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Ubiquitous Points of Control over Regulatory T cells

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Abstract

Posttranslational modification by ubiquitin tagging is crucial for regulating the stability, activity and cellular localization of many target proteins and processes including DNA repair, cell cycle progression, protein quality control and signal transduction. It has long been appreciated that ubiquitin-mediated events are important for certain signaling pathways leading to leukocyte activation and the stimulation of effector function. It is now clear that the activities of molecules and pathways central to immune regulation are also modified and regulated through ubiquitin. Among the mechanisms of immune control, regulatory T cells (or Tregs) are themselves particularly sensitive to such regulation. E3 ligases and deubiquitinases both influence Tregs through their effects on signaling pathways pertinent for these cells or through the direct, posttranslational regulation of Foxp3. In this review we will summarize and discuss several examples of ubiquitin-mediated control over multiple aspects of biology of Tregs including their generation, function and phenotypic fidelity. Fully explored and exploited, these potential opportunities for Treg modulation may lead to novel immunotherapies for both positive and negative fine-tuning of immune restraint.

Keywords

E3 ligase; Foxp3; Treg; Ubiquitin; Deubiquitinase

Introduction

Defending the host from invading pathogens and malignant threats from within demands the capacity for robust immune system activation and mobilization. While the effector mechanisms responsible for mounting an adequate immune response are critical, equally important are regulatory safeguards keeping the immune system in check. These prevent collateral tissue damage and autoimmunity by limiting the extent of immune responses and suppressing the activation of self-reactive immune cells, respectively. Among the foremost cellular mediators of immune control are regulatory T cells (Tregs), a phenotypic hallmark of which is the ability to suppress activation of other leukocytes [1].

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While numerous T cell subsets display suppressive function, the most recognized and physiologically important are those marked by characteristically high expression of CD25 and the transcription factor Foxp3 (CD4⁺/CD25^{HIGH}/Foxp3⁺). These cells deploy a number of mechanisms to counteract immune activation including the anti-inflammatory cytokines IL-10, TGF β and IL-35 and the coinhibitory molecules CTLA-4 and LAG3 [2]. The importance of these cells and their action in maintaining immune homeostasis is clearly evidenced by the widespread autoimmunity arising from deficient Treg numbers or function [1].

Our understanding of Foxp3⁺ Tregs has become much more nuanced of late. It is now clear that within this population, considerable heterogeneity exists not only in terms of their tissue of origin (i.e. thymic vs. peripheral), but their suppressive function, proliferative capacity and phenotypic stability as well [3, 4]. Tregs are capable of specialization marked by limited acquisition of helper T cell lineage attributes that may afford enhanced suppressive function under specific conditions [5, 6], and the importance of Tregs beyond immune suppression is becoming clear. For instance the intriguing abilities of Treg subsets to affect the composition of the gut commensal community and to paradoxically prime immune responses have been recently described [7, 8].

Our grasp of Foxp3 function has grown considerably more detailed as well. While the central importance of this molecule's sustained expression as a marker and necessary master regulator of the Treg lineage is clear [9], recent studies show that Foxp3-mediated enforcement of the signature Treg gene expression profile of Tregs relies on a network of co-regulator molecules with potentially overlapping function [10–12], and altering the ability of these coregulators to interact with Foxp3 has significant and potentially context-specific consequences for immune regulation [13, 14].

These additional facets of Treg biology offer fresh opportunities for therapeutic intervention aimed at tightening or relaxing Treg-mediated restraint of the immune response. So too do recently uncovered posttranslational mechanisms that hold considerable sway over Tregs and their suppressive function.

One such mechanism hinges on the ubiquitin-tagging of Treg-critical molecules. This process occurs through the cascading action of the three enzyme classes of the “ubiquitin system”. In an ATP-dependent manner, the activator of ubiquitin (E1) modifies the C-terminus of small (8kD) ubiquitin molecules to generate highly reactive thioesters that permits linkage to ubiquitin-transfer/conjugation enzyme (E2). An E3 ubiquitin ligase then facilitates the transfer of activated ubiquitin from the E2 carrier protein and attachment of the ubiquitin C-terminus to target protein lysine residues by an isopeptide bond. Additional ubiquitin monomers can also be delivered to the lysines of already tethered ubiquitin molecules as well to extend polyubiquitin chains [15–17]. This sort of modification, especially when ubiquitin monomers are joined at their lysine 48 (K48 linkage), leads to degradation of target protein via the 26S proteasome. Monoubiquitin-tagging or K63-linked polyubiquitin chains on the other hand tend to affect target protein localization or signaling activity [18]. A number of additional, noncanonical linkage types exist as well with distinct functional consequences [19].

E3 ligases are sub-classified based on their prominent structural domains. Notable examples E3 ligases families are the RING finger domain and HECT domain type ligases distinguished by consensus sequences of cysteines and histidines (the former) or catalytic domains homologous to that of E3 ligase E6AP (the latter). Members of these families and other unique E3 classes play major roles in a number of immune cells and diverse processes [19].

Opposing the ubiquitination process are the deubiquitinating enzymes (DUBs). These enzymes cleave ubiquitin and ubiquitin-like proteins from modified substrate residues. DUBs have been grouped into five families based on their enzymatic nature [20, 21]. Of these, the Ubiquitin Specific Protease family (USP) has considerable immune relevance. The highly conserved “USP domain fold” shared by these DUBs has been likened to a hand with “thumb”, “palm” and “finger” elements gripping the C-terminus of ubiquitin. In addition to catalytic and ubiquitin binding domains, USPs, and DUBs in general, often contain insertions and terminal extensions that facilitate protein-protein interactions necessary for the binding to cofactors that by regulate substrate accessibility and recognition and thereby account for specificity [21]. The action of these enzymes and their ubiquitin tagging counterparts control events crucial to immune processes ranging from anergy to activation that are essential for a functional immune system (recently reviewed in detail by Zinngrebe et al. [19]).

In the following sections we will summarize a number of degradative and nondegradative ubiquitin-mediated pathways that control Treg generation, function and phenotypic stability. While many of the enzymes and factors discussed herein play critical roles across and beyond the immune system, by determining the frequency and crucial restraining function of Tregs, ubiquitin-driven processes can have widespread consequences for immune control.

