

Ubiquitin-Dependent Regulation of Treg Function and Plasticity

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Abstract

Immunosuppressive regulatory T cells (Tregs) are critical in maintaining immune tolerance and homeostasis of the immune system. Treg dysfunction leads to autoimmunity, while active Tregs in the tumor microenvironment hinders anti-tumor immune responses. As a master transcription factor of Tregs, forkhead box P3 (Foxp3) coordinates with different proteins in regulating Treg development and function. Such events are controlled by a series of post-translational modifications (PTMs). Among them, ubiquitin-dependent modifications of Foxp3 regulate degradation, nuclear localization, signal transduction, and suppressive function of the protein. Research has discovered different partners involved in the ubiquitination and deubiquitination of Foxp3. Understanding the mechanisms of these key PTMs of Foxp3 shall expand our knowledge of Tregs and pave the way for targeting Tregs in treating autoimmune diseases and cancer.

Keywords: Autoimmunity, Clinical immunology, Immune cells and organs, Immunotherapy, Tumor immunology

Introduction

Delicately, our immune system eliminates exogenous and endogenous threats and prevents harmful immune responses against the host. Regulatory T cells (Tregs) are indispensable in controlling immune responses and inducing immune tolerance; thus, immune homeostasis is maintained [1]. As a subset of CD4⁺ T cells, Tregs have been extensively studied for decades. They are best known for their ability to suppress immune responses, induce self-tolerance and help tumor cells escape immune surveillance [2-5]. Tregs mediate immune suppression via several mechanisms: They constitutively express cytotoxic T lymphocyte antigen-4 (CTLA-4), which competes with the costimulatory molecule CD28 for binding CD80/86 to downregulate T cell activation [6]. Tregs also highly express CD25 (IL-2 receptor α chain), depriving effector

T cells of IL-2 [7]. Moreover, Tregs secrete immunosuppressive cytokines (e.g., IL-10 and IL-35) and molecules (e.g., adenosine) to inhibit effector T cell activation and function [8].

Transcription factor forkhead box protein 3 (Foxp3) is specifically expressed in Tregs and defines the Treg population [4,9]. Mutations in the *Foxp3* gene cause immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX) in humans and lead to fatal lymphoproliferative disorders in mice (Scurfy phenotypes) [10-12]. As a key regulator of Treg function, Foxp3 inhibits IL-2 production and upregulates CD25 and CTLA-4 expression [9,13]. Moreover, Foxp3 cooperates with a series of partners including NFAT [13], RUNX1 [14], ROR α [15], Rel A, c-Rel [16], IRF4 [17], Eos [18] and HIF-1 [19] in manipulating Treg cell function. These Foxp3-mediated regulatory events are carefully controlled by

different mechanisms to maintain immune homeostasis and post-translational modifications (PTMs) are one of them. Many studies have investigated the PTMs of Foxp3 protein, including phosphorylation, acetylation, methylation, glycosylation, and ubiquitination [20]. Ubiquitination is an ATP-dependent PTM that attaches ubiquitin to target proteins [21]. Ubiquitin-dependent regulation of Tregs in their function and plasticity was comprehensively reviewed in 2021 [22]; here, we shall revisit this topic and share some recent knowledge.

Ubiquitination and Deubiquitination

Ubiquitin is a 76-amino acid protein and can be initially activated by ubiquitin-activating enzymes (E1s) on the C terminus, then transiently carried by ubiquitin-conjugating enzymes (E2s) and eventually transferred to lysine residues of the target proteins by ubiquitin ligases (E3s) [23]. Monoubiquitination regulates protein-protein, protein-DNA, and protein-lipid interactions in different cellular processes [24] and polyubiquitination is often associated with protein degradation and cell signaling events [23]. Polyubiquitination

chains can be linked to seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) and methionine at position 1 (M1) of ubiquitin [25]. K48 and K63 polyubiquitination are best studied. K48 polyubiquitination induces degradation of target proteins by the 26S proteasome, while K63 polyubiquitination can regulate cell signaling and protein trafficking events [26]. Deubiquitination is catalyzed by deubiquitinating enzymes (DUBs), which cleave ubiquitin from ubiquitin-conjugated protein substrates; thus, DUBs regulate various critical cellular processes including TGF- β and NF- κ B signaling, proteasome function, chromatin structure and endocytosis [27]. Ubiquitination and deubiquitination regulate Foxp3 stability, turnover, protein-protein interactions, localization, Treg development and function [22]. Given the roles Tregs play in autoimmunity and tumors, understanding the mechanisms of these PTMs will provide better ideas for targeting Foxp3 in treating autoimmune diseases and cancer. Here we shall discuss some widely studied ubiquitin-associated enzymes and proteins, and a summary diagram is shown below (Figure 1).

