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T_{REG} STABILITY: TO BE OR NOT TO BE

Abigail E. Overacre¹ and Dario A.A. Vignali^{1,2}

¹Department of Immunology, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15213, USA.

²Tumor Microenvironment Center, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15232, USA.

Abstract

Regulatory T cell (T_{reg}) stability has been primarily determined by the maintained expression of the transcription factor *Forkhead box P3* (*Foxp3*). However, T_{regs} can exhibit instability while maintaining *Foxp3* expression, requiring a re-examination of what defines T_{reg} stability. Recent work suggests that the establishment and stability of T_{regs} is mediated by a number of mechanisms besides *Foxp3* expression, such as epigenetic modifications, Foxo1/3a localization, expression of *Eos* and signaling via Neuropilin-1. Additional studies may help to define approaches that can undermine T_{reg} stability in cancer or enhance T_{reg} stability in transplantation, autoimmune or inflammatory diseases and therefore have substantial therapeutic utility. In this review, we will discuss how T_{reg} stability is defined and the mechanisms utilized to maintain stability.

INTRODUCTION

Regulatory T cells (T_{regs}) are essential for maintaining immune homeostasis via a wide variety of suppressive mechanisms [1–3]. Lethal autoimmune disease develops in the absence of T_{regs} highlighting their critical role in preventing autoimmunity. However, they can be detrimental in other diseases, such as cancer and chronic infection [3]. T_{regs} are characterized by the transcription factor *Foxp3*, which is required for their development, function and stability. A critical concern in targeting or utilizing T_{regs} therapeutically is their stability, as defined by the maintenance of *Foxp3* expression and suppressive activity, while not exhibiting pro-inflammatory effector function. While continued *Foxp3* expression has been the primary determinant of T_{reg} stability, it has become apparent that this alone does not define ‘stability’. Indeed, there are many additional factors and mechanisms that need to be considered, including epigenetic modifications and expression of activating or regulatory factors, as examples of T_{reg} instability and loss of function have been described despite continued *Foxp3* expression [5, 6]. In this review, we will discuss what T_{reg} ‘stability’ means and whether T_{regs} are stable, outline the mechanisms that are currently known to

Corresponding author: Vignali, Dario AA (dvignali@pitt.edu).

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impact and maintain T_{reg} stability, and define what remains to be determined. In the interest of focus, we will restrict this review to thymically-derived T_{regs} [7]. The stability of induced T_{reg} populations, such as peripherally derived T_{regs} , has been discussed elsewhere [5, 8, 9***]

WHAT DOES T_{REG} STABILITY MEAN AND HOW IS IT ASSESSED?

Fundamentally, we would argue that T_{reg} stability should be defined by the maintenance of all of the following characteristics: (1) sustained *Foxp3* expression, (2) potent suppressive activity, and (3) lack of effector activity (eg. production of IL2 and pro-inflammatory cytokines, cytotoxicity, B cell help, etc). While there are observations that challenge this definition, such as the expression of lineage defining transcription factors *T-box 21* (*Tbet*) [10], *Interferon regulatory factor 4* (*IRF4*) [11] and *Signal transducer and activator of transcription 3* (*STAT3*) [12] by T_{regs} , these fundamental characteristics are nonetheless maintained.

Stable *Foxp3* expression is thought to be maintained by specific epigenetic modifications. For instance, a Conserved Noncoding Sequence 2 (CNS2, also known as TSDR) in the 5' UTR of *Foxp3*, comprised of a series of CpG motifs is hypomethylated in T_{regs} but hypermethylated in naive or effector T cells [5, 13***]. This hypomethylated pattern is maintained by an IL-2 dependent manner [5]. A fully methylated locus is considered to be closed and thus not transcriptionally active, while an unmethylated locus is open and active [14]. Indeed, the increased CNS2 methylation seen in TGF β -induced T_{regs} is thought to underlie their instability [9***]. A genome wide analysis has suggested that hypomethylation at other loci such as *Il2ra* (CD25), *Ikzf4* (Eos), *Ctla4* (Cytotoxic T-lymphocyte-associated protein 4) and *Tnfrsf18* (GITR), may also be important for T_{reg} stability [9***]. Although all of these loci show a similar hypomethylation pattern to *Foxp3* in T_{regs} , *Foxp3* CNS2 demethylation is reliant on DNA methyltransferases, such as DNMT1 [15], while the other loci are not. Furthermore, it was recently shown that Tet methylcytosine dioxygenase 2 (Tet2) is required for *Foxp3* CNS2 hypomethylation [16, 17]. In fact, overexpression of Tet2 led to hypomethylation and stability of *Foxp3* regardless of IL-2 expression. These reports suggest a role for epigenetic alterations in maintaining *Foxp3* expression and T_{reg} stability.