Ubiquitin-mediated regulation of extrathymic Treg induction

While a large percentage of functionally suppressive, Foxp3⁺ Tregs arise in the thymus (so called thymic Tregs or tTregs), Tregs can also be induced outside this site (peripheral or pTregs) as well as *ex vivo* (so called induced or iTregs) under conditions typically including suboptimal activation and the presence of TGF β and IL-2 [22]. The process of extrathymic Treg induction is heavily influenced by the action of several ubiquitin E3 ligases.

Cbl-b

The RING finger domain containing E3 ligase Casitas-B-lineage lymphoma protein-b (Cbl-b) has been linked to the induction of T cell anergy. Additionally, it also moderates TCR signaling by fine-tuning the threshold for activation [23]. Correspondingly, mice lacking functional Cbl-b are prone to T cell hyperactivation and autoimmune disease [24]. Interestingly, while tTregs from these mice develop and function similar to those of wild type mice, effector T cells lacking Cbl-b are resistant to the suppressive function of Tregs [25, 26].

While Cbl-b does not appear critical for thymic generation of tTregs, this ligase is important for the induction of their extrathymic counterparts. It has been shown repeatedly that robust

activation of the PI3K/AKT/mTOR signaling pathway negatively impacts the TGF β -driven differentiation of iTreg from naïve CD4⁺ T cell precursors [27, 28]. Sustained or excessive PI3K/AKT signaling precipitates the inhibitory phosphorylation of the Foxo proteins, which are important drivers of Foxp3 expression [29, 30]. Cbl-b targets the SH3 domain of the regulatory subunit of PI3K (p85) [31]. This nonproteolytic modification disrupts the subunit's association with the TCR and CD28 and its activity preventing downstream phosphorylation and activation of AKT. As a result of this disruption by Cbl-b, Foxo proteins are not inactivated by AKT and freely translocate to the nucleus to enhance Foxp3 expression [32]. Demonstrating this role, Foxp3 upregulation is deficient in the face of enhanced AKT phosphorylation in Cbl-b^{-/-} T cells, but treatment of these cells with the PI3K inhibitor LY294002 can rescue the ability induce Tregs from naïve precursors [30].

Cbl-b has also been implicated as a positive regulator of TGF β signaling. In the absence of Cbl-b, TGF β signaling is reported to be defective [33]. This E3 ligase was recently shown to target Smad7, which is an inhibitor of TGF- β receptor signaling and iTreg generation, for degradation [34, 35]. Given the importance of TGF- β and its down stream mediators in the upregulation of Foxp3 transcription, as well as the need to curtail PI3K/AKT activation during Treg induction, it is clear how the absence of this E3 ligase so negatively affects the generation of iTregs.

ITCH

Another ligase important in Treg induction is named for the “Itchy” phenotype displayed by mice lacking it. ITCH, a HECT-domain type E3 ligase, also promotes the generation and maintenance of Tregs. Like those of Cbl-b^{-/-} mice, ITCH deficient T cells are hyperproliferative. As a result these mice are disposed to excessive, Th2-biased responses, and their conventional T cells resist the suppressive effects of Tregs and TGF β exposure [36] suggesting multi-tiered involvement in the maintenance of immune tolerance.

TGF β signaling is critical during the conversion of naïve CD4⁺ T cells into potentially suppressive Foxp3 expressors and ITCH has been implicated as an important participant in this cascade. ITCH has been linked to the phosphorylation and activity of Smad2 [37]. ITCH also monoubiquitinates a transcription factor known as TGF β -inducible early gene 1 product (TIEG1) promoting its activity rather than its degradation. Nuclear translocation of modified TIEG1 is capable of binding and transactivating the Foxp3 promoter. Reflecting this, activation of ITCH^{-/-} naïve T cells in the presence of TGF β results in poor induction of Foxp3 and a heightened frequency of IL-4⁺ cells. TIEG1 null T cells show similar defects supporting the involvement of this factor in ITCH mediated Treg induction. In these mice the function of iTregs is also adversely affected as they fail to suppress airway inflammation *in vivo* [38]. Interestingly, upon IL-6 induced phosphorylation, TIEG1 is subject to inhibitory, K27-type polyubiquitination by ITCH inhibiting Foxp3 expression and tumor-induced immune tolerance [39].

Another study suggested that ITCH stabilizes Foxp3 expression during the Treg skewing process through a separate mechanism also dependent upon TGF β . Here the authors found that deficiency in either ITCH or Nedd4 family-interacting protein 1 (Ndfip1), a TGF β -upregulated adaptor molecule necessary for ITCH mediated polyubiquitination, resulted in

reduced Treg generation in the gut (pTregs) as well as *in vitro* (iTregs) while tTregs were unaffected. This defect was found to stem from ITCH's importance as a suppressor of key Th2-associated genes during iTreg commitment. By marking the transcription factor JunB for degradation, ITCH negatively regulates this driver of Th2 gene expression. In the absence of the ITCH adaptor Ndfip1, differentiating iTregs display enhanced expression of Th2 genes such as IL-4, which interferes with iTreg differentiation [37].

Skp2

Another E3 ligase inversely related to both Foxp3 expression and Treg suppressive function is known as S-Phase kinase-associated protein 2 (Skp2), a member of the multi-subunit SCF (Skp1, Cullin, F-box containing) E3 ligase complex family. Knockdown of this Skp2 in effector CD4⁺ T cells converts them into Foxp3-expressing, functionally suppressive Treg cells complete with enhanced IL-10 and TGF β levels and reduced proinflammatory cytokine production. Downregulation of Skp2 by conventional T cells also induces a Treg-like resistance to activation-induced cell death that in addition to suppressive capabilities imparts them with the ability to prevent spontaneous development of type I diabetes upon injection into NOD mice. On the other hand, overexpression of Skp2 in Tregs reduces both Foxp3 expression and Treg suppressive function. In this study, the mechanism behind Skp2's antagonism of Foxp3 expression and Treg function was linked to its suppression of cell cycle inhibitors (p21, p27) as well as that of the Foxo proteins, Foxo1 and Foxo3a [40], known transcriptional activators of the *Foxp3* gene [29, 30].