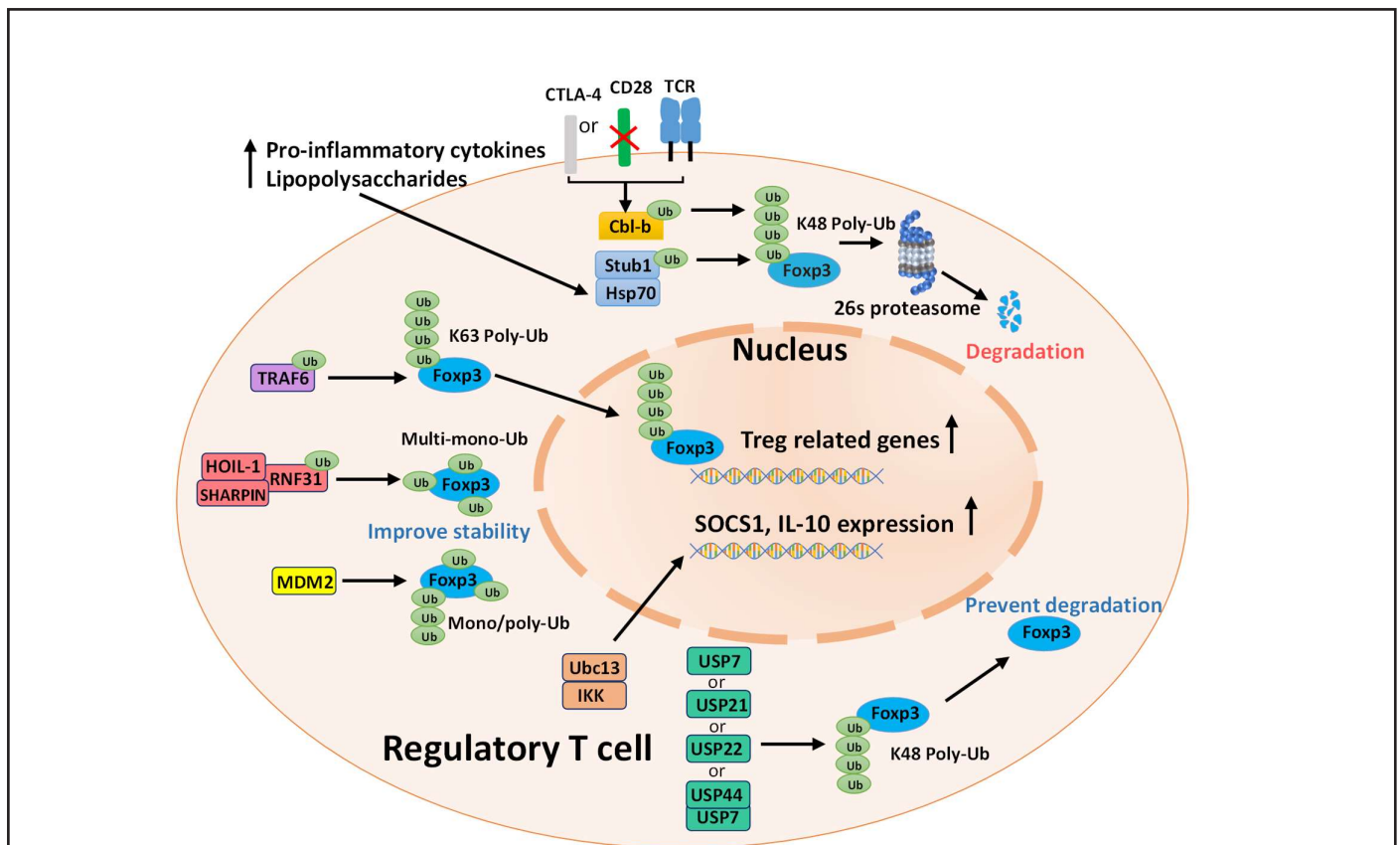


Figure 1. Ubiquitin-dependent regulation of Foxp3 in Tregs. Foxp3 is modified by E3 ligases and DUBs that regulate its stability, degradation, and function. In Tregs, inflammatory stress or T cell activation under attenuated co-stimulation favors K48 polyubiquitination of Foxp3, resulting in its degradation by the 26S proteasome. TRAF6 mediates Foxp3 K63 polyubiquitination that guides its nuclear transport. RNF31 and MDM2 induce Foxp3 monoubiquitination or polyubiquitination, improving its stability. Ubc13- IKK signaling axis promotes SOCS1 and IL-10 gene expression to prevent the conversion of Tregs into Th1 or Th17-like effector T cells. DUBs prevent Foxp3 degradation by reversing its K48 polyubiquitination.

Ubiquitin Ligase-dependent Regulation of Treg Stability and Function

Stub1

STIP1 homology and U-box containing protein1 (Stub1) is a U-box-type E3 ligase required for T cell receptor (TCR)-induced NF- κ B activation and IL-2 secretion [28]. Recruited by the Hsp-70 protein, Stub1 promotes Foxp3 degradation in Tregs by inducing its K48-type polyubiquitination under inflammatory stress [29]. However, such regulation can be held back. During Treg activation, Foxp3 phosphorylation induced by Nemo-like kinase (NLK) prevents the association of Stub1 and Foxp3, which subsequently stabilizes Foxp3 expression [30]. Moreover, a recent study reported that in patients with non-small-cell lung carcinoma (NSCLC), metastasis-associated lung adenocarcinoma transcript1 (MALAT1) binds the zinc finger (ZF) domain and leucine zipper (LZ) domain of Foxp3, thus masking these binding sites for Stub1 and inhibits Stub1-induced Foxp3 degradation [31]. Overall, Stub1 is a key regulator in Foxp3 stability.

TRAF6

