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Plasticity of CD4⁺ FoxP3⁺ T cells

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Summary

Regulatory T (Treg) cells play an essential role in maintaining immunological tolerance. The discovery of FoxP3 as a key Treg transcription factor combined with recent advances in the development of functional reporter mice have enabled new insights into Treg biology and revealed unexpected features of this lineage. In this review, we address the stability of this population, focusing on studies that suggest that Tregs can down-regulate FoxP3, lose regulatory activity and, under some conditions, become memory T cells capable of recognizing self-antigens and expressing effector cell activities including the production of IL-17 and IFN γ . The presence of these “exTregs” in multiple inflammatory settings suggests a potential role for these cells in a variety of disease settings ranging from autoimmunity to cancer and infectious disease.

Introduction

The immune system is designed to recognize and destroy foreign pathogens while preserving immune tolerance to self. Mechanisms of self-tolerance in the periphery are essential to control rare thymocytes with self-reactivity that escape central tolerance due to a lower threshold of affinity for self peptide or the lack of thymic expression of tissue-specific proteins. In recent years, we have learned that suppressor, or so-called “Regulatory T cells (Tregs)” play a prominent role in controlling peripheral autoreactive T lymphocytes. While the spectrum of regulatory/suppressor T cells includes multiple different T cell subsets, in this review, we will focus on FoxP3⁺ Tregs. Direct evidence for the essential role of FoxP3 comes from the observation that germline FoxP3 point mutations results in a lack of suppressive Tregs and fatal multi-organ autoimmune disease, termed scurfy disease in mice and Immunodysregulation Polyendocrinopathy, Enteropathy X-linked syndrome (IPEX) in humans [1,2]. By comparison, ectopic FoxP3 expression in CD4⁺ non-Treg cells is sufficient to confer suppressor function in vitro and in vivo [3,4,5].

FoxP3⁺ Tregs have the capacity to actively block immune responses, inflammation, and tissue destruction by suppressing the functions of an array of cell types including conventional CD4⁺ helper T cells, B cell antibody production and affinity maturation, CD8⁺ cytotoxic T lymphocyte activity, and antigen-presenting cell function and maturation state. There are over 15 different mechanisms of suppressor function that have been attributed to Tregs [6] which play a key role in regulating immune responses as a global “brake” on immunity. However, it

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has become increasingly clear that CD4⁺ T cell subsets are not stable, and display plasticity during development/differentiation and maintenance.

FoxP3 Expression and Tregs

Fate decisions for FoxP3 expression and thymically-derived, natural (n)Treg function are determined early in thymocyte development [7] with nTregs developing from FoxP3⁺, CD4⁺CD8⁻CD25⁺ mature thymocytes [8]. Using an elegant technique of cloning TCRs from nTregs, and retrovirally-transduced conventional T cells, Hsieh et al. (2004) reported that the peripheral Treg pool is skewed towards a self-reactive repertoire requiring that anergy and other Treg transcriptional programs restrain nTregs from becoming unstable under normal circumstances [9]. However, a recent report using similar techniques proposed that the nTreg population, although containing a self-reactive repertoire, is primarily specific for non-self antigens [10] and another study suggested that other factors in addition to TCR avidity to thymic peptides are required for nTreg development [11]. A number of extrinsic factors have been implicated in the development and stability of nTregs. For instance, STAT5 activation, likely driven by IL-2 in most settings, is required for FoxP3 expression in Tregs [12]. In the periphery, multiple extrinsic signals are required to maintain FoxP3 expression and Treg homeostasis. Continued TCR engagement of MHC in the periphery as well as CD28/B7-mediated co-stimulation is required for Treg development and nTreg homeostasis [13]. Mutant CD4⁺ T cells that are unable to respond to TGFβ have reduced peripheral Tregs, and succumb to a fatal autoimmune disease similar to scurfy mice [14]. Thus, continuous signals are required for stable FoxP3 expression in nTregs, and in the absence of these signals, FoxP3 expression is lost. As will be suggested below, the potential for an autoreactive regulatory T cell population to lose FoxP3 and thus its suppressive phenotype has important implications for immune homeostasis.

Treg stability/instability

The majority of nTregs are relatively stable in the healthy immune system. Floess et al. and Gavin et al. showed that most Tregs retain high FoxP3 expression following adoptive transfer in a non-pathogenic setting [15**,16**]. However, 10-15% of “stable” Treg cells were found to lose FoxP3 expression after adoptive transfer into lymphopenic hosts [16**]. There are two fates for Tregs that lose FoxP3 in lymphopenic hosts: death or de-differentiation. A recent study showed that half of the Tregs transferred into lymphopenic hosts did not die but rather began producing IL-2 and IFNγ [17**]. Upon losing FoxP3, the ‘exTregs’ no longer expressed high levels of CD25, GITR and CTLA-4, and were unable to suppress T effector cell proliferation in vitro. This work supported genetic lineage tracing studies performed in our laboratory which showed using a FoxP3-GFP-Cre × ROSA26-YFP dual reporter mice that about 10% of YFP⁺ T cells either never stably expressed FoxP3 or lost FoxP3 expression after a period as “bonafide” FoxP3⁺ Treg development [18**]. Moreover, there have been several recent studies in the inflammatory settings of autoimmunity [19**,20] using either FoxP3 expression or FoxP3-GFP reporter mice that have suggested that there is a loss of FoxP3 during inflammatory responses. Importantly, it has been demonstrated that low expression of FoxP3 predisposes for spontaneous autoimmune disease in susceptible mouse strains [21*]. Together, these results suggested that under certain conditions FoxP3 expression, and consequently Treg function, may not be stable. We have termed these cells ‘exTregs’ to describe this Treg population that were once FoxP3⁺.

