Review

# E3 ubiquitin ligases and their control of T cell autoreactivity

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## Abstract

A loss of T cell tolerance underlies the development of most autoimmune diseases. The design of therapeutic strategies to re-institute immune tolerance, however, is hampered by uncertainty regarding the molecular mechanisms involved in the inactivation of potentially autoreactive T cells. Recently, E3 ubiquitin ligases have been shown to mediate the development of a durable state of unresponsiveness in T cells called clonal anergy. In this review, we will discuss the mechanisms used by E3 ligases to control the activation of T cells and prevent the development of autoimmunity.

## Introduction

Autoreactive T cells are involved in the development of most autoimmune diseases. Consequently, the induction and maintenance of T cell tolerance to self-antigens is as important to the normal function of the immune system as is the activation of T cells in the presence of pathogens. Despite the enormous effort that has already been made to understand the biochemical and cellular mechanisms that lead to the development of immune tolerance in model systems, we do not yet understand how to re-institute immune self tolerance in individuals that have already developed autoimmune disease. Therefore, a better understanding of the molecular processes involved in this immunological decision-making offers the possibility of defining new therapeutic targets and designing new agents that can better promote a state of immunological self tolerance and more effectively treat clinical autoimmunity.

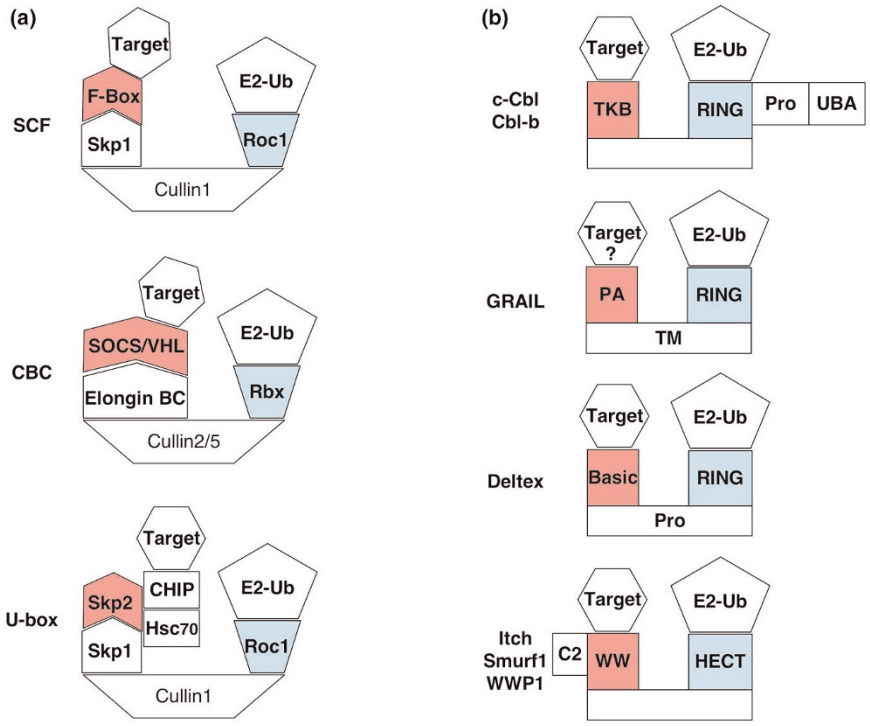
In this review, we will discuss a novel form of T cell regulation that involves a post-translational modification of proteins by ubiquitination. This system of protein ubiquitination plays a key role in many cellular processes, such as the regulation of the cell cycle, modulation of cell surface receptors, cellular differentiation, DNA repair, gene transcription, and cellular stress responses. In the innate immune system, ubiquitin-dependent proteasomal degradation of foreign proteins mediates antigen presentation. Furthermore, the activation of the proinflammatory cytokine gene transactivator nuclear factor κB (NFκB) relies on ubiquitin-mediated degradation of the IκBα inhibitory protein at sites of infection and/or inflammation. Recently, protein ubiquitination has been shown to mediate several important molecular functions in T cells that are linked to the avoidance or development of autoimmunity. Below, enzymes important to the regulation of protein ubiquitination in T cells will be described and their roles as negative regulators of autoimmunity will be considered in more detail.

