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A Degrading View of Regulatory T Cells

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Studies by van Loosdregt et al. and Chen et al. in this issue of *Immunity* provide evidence for previously unrecognized players that regulate FOXP3 degradation. These are interesting developments that point to unappreciated mechanisms by which inflammatory signals can impact expression of FOXP3 and possibly the stability of Treg cell phenotypes.

An essential aspect of the vertebrate immune response is the requirement for peripheral tolerance. An immune response that, by its nature, is self-reactive and poised to respond needs to be held in check and to not damage host tissues. There are multiple mechanisms that contribute to peripheral tolerance, and many types of immune cells can acquire regulatory properties in a contextual manner; however, one unquestionably critical component is regulatory T (Treg) cells. It has been a decade since the discovery that the forkhead transcription factor FOXP3 is critical for Treg cell development and homeostasis. Although other factors also contribute to Treg cell integrity, there is general agreement that FOXP3 is essential for tolerance, a fact that is vividly illustrated in both mice and humans by the severe autoimmune disease associated with the absence of FOXP3 and Treg cells. Elucidating how easily *Foxp3* expression is or is not extinguished and defining populations of FOXP3-expressing cells that represent distinct, stable "lineages" remain topics of ongoing study. In this issue of *Immunity*, studies by van Loosdregt et al. (2013) and Chen et al. (2013) point to new mechanisms through which this key transcription factor can be degraded. These studies reveal previously unrecognized pathways that control FOXP3 protein expression in response to inflammatory stimuli and how the disruption of these pathways impact Treg-cell-mediated immune suppression.

First, in order to put these papers in context, it is worth reviewing that, in the mouse, two general types of FOXP3-expressing Treg cells exist (Abbas et al., 2013). Thymic Treg (also termed natural or nTreg) cells are generated in a T cell-receptor-dependent manner. Under normal conditions, and in the setting of some infections and autoimmune disease, nTreg cells appear to have relatively stable expression of FOXP3, the *Foxp3* locus being fully demethylated (Floess et al., 2007; Miyao et al., 2012; Rubtsov et al., 2010). FOXP3 can also be induced by cytokines, especially IL-2 and TGF-β—such regulatory cells are referred to as induced or iTreg cells and are generated under normal conditions at mucosal barriers. These cells have more flexible FOXP3 expression, the *Foxp3* locus being incompletely demethylated. Whether iTreg cells can lose FOXP3 expression and may become pathogenic is not under debate. However, whether there is a definable stable lineage of Treg cells that permanently expresses FOXP3 and always maintains its suppressor phenotype irrespective of the host environment has been the topic of considerable investigation. The practical implication is that, if we can isolate or create such a population, then these cells could be effective therapies for autoimmune disease and allotransplantation. Indeed, several clinical trials are underway.

In the nTreg cell plasticity camp, several groups have reported that "stable" Treg cells can lose FOXP3 expression in the setting of inflammation (Oldenhove et al., 2009; Zhou et al., 2009). This has been documented either directly by the measurement of FOXP3 expression with intracellular staining or indirectly with different *Foxp3* reporter mice. Typically, the loss of FOXP3 is associated with the acquisition of the capacity to produce effector cytokines. Challenging these conclusions, rival groups have drawn opposite conclusions with similar reporter mice (Miyao et al., 2012; Rubtsov et al., 2010). Supporting their work is the

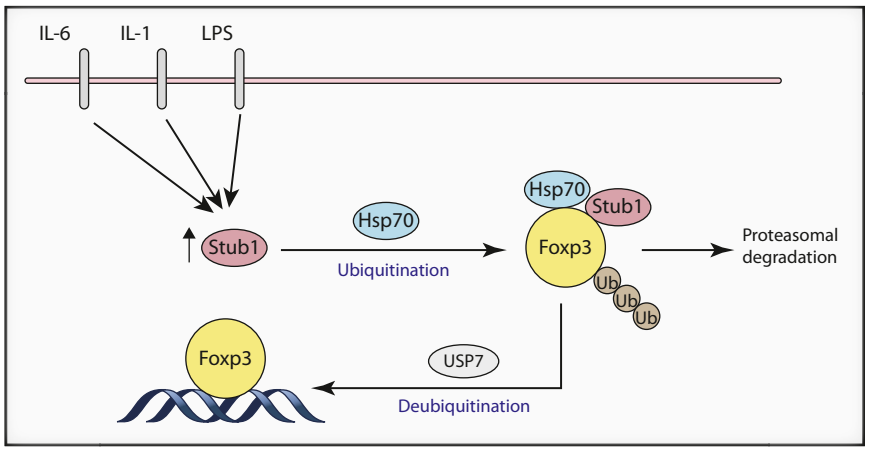

Figure 1. New Players in the Posttranslational Regulation of FOXP3 Stability in the Setting of Inflammation