TNF receptor-associated factor 6 (TRAF6) also directly interacts with and modifies Foxp3. This broadly expressed RING-type E3 ligase acts as an adapter molecule downstream of the IL-1 receptor, Toll-like receptor (TLR) family, and TNF receptor superfamily signaling pathways [32]. It was found that TRAF6 interacts with Foxp3 and mediates K63-type polyubiquitination at lysine 262, which is indispensable for nuclear Foxp3 localization and regulatory function [33]. Treg-specific deletion of TRAF6 abrogates Treg-mediated *in vivo* suppression and enhances anti-tumor responses [33]. Consistently, another group reported that when the E3 ligase of TRAF6 is inactivated, mice develop autoimmunity and inflammations in the skin, liver, and lung [34]. Treg cells expressing this E3 ligase inactive TRAF6 mutant are likely non-functional and incapable of restraining autoimmune responses in mice [34].

RNF31

The linear ubiquitin chain assembly complex (LUBAC) has three subunits, including ring finger protein 31 (RNF31/HOIP), RanBP-type and C3HC4-type zinc finger containing 1 (RBCK1/HOIL-1), and SHANK-associated RH domain interacting protein (SHARPIN/SIPL1) [35,36]. LUBAC regulates downstream signaling pathways of TCR, BCR, TNFR1, TLR, and NLRP3 [37-41]. All three LUBAC components play roles in thymic Treg development, and deletion of RNF31 results in massive loss of peripheral Tregs [42]. RNF31 is a RING-type E3 ligase, and a recent study indicates that RNF31 directly interacts with Foxp3 and stabilizes the protein via multi-monoubiquitination [43]. Moreover, upregulated RNF31 expression was observed in

human gastric tumor-infiltrated Tregs, suggesting RNF31 may be a potential target for treating gastric cancer [43].

MDM2

Mouse Double Minute 2 Homolog (MDM2) is an oncogenic RING-type E3 ligase that ubiquitinates and degrades p53, a tumor suppressor [44]. Numerous studies have been focused on developing inhibitors to block MDM2-p53 interactions for cancer treatment [45]. In T cells, USP15 stabilizes MDM2, which promotes the degradation of transcription factor NFATc2 and downregulates T cell activation [46]. A later study showed that MDM2 interacts with Foxp3 and catalyzes various types of ubiquitination of Foxp3 at ten different lysine residues, stabilizing the protein [47]. These data reveal a new role of MDM2 in maintaining Foxp3 stability.