## Ubiquitin biochemistry

Ubiquitin is a highly conserved 76 amino acid globular protein that is attached to substrate proteins to modify a variety of cellular processes. Although first described as a mechanism for proteolysis of misfolded or damaged proteins, ubiquitination is now appreciated as an important modification for cellular trafficking and transcriptional activation, as well as for proteasomal- and lysosomal-mediated degradation of signaling intermediates in the regulation of normal cell function. Ubiquitination is accomplished through a series of enzymatic steps involving a ubiquitin-activating enzyme (called E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3), resulting in the transfer of covalently bound ubiquitin from the E2 protein to a lysine residue on the target protein [1]. While mammals have only one confirmed E1, there are over 30 E2 enzymes and many more E3 ligases, and this allows for the ubiquitination system to confer

AP-1 = activating protein 1; APECED = autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy; CBC = Cullin-Elongin BC-SOCS/VHL; CHIP = carboxyl terminus of Hsc70-interacting protein; E1= ubiquitin-activating enzyme; E2 = ubiquitin-conjugating enzyme; E3 = ubiquitin ligase; GRAIL = gene related to anergy in lymphocytes; HECT = homologous to E6-associated protein carboxyl terminus; HIF = hypoxia inducible factor; IFN = interferon; IL = interleukin; NFκB = nuclear factor κB; NF-AT = nuclear factor of activated T cells; PA = protease-associated; PI3K = phosphoinositide 3-kinase; PLC = phospholipase C; RING = really interesting new gene; SCF = Skp1-Cullin-F-box; SOCS = suppressor of cytokine signaling; STAT = signal transducer of activated T cells; TCR = T cell receptor; TGF = transforming growth factor; Treg = T-regulatory; VHL = von Hippel-Lindau.

**Figure 1**




Multi-subunit and single-chain E3 ligases that regulate T cell function. **(a)** Multi-subunit E3 ligases (Skp1-Cullin-F-box (SCF), Cullin-Elongin BC-SOCS/VHL (CBC), and U-box) are anchored by a Cullin scaffold protein and recruit an E2 ubiquitin-conjugating enzyme via a Roc1 or Rbx RING protein (as shown in blue). Substrate specificity is determined by the binding of the target protein (either with or without the carboxyl terminus of Hsc70-interacting protein (CHIP) and Hsc70 containing pre-ubiquitin complex) to a particular F-box (e.g., Skp2), suppressor of cytokine signaling (SOCS), or von Hippel-Lindau (VHL) protein (red), and is mediated by a Skp1 or Elongin BC adapter protein [6,15,29]. **(b)** Single-chain E3 ligases contain RING or homologous to E6-associated protein carboxyl terminus (HECT) E2 recruitment (blue) and substrate binding (red) domains within one polypeptide [31,50,93]. The question mark on the putative GRAIL target protein indicates that no substrate has yet been identified. C2, $Ca^{2+}$ binding; PA, protease-associated; Pro, proline rich; TKB, tyrosine kinase binding; TM, transmembrane; UBA, ubiquitin-associated; WW, two tryptophan repeat.

substrate specificity on the many cellular processes controlled by ubiquitin modification [2].

Different patterns of covalent attachment of ubiquitin to target proteins provide a further level of specificity to regulation of cellular processes by ubiquitination. E3 ligases may attach one or more ubiquitin polypeptides to lysine residues of the target protein in order to direct degradation, transport, or function. For recognition and degradation by the 26S proteasome, substrates are polyubiquitinated in that four or more ubiquitins form a chain by ligating the carboxyl terminus of free ubiquitin to Lys48 of the previously attached ubiquitin protein [3]. In contrast, monoubiquitination of target protein lysine residues results in altered trafficking to the endosome or lysosome [4]. Substrate proteins may also be multi-ubiquitinated with the ubiquitin chains ligating the Lys63 or Lys39 residue of the previously attached ubiquitin, resulting in altered transport or function of the target protein [5].

All E3 ligases are functionally similar in that they contain a domain for recognition and binding of the E2 ubiquitin conjugating enzyme, a catalytic domain for the transfer of ubiquitin from the E2 to the target protein, and one or more protein-protein interaction domains for substrate recognition and binding (Fig. 1) [2]. E3 ligases, however, may be either multi-protein complexes or single proteins containing all of these functional domains. There are three types of E3 ligases known to function in the immune system: the really interesting new gene (RING) proteins, homologous to E6-associated protein carboxyl terminus (HECT), and U-box. These enzymes can act either to enhance immunity or dampen T cell responses.

## Polymeric RING E3 ligases and the positive regulation of T cell function

### SCF

Skp1-Cullin1-F-box (SCF) represents the prototypical multi-protein E3 ligase complex composed of a Cullin1 backbone linked to the Skp1 adaptor protein and an F-box protein that acts as a substrate receptor to recruit specific target proteins [6]. Cullin1 also binds the RING protein Roc1, which recruits an E2 ubiquitin-conjugating enzyme. Specificity is achieved

through the orchestrated expression of a unique F-box protein and the activation-dependent phosphorylation of the particular substrate protein.

