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Review Article

Regulation of T cell receptor complex-mediated signaling by ubiquitin and ubiquitin-like modifications

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Abstract: Post-translational protein modifications are a dynamic method of regulating protein function in response to environmental signals. As with any cellular process, T cell receptor (TCR) complex-mediated signaling is highly regulated, since the strength and duration of TCR-generated signals governs T cell development and activation. While regulation of TCR complex-mediated signaling by phosphorylation has been well studied, regulation by ubiquitin and ubiquitin-like modifiers is still an emerging area of investigation. This review will examine how ubiquitin, E3 ubiquitin ligases, and other ubiquitin-like modifications such as SUMO and NEDD8 regulate TCR complex-mediated signaling.

Keywords: c-Cbl, NEDD8, MLN4924, SUMO, SLAP, T cell, ubiquitin

Ubiquitin as a post-translational modification

Post-translational protein modifications, or the covalent addition of a chemical moiety to a protein after its translation, permit seemingly endless possibilities for regulatory control of a protein. These reversible changes in protein structure provide a quick and dynamic means of responding to physiologic changes or environmental signals. By adding or removing chemical groups to one or more amino acid residues of a protein, a cell can regulate protein structure, activation, complex assembly, location, and half-life. To date, it is estimated that within the human body, 50-90% of proteins are post-translationally modified [1], and over 200 forms of post-translational protein modifications have been identified [2].

Ubiquitination is an enzymatically-catalyzed reversible post-translational modification that can be conjugated to multiple lysine residues of a protein. In contrast to phosphorylation, ubiquitination involves the addition of a 76 amino acid (8.5 kD) polypeptide, rather than an ion or other small molecule. Ubiquitin moieties are attached to lysines of proteins through a peptide bond to a C-terminal glycine and are often repeatedly conjugated to lysines of the previous ubiquitin to form a polyubiquitin chain on target proteins [3]. Since it's discovery in the late 1970's, ubiquitination has been found to play a critical role in most aspects of biology, both through its effects in regulating protein proteasomal and lysosomal degradation, as well as its involvement in protein trafficking, complex assembly, and downstream signaling [3].

Protein ubiquitination involves a complex enzymatic cascade, providing the cell multiple layers for controlling how a specific protein is ubiquitinated (**Figure 1**). Initiation of protein ubiquitination begins with an E1, or the ubiquitin-activating enzyme (UAE). The E1 first utilizes ATP to create an adenylate-ubiquitin intermediate, before it can form a high-energy thioester intermediate between its own cysteine residue and the C-terminal glycine of ubiquitin. After this ubiquitin activation step, the E1 enzyme

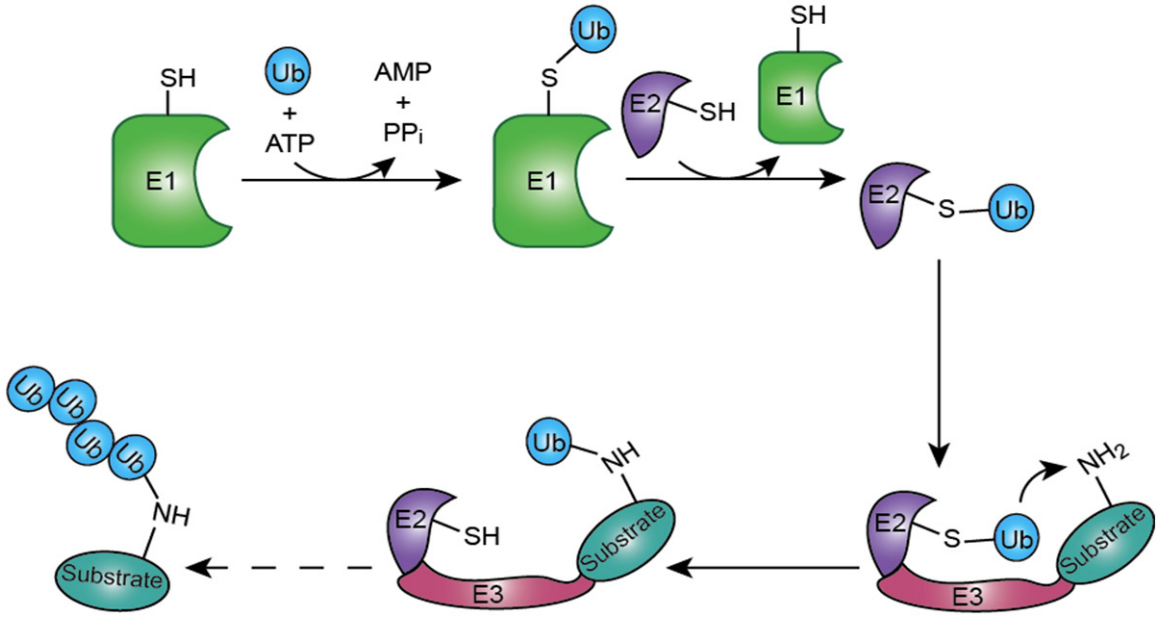

Figure 1. Ubiquitination enzymatic cascade. An E1, or a ubiquitin-activating enzyme (UAE), activates ubiquitin by first converting it to a high-energy thioester in an ATP-dependent manner. The activated ubiquitin can subsequently be transferred to an E2, or a ubiquitin-conjugating enzyme (UBC). The E2 enzyme then binds a recruitment domain of an E3 ubiquitin ligase that is already bound to a specific substrate. The E3 then facilitates transfer from the E2 to a lysine of the target substrate. The process can then be repeated to polyubiquitinate a protein, as new ubiquitin moieties can be covalently attached to lysines on the previous ubiquitin.

transfers ubiquitin to an active site cysteine of an E2 enzyme, also known as a ubiquitin-conjugating enzyme (UBC). A specific E3 ubiquitin ligase, which is bound to a specific target substrate protein, will then recognize and interact with the E2 enzyme to facilitate the formation of an isopeptide bond between ubiquitin and a lysine of the target substrate [4, 5]. This three-step pathway allows either one or a few enzymes (E1s) to have the non-specific task of activating ubiquitin, several enzymes (E2s) to have the more specific task of creating a specific linkage for a polyubiquitinated chain, and then many enzymes (E3s) to target specific substrates.