T_{reg} stability has been a controversial topic for a number of years [18]. Many studies have suggested that T_{reg} development leads to a terminally differentiated population, albeit with a number of different suppressive functions determined by the surrounding milieu [19]. Indeed, some have suggested that T_{regs} are one of the more stable cell lineages, based on lineage tracing experiments with *Foxp3*^{CreERT2-GFP} *Rosa*^{LSL.YFP} mice. These mice allow for tamoxifen-inducible expression of *Foxp3*-driven Cre in T_{regs} , which permanently marks these cells with YFP regardless of subsequent *Foxp3* expression [13***]. Using this approach, very few if any T_{regs} appear to lose *Foxp3* expression and become “ex T_{regs} ” up to 5 months after tamoxifen injection. In addition, the majority of T_{regs} maintained *Foxp3* expression even under IL-2 deprivation, and although some cells had a minor decrease in *Foxp3* expression, they maintained their suppressive activity and exhibited a normal pattern of cytokine secretion. T_{reg} stability has also been tested in a limited number of disease

scenarios, such as *Listeria monocytogenes* and diabetes. When given a sub-lethal dose of *Listeria monocytogenes*, T_H1 transcription factors such as *Tbet* and *Cxcr3* were increased in both *Foxp3*⁺ and *Foxp3*⁻ T cells, but *Foxp3* expression was largely maintained [13***]. To assess T_{reg} stability in an autoimmune setting, pure GFP⁺ cells from BDC2.5 TCR-transgenic *Foxp3*^{GFP} NOD mice were transferred to lymphoreplete NOD Thy1.1 mice. Transferred T_{regs} infiltrated islets by 4 weeks after injection, and were almost exclusively *Foxp3*⁺.

However, other studies have suggested that T_{regs} can lose *Foxp3* expression and take on a pro-inflammatory, memory-like phenotype in certain disease environments [20]. Using a *Foxp3*^{Cre-GFP} *Rosa*^{LSL-YFP} BAC transgenic mouse in which Cre is expressed by all T_{regs} and YFP marks all cells that have *Foxp3*, it was noted that a small percentage of mature T_{regs} had lost *Foxp3* expression (10–20%) [21, 22***]. These exT_{regs} had increased *Foxp3* CNS2 methylation compared with stable T_{reg} counterparts, suggesting instability. These cells displayed an activated-memory phenotype as noted by increased CD44 expression and IFN γ secretion. When *Foxp3*^{Cre-GFP} *Rosa*^{LSL-YFP} NOD mice were analyzed to assess the role of exT_{regs} in a disease setting, a moderate percentage of exT_{regs} was observed in the islets (30–35%) [21]. These cells were also capable of inducing autoimmune diabetes, as shown following adoptive transfer of BDC2.5 TCR transgenic T_{regs}. Interestingly, when purified and cultured *in vitro*, up to 20% of the T_{regs} lost *Foxp3* expression.