Two F-box proteins associated with the SCF complex, Skp2 and βTrCP, positively regulate T cell activation. $SCF^{Skp2}$ catalyzes the ubiquitination of $p27^{kip1}$, which is a cyclin-dependent kinase inhibitor that negatively regulates cell cycle progression by binding to cyclin/cdk complexes and holding them inactive in quiescent cells [7]. When the cell cycle is initiated, $p27^{kip1}$ is phosphorylated, ubiquitinated by $SCF^{Skp2}$, subsequently degraded via the proteasomal pathway [8,9], and then cyclin/cdk is released. The end result is a cyclin/cdk-dependent $G_1$/S phase transition and T cell proliferation.

βTrCP is a second F-box protein that forms a complex with SCF and positively regulates T cell NFκB activation. NFκB family members form dimeric transcription factors that are rapidly induced by a number of stimuli and result in transcriptional activation of genes important for T cell activation and survival [10]. In resting T cells, cytoplasmic NFκB is bound by IκBα and held inactive. Upon stimulation via the tumor necrosis factor-α receptor or a combination of T cell antigen receptor (TCR) and CD28 ligands, IκBα is phosphorylated by IKK on Ser32 and Ser36, thus forming a docking site for the F-box protein $SCF^{βTrCP}$ [11,12]. Ubiquitinated IκBα is then targeted to the 26S proteasome for destruction, and NFκB is released to translocate to the nucleus [13]. Without $SCF^{βTrCP}$, T cells demonstrate defective IκBα degradation and reduced NFκB activation [14].

## Negative regulation of T cell function by polymeric RING E3 ligases

### CBC

Another multi-protein RING E3 ubiquitin ligase family is composed of Cullin-Elongin BC-SOCS/VHL (CBC) proteins and acts to negatively regulate the activation, differentiation, and function of T cells. CBC is composed of a Cullin scaffold bound to the adaptor proteins Elongin B and C, which in turn bind to the substrate receptors suppressor of cytokine signaling (SOCS) or von Hippel-Lindau (VHL) [6]. Cullin also binds the RING protein Rbx to recruit E2 proteins.

### SOCS

SOCS proteins function similarly to F-box proteins in that they bridge E3 ubiquitin ligase activity (RING protein Rbx2-Cullin5-Elongin B and C) via protein-protein interactions with target proteins [15,16]. The eight proteins of the SOCS family (SOCS1 to SOCS7 and CIS) contain a central SH2 domain for interaction with phospho-tyrosine residues in target proteins, and a conserved carboxy-terminal SOCS box to bind Elongin C and join the E3 complex [17]. SOCS proteins bind activated cytokine receptors, janus kinases (JAKs), and signal transducers of activated T cells (STATs), and mediate their degradation [18]. SOCS proteins are expressed in T cells in response to TCR or cytokine receptor stimulation, and are thought to provide negative feedback inhibition to cytokine receptor signaling and thereby play a role in T cell proliferation as well as in $Th_1/Th_2$ differentiation.

SOCS3 mRNA is present in resting CD4 T cells, but is down-modulated upon TCR stimulation [19,20]. Remarkably, T cells transgenic for SOCS3 demonstrate decreased IL-2 production in response to TCR and CD28 costimulation, perhaps relating to the ability of over-expressed SOCS3 to inhibit nuclear factor of activated T cells (NF-AT) activation and *Il2* gene transcription [21,22]. Consistent with this, the depletion of SOCS3 enhances T cell proliferation [20]. Unlike antigen stimulation, cytokines enhance the expression of SOCS3 in a STAT5a-dependent manner, and it then interacts with phosphorylated IL-2 receptor (IL-2R)β to reduce the level of phosphorylation of STAT5b and inhibit IL-2-dependent proliferation [19,23]. Finally, IL-12-dependent induction of Th1 differentiation and resultant IFNγ production depends on the activation of STAT4, and this activation event is also antagonized by SOCS3 [24]. Taken together, the results suggest that SOCS3 may play a role in maintaining CD4 T cells in a quiescent state in the absence of antigen, while TCR-mediated down-regulation of SOCS3 protein early during antigen recognition allows for the initiation of an activation-induced proliferative response. In contrast to SOCS3, SOCS1 and SOCS2 are normally expressed at only low levels in naïve T cells and are up-regulated during the course of antigen stimulation [19,20]. SOCS1 expression is induced by IL-2, IL-4, IL-7, IL-12, IL-15 and IFNγ, and T cells deficient in SOCS1 are hyper-proliferative to IL-2 and IL-4 [19,25], thus establishing SOCS1 as an additional feedback inhibitor of cytokine receptor signaling in T cells.