There are two known E1 enzymes encoded in the human genome for ubiquitin, while there are over 30 genes for E2 enzymes and over 600 genes for E3 enzymes [6]. The two most studied families of E3 ubiquitin ligases are the homologous to E6-associated protein carboxyl terminus (HECT) domain family and really interesting new gene (RING) finger family. HECT-type ligases receive activated ubiquitin from an E2 enzyme and bind it covalently to a conserved cysteine residue in the HECT domain before transferring it to the substrate protein [7]. In contrast, the RING finger-type lacks an intrinsic catalytic domain but contains a RING finger domain featuring cysteine and histidine residues coordinating two zinc ions. These are required for associating with E2 enzymes and facilitating the transfer of ubiquitin to protein substrates [7].

Ubiquitination can also be reversed by deubiquitinases (DUBs) that can remove ubiquitin either from a monoubiquitinated substrate or from a polyubiquitin chain. DUBs remove ubiquitin from protein targets for the purpose of recycling ubiquitin from proteins undergoing proteolysis in the 26S proteasome, to rescue proteins from proteolytic destruction, or to regulate either the cellular location or enzymatic activity of the target protein [8]. At least 98 putative DUBs have been identified in the human genome to remove ubiquitin linked to specific targets to add to the complexity for regulating protein degradation and signaling by ubiquitination [8, 9].

Ubiquitin in TCR-mediated signaling

E3 ubiquitin ligases are involved in the elaborate regulation of TCR complex signaling, serving as important controls to attenuate or enhance responses. We have outlined a few

Table 1. E3 Ubiquitin ligases that play an important role in TCR signaling

E3 ubiquitin ligase	Domain organization	Putative substrates in T cells	Role in TCR signaling	References
c-Cbl	RING	TCRζ	Negative regulator of T cell activation.	[9-16]
Cbl-b	RING	PI3K (p85), Vav1, PLCγ1, PKCθ	Negative regulator of T cell activation. Prevents autoimmunity.	[9, 10, 28-31]
CRLs (CUL1, CUL2, CUL3)	RING	IκBα, JAK2, RelA, PLZF	Negative regulator T cell activation, proliferation, and cytokine production.	[32-49]
GRAIL	RING	USP8, CD40L, CD83	Negative regulator of T cell activation, CD4 proliferation, and tolerance/anergy.	[50-54]
c-IAP1 and c-IAP2 (BIRC)	RING	RIP1, NIK, TRAF2 and TRAF3	Suppress noncanonical NFκB activation.	[55-57]
ITCH	HECT	JunB, RIP1, TIEG1	Negative regulator of NFκB and Th2 cell activation. Role in the induction of Tregs.	[58-61]
NEDD4	HECT	Cbl-b	Negative regulator of T cell effector proliferation and IL-2 production.	[62]
Peli1	RING	c-Rel	Negative regulator of T cell activation. Prevents autoimmunity.	[63, 64]
STUB1/CHIP	U-box	FoxP3, Carma-1	Positive regulator of T cell activation promoting NFκB signaling and IL-2 production. Negatively regulates FoxP3.	[65, 66]
TRAF6	RING	LAT, TAK1/TAB complex, MALT1, NEMO	Enhance TCR enduced NFAT activation.	[67-71]
TRIM21/Ro52	RING	IRF3, IRF5, IRF7, IRF8	Negative regulator of proinflammatory cytokine production (IL-23 Th17 pathway).	[72]
TRIM27	RING	IRF3, IRF5, IRF7, IRF8	Negative regulator of CD4 T cells.	[73]

select molecules that have been implicated, both *in vitro* and/or *in vivo*, in T cell development, differentiation, activation, inflammation, anergy, and autoimmunity (**Table 1**).

The role of c-Cbl and SLAP

Currently, the most well studied E3 ubiquitin ligases linked to the ubiquitination of TCR proximal signaling components are the members of the Casitas B-lineage lymphoma protein (Cbl): c-Cbl and Cbl-b [10, 11]. Cbl family E3 ubiquitin ligases were initially shown to be integral in ubiquitinating and downregulating receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) [12, 13].

With respect to c-Cbl, thymocytes from c-Cbl-deficient mice have enhanced zeta-chain-associated protein kinase-70 (ZAP-70) activation, elevated levels of surface TCR, and enhanced thymic positive selection in the 5C.C7 pigeon cytochrome *c* transgenic model [14, 15]. An overexpression system demonstrated that the tyrosine kinase ZAP-70 could potentially recruit c-Cbl to the TCRζ chain, and that c-Cbl was necessary to target TCRζ for polyubiquitination [16]. Subsequent studies have shown that thymocytes with a tyrosine 292 to phenylalanine point mutation (Y292F) in ZAP-70, which prevents ZAP-70 assembly with the tyrosine kinase-binding domain of c-Cbl, have increased proximal TCR complex signaling and slower rates of antigen-induced TCR down-modulation [17]. However, thymocytes with this point mutation still demonstrated TCR stimulation-induced c-Cbl phosphorylation, indicating that another adaptor would be required for c-Cbl activation [17]. Moreover, although the signaling deficiencies found in mice completely lacking ZAP-70 prevent thymocyte maturation, the existing thymocytes do not have elevated levels of surface TCR expression [18, 19], demonstrating the necessity of an additional molecule to recruit c-Cbl in targeting TCRζ for ubiquitin-dependent degradation.

Research over the last decade has pointed to Src-like adaptor protein (SLAP) as being a crucial adaptor in bringing c-Cbl to the TCR complex and targeting the TCRζ chain for ubiquitination and subsequent degradation [19-24]. In thymocytes, SLAP protein levels are highest in thymocytes during the $CD4^+CD8^+$ double positive (DP) stage of development [22]. SLAP protein levels are lower both in thymocytes at later stages of development, as well as in peripheral T cells. However, SLAP expression in peripheral T cells can be upregulated with phorbol myristate acetate (PMA) and ionomycin stimulation and by α-CD3 or α-CD3/α-CD28 stimulation [22, 25]. Studies in cell lines demonstrated that SLAP overexpression diminished nuclear factor of activated T cells (NFAT), AP-1, and interleukin 2 (IL-2)-mediated gene transcrip-

tion. Furthermore, DP thymocytes from SLAP-deficient mice have enhanced phosphorylation of signaling proteins when stimulated through the TCR with α-CD3 [25]. These studies suggested that SLAP could serve as a negative regulator of TCR complex-mediated signaling [20, 21].

