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Regulation of immune responses by E3 ubiquitin ligase Cbl-b

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Abstract

Casitas B lymphoma-b (Cbl-b), a RING finger E3 ubiquitin ligase, has been identified as a critical regulator of adaptive immune responses. Cbl-b is essential for establishing the threshold for T cell activation and regulating peripheral T cell tolerance through various mechanisms. Intriguingly, recent studies indicate that Cbl-b also modulates innate immune responses, and plays a key role in host defense to pathogens and anti-tumor immunity. These studies suggest that targeting Cbl-b may represent a potential therapeutic strategy for the management of human immune-related disorders such as autoimmune diseases, infections, tumors, and allergic airway inflammation. In this review, we summarize the latest developments regarding the roles of Cbl-b in innate and adaptive immunity, and immune-mediated diseases.

Keywords

Cbl-b; ubiquitination; innate and adaptive immune responses; T cell tolerance; immune-related disorders

1. Introduction

Ubiquitination, the covalent conjugation of ubiquitin (Ub) (a 76 amino-acid peptide) to protein substrates, is an essential mechanism of post-translational modification, which modulates various cellular pathways. Ub modification of proteins can be realized by three classes of enzymes termed E1, E2 and E3 [1]. By targeting and binding the protein substrates, E3 Ub ligases have emerged as key regulators of immune responses [2]. E3 Ub ligases can be generally divided into three subgroups: the homology to E6-associated protein carboxyl terminus (HECT) domain, the really interesting new gene (RING) type E3 ligases, and the RING between RING (RBR) E3 ligases [3].

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Declaration of Interest

The authors declare no competing interest.

Casitas B lymphoma-b (Cbl-b) is a member of the Cbl family proteins. c-Cbl, a cellular homologue of murine v-Cbl, was first identified in 1989 as a proto-oncogene [4]. These studies led to the subsequent identifications of Cbl-b [5] and Cbl-3 [6]. Although Cbl proteins had been shown to negatively regulate the signaling derived from receptor protein tyrosine kinases (RPTKs) [7–9], the mechanisms for this inhibitory function had been unknown until Cbl was characterized as a RING-type, E2-dependent Ub ligase [10]. The most widely studied Cbl family member in the immune system is Cbl-b. Accumulating evidence demonstrates that Cbl-b acts as a negative regulator in signaling pathways that involve T cell receptors (TCRs), B cell receptors (BCRs), CD28, CD40, and C-type lectin receptors (CLRs), and modulate innate and adaptive immune responses [11–15]. In this review, we will focus on the most recent progress regarding the roles of Cbl-b in innate and adaptive immunity, and the involvement of Cbl-b in immune-mediated diseases.

2. The structures of Cbl family proteins

The Cbl family of proteins consists of a conserved N-terminal tyrosine kinase binding (TKB) domain, a short linker region, and a RING finger (RF) domain (Fig. 1). The highly conserved TKB, linker and RF domains play key roles in enabling Cbl proteins to function as E3 Ub ligases [16]. The TKB domain is composed of a 4-helix bundle (4H), a calcium-binding domain with an EF-hand fold, and a variant Src homology region 2 (SH2) domain, all three of which are required to form a unique phosphotyrosine-binding (PTB) module [17]. The TKB domain recognizes and binds substrate proteins containing specific phosphotyrosine motifs in proteins such as ZAP-70 and Syk. The highly conserved short linker domain and the integrity of the linker-TKB interface are necessary for E3 ligase activity and the transformation potential of c-Cbl [18]. Recently, we have shown that three N-terminal tyrosine residues (Y106 and Y133 within the TKB domain, and Y363 within the linker region) are essential for Cbl-b E3 Ub ligase activity [19]. Indeed, the phosphorylation of Cbl-b at Y363 has been shown to regulate its E3 Ub ligase activity by removing the masking of the RF domain from the TKB domain, or by generating a structural element adjacent to the RF domain that enhances its catalytic efficacy [20, 21]. The conserved RF domain which has the intrinsic E3 ligase activity, can recruit E2 Ub-conjugating enzymes, and mediate the transfer of Ub to target substrates [10]. The structural integrity of the RF domain is indispensable for the function of Cbl proteins as E3 Ub ligases [10].