Extrinsic control

There are a number of environmental or extrinsic factors that are likely to be involved in Treg stability. The earliest suggestion that Tregs could be destabilized by cytokines was reported by Medzhitov and colleagues who showed that Tregs incubated in vitro with IL-6 plus some

other factor(s) secreted by activated dendritic cells led to loss of Treg function [22]. Others have shown that inflammation can lead to loss of Treg and a shift in the Treg-effector T cell balance. After transfer into lymphopenic hosts, a significant percentage of Tregs lose FoxP3 expression following immunization with MOG and CFA [23**,24]. This Treg instability may be due to inflammatory cytokines, perhaps functioning synergistically with IL-1 and IL-23 to downregulate FoxP3 expression. IL-6-dependent FoxP3 mRNA downregulation has been shown to be STAT3 dependent, which may partly be explained by a recent observation that IL-6 induced re-methylation of the FoxP3 non-intronic upstream CpG-rich island and closed the chromatin structure of the FoxP3 locus [25].

Decreasing FoxP3 expression in Tregs has been observed in other autoimmune settings [19**,21*,26]. Flavell and colleagues showed a selective downregulation of intra-islet FoxP3 expression in the diabetic mouse consistent with our observation that Treg numbers decreased in NOD pancreases due to an IL-2 deficiency within the inflamed islets [19]. In fact, in this setting, the administration of low-doses of IL-2 increased CD25 expression and subsequently the number of Treg cells and prevented diabetes. Although it is possible that the loss of Tregs was due to apoptosis, it remains possible that failing to receive enough IL-2 signals may have resulted in Treg instability in inflamed tissues.

Intrinsic control

FoxP3 has been suggested to play a key feedback role in the intrinsic stability of Tregs. Williams et al. showed that the disruption of FoxP3 function in fully committed Tregs resulted in reduced FoxP3 transcription [27]. Similarly to what was observed *in vitro*, Gavin et al. showed that FoxP3 maintains a positive feedback loop on itself. In this regard, ablation of a FoxP3 binding protein, Runx1, leads to decreased expression of FoxP3 in Tregs, suggesting that the FoxP3-Runx1 complex cooperates to maintain high FoxP3 expression levels under homeostatic conditions [28]. In fact, other signaling pathways have also been implicated in Treg stability. Rapamycin, which alters PI3K/mTOR signaling, stabilizes FoxP3 [29]. Conversely, continued TCR stimulation and constitutive activation of the PI3K/mTOR pathway inhibits FoxP3 expression [29, 30]. Similar antagonistic roles for GATA-3 [31], STAT6 [32], and ROR γ t [33,34] have been proposed. Finally, there is now ample evidence that the proteome in Treg is also controlled by miRNAs. Treg cells and conventional CD4⁺ T cells have different microRNA profiles and a significant part of the Treg-specific microRNA signature actually can be conferred by enforced expression of FoxP3 in conventional CD4⁺ T cells [35]. Multiple studies have shown that the ablation of the critical microRNA processing enzymes, Dicer and Drosha, can alter Treg development and function. In early studies by Merkenschlager and colleagues and Muljo et al., conditional ablation of Dicer during thymocyte development revealed significant alterations of thymocyte development and a very prominent defect in Treg development suggesting a key role for microRNAs prior to FoxP3 expression and Treg lineage commitment. Equally important, a number of studies by ourselves and others have shown that Dicer and Drosha-disruption after Treg lineage commitment led to instability of FoxP3 [18**,36,37]. Although Treg development in the thymus was largely intact, shortly after birth and over a few weeks, the Tregs became unstable leading to rapid loss of their immunosuppressive activity and development of lethal lymphoproliferative disease. Although there appeared to be a direct effect on FoxP3 expression and the FoxP3-driven Treg gene expression program, a number of unique mRNAs were disrupted that may not be directly regulated by FoxP3. Thus, microRNAs contribute to the stability of Treg lineage by stabilizing the Treg developmental program and subsequent functional program by stabilizing FoxP3 expression, repressing the expression of Th1 and Th2-driving genes, and promoting downstream suppressive functions.