As with c-Cbl, SLAP-deficient (*SLAP*$^{-/-}$) mice have increased surface TCR expression, and increased expression of CD3, CD4, CD5, and CD69 in DP thymocytes [14, 22]. *SLAP*$^{-/-}$ DP thymocytes are also more efficiently positively selected in mice expressing the DO11.10 transgenic TCR specific for ovalbumin (OVA) [22]. *SLAP*$^{-/-}$ DP thymocytes also have diminished TCRζ degradation and enhanced TCR recycling, as TCRζ degradation would otherwise prevent fully assembled TCR-CD3 complexes recycling back to the plasma membrane [23]. Lastly, mice deficient in both SLAP and c-Cbl have similar defects in TCRζ degradation as mice deficient in only one molecule [19, 22]. Thus, the lack of additive or synergistic effects suggests that SLAP and c-Cbl function in the same biochemical pathway in targeting TCRζ for degradation.

Once TCRζ is phosphorylated, it becomes a target for ubiquitination. It is likely that SLAP and ZAP-70 can compete for the phosphorylated ITAMs of TCRζ at the cell surface, with SLAP engagement leading to the intracellular retention and ultimate degradation of TCRζ through a lysosomal dependent pathway [23]. Evidence supporting TCRζ degradation within lysosomes has come from experiments using lysosomal inhibitors [23], as well as a study demonstrating that deficiency of lysosomal-associated protein transmembrane 5 (Laptm5) increases TCRζ expression in DP thymocytes [26, 27].

During thymocyte development, SLAP deficiency enhances the positive selection especially of CD4^{+} cells that would otherwise die by neglect as a result of weak TCR complex-mediated signaling. Additionally, studies using the SKG mouse model of inflammatory arthritis revealed that SLAP deficiency increases development of regulatory CD4^{+} T cells (Tregs). These Tregs have enhanced function and suppress the development of inflammatory arthritis [25]. Thus SLAP deficiency also improves agonist selection of thymocytes on the border of positive and negative selection. Recently, SLAP has also been found to play a role in both the deletion of CD8^{+} thymocytes and secondary α-chain rearrangement during thymocyte development [28]. *SLAP*$^{-/-}$ mice with the MHC class I-restricted transgenic TCR, OT-1 have fewer CD8^{+} thymocytes and more CD8^{+} splenocytes that were specific for a non-cognate antigen. Moreover, *SLAP*$^{-/-}$ mice, expressing only the OT-1 TCR-β chain paired with endogenous α-chains, have fewer cognate antigen-specific T cells and more specific α-chain usage in non-cognate antigen-specific peripheral CD8^{+} T cells [28]. Thus, impairing TCR ubiquitination, and enhancing TCR avidity, can alter thymocyte development and the peripheral CD8^{+} T cell repertoire.

Cbl-b

Both c-Cbl and Cbl-b are widely expressed, although they are highly upregulated in thymocytes and peripheral T cells, respectively [11, 29]. Genetic studies of mice deficient in Cbl-b have demonstrated T cells that are resistant to anergy with hyperactive Vav1 signaling in peripheral T cells that is independent of CD28 co-stimulation. These findings have been attributed to Cbl-b ubiquitinating the p85 subunit of phosphoinositide 3-kinase (PI3K), which would otherwise phosphorylate and activate Vav1 [30, 31]. However, in overexpression systems, PI3K ubiquitination did not result in its degradation, and the mechanism of this negative regulation has yet to be defined [32]. With this hyperactive Vav1 signaling, T cells from *Cbl-b*$^{-/-}$ mice have an increased proliferative response to TCR signaling. Moreover, *Cbl-b*$^{-/-}$ mice show signs of autoimmune reactivity with T cell and B cell infiltration into vital organs, elevated autoantibody production, and increased susceptibility to experimental autoimmune encephalitis [30, 31].

CRL E3 ubiquitin ligases

Cullin (CUL)-RING E3 ubiquitin ligases (CRLs) were identified as recently as 1996 and are the largest ubiquitin E3 subclass [33, 34]. CRLs are composed of multiple, modular subunits assembled on a CUL scaffold. Humans and mice express seven CUL variants: CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, and CUL7. CULs are activated by neddylation [35, 36] and negatively regulated both by the constitutively photomorphogenic 9 (COP9) signalosome that removes neural precursor cell expressed devel-

opmentally down-regulated 8 (NEDD8) [37], as well as by cullin-associated NEDD8-dissociated protein 1 (CAND1) that binds to deneddylated CULs to prevent both CRL assembly and reactivation by neddylation [38]. In the last decade, CRLs have been implicated in a wide range of cellular processes, including cell cycle, limb patterning, and signal transduction [39-41].

All CRLs share a common molecular structure, whereby each CUL serves as a scaffold for the either RING box protein 1 (RBX1) or RING box protein 2 (RBX2), which binds the CUL C-terminal globular domain [42, 43]. Substrate recruitment to CRLs occurs through specific linker and adaptor proteins that are recruited to CULs through the N-terminal domain. Substrate specificity is determined not only by which CUL is involved in the CRL, but also by the presence and abundance of specific substrate adaptors that regulate CRL ubiquitin ligase activity [44, 45]. Over 300 substrate adaptors have been identified in human cells [46, 47]. Using 293T cells, it has been shown that each CUL binds to a distinct set of substrate adaptors, including at least 26 F-box adaptors with CUL1, 12 BC-box adaptors and 14 suppressor of cytokine signaling (SOCS)-box containing adaptors associated with either CUL2 or CUL5 respectively, 53 bric-a-brac (BTB)-containing proteins with CUL3, and 24 different damage-specific DNA binding protein-1 (DDB1) factors associated with CUL4a or CUL4b [44]. However, whether these interactions are cell type specific is not known. Therefore, a greater understanding of which specific substrate adaptors are associated with each CUL in T cells is needed to understand their role in the ubiquitin-dependent control of T cell effector fate and function.

Most investigations regarding how CRLs regulate the immune system have involved determining which individual CRLs target specific immunological substrates for ubiquitination. One of the most well known examples within the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway includes the inhibitor of NF-κB alpha (IκBα), which is known to be a target of the SKP1-CUL1-F-box (SCF) complex that contains CUL1 [39]. Other examples of proteins targeted by CRLs include Janus kinase 2 (JAK2) in the JAK/signal transducers and activators of transcription (STAT) signaling pathway, which is known to be a target of SOCS box adaptors interacting with CUL2 and CUL5 [48], and the invariant natural killer T (iNKT) cell transcription factor promyelocytic leukemia zinc finger (PLZF), which is known to be targeted by CUL3 [49]. Alterations in CRL function and expression have also been associated with T cell dysfunction, including malignant transformation, rheumatoid arthritis susceptibility, and rapid $CD4^+$ T cell death upon HIV infection [50, 51]. These studies suggest that CRLs may have a critical role in regulating T cell signaling, activation, and effector functions, which inevitably will relate to disease pathogenesis, but the mechanisms defining how CRL functions are regulated are poorly understood.