The examples described above illustrate how the modification of numerous targets by E3 ligases affects the process of Treg induction through a variety of pathways. Additionally, an element most central to the acquisition of the Treg phenotype – the Treg master regulator Foxp3 – also answers to ubiquitin-mediated sway at a critical point in the shaping of a T cell response.

VHL and HIF-1

While functionally opposite, the suppressive iTreg and the proinflammatory Th17 lineages share common elements in their differentiation pathways. Particularly, TGF β is required for commitment to either fate, and this cytokine can induce both Foxp3 and ROR γ t in freshly activated naïve T cells [41]. High concentrations of TGF β among other factors can result in sustained Foxp3 expression and direction to the iTreg fate. An abundance of STAT3-activating cytokines, such as IL-6, on the other hand favors dominance of ROR γ t and the Th17-associated gene program typified by IL-17 [41, 42].

For optimal Th17 commitment, timely removal of Foxp3 protein from cells poised at the crossroads of these divergent lineages is necessary. We found that the oxygen sensor and mediator of the cellular response to hypoxia known as hypoxia-inducible factor -1 (HIF-1) plays a key role in this process. The ability of HIF-1 to act as a transcriptional activator is regulated by oxygen-induced, ubiquitin-mediated degradation of the HIF-1 α subunit. In the presence of oxygen, critical proline residues on HIF-1 α are hydroxylated by the prolyl hydroxylases or PHDs (which are themselves regulated by the E3 ligases Siah1a and Siah2 [43]), and are recognized by the Von Hippel-Lindau protein (VHL), which recruits the

Elongin-C-Elongin-B-Cullin-2-E3- ubiquitin ligase complex. Polyubiquitination of HIF-1 α by this complex then leads to its degradation via the 26S proteasome, negating its expansive transcriptional impact (e.g. regulating the machinery for glycolytic metabolism). At low cellular oxygen levels, unmodified HIF-1 α is spared from modification and is thus stabilized [44, 45].

Besides hypoxia-driven stabilization of HIF-1 α protein, normoxic stimuli can upregulate this molecule including the Th17-promoting IL-6/STAT3 signaling pathway. Indeed during Th17 differentiation, HIF-1 is induced, even at atmospheric oxygen levels in a STAT3-dependent process. Hypothesizing that HIF-1 is important for Th17 fate commitment, we found that under Th17-inducing conditions *in vitro*, naïve CD4⁺ T cells lacking HIF-1 α (from CD4^{cre}+/HIF-1 α ^{flox/flox} mice) are substantially less effective than wild type cells at upregulating Th17 genes, including those encoding ROR γ t and IL-17. Instead, these T cells show reciprocal upregulation of Foxp3 protein – but not transcript [46], suggesting a HIF-1 dependent mechanism for rapid downregulation of Foxp3 at the divergence of the Th17 and iTreg lineage.

The mechanism behind this effect is linked to HIF-1's ability to physically interact with Foxp3 as well as its own ubiquitin-mediated degradation pathway. Mutant HIF-1 molecules rendered insensitive to oxygen-dependent modification and degradation failed to facilitate Foxp3 protein loss. Similarly knocking down components of the HIF-1 degradation machinery and inhibition of the proteasome also stabilized Foxp3 levels. Furthermore, Foxp3 itself was observed to be ubiquitinated in iTregs suggesting a model of “piggy-back” co-modification/co-degradation of interacting HIF-1 and Foxp3 molecules. In addition to its role in marking Foxp3 protein for degradation, HIF-1 was also found to enhance both the transcription of ROR γ t itself as well as its activation of Th17-associated loci [46].

In accord with the finding that HIF-1 promotes Th17 commitment at the expense of iTreg generation, another group found that HIF-1 deficiency tilts the balance between these lineages in favor of Foxp3-expressing cells although the effect was attributed to HIF-1's role in metabolic reprogramming [47]. More recently, a study contrasting the differentiation requirements of newly appreciated “natural Th17 cells” to those of traditional or “induced Th17 cells” found that in naïve T cells lacking HIF-1 (conditional knockouts of the HIF-1 β subunit), *in vitro* and *in vivo* iT17 levels are reduced and these cells preferentially upregulate Foxp3 [48].

Interestingly, others have reported that HIF-1 has a positive effect on the transcription of the *Foxp3* gene [49]. While in our study, any contribution of HIF-1 to Foxp3 levels by transcriptional augmentation was likely dwarfed by its negative effect on the Foxp3 protein pool and its reciprocal engagement of the Th17 transcriptional program. It is possible that under certain experimental or physiological conditions, the outcome of the apparent tug-of-war between HIF-1's transcriptional and posttranslational roles may be quite different.

For instance, it is possible that extended inactivity of the PHD/VHL/ubiquitin-driven HIF-1 α degradation machinery, as may occur under prolonged hypoxia, results in a stalling of putative Foxp3/HIF-1 co-degradation. This would likely lead to accumulation of both

proteins and transcriptional effects may become more prominent. Unpublished results from our group are in line with this. We find that transgenic mice constitutively expressing a hydroxylation-resistant HIF-1 α molecule (impervious to PHD/VHL-driven degradation) do, relative to wild type controls, display modestly enhanced Foxp3 levels under select conditions *in vitro* and *in vivo*. This suggests that HIF-1 α turnover rather than its expression is more central to its role as down-modulator of Foxp3 protein. Further study is needed to delve deeper into the consequences of HIF-1 α stabilization for Treg function and stability.

The physiological niches generated within tumors are likely to witness prolonged hypoxia. Indeed Foxp3⁺ CD4⁺ T cells are enriched among tumor infiltrates [50], suggesting that Foxp3 levels are stabilized there along those of HIF-1. However, revelations that tumors may be more adept at recruiting and/or expanding pre-existing Tregs from other locales than inducing *de novo* Foxp3 expressors [51, 52] allude to a more complex explanation for the high Treg presence within tumors.

Another distinction of prolonged hypoxia is the potentially altered representation of HIF-1 and HIF-2 resulting from differential sensitivity to an alternative route for HIF decay that is known to be both active in non-T cells under prolonged hypoxia and independent of the traditional PHD/VHL/Elongin-mediated pathway [53]. Indeed the importance of oxygen-independent HIF-1 α degradation in T cells is, while unknown at present, most germane to the dynamics of the Treg/Teffector balance.