The C-terminal region of Cbl proteins is less conserved and contains proline-rich (PR) regions which mediate its binding to SH3-containing proteins. c-Cbl and Cbl-b are well-known substrates of PTKs and can be phosphorylated after the stimulation of various cell-surface receptors [22]. c-Cbl contains 22 tyrosine residues, with tyrosine residues 700 (Y700), Y731 and Y774 in the C terminus being the most prominent phosphorylation sites. Similarly, Cbl-b possesses phosphorylation sites including Y655 and Y709 whose sequences are homologous to Y700 and Y774 of c-Cbl, respectively [23]. Therefore, c-Cbl and Cbl-b are specific substrates for a similar range of PTKs and can interact with SH2 domain-containing proteins [23]. Notably, the tyrosine residue at Y731, which can bind to the SH2 domain of the p85 subunit of PI3K and then regulate function of PI3K, is unique to c-Cbl since it is not present in Cbl-b [24]. However, PI3K p85 may associate with Cbl-b via the interaction between its PR region and the SH3 domain of p85 [25]. The phosphorylation of

C-terminal tyrosine residues of Cbl-b does not appear to regulate its E3 Ub ligase activity [19], but Y709 and Y655 residues of Cbl-b seem to be the binding sites for Crk-L, with the latter being the primary binding site [23].

The C-termini of c-Cbl and Cbl-b, but not Cbl-3, have a conserved domain termed the ubiquitin-associated (UBA) domain [26]. UBA domains are capable of interacting with each other, which enables the homodimerization and heterodimerization between c-Cbl and Cbl-b [27]. The UBA domains are not required for the E3 Ub ligase activity of c-Cbl or Cbl-b, and their function and importance are still not clear. It has been showed that only the UBA domain of Cbl-b, but not c-Cbl, can interact with ubiquitinated proteins [26, 28]. Moreover, the UBA domain of Cbl-b has a greater affinity for free poly-Ub than mono-Ub, and restrains a number of Ub-mediated processes, such as degradation of ubiquitinated proteins [26]. This difference in Ub-binding ability reveals an additional distinction between c-Cbl and Cbl-b (Fig. 2).

The E3 Ub ligases act as crucial components in the ubiquitination system by recognizing specific substrates, thus facilitating Ub transfer from the E2 to the lysine (K) residues of the protein substrates [16, 29]. As an E3 Ub ligase, Cbl-b can conduct the final step of Ub transfer, characterized by the formation of an isopeptide bond between the C-terminus of Ub and lysine residues on the substrates [27, 29]. Proteins can be ubiquitinated on a single or multiple lysine residues, leading to monoubiquitination or multi-monoubiquitination, respectively [30]. Ub has seven lysines in positions 6, 11, 27, 29, 33, 48 and 63, all of which can be involved in poly-Ub chain formation. Monoubiquitination serves as important modulator in processes such as endocytosis and protein localization. K48-linked ubiquitination is usually associated with proteasomal degradation, whereas K63-linked Ub is related to signaling and endocytosis [31, 32] (Fig. 2). However, much less is known about the functions of K6-, K11-, K27-, K29-, and K33-linked ubiquitination. It has been shown that K6-, K11-, and K29-linked ubiquitination may promote proteasome-mediated degradation, whereas K27- and K33-linked ubiquitination may be involved in changes to protein functions [33–35]. In addition to the above lysine residues, the N-terminal methionine (M) residue of Ub can modulate the conjugation of Ub molecules in a novel head-to-tail fashion to form a linear Ub chain [36]. Cbl-b is centrally involved in both poly- and mono-ubiquitination processes, and plays critical roles in the regulation of immune responses. Furthermore, Cbl-b is itself a target of E3 Ub ligases and this and other post-translational modifications are discussed below.