GRAIL

The gene related to anergy in lymphocytes (GRAIL), also referred to as RNF128, is expressed in naïve mouse and human $CD4^+$ T cells and was shown to be an essential signaling regulator in these cells [52]. Costimulation of $CD4^+$ naïve T cells with α-CD3/α-CD28 resulted in GRAIL degradation, T cell proliferation, and IL-2 production [53], and *GRAIL*$^{-/-}$ $CD4^+$ T cells displayed hyperproliferation and excess cytokine production compared to wild type (WT) T cells upon CD3/CD28 engagement or CD28 stimulation alone [54]. $CD4^+$ T cells had increased CD3 ubiquitination when GRAIL was present compared to *GRAIL*$^{-/-}$ $CD4^+$ cells, and upon stimulation, CD3 downregulation was reduced in *GRAIL*$^{-/-}$ naïve T cells and Tregs compared to their WT counterparts [55]. Additionally, GRAIL expression in T cells had a negative effect on actin cytoskeleton rearrangement during interactions with antigen presenting cells [56]. These studies together demonstrate that GRAIL is a negative regulator of T cell activation and proliferation and plays an important role in anergy and tolerance.

c-IAP1 and c-IAP2

The cellular inhibitors of apoptosis 1 and 2 (c-IAP1 and c-IAP2) are key E3 ubiquitin ligases for the suppression of the noncanonical NF-κB pathway. In normal cells prior to NF-κB activation, an inhibitory complex forms between NF-κB inducing kinase (NIK), tumor necrosis factor receptor associated factor-3 and (TRAF-3), tumor necrosis factor receptor associated

factor-2 (TRAF-2), and either c-IAP1 or c-IAP2. Within this inhibitory complex, c-IAP1 and c-IAP2 will constitutively ubiquitinate NIK for proteasomal degradation. However, upon activation of the noncanonical NF-κB pathway, TRAF-2 will mediate K63 linked ubiquitination of c-IAP1 and c-IAP2, such that they in turn ubiquitinate TRAF-3 instead of NIK. This process allows NIK to activate IKKα to promote further downstream gene transcription [57].

In T cells, the function of c-IAP1 and c-IAP2 are redundant to the extent that T cells from either *c-IAP1*$^{-/-}$ or *c-IAP2*$^{-/-}$ mice appear normal. However, the E3 inactive mutant form of c-IAP2 (c-IAP2^{H570A}) has been demonstrated to interfere with the E3 activity of c-IAP1 through a dominant negative mechanism, and thus has been used to study the function of these proteins. Mice expressing *c-IAP2*^{H570A} had an excessive Th1 response to *Toxoplasma gondii* infection, demonstrated by high amounts of interferon (IFN)γ and IL-10 and death in 9.5 days compared to WT and *cIAP2*$^{-/-}$ mice. *c-IAP2*^{H570A} T cells had increased levels of NIK and displayed constitutive activation of the NF-κB noncanonical pathway. These *c-IAP2*^{H570A} T cells required only α-CD3 stimulation for proliferation and both IL-2 and IFNg production, compared to WT and cIAP2$^{-/-}$ T cells, which required both α-CD3 and α-CD28 costimulation to achieve the same response [58]. T cells from mice that expressed the E3 inactive form of c-IAP1 (*c-IAP1*^{H582A}) were also able to proliferate with only α-CD3 stimulation, though to a lesser extent than *c-IAP2*^{H570A} T cells. Similarly, activation of the noncanonical NF-κB pathway also resulted in a slight elevation of NIK expression in resting *c-IAP1*^{H582A} cells [59].

NEDD4 and ITCH

Neural precursor cell expressed developmentally down-regulated 4 (NEDD4) and Itchy E3 Ubiquitin Protein Ligase (ITCH) belong to the NEDD4 HECT-domain E3 ligase family. ITCH has been implicated in a range of cellular processes including promotion of Treg development and negative regulation of Th2 differentiation [60-62]. *Itch*$^{-/-}$ mice had increased numbers of Th2 cells and increased production of IL-4 and IL-5, resulting in severe inflammation and continuous skin scratching. It was discovered that Itch ubiquitinated the transcription factor, JunB, that promotes the differentiation of Th2 cells [60, 61]. In humans, Itch deficiency results in multisystem autoimmune disease affecting the lungs, liver, and gut [63]. Recent findings have also demonstrated that while Treg specific ablation of ITCH does not change the percentage of T cells that express Foxp3, loss of ITCH does however promote Th2 cytokine production in Tregs that have lost Foxp3 expression irrespective of ITCH [62].

While T cells from *Itch*$^{-/-}$ mice were found to be hyperresponsive to TCR stimulation, T cells from *Nedd4*$^{-/-}$ fetal liver chimera mice were found to be hyporesponsive. In *Nedd4*$^{-/-}$ fetal liver chimera mice, T cells had impaired effector proliferation, impaired signal delivery to B cells, and decreased IL-2 production upon α-CD3/α-CD28 stimulation. These *Nedd4*$^{-/-}$ primary T cells also had diminished amounts of polyubiquitinated Cbl-b in Cbl-b immunoprecipitations [64]. Thus to promote T cell activation NEDD4 likely targets Cbl-b for ubiquitin-mediated destruction, such that Cbl-b can no longer negatively regulate TCR signaling.

Peli1

Pellino-1 (Peli1) is a member of the recently discovered Peli family of RING E3 ubiquitin ligases, which has been shown to catalyze K63, K48, and K11 ubiquitin chains [65]. Peli1 is highly expressed in T cells, and its levels further increase upon α-CD3/α-CD28 stimulation [66]. Peli1 also plays a role as a negative regulator of T cell activation, as CD4^{+} T cells from *Peli1*$^{-/-}$ mice have increased production of IL-2 and IFNg upon CD3/CD28 activation. These effects were even more pronounced in *Peli1*$^{-/-}$ CD8^{+} T cells, including increased proliferation in the absence of α-CD28 co-stimulation. *Peli1*$^{-/-}$ CD4^{+} T cells were less sensitive to suppression by Tregs and TGFβ, and they spontaneously developed autoimmunity. NF-κB activation was increased in *Peli1*$^{-/-}$ CD4^{+} and CD8^{+} T cells, and while proximal TCR signaling and nuclear protein levels of RelA/RelB were unaffected, c-Rel levels in nuclear extracts were increased upon TCR/CD28 stimulation, suggesting that c-Rel is hyperactivated with Peli deficiency. Furthermore, *in vitro* studies demonstrated that Peli1 is involved in K48 ubiquitination of c-Rel to inhibit its activation and regulate the threshold of TCR-mediated responses involving CD28 co-stimulation [66].