### VHL

While SOCS proteins bind an Elongin BC-Cullin5-Rbx2 complex to generate a CBC E3 ubiquitin ligase, the substrate-binding protein VHL interacts with an Elongin BC-Cullin2-Rbx1 complex to exert its function [15]. VHL has been shown to promote the ubiquitin-mediated degradation of the hypoxia inducible factor (HIF)-1α part of a transcription factor complex that mediates the cellular response to hypoxia, to maintain homeostasis in normoxic conditions [26,27]. Sites of inflammation, which are known to be hypoxic, are areas of intense T cell effector function. HIF-1α has been shown to be upregulated in the synovium of a patient with rheumatoid arthritis [28], perhaps indicating a loss of VHL-mediated degradation of HIF-1α in autoimmune disease.

### U-box

A novel type of multi-chain E3 ubiquitin ligase has recently been described that incorporates the U-box protein carboxyl terminus of Hsc70-interacting protein (CHIP) into the $SCF^{Skp2}$ complex. CHIP was identified in a yeast two-hybrid screen for novel E3 ligases based on its ability to bind the E2A transcription factor E47, an important mediator of Notch signaling in lymphoid cell lineage commitment, and Smad1, a

transforming growth factor (TGF)β receptor-regulated transcription factor [29,30]. CHIP has been proposed to function by assembling a pre-ubiquitin complex composed of CHIP, its co-chaperone Hsc70, Skp2, and the target protein E47 [29]. This complex can then bind to Skp1-Cullin1-Roc1 to form a functional E3 ubiquitin ligase.

## Single-chain E3 ligases

The single chain RING and HECT E3 ubiquitin ligases perform a similar role as the multi-chain E3s, but all of the functional domains are contained within a single polypeptide (Fig. 1b). The catalytic RING domain transfers ubiquitin from the E2 ubiquitin-conjugating enzyme directly to the target protein, whereas HECT proteins themselves accept the ubiquitin polypeptide prior to its transfer to a target protein. Specificity is achieved through the recognition of target substrates via protein-protein interaction domains.

### Cbl

The Cbl family E3 ligases are composed of an amino-terminal tyrosine kinase binding domain for substrate recognition, a RING domain, a proline-rich domain, and a carboxy-terminal ubiquitin-associated domain [31]. Before the function of Cbl proteins as E3 ubiquitin ligases was known, c-Cbl was recognized as a negative regulator of TCR-mediated $p56^{Lck}$ phosphorylation [32,33]. c-Cbl was subsequently shown to ubiquitinate both TCRζ and phosphorylated $p56^{Lck}$ [34,35]. TCR down-modulation is reduced in c-$Cbl^{-/-}$/Cbl-$b^{-/-}$ T cells, suggesting that these E3 ligases mediate ligand-dependent TCR internalization [36]. T cells deficient in both Cbl-b and c-Cbl show enhanced proliferation and IL-2 production in response to TCR stimulation, and the spontaneous development of autoimmunity [36]. Therefore, Cbl proteins appear to dampen TCR/CD28 signaling via ubiquitination of signaling intermediates or the TCR itself.

Cbl-b and ubiquitinated target proteins accumulate at the immunological synapse during T cell activation [37]. This has suggested an important role for Cbl-b in the regulation of TCR signaling. Cbl-b can physically interact with $p56^{lck}$, Slp76, Zap70, phospholipase C (PLC)γ1, Vav, and the p85 subunit of phosphoinositide 3-kinase (PI3K); however, resting *Cblb*$^{-/-}$ T cells show no notable changes in their expression of these proteins [38]. Nevertheless, Cbl-b does ubiquitinate p85 during T cell activation, and this reduces its association with TCRζ [39,40]. As CD28 costimulation has also been linked to the activation of PI3K, Cbl-b may normally act to antagonize CD28 downstream signaling [41]. Loss of Cbl-b in T cells does relieve the requirement for CD28 co-stimulatory signals to achieve maximal TCR/CD3-mediated receptor clustering, reorganization of membrane rafts, and filopodia formation [42]. Also consistent with this model, *Cblb*$^{-/-}$ T cells show enhanced activation of Vav [43]. Despite these data supporting a role for Cbl-b in the counter-regulation of CD28 signaling, genetic deficiency of CD28 cannot block the development of spontaneous autoimmunity in *Cblb*$^{-/-}$ mice, suggesting that Cbl-b also antagonizes other signaling pathways [44].