The maintenance of intrinsic Treg stability likely involves the epigenetic modification of the FoxP3 locus, especially the two highly conserved CpG-rich regions located in the FoxP3 first intron and up-stream FoxP3 promoter. The methylation status of these two regions are tightly associated with stable gene expression of FoxP3, thus, the CpG's of naive T cells are highly methylated, whereas in FoxP3⁺ Tregs CpGs in these regions are fully un-methylated. Moreover, in vitro TGF- β -induced unstable FoxP3⁺ T cells retain only a few demethylated CpG motifs in both regions suggesting a potential role of this process in regulating FoxP3 stability [15,25].

In summary, based on a variety of cell markers, intracellular signaling and in vivo proliferative potential, it appears that Tregs are not resting but rather in a constant state of activation. Thus, at any given point in time, intrinsic and extrinsic inputs are responsible for stabilizing, or potentially destabilizing, FoxP3 expression and thus, Treg stability. Positive feedback loops likely drive FoxP3 expression to achieve highly stable FoxP3 expression under homeostatic conditions. It is tempting to speculate that FoxP3 expression levels are the sum of external signals and intracellular integration of these signals. If enough pro-active signals reach a certain threshold for turning on FoxP3 expression, FoxP3 itself provides a positive feedback loop to further enhance and stabilize Treg cells. miRNAs might play a critical role in fine-tuning and balancing the positive and negative stability signals in Tregs to control FoxP3 expression. Ultimately, this stable FoxP3 expression translates into epigenetic changes of the FoxP3 locus to establish and further solidify a stable Treg lineage. Most importantly, the data suggest that this meta-stable state, at least for a significant subset of FoxP3⁺ cells, can be altered in certain inflammatory settings leading to loss of FoxP3 expression, associated expression of the key gene expression signature and loss of Treg function.

What are the consequences of loss of FoxP3 expression and Treg stability?

Early reports suggested that loss of FoxP3 and its associated phenotypic signature would lead to increased Treg cell death and loss of FoxP3-expressing Tregs. Unless there was a catastrophic loss of the regulatory T cell subset, the limited Treg-deficiency would have minimal impact due to the stable FoxP3⁺ Tregs still remaining. However, there is increasing evidence that the FoxP3-deficient “exTregs” survive in multiple settings and may have an important biologic function themselves. Yang et al. showed under IL-6 and TCR stimulation, a subset of nTregs from both thymus and the periphery that downregulated FoxP3 converted to Th17 cells [23]. Consistent with this result, Hori and colleagues showed that adoptive transfer of Tregs into lymphopenic hosts resulted in the loss of FoxP3 expression and those FoxP3 negative cells could produce strong pro-inflammatory cytokines including IL-17 and IFN γ [17]. Thus, FoxP3 functions not only to initiate and maintain the suppressor cell program that allows this small subset of T cells to efficiently preserve immune tolerance, but functions as a transcriptional suppressor to prevent Treg production of multiple pro-inflammatory cytokines and cytolytic proteins. In this regard, it is important to remember that the majority of studies suggest that a significant proportion, if not the vast majority of Tregs are skewed towards self-reactivity [9]. Given the potential pathogenic cytokine and destructive intracellular proteins that might target tissues, the loss of FoxP3 expression can have a dramatic effect. If, as has been postulated above, a significant percentage of Treg lose FoxP3 expression under certain inflammatory conditions, these cells can develop into “effector-like” exTregs that might play an important role in immunity and autoimmune disease. In this regard, it is interesting that several investigators have suggested that there may be Treg subsets (based on T-bet or IRF4) that may selectively track with and suppress Th1 and Th2 helper subsets, respectively (Daniel Campbell, personal communication, [38]).

But, why should such a potentially dangerous subset exist? We would like to postulate that exTreg may be critical in fighting infectious disease and cancer surveillance in an innate-like

manner. Lund et al. have recently shown an unexpected benefit of regulatory T cells for viral clearance during infection. Indeed elimination of regulatory T cells rendered mice more susceptible to replication of herpes simplex virus in vaginal epithelial cells [39]. It will be interesting to test the possibility that exTreg, may be de-differentiating at local infected tissue sites as a consequence of microbial-derived or induced factors leading to microbial or autoreactive tissue-specific Tregs that actually help initiate clearance viral infection before the adaptive immune response develops.

Conclusions

Increasing evidence suggests that Tregs might be a dynamic population that under certain conditions can become unstable. A better understanding of the extracellular and intracellular signals that maintain or destabilize FoxP3 may have important therapeutic applications in a variety of disease settings ranging from autoimmunity to cancer and infectious disease.

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* of special interest

** of outstanding interest

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FoxP3 stability versus instability

Figure 1. FoxP3 stability vs. instability

Treg stability is controlled by a positive feedback loop driven by miRNAs, Runx1 and FoxP3, and further affected by various ex-cellular stimuli. TGF- β , IL-2, and other signals upregulate FoxP3 expression through induced phosphorylation of SMAD complex and STAT5, whereas IL-6, IL-4 and strong TCR signals downregulate Foxp3 through STAT6, GATA3 and mTOR, respectively.