Chen et al. (2013) and van Loosdregt et al. (2013) provide evidence for Stub1 and USP7—two factors that control amounts of FOXP3 by influencing ubiquitination and proteasomal degradation. In investigating means by which inflammatory signals downregulate FOXP3 protein, Chen et al. (2013) note that these stimuli induce Stub1; in conjunction with its partner, HSP-70, Stub1 promotes FOXP3 ubiquitination and degradation. In contrast, van Loosdregt et al. (2013) identify another player, USP7, that deubiquitinates FOXP3 and impedes its degradation. Normally, Treg cells express USP7, but IL-6 inhibits its expression. Loss of USP7 accelerates FOXP3 degradation.

realization that not all nTreg cells are truly thymically derived, and small numbers of contaminating, peripherally generated iTreg cells may be responsible for the discrepancy (Miyao et al., 2012; Weiss et al., 2012). Furthermore, it has been appreciated that the expression of FOXP3 alone is not sufficient to maintain Treg cell integrity; in addition to FOXP3, extrinisic factors are important, as illustrated by the role of IL-2 receptor (Miyao et al., 2012) along with other possible intrinsic factors, including Neuropilin 1 (NRP1) (Weiss et al., 2012). Thus, the isolation and investigation of $CD25^+$ $NRP1^+$ $FoxP3^+$ nTreg cells has cemented the orthodoxy that stable nTreg cells exist and that this stability is critically dependent on the methylation status of the *Foxp3* gene locus (Sakaguchi et al., 2013).

The two studies in this issue of *Immunity* shed new light on mechanisms that control FOXP3 expression. Importantly, the focus is not the transcriptional or epigenetic regulation of *Foxp3*; rather, they investigate how Treg cell stability might be influenced by factors that impact FOXP3 protein (Figure 1). Though not definitively established, especially with respect to in vivo control, the findings nonetheless may provide another way of thinking about FOXP3 stability and Treg cell plasticity. The basic finding by Chen et al. (2013) is that inflammatory stimuli result in Myd88- and proteasome-dependent degradation of FOXP3 in transfected Jurkat cells as well as primary mouse and human Treg cells. The authors used mass spectrometry to find FOXP3 partners and identify Hsp70. They note that Hsp70 associates with the stress-activated E3 ubiquitin ligase Stub1 and show that these proteins bind and promote Foxp3 K48 ubiquitination. Knocking down Stub1 and Hsp70 prevents the degradation of FOXP3 protein, and, conversely, the generation of *Foxp3* mutants that do not bind these factors are resistant to degradation.

van Loosdregt et al. (2013) looked at the other side of the coin, identifying factors that preserve FOXP3 protein. The present study starts off by using a deubiquitination (DUB) inhibitor, which inhibits in vitro and in vivo suppressive activity of Treg cells. Consistent with the findings of Chen et al. (2013), the Coffer group's previous work and the current van Loosdregt et al. (2013) study also show that FOXP3 is ubiquitinated and degraded in a proteasome-dependent manner. Using mass spectrometry, the authors identify ubiquitination sites on FOXP3 and also identify USP7 as a DUB prominently expressed in Treg cells. They go on to provide evidence that FOXP3 is a substrate of USP7 and that USP7 regulates FOXP3 turnover in transfected cells. The authors also show that USP7 levels are reduced by inflammatory stimuli, and this correlates with a reduction in FOXP3 protein levels. Functionally, they show that knocking down USP7 interferes with Treg cell function.