Intermittent or transient hypoxia, or normoxic HIF-1 induction, however should all result in highly augmented Th17 differentiation and simultaneously stunted Treg generation due to the parallel upregulation of HIF-1 and the active turnover of HIF-1/Foxp3 complexes by the PHD/VHL/Elongin pathway. Indeed, this notion is supported by differentiating naïve T cells *in vitro* under hypoxic culture conditions interrupted by periodic normoxic respite [46], as well as transient hypoxic culture [54].

One physiological setting for this scenario of interrupted hypoxia may occur in obstructive sleep apnea syndrome (OSAS) patients. OSAS is typified by repeated airway obstruction that interrupts nocturnal ventilation inducing bouts of low blood oxygen levels and reducing sleep quality [55]. In a recent study, Th17 frequencies were elevated in the peripheral blood of OSAS patients compared to non-afflicted controls while Treg levels were reciprocally diminished – both to an extent commensurate with OSAS severity [56]. This association between intermittent oxygen deprivation and skewed Th17/Treg balance is in keeping with prior links between hypoxia and an enhanced Th17 program [46, 54] as well as the hypothesis suggested above.

Thymic Treg generation

While many ubiquitin-regulated processes are active during conversion of naïve CD4⁺ precursors into iTregs, E3 ligases are also important for the thymic generation of Tregs. Initiation of Foxp3 expression in the thymus requires the triggering of TCR and CD28 signaling. This critical activation leads to the binding of several transcriptional activators to the *Foxp3* locus including NFAT, AP-1 and CREB [57]. TCR-induced activation of the NF κ B signaling pathway has also been shown to be critical for Foxp3 induction by fledgling

tTregs. Reflecting this, mice lacking various components of this pathway (including CARMA1, PKC θ , Bcl-10) have reduced Foxp3-expressing thymocytes as well as mature Tregs compared to wild type mice [58–61].

The pivotal role of the ubiquitin system in the activation of NF κ B-type signaling is perhaps one of the most recognizable instances of regulation by modification in the immune system (reviewed in reference [62]). TCR/CD28 engagement triggers a cascade of protein-protein interactions and posttranslational modification events that culminate in the degradation of inhibitor molecules that, in resting cells, curb the transcriptional activity of NF κ B family members by sequestering them in the cytoplasm.

Peli1/c-Rel

One NF κ B family member in particular, called c-Rel, drives development of Tregs in the thymus [63–65]. c-Rel deficient mice harbor reduced frequencies of Treg precursors (identified as CD25⁺GITR^{hi}Foxp3⁻CD4⁺) among their thymocytes relative to wild type mice [66]. c-Rel appears to act as a “pioneer factor” – initiating the process of turning on Foxp3 transcription by binding the conserved non-coding sequence 3 of the *Foxp3* gene, which is critical for Foxp3 induction [63–65, 67–69]. Interestingly, while c-Rel is critical for initiating *Foxp3* transcription, it appears dispensable for continued expression of the transcription factor and may be inactive later during Treg ontogeny [70]. Indeed, NF κ B activation in general may be selectively important for Foxp3 induction in the thymus since robust activation of this pathway can interfere with iTreg induction [71], and some elements are either not necessary for [58, 59] or may adversely impact the functional stability of mature Tregs [72].

In addition to its activation, c-Rel expression is also regulated by the ubiquitin system. The E3 ligase known as Peli1 mediates the K48-ubiquitination of c-Rel, bringing about its degradation via the proteasome [73]. In mice, deleting the gene encoding Peli1 results in over-activation and autoimmunity clearly demonstrating its importance for restraining T cell activation by preventing the accumulation of c-Rel protein. Interestingly, Tregs from these mice are functional *in vitro* and only slightly increased without this mediator of c-Rel downregulation [74]. It is unclear whether any Treg function or stability defect contributes to the hyperactive phenotype seen.

MARCH1

Another E3 ligase involved in tTreg generation is Membrane-associated RING-CH1 (MARCH1). This ligase is known to target MHC II and CD86 for degradation making it an important modulator of antigen presentation and costimulation [75]. In MARCH1^{-/-} mice, SP thymocytes contain reduced frequencies and absolute numbers of Foxp3⁺ cells. Interestingly, MARCH1 deficiency in hematopoietic, but not Tregs themselves was found to be responsible for this paucity. The authors of this study went on to show that by ubiquitinating MHC II molecules in dendritic cells (DCs) MARCH1 downmodulates the process of antigen presentation, which allows greater Treg frequencies. Dampening antigen presentation in the thymus could theoretically spare more strongly self-reactive, potential Tregs from deletion. However, with no obvious alteration in thymocyte deletion, the authors

suggest that a dynamic expression of surface MHC molecules imparted by MARCH1 action may favor Treg generation in the thymus [76]. Reconciling this finding with the notion that strong TCR signaling aids tTreg development is an intriguing topic that may involve unappreciated subtleties of the TCR-antigen-MHC II triumvirate.

Traf6

Traf6 is a K63-type, RING finger domain containing E3 ligase involved in a number of receptor initiated signaling pathways and the activation of transcription factors including NF κ B and AP-1 [77]. Suggestive of a defect in immune control, Traf6 deficient mice are prone to autoimmunity [78, 79]. Indeed, Traf6 plays an important role in the generation of Treg precursors in the thymus as knocking out this ligase reduces the frequency of Foxp3-expressing single positive CD4⁺ thymocytes [80].

Despite this role in Foxp3 upregulation by pre-tTregs, Traf6 does not appear as critical for induction of Tregs outside the thymus. Peripheral Treg numbers in Traf6^{-/-} mice are reported to be similar to wild type levels [80]. Some report that Traf6 deficiency does not impair TGF β mediated up-regulation of Foxp3 expression in naïve CD4⁺ T cells *in vitro* – in fact Traf6^{-/-} T cells can induce Foxp3 expression more robustly than wild type controls [80]. Interestingly, a role for Traf6 in maintaining the phenotypic stability of Tregs has recently come to light as well that will be discussed later.