ITCH

Some other ubiquitin ligases play roles in regulating signaling pathways that indirectly mediate Treg stability and function. The Itchy E3 ubiquitin ligase (ITCH) is a HECT-type E3 ligase that plays a role in mediating allergic responses, as Th2 cells from ITCH^{-/-} mice are resistant to anergy induction [48]. A subsequent study reported that TGF- β -mediated conversion of naïve T cells into induced Tregs (iTregs) is largely impaired in ITCH^{-/-} mice. Further investigations confirmed that ITCH regulates Foxp3 expression by promoting ubiquitination of a transcription factor TIEG1 (TGF- β -inducible early gene 1), resulting in its nuclear translocation and transactivation of the Foxp3 promoter [49].

Cbl-b

Casitas-B-lineage lymphoma protein-b (Cbl-b) is a RING-type E3 ligase involved in regulating T cell activation thresholds and inducing immune tolerance [50]. Cbl-b deficient mice are prone to develop autoimmune diseases, and their T cells can produce IL-2 without CD28 co-stimulation [50,51]. It was discovered that in normal T cell activation, CD28 co-stimulation selectively induces Cbl-b K48-type ubiquitination and degradation by the 26S proteasome [52]. On the contrary, when T cells are activated in the absence of CD28 co-stimulation or with CTLA-4-B7 co-inhibition, Cbl-b expression is stabilized [51,53]. In terms of regulating Treg stability, Cbl-b promotes the conversion of CD4⁺ naïve T cells into iTregs by suppressing Akt2 of the PI3K/Akt/mTOR pathway [54]. In tTreg development, Cbl-b can be recruited by Stub1-induced Foxp3 ubiquitination. Cbl-b binds Foxp3 via its UBA domain and together with Stub1, promotes Foxp3 ubiquitination and degradation, which maintains Foxp3 at a steady state [55].

Ubc13

Ubc13 is a K63-type E2 ubiquitin-conjugating enzyme that plays a role in TNF receptor and TLR signaling pathways

[56]. Treg-specific deletion of Ubc13 in mice impairs *in vivo* immunosuppressive function of Tregs, and Ubc13-deficient Tregs acquire effector function to produce interferon- γ (IFN- γ) and IL-17 [57]. The I κ B (inhibitor of NF- κ B) kinase IKK is a major downstream target of Ubc13. The Ubc-IKK signaling axis maintains Treg stability and immunosuppressive function by regulating the expression of suppressor of cytokine signaling 1 (SOCS1), a key regulator known to prevent the conversion of Tregs into Th1 or Th17-like effector T cells [58], and IL-10 [57].

A20

A20 or TNF- α induced protein 3 (TNFIP3) is a widely expressed anti-inflammatory protein with both E3 ligase and DUB function, which regulates NF- κ B signaling and other ubiquitin-dependent activities [59]. TRAF6 and receptor-interacting protein 1 (RIP1) are two known targets of A20 to restrict downstream NF- κ B signaling [60,61]. A20 inhibits tTreg development by limiting the generation of CD45⁺ CD25⁺ GITR⁺ Foxp3⁺ tTreg progenitors [62]. Moreover, A20 regulates Th17/Treg balance in rheumatoid arthritis (RA). A20 expression was found to be downregulated in the bone marrow mesenchymal stem cells (BM-MSCs) of RA patients. Overexpression of A20 in human umbilical cord MSCs (UC-MSCs), a similar cell type of BM-MSCs, is able to inhibit IL-6 expression and reverse Th17 up-regulation [63].

HIF-1

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor formed from an O₂-regulated HIF1- α and a HIF-1 β subunit found in mammalian cells under hypoxic conditions [64]. In the hypoxic tumor microenvironment, HIF1 contributes to tumor evasion by inhibiting differentiation and function of NK and dendritic cells, mediating T cell exhaustion, and upregulating the expression of immune checkpoint molecules (e.g., PD-L1) [65]. Under normoxia, HIF-1 α is both synthesized and degraded, while under hypoxia HIF-1 α degradation is inhibited, resulting in its accumulation and dimerization with HIF-1 β [66]. When HIF-1 is hydroxylated at prolines 402 and 562 by the prolyl hydroxylase domain protein (PHD), it binds the von Hippel-Lindau protein (VHL), which subsequently recruits the Elongin-C-Elongin-B-Cullin-2-E3-ubiquitin ligase complex and leads to HIF1 degradation [66]. Notably, HIF-1 regulates Th17/Treg balance. Under Th17-skewing conditions, HIF-1 interacts with Foxp3 and mediates their co-degradation. Meanwhile, in a STAT3-dependant manner, HIF-1 enhances Th17 differentiation by directly inducing ROR γ t transcription and cooperating with ROR γ t and p300 [19]. Consistently, it was reported that a proinflammatory cytokine IL-1 β downregulates iTreg differentiation by increasing HIF-1 α expression via the mTORC1 pathway [67]. Chemically stabilizing HIF-1 α by dimethyloxalylglycine (DMOG) abrogates iTreg differentiation [67], and promoting HIF-1 α degradation by Deltex1 (DTX1) increases Foxp3 stability [68]. Together, these data suggest HIF-1 is a potential target to regulate Treg stability.