The topic of *Foxp3* stability remains controversial. However, there are a number of potential reasons why different conclusions may have been reported. First, the mouse strains used to assess T_{reg} stability are designed differently. Whereas a *Foxp3*^{Cre} BAC transgenic mouse labels all cells that express *Foxp3* early in development, *Foxp3*^{CreERT2} mice label *Foxp3*⁺ cells only upon tamoxifen injection. In addition, BAC transgenic mice sometimes don't exhibit entirely faithful promoter expression that is identical to the endogenous promoter due to the location of insertion and the influence of local control elements. Second, different methods were used to isolate T_{regs}, such as double versus single sorting, before transfer into a new host. It is possible that the observed exT_{regs} could have been generated by cellular stress or derived from contamination, either from *Foxp3*⁻ cells or cells that transiently upregulated *Foxp3* during development. Third, it is also possible that T cells transiently upregulate *Foxp3* during development leading to 'false labeling' of cells that are not destined to become *bona fide* T_{regs}, as indicated by hypermethylation at the *Foxp3* CNS2. Some *Foxp3*⁺ T cells also transiently lose *Foxp3* expression, which leads to a partially methylated CNS2. However, upon reactivation, a portion of these cells reacquires a hypomethylated CNS2, indicating lineage commitment [23*]. Indeed, it was previously shown that a small subset of naïve cells transiently co-expresses ROR γ t and *Foxp3*, producing either T_H17 or T_{reg} cells depending on the surrounding environment [24]. Transient *Foxp3* expression has also been observed in human T cells, although these cells have no suppressive function [25]. While a few reports suggest that a small percentage of exT_{regs} exist, two points remain: (1) Are they simply undergoing transient expression of *Foxp3*? (2) If they are a separate subset, do they impact specific diseases? Regardless of whether one considers T_{regs} stable or occasionally unstable, an overriding question is how is T_{reg} stability maintained?

HOW IS T_{REG} STABILITY MAINTAINED?

While *Foxp3* has been shown to be critical for maintaining T_{reg} stability, there are several reports suggesting that T_{reg} stability is maintained and enforced by other factors, such as IL-2, *Foxo1/3a*, *Eos* and *Nrp1*.

Once *Foxp3* is upregulated, expression must be maintained to preserve T_{reg} function and stability. Many factors are involved in this, including the IL-2/STAT5 pathway. In fact, *Foxp3* expression is significantly diminished in *Il2rb*^{-/-} (CD25) mice, while STAT5 deletion prevents T_{reg} development. Indeed, STAT5 binds to the *Foxp3* promotor region, leading to stabilization of the locus [26]. *Foxp3* is also modulated by other members of the Forkhead box family, *Foxo1* and *Foxo3a* [27]. *Foxo1/3a* prevent T_{regs} from taking on effector functions [28, 29]. In the absence of *Foxo1*, T_{reg} development is diminished, and those that do develop are unable to suppress *in vitro*. Indeed, in the absence of *Foxo1*, T cells express more IFN γ , leading to severe autoimmunity *in vivo* [30*]. This is further exacerbated in the absence of both *Foxo1* and *Foxo3a* [31]. Stable T_{regs} show reduced Akt expression, leading to enhanced *Foxo1* and *Foxo3a* in the nucleus, where they stabilize *Foxp3* expression by binding to the promotor region and regulating other transcription factors [32].

Eos, a zinc-finger transcription factor, was identified as a critical facilitator of T_{reg} stability [33*]. Indeed, *Eos*-limited T_{regs} exhibited enhanced expression of IL-2 and IFN γ along with reduced suppressive capacity, but showed no difference in the expression of *Foxp3*. Forced overexpression of *Eos* prevented T_{reg} reprogramming, even in destabilizing environments, maintaining CTLA-4 expression and preventing CD40L, IL-2 and IL-17 expression. *Eos*⁻*Foxp3*⁺ T_{regs} (*Eos*-labile) were later identified *in vivo* and were also shown to have reduced regulatory function [34*]. *Eos*-labile T_{regs} were capable of functional reprogramming, shown through adoptive transfers into *Cd40lg*^{-/-} mice, which lack efficient priming of naïve CD8 cells. Indeed, when transferred into a CD40L-deficient hosts, a subset of T_{regs} downregulated *Eos* and upregulated CD40L to provide help for CD8 priming. Therefore, both *Eos*-labile and *Eos*-stable T_{regs} suppress and maintain *Foxp3* expression, but the former maintain the ability to reprogram and display helper activity in certain environments. However, whether *Eos*-labile and *Eos*-stable T_{regs} are two distinct subsets, or if *Eos* expression enhances stability, is unknown.