Ubiquitin-mediated control of Treg functional stability

The stability of the Treg lineage is a subject fraught with controversy. A number of studies have reported that under conditions such as extreme inflammation, Tregs may lose Foxp3 expression and abandon suppressive function in favor of effector T cell attributes [81–83]. In contrast, it was recently shown that most bona fide, thymically derived Tregs display remarkable fidelity to the Treg phenotype (i.e. Foxp3 expression and suppressive function) [84, 85]. Despite this, additional reports of significant inflammation-induced instability [86–88] and expansive heterogeneity in human and mouse Tregs [4, 89] preclude a closing of this case. While the plasticity of the Treg lineage (i.e. the ability to completely reprogram a fully committed Treg) remains to be completely sorted out, a new, but related conversation has emerged concerning the functional stability of Tregs and its responsiveness to external inputs.

A growing number of studies suggest that Treg function is responsive to cues from the microenvironment. While a number of stabilizing factors have been identified [90–94], proinflammatory cytokines and other hallmarks of inflammation can disrupt Treg suppressive function [95–102]. Interestingly, the mechanisms responsible for these stabilizing and destabilizing effects appear to operate at both the transcriptional as well as the posttranslational level.

The known Treg-antagonist cytokine, TNF α precipitates the dephosphorylation of Foxp3 hindering its regulatory activity and broader Treg suppressive function [95]. In contrast, acetylation of Foxp3 promotes both its stability and effectiveness as a transcriptional regulator [103, 104]. These findings highlight how posttranslational mechanisms can be at

play in the modulation of Tregs by the microenvironment. Ubiquitination of Foxp3 and other targets provides yet another pathway for such protein-level regulation.

ITCH

Previously mentioned as important for Treg induction by TGF β , ITCH is also important for the function of established Tregs. Mice with Foxp3-restricted ITCH deficiency were recently found to be less fit than their wild type counterparts and show signs of widespread autoimmunity. Even though Tregs in these mice were as frequent as those of their wild type counterparts and as functional *in vitro*, ITCH deficient Tregs failed to curtail Th2-type inflammation *in vivo*, particularly in the lung and gut. This *in vivo* defect was highly specific as ITCH^{-/-} Tregs were, like their wild type counterparts, able to suppress the predominantly Th1-mediated colitis of a widely used adoptive transfer induced IBD model [105].

Fate tracking experiments also revealed comparable stability of Foxp3 expression in the presence or absence of ITCH. Deficiency in this molecule did, however, result in the acquisition of Th2-like attributes (increased STAT6 activation, and expression of GATA-3 and IL-4) by Tregs and “exTregs” – uncharacteristic attributes that interestingly enhanced commitment of naïve CD4⁺ T cells to the Th2 lineage and Th2 inflammation [105]. This is one of several recent studies demonstrating the importance of suppressing inappropriate expression of helper T cell genes for maintaining the Treg phenotype.

Ubc13

Another suppressor of inappropriate gene expression in Tregs can be found among the E2 enzymes of the ubiquitin system. Ubc13, an E2 protein and known catalyst of K63-linked polyubiquitin tagging was previously implicated as a mediator of TNF α and NF κ B activation [106]. Recently, however, Ubc13 was shown to promote Treg suppressive function *in vivo* and prevent acquisition of uncharacteristic T effector-like gene expression by Tregs [107]. While having little effect on *in vitro* function and the frequency of Tregs at baseline, Foxp3-driven *Ubc13* deletion does result in pronounced *in vivo* defects in Treg function. Compared to controls, mice lacking Ubc13 in their Tregs displayed reduced body weight, hyperactivation of T cells, abundant proinflammatory cytokine production and multi-organ lymphocytic infiltration [107].

Importantly, in the absence of Ubc13, Tregs exhibited abnormal proliferative capacity and expression of the effector cytokines IFN γ and IL-17. Furthermore Tregs lacking Ubc13, unlike their wild type counterparts, fail to prevent progressive colitis *in vivo*, and may themselves contribute to the severity of disease in this model. While Ubc13 deficiency did not hinder expression of Treg-associated factors (CTLA-4, GITR, CD25), expression of SOCS1 and IL-10 were reduced compared to wild type Tregs [107]. These deficits likely account for the perturbed phenotype seen in Ubc13^{-/-} Tregs as IL-10 is a well-known means of immune regulation, and SOCS1 has recently emerged as a buttress of Treg stability capable of restricting conversion into Th17- and Th1-like cells [108].

Promotion of SOCS1 expression is mediated by the Ubc13 target, IKK2. Indeed, in this study Treg function in Ubc13 knockout Tregs could be rescued to some degree by

constitutive activation of IKK2 or administration of a SOCS1 mimetic peptide [107]. In this way, Ubc13 contributes to the focusing of Treg gene expression.

GRAIL

The ubiquitinating enzyme known as “gene related to anergy in lymphocytes” (GRAIL) is another RING finger E3 ligase influencing the generation of suppressor T cells. GRAIL is upregulated by anergic T cells and is important for their characteristic hypoproliferative state. Knocking out the gene encoding GRAIL in mice prevents the induction of T cell anergy, setting the stage for unchecked T cell proliferation and dysregulated cytokine production [109]. Since GRAIL targets components of the TCR complex, naïve T cells from GRAIL^{-/-} mice fail to downregulate CD3 and are therefore hyperresponsive to TCR stimulation [110]. Reflecting this, these mice are poor inducers of tolerance and are susceptible to autoimmune disease.

Interestingly, while GRAIL is also upregulated by Tregs [111], populations of these regulators in GRAIL^{-/-} mice appear normal as does the process of *in vitro* Foxp3 induction in TGFβ-treated naïve CD4⁺ T cells. However, Tregs from these mice are less suppressive than their wild type counterparts, and despite expressing wild type levels of Foxp3 mRNA, these cells express genes typical of Th17 cells. This uncharacteristic effector T cell gene expression was attributed to the abnormally high levels of IL-21 made in the absence of GRAIL-mediated restraint of NFAT signaling [110]. Therefore it seems that in addition to an important role in the induction of anergy, GRAIL preserves Treg function by restraining inappropriate gene expression capable of undermining their suppressive phenotype.