The strength of both studies is that they provide evidence of previously unrecognized modes of regulating FOXP3. Both studies provide provocative biochemical data and reasonably solid in vitro data. Given that the mechanism of action is posttranslational, it is unlikely that nTreg cells will be spared any more than iTreg cells, and much of the work by Chen et al. (2013) was performed with nTreg cells, albeit ones that were isolated with simple expression of CD25. It is likely that future work will focus on subsets of Treg cells and other factors that influence susceptibility to degradation. Additionally, these are not the only studies to invoke FOXP3 ubiquitination as a regulatory mechanism. That is, metabolic factors also control Treg cell function and expression of Foxp3. HIF-1α has been shown to inhibit FOXP3 through ubiquitination (Dang et al., 2011), and, like Stub1, HIF-1α is induced by IL-6 in conjunction with T cell receptor stimulation. However, Chen et al. (2013) show that HIF-1α is not required for lipopolysaccharide (LPS)-induced Foxp3 downmodulation in Treg cells, suggesting that the LPS- and Stub1-mediated FOXP3 depletion is distinct from the process previously observed under hypoxic stress (Dang et al., 2011). As a corollary, van Loosdregt et al. (2013) noted that IL-6 inhibits USP7 protein expression, further inhibiting the stability of FOXP3.

The biggest limitations of both studies pertain to the in vivo data. Using the T cell transfer colitis model, the authors of both studies link the function of Stub1 and USP7 to Treg cell integrity. That is, Stub1 overexpression and USP7 knockdown interfere with Treg cell function in this standard model. Ideally, in the future, we will learn more definitively what the consequences are of specific Treg cell deletion of Stub1, Hsp70, and USP7. The use of fate mapping models and of additional markers have been important in trying to sort out bona fide ex-Tregs from cells that arise as nTreg versus iTreg cells; clearly, these strategies will be employed in order to understand

what circumstances are associated with proteasome-dependent degradation of FOXP3. Whether the findings of the present papers speak more to the prevention of maximal induction of FOXP3 or the effective loss of FOXP3 in fully differentiated nTreg cells remains to be established.

Regardless, the implication that inflammatory cytokines can strip FOXP3 from a Treg cell does not simply provide more fuel for an academic fire; what makes this work of real clinical importance is the possibility of manipulating FOXP3 and Treg cells in autoimmunity and transplantation. Conversely, the therapeutic utility of attenuating regulatory immune mechanisms is one of the most exciting developments in cancer immunology. Both groups were mindful in demonstrating that ubiquitination is equally as important in human and murine Treg cells. Proteasome inhibitors are already used in the treatment of myeloma and mantle cell lymphoma, and it may not be long before they are used in the regulation of autoimmune disease.

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Generating CD8 T Cell Heterogeneity: Attack of the Clones

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Pathogen-induced inflammation modulates CD8 T cell effector and memory differentiation. In this issue of *Immunity*, Plumlee et al. (2013) demonstrate that clonally distinct CD8 T cells have the ability to generate numerous types of effector cell fates based on extrinsic pathogen-induced environmental cues.

During infection, individual naive pathogen-specific T cells receive signals that incite exponential growth and effector differentiation in order to rid the body of the pathogen. After pathogen clearance, most of the effector T cells undergo apoptosis, but a small proportion of cells survive to differentiate into mature memory T cells that, together with long-lived plasma cells and memory B cells, provide protection upon reinfection. As effector CD8 T cells expand and differentiate, they give rise to numerous phenotypically, functionally, and anatomically distinct subsets, which in turn give rise to diverse pools of memory CD8 T cells. Some effector cell subsets are inherently more fit to persist long-term and populate the memory cell pool, and in many cases these cells can be identified based on increased expression of interleukin-7Rα (IL-7Rα, CD127), CD27, and B cell lymphoma 2 (Bcl2) (Kaech and Cui, 2012). Understanding the basis of diversity in effector CD8 T cell function, migration, and memory cell potential might help inform the generation of more efficacious vaccines against pathogens and cancers. In the current issue of *Immunity*, Plumlee et al. (2013) establish that extrinsic pathogen-induced environmental cues shape the differentiation of individual naive CD8 T cell clones during infection.

T cell effector and memory differentiation is influenced by the type, timing, strength, and duration of antigenic (signal 1), costimulatory (signal 2), and cytokine (signal 3) signaling. Different infections modulate these signals by infecting