A pathway that is important for maintaining T_{reg} stability in a manner that appears to be independent of *Foxp3* expression is the Neuropilin-1:Semaphorin-4a axis [35***]. The majority of mouse T_{regs} express high levels of *Nrp1* [38] and this contributes to their function and stability [35***, 39]. *Nrp1* has also been shown to increased angiogenesis and CD8 activation in a VEGF-dependent manner [35***, 39]. When *Nrp1* was blocked *in vitro*, T_{regs} were capable of suppressing in classic contact-dependent suppression assays, but were incapable of suppressing across a permeable transwell, where suppression is mediated by IL-10 and IL-35 [40]. *Nrp1*^{L/L}*Foxp3*^{Cre} mice showed no signs of autoimmunity and remained healthy well past one year of age, indicating that *Nrp1*-deficient T_{regs} were capable of maintaining immune homeostasis. However, when injected with transplantable tumors (B16, EL4, MC38), tumor growth was substantially reduced. While tumor clearance

was also observed in most cases, no autoimmunity or inflammatory lesions were observed. Therefore, Nrp1-deficient T_{regs} lost stability and suppressive capacity in the tumor microenvironment, while maintaining stability in the periphery, likely due to destabilizing factors that were confined to the tumor microenvironment. Blocking antibodies to Nrp1 or Sema4a were also found to limit tumor growth. Nrp1-deficient T_{regs} maintained *Foxp3* expression, but showed alterations in other critical T_{regs} markers, such as a decrease in ICOS, IL-10 and CD73. Further mechanistic analysis showed that Nrp1:Sema4a ligation leads to the recruitment of PTEN, which limits Akt activity, leading to increased nuclear translocation of Foxo1 and Foxo3a, thereby stabilizing *Foxp3* expression. This leads to an enhancement of quiescence and survival factors (Klf2, Bcl2) and effector molecules (IL-10, CD73), while limiting lineage-defining transcription factors (T-bet, IRF4, Ror γ t) and effector molecules (IFN- γ). This suggests that T_{reg} stability can occur independently of *Foxp3* expression.

CONCLUSIONS

In summary, we would argue that T_{reg} stability can be defined by the maintenance of three critical traits: (a) stable *Foxp3* expression, (b) efficient suppressive activity, and (c) a lack of effector activity. Several factors appear to contribute to and are required for the maintenance of this stable phenotype, including demethylation of the *Foxp3* CNS2 locus, the transcription factors Foxo1/3a and Eos, and the Nrp1:Sema4a axis.

However, many questions remain. (1) Are there other factors or mechanisms that are required for the maintenance of T_{reg} stability beyond those described in this review? (2) What is the functional relevance of *Foxp3* CNS2 hypomethylation, given that this is not always required for *Foxp3* expression? (3) Is T_{reg} stability engrained during development or does it have to be continually and actively reinforced in the periphery? (4) Do exT_{regs} or destabilized *Foxp3*⁺ T_{regs} have distinct and unique functions, and if so, how can we track or target these cells in humans? (5) How can we utilize or target T_{reg} stability in disease?

Limiting or enhancing T_{reg} stability provides a novel therapeutic strategy for a wide variety of diseases. For example, inducing T_{reg} instability in tumors could lead to an improved response to immunotherapies, while enhancing T_{reg} stability could limit transplant rejection, autoimmunity, and inflammatory disease. Clearly, more research is required to further understand how T_{reg} stability is maintained and its functional relevance in various disease settings.

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HIGHLIGHTS

- T_{reg} stability remains a controversial topic and thus requires further study.
- T_{regs} can lose suppressive function while maintaining *Foxp3* expression.
- T_{reg} stability maintained in part by epigenetic modifications, FOXO, Eos and Nrpl.
- T_{reg} stability may be therapeutically manipulated to treat cancer and autoimmunity.

Figure 1.
Mechanisms of maintaining T_{reg} stability *in vivo*.