Deubiquitination-mediated Regulation of Treg Stability and Function

USP7

Ubiquitin-specific-processing protease 7 (USP7) is a DUB found to stabilize Foxp3 expression.

van Loosdregt *et al.* [69] initially reported that USP7 expression is upregulated during iTreg differentiation, and USP7 interacts with and deubiquitinates Foxp3. They also showed that USP7-mediated deubiquitination prevents polyubiquitination-induced degradation of Foxp3 and improves Treg-mediated suppression *in vitro* and *in vivo* [69]. A later study showed that specific deletion of USP7 in murine Tregs triggers lethal systemic autoimmunity, and USP7 also regulates Treg function by promoting the multimerization of Tip60 and Foxp3 [70]. These data indicate that USP7 is a critical regulator of Treg function.

USP18

USP18 may also be involved in regulating Treg differentiation. USP18 is a multifunctional enzyme that negatively regulates type I, type III interferon, and NF- κ B signaling [68] and DC and Th17 cell development [71,72]. A recent study revealed that USP18 deficiency enhances Treg differentiation by upregulating CD25 expression but downregulates Treg-mediated suppression [73]. Since a USP18 germline-knockout mouse model was used in this study, impacts of other USP18 deficient immune cells were not excluded. Further studies using Treg-specific USP18 knockout mice were required to further confirm the roles of USP18 in controlling Treg stability and function.

USP21

USP21 is a multifunctional DUB that regulates NF- κ B, Hippo, MAPK/ERK, GATA3, Wnt, and Hedgehog signaling pathways [74]. GATA3 is a transcription factor required for Treg function [75]. An early study showed that USP21 interacts with and stabilizes GATA3, which is pivotal for Treg suppressive function, through deubiquitination [76]. Later the same group discovered that USP21 prevents Foxp3 degradation *via* direct deubiquitination and the generation of Th1-cell-like Tregs [77]. Thus, upon TCR activation, Foxp3 binds the USP21 gene promoter and induces its expression; in return, USP21 prevents Foxp3 degradation thus maintaining Treg stability, and forming a positive feedback loop [76, 77].

USP22

USP22 is a cancer stem cell marker and enzymatic subunit of the human SAGA transcriptional cofactor complex required for cell-cycle progression [78]. Using a CRISPR-based pooled screening method, USP22 was found to be a positive regulator

of Foxp3 expression [79]. USP22 deficiency in Tregs dampens Foxp3 expression and Treg-mediated suppressive function, resulting in spontaneous autoimmunity and enhanced anti-tumor responses [79]. Moreover, E3 ubiquitin ligase RNF20 was identified as a negative regulator of Foxp3, and ablation of RNF20 was able to rescue impaired Foxp3 transcription in USP22 deficient Tregs, suggesting reciprocal regulation of Foxp3 by USP22 and RNF20 [79].

USP44

USP44 is involved in regulating cell cycle [80], stem cell differentiation [81], and tumorigenesis [82]. Recently, one study identified another role of USP44 in modulating Foxp3 expression and Treg function [83]. Initially, USP44 expression was found to be upregulated in both natural Tregs (nTregs) and iTregs. Further investigations confirmed that USP44 interacts with, deubiquitinates, and stabilizes Foxp3. Moreover, USP44 cooperates with USP7 in deubiquitinating Foxp3, while USP44 mainly targets K48-linked polyubiquitinated Foxp3. In mice, Treg-specific deletion of USP44 largely impairs their immunosuppressive function in vitro and in vivo. Notably, in three types of tumor models (MC38 colon carcinoma, B16F10 melanoma, and EL4 thymoma), Treg-specific USP44 deficiency inhibits tumor growth and enhances anti-tumor immune responses [83]. These data reveal a new USP44-dependent pathway in regulating Treg development and function and suggest USP44 be a drug target in treating autoimmune diseases and cancer.