MARCH1

Recently an E3 ligase was shown to underlie a major avenue of Treg suppression. Interactions between DCs and Tregs can downregulate MHC II and costimulatory molecules on the antigen presenting cells rendering them less capable of activating T cells. Chattopadhyay and Shevach found that in DCs, IL-10 produced by iTregs induces MARCH1 [112], an E3 ligase involved in tTreg selection and previously shown to trigger the degradation of MHC II and CD86 [76][75]. Furthermore, exogenous and iTreg-derived IL-10 can downregulate CD83 – an inhibitor of the interaction between MARCH1 with its targets [112][75] suggesting that this cytokine is a potent activator of this ubiquitin-mediated mechanism of Treg suppressive function. While most of the ubiquitin-sensitive events described in this review occur within developing or established Tregs, MARCH1 provides an example of a ligase in other cells that both fosters tTreg generation and facilitating iTreg function.

Traf6

Traf6 has recently emerged as a multi-stage player in Treg biology. In a report by Muto et al., mice with Treg-specific Traf6 deficiency display enhanced Treg frequencies, but nevertheless develop spontaneous autoimmunity. Interestingly, while functionally competent *in vitro*, Traf6 knockout Tregs, unlike wild type controls, are completely ineffective at preventing colitis in Rag2^{-/-} recipients of naïve CD4⁺ T cells. Unstable Foxp3 expression and inappropriate Th2 gene expression in the absence of Traf6 were deemed responsible for

this specifically *in vivo* defect [113], in keeping with earlier characterization of Traf6 as a specific suppressor of Th2 autoimmunity [79, 114].

While it is clear that this molecule is needed to maintain Treg function, the precise mechanism at play remains to be elucidated. Speaking to this, unpublished findings from our lab suggest that Traf6-driven, non-degradative modification of Foxp3 may affect its cellular distribution in a manner capable of promoting the effectiveness and stability of the transcription factor (FP, JB unpublished results). While in this case direct modification of Foxp3 appears to have a positive effect on Treg function, prior work has demonstrated the potential for negative regulation of Foxp3 by another mode of ubiquitination during naïve T cell differentiation [46]. More recent studies suggest that ubiquitin-mediated Foxp3 downregulation affects Treg stability as well.

Stub1

While transcription of the *Foxp3* gene in some or most Tregs can be highly stable, the Foxp3 protein pool appears to be more dynamically controlled. It has recently come to light that Foxp3 protein has a high turnover rate with Foxp3 loss regulated by K48-type polyubiquitination and proteasomal degradation [115, 116]. In keeping with reports that Treg function can be disrupted by inflammatory cues, the cellular half-life of Foxp3 can be markedly shortened and the bulk Foxp3 pool reduced upon exposure to a range of inflammatory stresses *in vitro* [116]. With our colleagues we set out to determine the molecular events involved in this regulation.

Mass-spectrometry analysis of the Foxp3 protein complex identified the heat shock 70kDa protein (Hsp70) to be a binding partner of this critical Treg regulator. Both Hsp70 and the closely related Hsc70 are known to act as chaperones for a stress-activated, U-box domain type E3 ubiquitin ligase known as C-terminal of Hsp-70-interacting protein (CHIP) or Stub1 [117]. Since Stub1 is recruited to mediate the degradation of target proteins that include important transcription factors such as Runx2 and HIF-1 α [53, 118], we suspected this ligase to be the driving force behind degradative Foxp3 polyubiquitination. Interestingly, Stub1, while not highly expressed in resting Tregs is upregulated in mouse Tregs under the same conditions downmodulating Foxp3 *in vitro*. This as well as an observed physical interaction between Foxp3 with Stub1 further implicated this E3 ligase in the process [116].

Indeed, retroviral overexpression of Stub1 in Tregs undermined their Foxp3 levels and suppressive function both *in vitro* as well and *in vivo*. Compromised expression of several Treg-associated genes and uncharacteristic effector cytokines were detected in contrast to control cells. Conversely, hampering Stub1 induction by siRNA knockdown stabilized Foxp3 expression and Treg function [116]. These studies identify an unappreciated pathway for the regulation of Treg function by environmental cues working at the protein level.

Whether physiologic induction by Stub1 can similarly impinge upon Treg function, and, in particular, whether such an effect is reversible are unanswered questions in need of further investigation. Also Stub1's role during the generation of Tregs is worth investigating. In light of the unique differentiation requirements of the Treg subsets, it is possible that thymic development of Tregs may actually benefit from Stub1's recently described role in NF κ B

activity [119] while iTreg induction, like the function of established Tregs, may be adversely affected.

Interestingly, in our study we found that among the inflammatory cues tested, LPS could both upregulate expression of *Stub1* and induce the loss of *Foxp3* from purified Tregs. Moreover, this pathway for *Foxp3* downregulation was not functional in Tregs from mice deficient in the TLR signaling mediator *Myd88* [116] lending credence to the notion of inflammation induced inhibition of Tregs. While these observations are in accord with a number of studies reporting a Treg antagonizing role for TLR signaling in general [120–125] or specifically *Myd88* or *TLR4* signaling [126–130], others report starkly contrasting effects of TLR activation on Tregs [131–134]. It is possible that these divergent conclusions reflect distinct outcomes possible along a common chain of events.

Evolutionarily speaking, mechanisms for rendering Tregs permanently inactive in the face of the inflammation they must suppress seems incongruous with the obvious need for immune homeostasis. However, temporary or reversible Treg-disarmament may be highly advantageous. Transient pausing of the Treg phenotype may permit both the initiation of highly effective immune responses (unleashed from the suppression of Tregs) as well as the proliferation of Tregs themselves leading to a larger pool of potential suppressors perhaps active with the waning of inflammatory triggers (i.e. microbial products at the resolving of infection). Indeed such a scenario was suggested by the effects of *TLR2* agonists on Tregs [121, 122]. An extension of this model suggests that a fine line may be walked by Tregs responding to inflammatory stresses. Modest or temporary insult may result in brief loss of function and effective expansion of the Treg pool capable of eventually subduing a robust effector response while excessive or prolonged inflammation leads to irreversible quenching of Treg function. It has also been proposed that the destabilizing of Tregs under intense activation or inflammation can actually select more stable subpopulations of Tregs to enhance the long-term suppressive ability of an embattled Treg pool [86]. In this model it is also conceivable that the outcome may differ should the degree and duration of the selective, destabilizing pressure prove extreme.