### Deltex

Notch is particularly important for T lymphocyte maturation and lineage commitment in the thymus [45]. In the periphery, the ligation of Notch by ligands Delta or Jagged during antigen presentation promotes $Th_1$ or $Th_2$ differentiation, respectively [46]. Notch signaling appears necessary for optimal T cell activation, as CD3/CD28 costimulation up-regulates the expression of Notch, and inhibition of Notch signaling blocks T cell proliferation and IL-2 production [47,48]. Nevertheless, Notch signaling has also been shown to upregulate the expression of Deltex1 [49]. Deltex1 functions as a RING-type E3 ubiquitin ligase that targets MEKK1 for ubiquitination and proteasomal degradation resulting in the negative regulation of TCR/CD28 signaling to IL-2 production [50]. Interestingly, Deltex1 has been shown to be highly expressed in unstimulated $CD4^+25^+$ T-regulatory (Treg) cells. Both Notch4 and the Notch ligand Delta1 are upregulated by CD3/28 stimulation of Tregs, perhaps suggesting a mechanism whereby T-T interactions via Notch-dependent Deltex1 induction suppress T cell activation [51].

### Smurf and WWP1

The single-chain HECT E3 ligase family includes NEDD4-1, NEDD4-2, Itch, Smurf1, Smurf 2, WWP1, WWP2, and NedL1, in humans and mice [52]. Besides a carboxy-terminal HECT domain for transfer of ubiquitin, these proteins contain an amino-terminal C2 domain, which is a binding site for $Ca^{2+}$ that directs phospholipid interactions at the membrane, and multiple two-tryptophan (WW) repeat domains, which are important for binding to proline-rich regions of target proteins [52].

Smurf1 and WWP1 negatively regulate signaling through the TGFβ receptor via ubiquitin-mediated degradation of receptor-regulated effector proteins Smad1, Smad2, Smad3, Smad5 and Smad8 as well as the TGFβ receptor itself [52]. Signaling through the TGFβ receptor is required for the maintenance of T cell homeostasis and functions through Smad3 to attenuate TCR/CD28-mediated IL-2 production and proliferation [53,54]. Likewise, TGFβ production by $CD4^+25^+$ Tregs suppresses the activation of $CD25^-$ T cells through an activation of a TGFβ receptor-Smad2 pathway [55]. The activation of Smurf1 E3 ligase activity leads to a ubiquitination and degradation of both Smad proteins and TGFβ receptors and releases the blockade of T cell proliferation. Interestingly, cells from Smurf1-deficient mice have recently been shown to accumulate phosphorylated MEKK2 and JNK, indicating a physiological role for Smurf1 ubiquitination and degradation of these signaling molecules [56]. Finally, WWP1 ubiquitinates lung Kruppel-like factor (LKLF or KLF2) [57,58]. This protein maintains homeostasis in $CD4^+$ and $CD8^+$ T cells. KLF2 levels decrease upon T cell activation and ubiquitin-mediated degradation of the protein by WWP1 provides a potential mechanism [59].

### NEDD4 and Itch

NEDD4 and Itch are HECT E3 ubiquitin ligases responsible for a ubiquitin-mediated counter-regulation of NFκB in T cells. Ligation of TCR/CD28 recruits an IKK complex to the immunological synapse where the scaffold molecules MALT1, Carma1, and Bcl10 bridge PKCθ activation to the induction of NFκB [60-65]. NEDD4 and Itch can ubiquitinate Bcl10 and promote its translocation to the lysosome, where Bcl10 is then marked for destruction, and the activation of NFκB is aborted [66]. Itch has also been shown to ubiqutinate c-Jun and JunB and to target these nuclear factors to the lysosome [67]. This is dependent on JNK-mediated phosphorylation and activation of Itch [68]. Both c-Jun and JunB have the capacity to form dimers with c-Fos and transactivate at cytokine genes. Thus, ubiquitin-mediated degradation of these proteins represents a potentially important negative regulatory event.

## Anergy as a T cell tolerance mechanism

Clonal anergy has been postulated to be one important immune tolerance mechanism that relies on the inducement of mature T cells into an unresponsive state following their initial exposure to a peripheral self-antigen [69]. This outcome differs greatly from that seen during a protective immune response. For the case of T cells responding to dangerous pathogens, continued antigen responsiveness is ensured because antigen presentation is restricted to dendritic cells that have detected the presence of the pathogen and its associated toll-like receptor ligands. Consequently, antigen presentation is accompanied by the surface expression of a high level of 'costimulatory' ligands such as CD80 and CD86 on the dendritic cells. CD80 and CD86 specifically bind to the CD28 costimulatory receptor within the immunological synapse that forms between the T cell and the antigen-presenting cell during antigen recognition. The end result of this strong costimulatory interaction is a maintenance of the high level of antigen sensitivity that is required to clear the pathogen.