Stub1/CHIP

In vitro studies of stress-induced phosphoprotein 1 homology and U-box containing protein (Stub1), also referred to as CHIP, demonstrated that Stub1 ubiquitinates CARD-containing MAGUK protein-1 (CARMA1) through a K27 linkage. Stub1 is a U-box E3 ubiquitin ligase, where the U-box structure creates a RING finger-like E2 binding surface but contains hydrogen bonds and salt bridges rather than zinc coordination sites [67]. Knockdown of STUB1 with RNA interference in Jurkat T cells resulted in both diminished NF-κB activation and IL-2 production upon TCR stimulation. This suggests that Stub1 enhances NF-κB activation and downstream IL-2 production by ubiquitinating CARMA1 [68]. More recent findings have further demonstrated that Stub1 could promote an inflammatory response by potentially polyubiquitinating Foxp3 in Tregs through a K48 linkage. Although the discovery that Stub1 can ubiquitinate Foxp3 was made using an overexpression system in 293T cells, Stub1 was also demonstrated to be upregulated in primary mouse and human Tregs at the same time that Foxp3 was downregulated after treatment with inflammatory stresses, such as IL-1β, LPS, and heat shock. Knockdown of endogenous Stub1 also limited lipopolysaccharide (LPS)-stimulated degradation of Foxp3 in primary human Tregs, and improved the ability of primary mouse Tregs to suppress naïve T cell proliferation and T cell driven inflammation in a mouse colitis model [69]. These experiments further exemplify how E3 ligases could be targeted in the prevention of autoimmune disease.

TRAF6

Another well studied ubiquitin ligase, Tumor necrosis factor receptor associated factor-6 (TRAF6) produces K63-linked ubiquitination of substrate proteins and has been shown to also be a critical regulator of TCR signaling. *In vitro*, TCR stimulation induced an interaction between TRAF6 and mucosa-associated lymphoid tissue lymphoma translocation protein-1 (MALT1) to activate NF-κB [70]. Upon TCR stimulation of Jurkat cells, linker for activation of T cells (LAT) became ubiquitinated through a K63-linkage in the presence of TRAF6. However, this ubiquitination was reduced when TRAF6 was knocked down, demonstrating that TRAF6 plays a role in regulating proximal TCR signaling. Interestingly, this ubiquitination was also accompanied by increased LAT tyrosine phosphorylation, and in turn increased NFAT activation [71]. TRAF6 deficiency in T cells resulted in hyperactivation and resistance to Treg suppression [72], as well as increased Th17 differentiation and decreased IL-2 production [73]. TRAF6 was also found to be important for Treg suppression of Th2 cells [74]. Together these findings suggest that TRAF6 plays a critical role in TCR signaling.

TRIM21 and TRIM27

Tripartite motif containing protein (TRIM) family members, including TRIM21 and TRIM27, are also members of the RING family of E3 ubiquitin ligases that regulate T cell immune responses. TRIM21, also referred to as Ro52, plays a significant role as a negative regulator of the Th17/IL-23 pathway. Studies in *Ro52*$^{-/-}$ GFP reporter mice demonstrated expression of TRIM21 in spleen, lymph nodes, and thymus, though very little expression was found in other organs and tissues. Interferons induced expression of TRIM21, whereas other cytokines, such as tumor necrosis factor (TNF)α and TGFβ, did not. Tissue injury in *Ro52*$^{-/-}$ mice by ear tagging, induced severe dermatitis, as well as systemic autoimmunity with both production of autoantibodies to DNA and renal pathology. Furthermore, when treated with the contact sensitizing agent, oxazolone, *Ro52*$^{-/-}$ mice displayed a higher degree of contact sensitivity, while their lymph nodes had increased activated T cells producing the proinflammatory proteins IL-6, IL-12/IL23p40, TNFα, IL-17, and IL-23R than WT mice, as well as increased Th17 cells [75]. The mechanism by which TRIM21 is purported to regulate inflammation through the Th17/IL-23 pathway is by ubiquitinating interferon regulatory transcription factors (IRFs) that would otherwise promote proinflammatory cytokine expression. Overexpression studies in 293T cells demonstrated that Ro58 polyubiquitinates IRF3, IRF5, and IRF8. Moreover, co-transfection of Ro52 and 4xGal4-IRF luciferase reporters in 293T cells, showed a decrease in IRF3 and IRF5 transcriptional activity upon TLR stimulation, indicating that Ro52 may serve as negative regulator of IRF3 and IRF5 within the Th17/IL-23 pathway [75].

TRIM27, also referred to as Ret finger protein, was recently shown through overexpression in COS cells to K48 polyubiquitinate PI3K-C2β. This ubiquitination of PI3K-C2β surprisingly inhibited its kinase activity rather than trigger-

ing degradation. The activity of PI3K-C2β is required for KCa3.1 channel activity, which in turn is required to activate T cells. KCa3.1 activity increased in Jurkat cells when TRIM27 was knocked down by short interfering RNA (siRNA). Furthermore, overexpression of TRIM27 led to a decrease in KCa3.1 channel activity, and dialyzing cells with PI3P rescued channel activity. In *TRIM27*$^{-/-}$ mice, CD4^{+} T cells demonstrated increased PI3K-C2β activity compared to WT cells, and *TRIM27*$^{-/-}$ CD4^{+} T cells had enhanced production of IFNγ and TNFα when stimulated with superantigen staphylococcal enterotoxin E. Signaling pathways downstream of the TCR were unaffected, as demonstrated by similar levels of phosphorylated proteins, AKT activation, and Erk MAP kinase activation between WT and *TRIM27*$^{-/-}$ CD4 T cells. This demonstrates that TRIM27 functions as a negative regulator of CD4 T cells [76].

Ubiquitin-like modifications in TCR-mediated signaling

Post-translational modifications such as SUMO and NEDD8, which are structurally similar to ubiquitin, are emerging as important regulators of TCR signaling networks and T cell effector functions. However, we are only at the earliest stages of understanding how these ubiquitin-like modifications regulate TCR signaling, let alone how we can target these regulatory pathways.