The Deubiquitinases: The other side of the story

It is clear that the action of several E3 ligases affect the generation and function of Tregs on multiple levels. The process of protein ubiquitination is, however, a highly reversible one due to the cleavage of ubiquitin from target proteins that is catalyzed by substrate-specific DUBs [135]. Given the wide involvement of E3 ligase in Treg generation and function, it should not surprise that enzymes capable of removing these tags play an equally important role in the biology of Tregs and immune control.

USP18

A DUB relevant to the T cell response is USP18, which is expressed by naïve CD4⁺ T cells as well as effector memory T cells and nTregs (although it is curiously downregulated under iTreg inducing conditions). This DUB targets TGF- β -activated kinase 1 (TAK1), a critical mediator of NF κ B activation in CD4⁺ T cells stimulated by TLR ligands, IL-1 or TNF. USP18 is therefore a potent negative regulator of signaling events downstream of the TCR.

In its absence, IL-2 production is enhanced by CD3/CD28 cross-linking on T cells [136]. Abundance of this cytokine, which is required to sustain and stabilize Tregs [92, 137], inhibits Th17 differentiation efficiency [138, 139]. Reflecting this relationship, knocking out the gene for USP18 favors the generation of iTregs *in vitro* while dampening Th17 commitment. Furthermore, these mice experience less severe symptoms in the EAE model accompanied by reduced frequencies of IL-17 producing cells in the CNS [136].

CYLD

Another DUB with noted effects on immune regulation is the cylindromatosis tumor suppressor (CYLD). This DUB with a penchant for cleaving K63-linked ubiquitin chains from its substrates regulates several signaling pathways (NF κ B, TLR, TNF) by targeting the intermediates TRAF2 and NEMO and appears to influence multiple aspects of Treg biology. CYLD negatively regulates TGF β responsiveness, adversely affecting extrathymic Treg induction and the pTreg pool. This occurs by the countermanding of Smad7 K63-polyubiquitination and the resulting downstream Foxp3 gene transcription. In the absence of CYLD, mice display enhanced peripheral Tregs and their T cells are permissive to the upregulation of Foxp3 *ex vivo* [140]. The stability of another important mediator of TGF β signaling, Smad3, is also inhibited by CYLD, albeit indirectly, through the removal of the K-63 polyubiquitin tag from AKT [141].

CYLD also limits tTreg generation due to antagonism of NF κ B signaling [142]. Mice expressing a mutant CYLD that is incapable of binding the targets TRAF2 and NEMO displayed hyperactive T cell proliferation and activation accompanying poorly restrained NF κ B activity. As with CYLD deficiency, Treg frequencies in these mice were elevated in the absence of functional CYLD [140] – an observation perhaps expected due to the importance of both TGF β and NF κ B signaling in early tTreg development [143]. However, Tregs with inactive CYLD were less effective suppressors than their wild type counterparts [142], a potential example of the distinct consequences of robust NF κ B activation for Treg development and Treg function. It was recently revealed that PKC θ , a facilitator of TCR/CD28-induced NF κ B signaling, binds to CYLD in T cells antagonizing the DUB's inhibition of NF κ B and NFAT transactivation. This may be due to sequestration of CYLD from its targets [144]. The existence of this antagonism in Tregs has yet to be established, but in light of reported inhibition of Treg function by PKC θ [72], it may hold implications for regulation of Treg function.

USP21

GATA3, a transcription factor traditionally thought of as key for commitment to the Th2 lineage, is also upregulated in Tregs upon TCR activation. Furthermore, expression of this transcription factor by Tregs appears linked to sustained Foxp3 expression, effective repression of Teffector gene expression and commitment to the Treg phenotype [145, 146]. Another DUB, USP21, which is itself activation induced, interacts with and deubiquitinates GATA3 to stabilize its expression in cell lines and primary human Tregs. Moreover, Foxp3 directly promotes USP21 expression and siRNA mediated USP21-knockdown reduces GATA3 and Foxp3 levels. This suggests USP21 upregulation by Foxp3 and TCR activation enhances GATA3 levels, which in turn stabilizes or promotes Foxp3 function [147]. While

the impact of USP21 on the broader functional stability of Tregs *in vitro* or *in vivo* remains to be tested, given the Treg stabilizing role of GATA3 [145, 146], it seems likely that the activity of USP21 similarly aids Treg phenotypic stability.

USP7

Since direct ubiquitination of Foxp3 can drive its degradation, it stands to reason that counteracting this process should preserve levels of this important regulatory hub of the Treg phenotype. Indeed, van Loosdregt and colleagues recently discovered a specific DUB capable of reversing Foxp3 polyubiquitination, preserving both its expression and Treg function [115].

Based on past observations of Foxp3 polyubiquitination and its negative association with Foxp3 stability [46, 103, 148], the authors set out to identify the DUB responsible for modulating Tregs. Confirming the importance of deubiquitination for stable Treg function, treatment of human Tregs with a pan-DUB inhibitor reduces their effectiveness in an *in vitro* suppression assay. Furthermore, mass spectroscopy analysis identified USP7, which is enriched in iTregs, as a DUB capable of both interacting with Foxp3 and mediating its deubiquitination. Linking the specific activity of this DUB to stable Treg function, the authors of this study showed that, knocking down USP7 diminishes both Foxp3 levels and Treg suppression *in vitro*, while its overexpression stabilizes and augments the Foxp3 protein pool and suppressive function [115]. Strikingly, the ability of murine Tregs to suppress colitis *in vivo* was found to be significantly reduced upon pre-treatment with either the pan-DUB inhibitor or shRNA against USP7 prior to lymphopenic recipients of colitogenic naïve CD4⁺ T cells [115].

This study demonstrates the dynamic nature of Foxp3 protein expression, reinforces the importance of protein-level Treg modulation, and reveals a potent mechanism for offsetting inflammation induced disruption of the Treg phenotype. It also suggests an exciting avenue for immunotherapy. Specifically, the targeting of Foxp3-preserving DUBs may be an effective means to break tolerance and muster an effective anti-tumor immune response in cancer patients.