In contrast to antigen recognition during infection, the delivery of a strong TCR signal as a consequence of self-antigen recognition is normally unaccompanied by sufficient co-stimulatory signaling to maintain a high level of antigen responsiveness [70]. This development of clonal anergy results in an inability of these cells to efficiently produce the autocrine growth factor IL-2 and to proliferate upon re-exposure to antigen. Unresponsiveness is actively induced by an increase in intracellular $Ca^{2+}$, and new proteins must be made in order to establish the anergic state [71,72]. We have also demonstrated that a fusion of anergic T cells to normal cells fails to restore antigen responsiveness, indicating the presence of dominant-acting repressor molecules within anergic T cells that inhibit signal transduction to the *Il2* gene [73]. Macian *et al*. [74] reported that $Ca^{2+}$ signaling using the calcium ionophore ionomycin could induce a limited set of anergy-associated genes in a NF-AT dependent manner to render T cells tolerant of antigen. Some of these genes appear to be involved in protein ubiquitination and, consequently, there has recently been great interest in the roles of E3 ubiquitin ligases as anergy factors.

## Single-chain E3 ligases are newly expressed during the induction of anergy

### GRAIL

The E3 ligase called gene related to anergy in lymphocytes (GRAIL) has been shown to be up-regulated in T cells following clonal anergy induction [75,76]. GRAIL protein contains a protease-associated (PA) conserved domain, a transmembrane region, and a RING. Over-expression of GRAIL in T hybridoma cells was initially shown to inhibit IL-2 and IL-4 secretion [75]. Similarly, constitutive expression of the *GRAIL* gene renders naive $CD4^+$ T cells anergic to antigenic challenge [76]. Remarkably, an enzymatically inactive form of GRAIL (called H2N2, based on mutations in its highly conserved RING) functions as a dominant negative mutant capable of inhibiting endogenous GRAIL function and blocking the development of anergy [76]. Such H2N2 RING mutants also fail to suppress IL-2 secretion in transfected T cells, thus predicting a role for the GRAIL RING domain and its associated E3 ligase activity in the counter-regulation of *Il2* gene expression following anergy induction [76]. As yet, no GRAIL target proteins have been identified in T cells, and the mechanism for substrate recognition has not been elucidated. Nevertheless, GRAIL protein has been localized to a transferrin-recycling endocytic pathway and the pharmacological blockade of endocytic trafficking reduces the inhibitory actions of GRAIL [75]. Therefore, GRAIL may function by targeting signaling proteins through its PA domain for binding and/or ubiquitination within this endocytic pathway.

### Cbl-b

Cbl-b has been shown to antagonize TCR and CD28 signaling in T cells. The spontaneous development of autoimmunity in $Cblb^{-/-}$ mice further suggested its potential as an anergy factor responsible for maintaining self-tolerance [38]. Subsequently, $Cblb^{-/-}$ $CD4^+$ T cells were found to be resistant to clonal anergy induction [77]. Anergic wild-type T cells demonstrate only transient and abortive immunological synapse formation during antigen recognition, whereas $Cblb^{-/-}$ T cells pre-treated with a calcium ionophore to promote the development of unresponsiveness have a much more stable interaction with the antigen-presenting cell [78].

### Itch

*Itchy* mutant mice deficient in Itch protein activity spontaneously develop autoimmunity, as discussed in more detail below [79]. This apparent loss of immune self-tolerance in mutant mice may relate to an inability to functionally inactivate autoreactive lymphocytes, since $Itch^{-/-}$ T cells have been found resistant to the induction of anergy by low doses of ionomycin [78].

## Single chain E3 ubiquitin ligases maintain anergic T cells in an unresponsive state

In normal resting T cells, the protein levels of Cbl-b, GRAIL, and Itch are relatively low, and these E3 ligases normally appear not to interfere with signaling cascades leading to IL-2 secretion and proliferation when costimulatory signals are abundant. Within anergic T cells, however, E3 ligase expression is increased and/or E3 enzymes are directed to unique cellular compartments during antigen stimulation. It appears they then cooperate in the ubiquitination of tyrosine-phosphorylated proteins that leads to their degradation in lysosomes.