SUMO

The small ubiquitin-like modifier (SUMO) family of proteins is very similar to ubiquitin in structure though only remotely similar to ubiquitin in sequence. SUMO proteins are covalently linked to lysine residues using an enzymatic cascade much like that of ubiquitin. SUMOylation is not known to target proteins for degradation, but instead has a range of functions including the altering of transcriptional activity, protein localization, protein interactions, and structural stability [77]. Regulation of TCR-mediated signaling by SUMOylation remains largely unknown, although a few studies have found a role for this ubiquitin-like modification in transcription, T cell immune responses, and lymphoid development. In the absence of a SUMO-2 specific protease, STAT5 was unregulated which led to a block in early lymphoid development [78]. Transcriptional activity of musculoaponeurotic fibrosarcoma proto-oncogene (c-Maf) was abrogated upon SUMOylation, which decreased IL-4 production in Th2 cells, thus implicating SUMOylation as an important player in Th differentiation [79]. In addition, blocking the SUMOylation of JunB, an AP-1 family member, was shown to diminish the activation of IL-2 and IL-4 reporter genes [80]. Upon PMA and ionomycin stimulation, the NFAT1 became SUMOylated by SUMO1, resulting in nuclear retention of NFAT1 and increased NFAT1 transcription [81].

Removal of SUMO has also been demonstrated to play an activating role in TCR signaling. Upon TCR stimulation, ubiquitin-specific cysteine protease 2a (USP2a) was found to deSUMOylate TRAF6, which mediated the interaction between TRAF6 and MALT-1. This interaction increased TRAF6 E3 ligase activity and resulted in downstream NFκB activation [82]. SUMO proteins can also block the activation of NFκB by directly modifying IκBα. SUMO2 modification of IκBα blocked ubiquitin-mediated degradation and decreased the translocation of RelA into the nucleus in dendritic cells, though this remains to be tested in T cells [83].

NEDD8

NEDD8 was originally reported as a novel mRNA found to be enriched in fetal mouse brain, but then downregulated during brain development [84]. It has since been found to be both highly conserved in most eukaryotes and widely expressed in most tissues [85]. NEDD8 has approximately 60% sequence identity with ubiquitin [86]. However, we are only beginning to understand both the spectrum of proteins that are neddylated and the physiologic consequences of neddylation. While neddylation may not directly affect the stability of its target, similar to both ubiquitination and phosphorylation, neddylation can induce a conformational change of its target to modulate protein function and may also promote or prevent the assembly of protein complexes [85]. Close to 20 proteins have been identified as neddylated with the best studied being the CUL subunits of the CRL family of E3 ubiquitin ligases, as well as p53, p75, and EGFR [87, 88]. However, several proteomic-based screens have identified over 400 neddylated proteins in a variety of cell types [88-91]. Further investigation is clearly necessary to understand how neddylation regulates many cellular processes.

Like ubiquitin, NEDD8 is linked to lysines on target proteins through its C-terminal glycine [84], and its attachment to proteins involves a stepwise enzymatic cascade initiated by NEDD8 activating enzyme (NAE), an E1 heterodimer of NAE1 and ubiquitin-like modifier activating enzyme 3 (UBA3), also known as NAEβ [92]. NAE binds the mature NEDD8 and ATP to catalyze the formation of an adenylate-NEDD8 intermediate [93]. This intermediate then reacts with the thiol group of a cysteine molecule in the active site to form an NAE-NEDD8 thioester [92, 93]. NEDD8 is then transferred to one known E2 enzyme, NEDD8 conjugating enzyme (Ubc12), which contains a unique N-terminal extension sequence that prevents Ubc12 from receiving ubiquitin [94]. The E2-NEDD8 complex then binds to an E3 ligase, which facilitates the transfer of NEDD8 to target substrates [93]. Currently only a few E3 ligases for NEDD8 are known: RBX1 and defective in CUL neddylation-1 (DCN-1) that are believed to be required for CUL neddylation [95, 96], as well as both Mdm2 and c-Cbl. The latter two have both recently been reported to not only be ubiquitin E3 ligases, but also have the capacity to neddylate other proteins [88]. Deneddylation can be achieved by at least two NEDD8 isopeptidases: COP9 signalosome homolog subunit 5 (CSN5) and NEDD8-specific protease (NEDP1) [37, 97, 98].

Pharmaceutical modulation of neddylation/CRL activation with MLN4924

MLN4924 is a cell permeable, first-in-class, investigational small molecule that selectively inhibits neddylation through substrate-assisted inhibition of NAE. MLN4924 reacts with the thioester bond between NEDD8 and the cysteine residue of NAEβ, creating an MLN4924-NEDD8 adduct that stably associates with NAEβ to prevent further reactions [99]. MLN4924 is highly selective for NAE with a half maximal inhibitory concentration (IC_{50}) of 4.7 nM compared to the UAE, which has an IC_{50} of 1.5 mM when tested in a purified enzyme assay. MLN4924 also shows selectivity for NAE vs. UAE in cell-based assays [100]. Consistent with the inhibition of NAE, MLN4924 treatment of cultured tumor cells results in a decrease in NEDD8-cullin levels and a reciprocal increase in the levels of known CRL substrates [100]. Preclinical data demonstrates that MLN4924 can induce apoptosis in tumor cell lines by inducing DNA re-replication during S phase with consequent DNA damage, as well as by inhibiting the proliferative and anti-apoptotic response of the NF-kB pathway in some cell lines [101-103]. MLN4924 has demonstrated antitumor activity in xenograft models of solid tumors, diffuse large B-cell lymphoma, and acute myelogenous leukemia (AML) [100-103].

MLN4924 has been evaluated in phase I clinical trials in patients with a variety of malignancies, including advanced solid tumors, melanoma, Hodgkin's and non-Hodgkin's lymphoma, multiple myeloma, and AML[104], and phase 1 combination trials are ongoing in AML and solid tumors (NCT01814826 and NCT01862328). However, little is understood regarding how global inhibition of neddylation will affect immune function, which could have widespread implications for treating cancer in terms of immune surveillance of malignant cells and immunological response to infection during the course of treatment. As MLN4924 is being given to patients systemically, it may be important to define its biological effects on cells of the immune system.