Summary and concluding thoughts

From the findings summarized in this review it is clear that the generation of Foxp3⁺CD4⁺ Tregs and their functional stability is significantly influenced by the ubiquitin-mediated regulation of numerous molecules and pathways. The signaling pathways promoting or antagonizing induction of Foxp3 in the thymus or periphery are sensitive to the action of numerous E3 ligases and functionally opposite DUBs (Figure 1). These enzymes are similarly crucial in the processes determining the functional stability of established Tregs (Figure 2; also see Table 1).

The identification of Foxp3 as target of ubiquitin-dependent regulation is a particularly interesting development in the unraveling of how Tregs function and are themselves regulated. While a number of studies mentioned here call attention to the importance of ubiquitin-mediated events in determining broader Treg function and even their potential as

starting points for future therapeutics, there is, however, much still to be explored. For instance the *in vivo* conditions regulating the expression and activity of Foxp3-targeting ligases and Foxp3-preserving DUBs, as well as the potential for cross-talk between them are intriguing future directions.

Another unresolved issue concerns how ubiquitination as a process interacts with other posttranslational means of regulating Foxp3 protein stability and function. Phosphorylation of some proteins, for instance, can speed subsequent ubiquitination [149]. However, modification of Foxp3 in this manner has been reported to facilitate its activity [95]. That modification of distinct residues has different consequences is possible [149] but remains to be thoroughly explored. Meanwhile lysine residues can be substrates for modification by acetylation as well as ubiquitination. It is possible that competition between these processes affords another layer of control over Foxp3 stability and function. Acetylation of Foxp3 positively affects both the stability as well as the activity of the transcription factor and has been observed to be inversely associated with polyubiquitination [103, 104, 148]. Indeed a recent study supports the notion that Foxp3-stabilizing/enhancing acetylation can compete with the process of ubiquitination for lysine targets and supporting a Treg phenotype by preventing the latter modification [150].

An important aspect of ubiquitin-mediated mechanisms of control over the Foxp3 protein pool is their potential for speed and reversibility. By inducing the loss of functionally active transcription factor while minimally affecting the production of new message and protein, this manner of regulation can weaken the effectiveness of Tregs to suppress the immune response while likely maintaining the transcriptional output preventing the complete or prolonged loss of Tregs. This rapidly deployed yet transient Treg modulation is like to muster considerable enthusiasm among those pursuing the development of novel immunotherapies designed to break tolerance during cancer, for instance. While strategies involving the depletion of Tregs can successfully unleash the immune response for improved anti-tumor responses, the specter of autoimmune side effects is a concern. Strategies taking advantage of existing, temporary pathways for Treg pausing, in contrast, should be less prone to this drawback.

Targeting the machinery responsible for the ubiquitin-mediated depletion of the Foxp3 protein pool should, on the other hand, allow for effective stabilization of this transcription factor and reinforcement of Treg identity and function. Certainly, a number of autoimmune diseases marked by Treg ineffectiveness may be ameliorated by adapting this strategy into therapies. In this way, exploiting the dependence of Tregs on ubiquitin-mediated events may permit unprecedented therapeutic control of immune regulation that is at once rapid, temporary and precise.

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Tolerance

Inflammation

Figure 1. Ubiquitin regulated players in T cell differentiation decisions

Induction of Foxp3 and commitment to the iTreg lineage (left side) is antagonized by strong TCR and costimulatory activation relying on the PI3K/AKT/mTOR signaling axis, which is sensitive to ubiquitin, mediated regulation. On the other hand, distinct ubiquitin modifications that enhance the activity of TGFβ signaling can drive Treg induction. Foxp3 protein is also directly regulated through a process linked to HIF-1α degradation during Th17 differentiation.

Figure 2. Modulation of Treg functional stability by ubiquitin ligases and deubiquitinases
 Direct polyubiquitination leads to degradation of Foxp3 and disruption of Treg function. Conversely, deubiquitination of Foxp3 by the DUB known as USP7 stabilizes the Foxp3 protein pool and preserves Treg function. Enzymatic addition or removal of the ubiquitin modification from other targets indirectly influences Treg stability.

Table 1

Summary of Ubiquitin System Machinery Influencing Treg Biology

Ubiquitination					
Factor	Abbreviation	Description	Relevant Target(s)	Impact on Tregs	Reference(s)
Casitas-B-lineage lymphoma protein-b	Cbl-b	RING Finger domain	PL3K (p85)	↑Development	23–32
			Smad7	↑Development	33–35
ITCH	ITCH	HECT domain	TIEG1	↑Development, ↑Function	38
			JunB	↑Function	37
S-Phase Kinase associated protein 2	Skp2	SCF ligase/multisubunit	p21, p27	↑Development, ↑Function	40
Von Hippel-Lindau/Elongin-C-Elongin-B-cullin-2-Rxb1	VHL-E3 complex	multi-subunit	HIF-1alpha	↓ Development, ? Function	44,45
Pellino 1	Pell 1	Pellino family	c-Rel	? Function	73,74
TNF Receptor associated factor 6	Traf6	RING Finger domain	Foxp3 ?	↑Development, ↑Function	113, unpublished results
Ube2n ubiquitin-conjugating enzyme E2N	Ubc 13	E2 enzyme	IKK2	↑Function	107
gene related to anergy in lymphocytes	GRAIL	Ring Finger domain	TCR	↑Function	110
STIP1 Homology and U-Box containing Protein 1	Stub1 (Chip)	chaperone dependent	Foxp3	↑Function	116
Membrane-associated RING-CH 1	MARCH 1	MARCH Family	MHC II, CD86	↑Development, ↑Function	76,112

Deubiquitination				
Factor	Abbreviation	Relevant Target(s)	Impact on Tregs	Reference(s)
Ubiquitin-specific-processing protease 18	USP18	TAK1	↑Development, ? Function	136
cylindromatosis tumor suppressor protein	CYLD	Nemo/Traf2	↑Development, ? Function	140–142
Ubiquitin-specific-processing protease 21	USP21	GATA3	↑Function	147
Ubiquitin-specific-processing protease 7	USP7	Foxp3	↑Function	115