transcription in Foxp3- cells (conventional T cells; non-Treg) in the thymus and the periphery; and (ii) the effects of tonic TCR signals in Foxp3+ Treg.

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 NFAT and AP1 [47], the NF-κB components c-Rel and p65 [32, 48-50], Cyclic AMP response element-binding protein (CREB) [51], and Nr4a proteins [52] (**Figure 2**).

Nr4a proteins (Nr4a1, Nr4a2, and Nr4a3) bind to their target sequences as homodimers or heterodimers and regulate transcription [53, 54]. Foxp3+ Treg

differentiation is abolished in Nr4a1/2/3 triple knock out (KO) and Nr4a1/3 double KO, and these mice develop fatal autoinflammatory 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 not only Foxp3+ Treg but also most of double-positive (DP) cell population [52], which suggests that the Treg 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 dataset 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 Treg 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]. It is, however, 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 Stat5 by the associated kinases Jak1 and Jak3, which promotes cell cycle entry and proliferation of TCR stimulated T cells [59]. In addition to the role in T cell activation, CD25 is a surface marker for Treg in mice [60] and humans as well [61]. In fact, IL-2 signalling is functional in Treg. Phosphorylated Stat5 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 both during an early phase of Treg differentiation as well as the maintenance of both *Foxp3* transcription and the Treg 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 Treg 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 multifaceted 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 recognised 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 Th17 and Treg by IL-6 and IL-2, respectively, under the presence of TGF-β [71, 72]. TGF-β signal-activated 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 Treg 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 Treg 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 Treg populations, while inhibiting the differentiation of Treg in the $CD45RB^{hi}$ 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-Treg axis. In TGF-β-rich microenvironment, such as in the intestines, tumour, or destructed tissues with 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 Treg requires CNS2, which includes the widely studied Treg-specific demethylated region, TSDR [33]. The CpG motifs in the TSDR are methylated in non-Treg cells, and fully demethylated in thymic Treg [22, 33]. The genetic deletion of CNS2 results in the reduction of Foxp3 expression in thymic Treg 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 Stat5 [63].

Foxp3 binding to CNS2 is dependent on Runx1/CBF-β [32]. Importantly, the expression of Foxp3 in Treg is reduced in both CBF-β-deficient Treg [78] and CNS2-deleted Treg [34]. CNS2 is required for maintaining the number of Treg in the periphery during homeostasis and is also important for sustaining Foxp3 expression during inflammation [7, 34]. CNS2-deleted Treg 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 Treg 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 recognised to be stable, it is in fact dynamically regulated in Foxp3+ Treg during homeostasis and during immune responses.

Our recent investigations using Foxp3-Tocky have shown that, intriguingly, resting Treg have intermittent *Foxp3* transcription, while activated effector Treg with high expression of immunoregulatory molecules (including CTLA-4 and IL-10) have more sustained *Foxp3* transcription across time [26]. The phenotype of these effector Treg with temporally-persistent *Foxp3* transcription is in fact very similar to those of the effector Treg that are dependent on *Myb* [85] and the $CD44^{hi}CD62L^{lo}$ activated Treg that are dependent on TCR signals [35], which supports the model that TCR signals induce temporally persistent *Foxp3* transcription and thereby enhance the suppressive phenotype of Treg. 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 Treg 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 Treg-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/ Cbfβ complex in the maintenance of Foxp3 expression and the Foxp3-Runx1 interaction in Treg 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 (**Figure 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 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 likely 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 $CD24^{hi}$Foxp3+CD4SP 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 Treg differentiation when the *Foxp3* gene is still 'silenced', and we would therefore hypothesise 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 CRISPR-mediated mutation, the Zhao group showed that the 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 organising factor Satb1 is also involved in activating *Foxp3* transcription in the thymus, as the genetic deletion of Satb1 results in the marked reduction of Foxp3+ Treg and the accumulation of thymic $CD25^{+}Foxp3^{-}$ Treg 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 dynamically induced through the interactions between Foxp3 protein and key chromatin organisers and epigenetic regulators. In addition, since those chromatin organisers and epigenetic regulators control not only the *Foxp3* gene but also other genes, the chromatin remodelling of Foxp3-target genes may be also dynamically induced in activated Treg and differentiating Treg. Future studies, therefore, should investigate the role of Foxp3 protein and its cofactors 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 Treg to the investigation of temporal changes in Foxp3-mediated mechanisms in vivo. Our analysis of Treg in peripheral immune compartments show that, in the non-inflammatory conditions, *Foxp3* transcription is most likely modelled by intermittent gene activity [26]. This intermittent transcription may offer an explanation for the low frequency of Treg cells with detectable *Foxp3* transcripts in Treg cells analysed by single cell RNA-seq [93, 94], although these datasets have limitations due to shallow sequencing depths. Given that the temporal changes in *Foxp3* transcription control Treg function and effector Treg

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 Treg markers needs to be re-addressed. Our investigation using Foxp3-Tocky has confirmed that the expression of Neuropilin 1 [95] and Helios [96] are dynamically regulated in Treg according to *Foxp3* transcription dynamics [26], and therefore are not faithful markers of thymic Treg, as has been previously noted in the literature [97].

Importantly, clinical studies and immunotherapy development may be benefitted by the endorsement of the dynamic perspective. Whether targeting Treg 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 Treg by OX40 and TNFRII, which are specifically expressed in Treg 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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Figure Legends

Figure 1: Comparison of Tools to investigate Foxp3-expressing T-cells in vivo.

(**A**) Most *Foxp3* reporter mice use stable Fluorescent Proteins (FP), such as EGFP, the half-life of which is > 56 hours. (**B**) Foxp3 fate mappers such as *Foxp3*^{GFPCre}:*Rosa26*^{RFP} allow the identification of Treg with Foxp3 expression and ex-Treg that lost Foxp3 expression. Notably, both GFP and RFP are stable FPs. (**C**) Foxp3-Tocky uses Fluorescent Timer, the emission spectrum of which spontaneously and irreversibly changes from blue to red fluorescence. The half-life of blue fluorescence is ~4 hours, and thus reports the 'real-time' activity of *Foxp3* transcription. In contrast, the half-life of red fluorescence is ~120 hours 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).

Figure 2: Activation vs. consolidation and tuning of *Foxp3* transcription

We propose to classify *Foxp3* transcriptional regulation into two major mechanisms. (**A**) **Activation of *Foxp3* transcription** is mainly regulated by TCR signals and enhanced by IL-2, TGF-β, and retinoic acid (RA). This may lead to thymic Treg selection and peripheral Treg 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 the 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 Stat5. This mechanism may lead to temporally-persistent *Foxp3* transcription, which promotes effector Treg differentiation, and the dynamic regulation of epigenetic modifications during Treg differentiation.

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