The exact mechanism by which these E3 ubiquitin ligases maintain antigen unresponsiveness in anergic T cells remains uncertain. Immediately after clonal anergy induction, T cells demonstrate global defects in TCR signaling, including reduced phosphorylation of TCR ζ and ε chains, poor activation of $p56^{Lck}$, Zap70, Ras, JNK and ERK, and defective transactivation at the *Il2* gene by NFκB, activating protein 1 (AP-1), and NF-AT [70]. Following antigen re-stimulation, anergic T cells also demonstrate an aberrant down-regulation of phosphorylated PLCγ1, PKCθ, and RasGAP [78]. Remarkably, the activation of $Itch^{-/-}$ and $Cblb^{-/-}$ T cells fails to induce a degradation of these signaling molecules even after an anergy-inducing regimen [78]. Itch and its HECT family relative NEDD4 have also been observed to translocate into a detergent-resistant membrane fraction following their stimulation of anergic T cells [78]. Itch can mono-ubiquitinate PLCγ1, promoting its degradation within an endocytic compartment [78]. Taken together, these findings suggest a model in which the E3 ligases GRAIL, Cbl-b, Itch, and NEDD4 ubiquitinate and chaperone critical proximal signaling molecules into an endocytic pathway and direct them away from the immunological synapse and into a lysosomal compartment where they are subject to degradation.

Another plausible substrate for the Itch E3 ligase activity in anergic cells is the AP-1 component molecule JunB. Like GRAIL, Itch localizes to an endocytic pathway during T cell stimulation. Itch appears to specifically recognize JunB, leading to its ubiquitination and degradation [80]. Consistent with this, Itch and ubiquitinated JunB have been co-localized within a lysosomal compartment following stimulation [67,81]. $Itch^{-/-}$ T cells do, in fact, have a slower rate of JunB turnover, and higher JunB DNA-binding activity [80]. In anergic T cells, dysregulated Ras function and deficient activation of the mitogen-activated protein kinases ERK, JNK, and p38, can be expected to result in only a limited induction of JunB protein during antigen stimulation [82-84]. Therefore, a combination of defective *JunB* gene transcription and enhanced JunB protein turnover ultimately leads to a deficiency of AP-1-dependent transactivation at the *Il2* gene. Interestingly, JNK has been shown to enhance the degradation of JunB through a phosphorylation-dependent activation of Itch itself [68]. Whether the defect in JNK activation that exists in anergic T cells tempers the ability of Itch to ubiquitinate JunB and promote its premature degradation in the lysosome remains unknown at this time.

By working cooperatively or sequentially, these E3 ligases appear to target activated signaling complexes in anergic T cells and disrupt the nascent immunological synapse and inhibit the ongoing TCR signaling cascade. Premature turnover of Jun family nuclear factors would also put a brake on TCR signaling and prematurely abort the IL-2 production and proliferative responses of anergic T cells (Fig. 2).

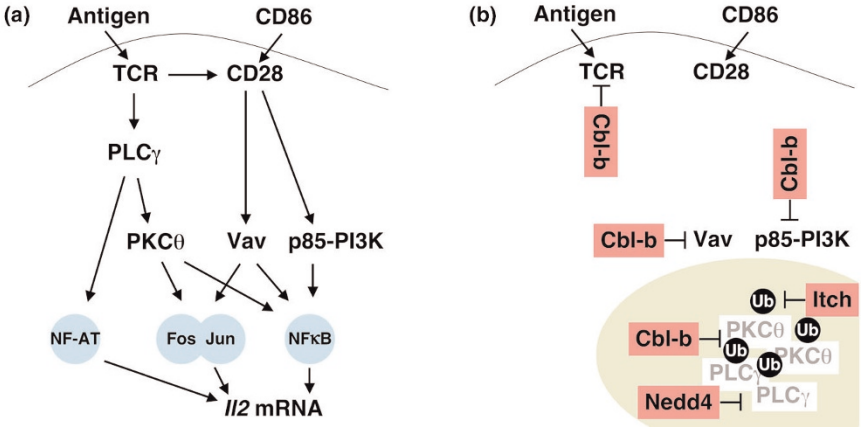
## Autoimmunity arises from insufficient E3 ligase activity