Dysregulated neddylation has been implicated in human neurodegenerative disorders [105], as well as in malignancy by regulating both CULs as well as p53 [106]. Hints that neddylation may impact immune function have emerged from mouse studies demonstrating that conditionally knocking out CSN5 severely impairs thymocyte development [107], while conditionally knocking out CSN subunit 8 (CSN8) limits peripheral T cell survival, proliferation, and activation [108]. As most, if not all of the COP9 signalosome subunits are required for efficient deneddylation [109], these results suggest that constitutive neddylation limits both T cell survival and activation. While both studies demonstrate defects in lymphocyte cell cycle progression, the exact mechanism for how these CSN subunits regulate cell cycle, or other aspects of T cell function, is yet to be determined [107, 108].

A relationship between TCR complex-mediated signaling and CUL neddylation

Recent studies have demonstrated that *in vitro* treatment of primary T cells with MLN4924 while stimulating with higher dose α-CD3/α-CD28 limits T cell activation [110, 111]. However, treatment of both T cell lines and puri-

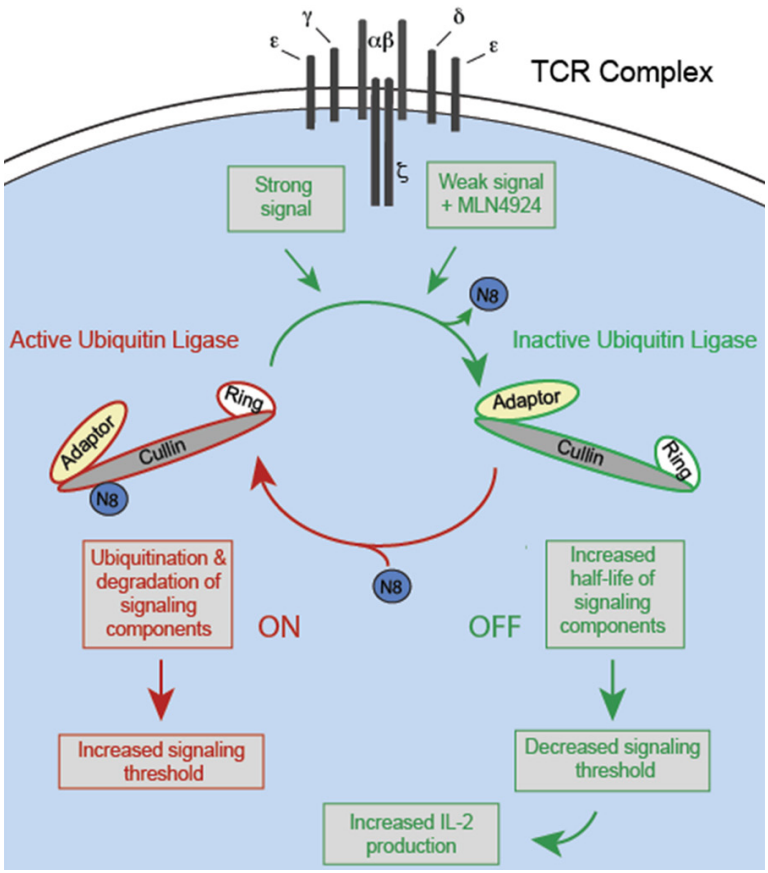

Figure 2. Model for regulation of TCR complex-mediated signaling and IL-2 production by CRLs. Either strong signaling initiated at the antigen receptor, or weak signaling with inhibition of neddylation by MLN4924, will trigger loss of CUL neddylation and inactivation of CRL ubiquitin ligase function. This in turn increases the half-life of signaling components to increase IL-2 production.

fied primary T cells with MLN4924 can increase TCR-stimulated cytokine production, proliferation, and iTreg development when T cells are stimulated with lower doses of α-CD3, as opposed to higher doses [111]. Moreover, loss of CUL neddylation was found to occur in T cell lines and purified primary T cells upon the initiation of TCR complex-mediated signaling. This loss of CUL neddylation was also seen in multiple cell types after treatment with pervanadate to create unopposed tyrosine kinase-based signaling [111]. Thus, the increase in cytokine production seen with MLN4924 is likely due to a loss of CRL activity, as CUL knockdowns demonstrated that CRLs derived from CUL1 and especially CUL2 and CUL3 can inhibit IL-2 production.

The loss of CUL neddylation upon TCR stimulation explains how MLN4924 can lower the threshold with which a weak signal can still produce a strong cytokine response. As MLN4924 inactivates CRLs by preventing CUL neddylation, MLN4924 lowers the threshold for TCR signaling mimicking what would normally occur upon strong TCR stimulation. From this work, we propose a model (**Figure 2**) whereby a reciprocal relationship exists between TCR signaling and CUL neddylation. In this model, CRL activity contributes to preventing low level signaling from initiating a potentially harmful inflammatory response, while strong TCR signaling abrogates CRL activity to prevent degradation of proteins essential for TCR complex signaling. In demonstrating this relationship between TCR complex signaling and neddylation of CRLs, these findings present a new paradigm in the regulation of T cell signaling thresholds.

Inhibition of neddylation with MLN4924 limits progression of T cell induced inflammatory arthritis in mice

The SKG mouse model of inflammatory arthritis was used to determine if MLN4924 affects T cell function *in vivo* and thresholds for autoimmune disease [112]. SKG mice develop inflammatory arthritis as a result of a defect in TCR complex signaling that leads to increased numbers of autoimmune (Th17) effector T cells that drive the development of arthritis. A single dose of zymosan triggers arthritis development in SKG mice with predictable kinetics [112]. To test whether MLN4924 treatment alters arthritis development, zymosan was administered to cohorts of SKG mice. Once the mice began to develop arthritis (28 days post-zymosan injection), they were injected intraperitoneally with MLN4924 (60 mg/kg i.p.) or cyclodextrin as the vehicle control twice a day for 5 consecutive days, rested for 2 days, and then injected twice a day for another five days. SKG mice treated with MLN4924 had a reduction in disease progression in contrast to the cyclodextrin treated