Clinical Implication and Future Perspectives

Since the discovery of Treg cells, much attention has been drawn to elucidating the mechanisms of Treg development and Treg-mediated immune suppression. Recent research has shown the therapeutic potential of targeting Tregs to treat cancer and autoimmune diseases and prevent graft vs host disease (GvHD) in transplantation [20]. Depending on the disease settings, different Treg-target strategies are needed for better outcomes. In cancer immunotherapy, antibodies against surface molecules expressed by tumor Tregs such as CD25, CTLA-4, PD-1, GITR, 4-1BB, OX-40, LAG3, TIGIT, CCR4, and CCR8 can induce Treg-depletion in the tumor microenvironment, which enhances anti-tumor responses [5]. Adoptive transfer therapy (ACT) of Tregs has been widely used in clinical trials to prevent GvHD and treat autoinflammatory and autoimmune diseases [84]. Engineered Tregs are one of the main Treg products developed for the ACT. They express a chimeric antigen receptor (CAR) or specific TCR, which recognizes an antigen of interest. Recombinant CARs are MHC-independent with high and adjustable affinity; thus, CAR-Tregs have broader applicability than TCR-Tregs [85]. CAR-Tregs have shown their therapeutic potency in preclinical models of GvHD and autoimmune diseases, including type 1 diabetes, RA, and multiple sclerosis [86]. Overall, targeting Tregs in a

wide range of diseases is promising, and yet mechanisms of their suppressive function still require further investigation for better clinical applications.

As a key regulator of Treg differentiation and function, Foxp3 forms different large protein complexes which are controlled by PTMs. The idea of targeting Foxp3 is tempting but also challenging. Transcription factors are often regulated by interactions with DNA or multiple proteins, and they lack enzymatic activity and binding pockets for small molecules [87]. Therefore, interfering PTMs of Foxp3 may be more feasible in controlling Foxp3 activity. Overactive E3 ligases induce K48-linked polyubiquitination of Foxp3 that results in abnormal degradation of Foxp3 and impaired Treg function, sequentially leading to autoimmunity. Elevated expression of Stub1 in RA patients induces the imbalance of Th17/Tregs, via non-degradative ubiquitination of aryl hydrocarbon receptor (AHR) [88]. However, in the tumor microenvironment, E3 ligases stabilizing Foxp3 or DUBs reversing its K48-linked polyubiquitination amplifies Treg-mediated immunosuppression, compromising anti-tumor immunity. Upregulated RNF31 in Tregs of gastric cancer patient is associated with decreased survival of the patients [43]. Depleting USP22 or USP44 in Tregs impairs their suppressive function and impedes tumor growth [79,83]. These findings confirm that dysregulation of Treg stability and function mediated by E3 ligases and DUBs contributes to the development of autoimmune disorders and cancer.

Research targeting E3 ligases or DUBs in murine tumor models has shed light on therapeutic applications of ubiquitin-dependent regulation of Foxp3: A small molecule TRAF6 inhibitor 6877002 can reduce the number of intratumor Tregs by limiting their migration into the tumor. P5091, a USP7 inhibitor, impairs Treg suppressive function without dampening conventional T cell responses, which improves the efficacy of anti-tumor vaccines and anti-PD1 immune checkpoint inhibitors, and anti-tumor immunity [70]. Hence, small molecule drugs which only interfere with ubiquitin-dependent PTMs of Tregs could be a good approach to control Treg function.

Conclusion

In this article, we have reviewed some key ubiquitin-associated enzymes and proteins that regulate Treg function and plasticity in T cell development, autoimmune diseases, and cancer. Ubiquitin-mediated PTMs of Foxp3 regulate the transcription factor's expression, localization, and function and subsequently influence Treg-mediated immune suppression. Recent studies have largely broadened our knowledge of these PTMs, and some promising Foxp3-associated targets have come to light. In the future, modulating ubiquitination and deubiquitination of Foxp3 are likely to be efficient and specific means to tune Treg function when in need.

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Conflicts of Interest

The authors declare no conflicts of interest.

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