The induction of autoimmunity is a complicated process that generally involves the breaching of multiple checkpoints [85]. Nevertheless, the absence of a single E3 ligase activity can in some cases lead to the spontaneous development of autoimmune disease, perhaps via a loss of T cell tolerance to self antigens. Mice lacking Cbl-b are characterized by the production of autoantibodies, infiltration of activated T and B lymphocytes into multiple organs, and resultant parenchymal damage [38]. Furthermore, the resistance of Cbl-b-deficient mice to anergy induction during chronic and repeated exposure to antigen puts them at risk for high mortality due to toxic T cell activation [77]. The absence of Cbl-b also allows for the development of a destructive autoimmune arthritis that can be induced with type II collagen even in the absence of mycobacterial adjuvants [77]. Similarly, $Cblb^{-/-}$ mutant mice are highly susceptible to the induction of experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis [43]. A *Cblb* locus point mutation, which leads to the expression of a truncated form of the Cbl-b protein lacking E3 ligase activity, has been detected in Komeda diabetes-prone rats [86]. In one human study of patients with type I diabetes plus a second autoimmune disease, a *CBLB* exon 12 single nucleotide polymorphism was also shown to be significantly associated with disease occurrence [87].

*Itchy* mice demonstrate diverse immune disorders, including chronic inflammation of the pulmonary interstitia and alveolar proteinosis, inflammation of the glandular stomach tissue, as well as skin inflammation resulting in scarring due to constant itching. These mice also exhibit severe lymphoid hyperplasia and die at age 6 to 8 months [79,80]. Itch does not appear to be involved in T cell development in the thymus, but $Itch^{-/-}$ T cells become chronically activated as the mouse ages [80]. Similar to Cbl-b, $Itch^{-/-}$ T cells show resistance to clonal anergy induction [78]. No human autoimmune disease has yet been linked to the *ITCH* locus.

In mice, the homozygous genomic disruption of *Socs1* is lethal. However, $Socs1^{+/-}$ female mice, as well as $Socs1^{-/-}$ mice made transgenic for a low level of SOCS1 in the lymphoid compartment using a Eμ promoter, survive into adulthood but develop a lupus-like syndrome, including the

**Figure 2**




Ubiquitination of key signaling in anergic T cells. **(a)** *Il2* gene transactivation in normal T cells. TCR and CD28 signaling cascades synergistically activate phospholipase C (PLC)γ, PKCθ, Vav, and p85, which are responsible for the induction of transcription factors such as nuclear factor of activated T cells (NF-AT), activating protein 1 (AP-1: Fos and Jun), and nuclear factor κB (NFκB) leading to *Il2* gene transcription. **(b)** Sequestration or degradation of signaling intermediates in activated anergic T cells. Upon stimulation of anergic T cells, increased Cbl-b, Itch, and Nedd4 E3 ligase activities antagonize the normal function of the TCR, Vav, and p85, perhaps sequestering them within an endocytic pathway. Additionally, PLCγ and PKCθ appear to be ubiquitinated and degraded within an endosomal/lysosomal compartment during activation. Ub, ubiquitin.

expression of double-stranded DNA antibodies and immune-complex glomerulonephritis [88]. In these animals, $CD4^+$ T cells showed enhanced proliferative responses to IL-2. $CD4^{-/-}$ $Socs1^{-/-}$ double knockout mice lacking $CD4^+$ T cells were protected from autoimmunity. Thus, SOCS1 function in $CD4^+$ T cells may prove to facilitate an induction of anergy in response to self-antigen recognition.

Other E3 ligases have been genetically linked to autoimmune disease. A mutation in the autoimmune regulator (*AIRE*) gene is responsible for the development of autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), an organ-specific autoimmune disease with autosomal recessive inheritance [89,90]. Recently, AIRE protein was identified as an E3 ligase and APECED disease-causing mutations abolish its ubiquitin ligase activity [91]. Significant association of rheumatoid arthritis has also been observed with an intron 3 single nucleotide polymorphism from the *CUL1* gene. *CUL1* is important to proliferation and for the induction of IL-8 secretion during T cell activation [92]. Interestingly, the intron 3 sequence polymorphism found to be associated with rheumatoid arthritis demonstrates a greater DNA enhancer activity than an intron 3 sequence having no association with rheumatoid arthritis, perhaps suggesting that increased expression of this E3 ligase can contribute to the excessive T cell activation and loss of tolerance observed in this autoimmune disease.

In summary, these data indicate that the aberrant expression or function of any one of several E3 ubiquitin ligases is sufficient to initiate or prolong a T cell response that is directed against a self-antigen. As regulators of T cell activation, E3 ubiquitin ligases normally set an appropriate threshold for T cell activation to allow for a protective immune response against pathogens while preventing the onset of clinically important autoimmune disease. Dysregulation of one or more of these ubiquitination pathways in the human immune system, therefore, may pose the risk of a loss of immune self-tolerance.

## Competing interests

The author(s) declare that they have no competing interests.

## Authors' contributions

JLB and RZ contributed equally to this manuscript.

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