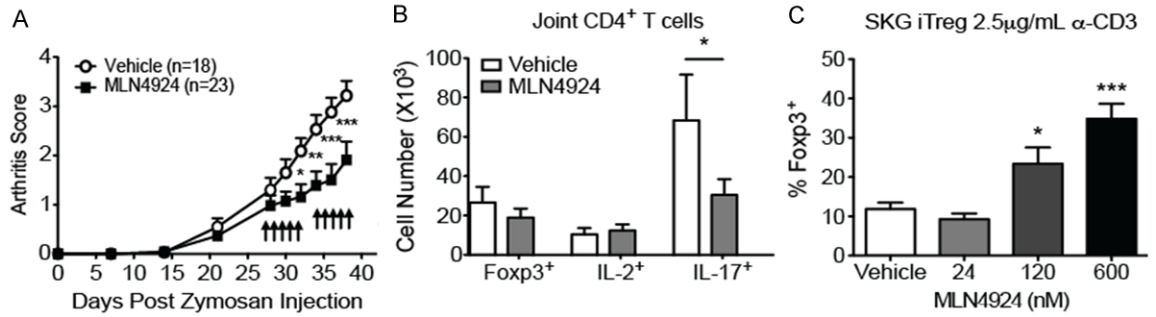

Figure 3. Inhibition of neddylation with MLN4924 limits the progression of T cell induced inflammatory arthritis, and alters effector differentiation of SKG $CD4^+$ $CD25^-$ T cells into inducible regulatory T cells (iTregs). (A) Progression of arthritis development in SKG mice (4 experiments with 3-5 mice per treatment group in each experiment) that were injected with 2 mg zymosan on day 0. 18 mice received 10% cyclodextran vehicle control (Vehicle: open circle) and 23 received 60 mg/kg MLN4924 (MLN: closed square). Injections were delivered i.p. twice a day and were started 28 days post-zymosan injection. Arthritis scores were measured by macroscopic observation. (B) Joints were analyzed by intracellular cytokine staining for the presence of $CD4^+$ cells expressing Foxp3, IL-2, and IL-17. Data shown is averaged from the staining from all mice shown in (A). (C) Percent $Foxp3^+$ iTregs after culturing $CD4^+$ $CD25^-$ T cells purified from SKG mice for 96 hours with 2.5 μg/mL plate-bound α-CD3, 2 μg/mL α-CD28, 5 ng/mL TGF-β1, 10 ng/mL retinoic acid, and increasing doses of MLN4924, reprinted with permission [111]. *p*-values in (A) and (B) were calculated using a 2-way ANOVA. Data in (C) are averaged from 3 independent experiments, and *p*-values were calculated using an unpaired two-tailed Student t test (± SEM); *p < 0.05; **p < 0.01; ***p < 0.001.

controls (**Figure 3A**). Analyses of T cell composition in the joints demonstrated that MLN4924-treated mice had decreased absolute numbers of effector $CD4^+$ T cells that express IL-17 (Th17) compared to controls (**Figure 3B**). No increase in Tregs was observed, but clearly the Th17:Treg ratio was shifted likely accounting for the effect on disease progression. MLN4924 was previously shown to increase the development of naïve BALB/c $CD4^+$ T cells into iTregs [111]. Thus, whether naïve SKG T cells have the same capacity to respond to MLN4924 was determined. Purified naïve SKG $CD4^+$ T cells were cultured with increasing doses of MLN4924 in conditions that favor the development of iTregs [113]. Indeed, MLN4924 treatment enhanced the development of $Foxp3^+$ SKG iTregs *in vitro* (**Figure 3C**) [111]. Collectively, these studies provide evidence that neddylation is an important regulator of T cell effector functions and thresholds of autoimmune disease. This demonstrates that MLN4924 treatment can influence arthritis progression and T cell effector function *in vivo*. However, since MLN4924 treatment should lead to the inhibition of neddylation in many cell types, it is unlikely that the arrest of arthritis progression can be attributed only to alterations in T cells. In addition, systemic treatment of SKG mice with MLN4924 may have opposing effects (pro- or anti-inflammatory) in different cell types, as well as at different times during the disease course, potentially limiting the efficacy of the treatment. Indeed, arrest of arthritis progression was only observed when MLN4924 was administered at or near the onset of arthritis. Initiation of treatment on day 7, 14 or 21 had no effect on arthritis progression, nor did initiation of treatment after an arthritis score of 3 had been observed (unpublished data). Thus, despite its potential as a cancer therapeutic, effects of MLN4924 seen in preclinical models suggest that MLN4924 may be a less attractive candidate for the treatment of arthritis. Future research could focus on identifying the CRLs, their adaptors, and ultimately the substrates responsible for the effects of MLN4924 on T cell effector functions and inhibition of arthritis.

Summary

This review discusses how ubiquitin and ubiquitin-like modifications regulate TCR complex-mediated signaling, as well as how these modifications influence T cell development, function, and effector differentiation. These findings highlight the essential role of E3 ligases in precisely regulating the T cell response both *in vivo* and *in vitro*. Given the growing number of targets for HECT and RING E3 ligases in TCR complex-mediated signaling, future studies will be important in not only identifying the regulatory role of these modifications, but further investigating these complex interactions in human T cells. E3 ligases are also involved in the innate

immune system, and identifying the overlap in function of these proteins will also be of critical importance when considering therapeutics that target these molecules.

Studies of ubiquitin-like modifications, such as SUMO and NEDD8 are just beginning to reveal the functions of these post-translational modifications in regulating TCR-mediated signaling. Meanwhile, two other ubiquitin-like modifications, ISG15 and FAT10, are known to be involved in TLR signaling in the innate immune response [114, 115]. Although the direct physiological relevance in T cells remains to be elucidated, it is possible that FAT10 and ISG-15 could also play a direct role in TCR-mediated signaling.

Our studies have also demonstrated that neddylation of CRLs is an important regulatory mechanism in setting the threshold for TCR-mediated signaling. MLN4924 treatment mimicking continuous TCR-mediated cullin deneddylation led to increased IL-2 production and T cell proliferation. MLN4924 inhibition of neddylation shifted TH17/Treg ratio in the SKG inflammatory arthritis mouse model, ultimately reducing disease progression. Moreover TCR stimulation alone reduces neddylation of the six CULs most highly expressed in T cells. Thus, which CRLs are expressed and active in T cell effector subsets must be determined in order to more specifically target this pathway. While studying the complexities of CRL substrate adaptors and CRL target substrates already opens up incredibly wide possibilities for new areas of investigation, the question still remains if other proteins in T cells, as well as other cells of the immune system, are post-translationally modified by neddylation.

Currently, the only drugs approved to target the ubiquitin pathway are the proteasomal inhibitors, bortezomib (VELCADE®) and carfilzomib (Kyprolis®). Undoubtedly, future studies will reveal the mechanisms behind E3 ligase and ubiquitin ligase regulation of TCR complex signaling that could be targeted to either enhance or inhibit T cell function, ultimately providing potential novel therapeutics for immune-mediated disease.

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Disclosure of conflict of interest

Allison J. Berger is an employee of Takeda Pharmaceuticals International Co.. All other authors declare that they have no conflict of interest.

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