3. Regulation of Cbl-b Expression

3.1. Post-Translational modifications of Cbl-b

3.1a. Ubiquitination and proteasome-mediated degradation of Cbl-b—In immune cells, c-Cbl is predominantly expressed in the thymus, while Cbl-b is highly expressed in peripheral lymphoid organs [4, 5, 31, 37]. Cbl-3 expression is mainly restricted to epithelial cells [6]. Optimal T cell activation not only requires signal 1 through the TCR, but also signal 2 through the CD28 costimulatory receptor, and that CD28 costimulation can amplify TCR signaling [38]. However, how CD28 costimulation amplifies TCR signaling was not fully understood. Our early studies demonstrated that CD28 costimulation enhances

ubiquitination and degradation of Cbl-b [39], whereas cytotoxic T-lymphocyte associated antigen-4 (CTLA-4)-B7 interaction induces Cbl-b expression which subsequently suppresses T cell proliferation [40] (Fig. 3). These results suggest that CD28 and CTLA-4 have opposing roles in T cell activation and proliferation and that they are regulated at least in part by the levels of Cbl-b protein. Our studies are supported by the fact that PKC-θ facilitates the ubiquitination and subsequent degradation of Cbl-b through the proteasomal pathway by phosphorylating Cbl-b on Ser282 in the TKB domain upon T cell stimulation [41]. The HECT family E3 Ub ligases Nedd4, and possibly Itch, have been shown to interact with Cbl-b, and modulate Cbl-b expression by targeting it for Ub-dependent proteasomal degradation [42]. Thus, these studies suggest that Nedd4 is the E3 Ub ligase for Cbl-b. This notion is further supported by the findings that *Nedd4*$^{-/-}$ T cells display increased levels of Cbl-b protein, and that Nedd4 is required for the polyubiquitination of Cbl-b induced by CD28 costimulation [43] (Fig. 3). In contrast to PKC-θ, glycogen synthase kinase-3 (GSK-3), a downstream target of Akt, facilitates Cbl-b expression by phosphorylating Ser476 and Ser480, two previously unreported phosphorylation sites in Cbl-b [44].

Cbl-b also undergoes auto-ubiquitination and therefore Cbl-b expression is regulated both by Nedd4-mediated ubiquitination and its auto-ubiquitination [45]. The latter is supported by our recent finding that Cbl-b specifically associates with the SH2 domain-containing protein tyrosine phosphatase 1 (SHP-1) upon TCR stimulation, whereas CD28 costimulation abrogates this association, which allows Src family protein tyrosine kinase Lck to phosphorylate Cbl-b, a process that is required for its ubiquitination [19].

3.2. Transcriptional regulation of Cbl-b

The precise mechanisms for the transcriptional regulation of Cbl-b are not fully understood. It was reported that early growth gene 2 (Egr-2) and Egr3 downstream of NF-AT are up-regulated, which correlates with enhanced expression of Cbl-b in anergic T cells [46], suggesting that Egr2 and Egr3 may transcriptionally regulate Cbl-b. However, subsequent studies showed that mice deficient for both Egr2 and Egr3 in T and B cells develop a lethal inflammatory autoimmune disease which is not due to reduced antigen receptor activation threshold, but rather is related to the uncontrolled production of inflammatory cytokines and dysregulated responses to proinflammatory cytokines mediated by Stat1 and Stat3 [47]. Therefore, it is unlikely that Egr2 and Egr3 directly regulate Cbl-b in T and B cells.

T cell activation causes the translocation of NF-κB dimers from the cytoplasm into the nucleus where NF-κB regulates inflammatory and immune response genes[48]. A recent study reported that T cells expressing an inactive form of NF-κB (IκBαΔN transgene) results in heightened expression of Cbl-b [49]. Further analysis indicated that NF-κB p65 specifically binds to an 11 bp NF-κB consensus sequence in the Cbl-b promoter, thus suppressing Cbl-b transcription [49].

4. Effects of Cbl-b on Innate and adaptive immunity

4.1. Regulation of T cell response by Cbl-b

4.1a. Cbl-b in T cell activation—As mentioned above, for T cells to be fully activated requires at least two signaling events, the first provided by the TCR complex after antigen recognition and the second through costimulatory molecules such as CD28. Lack of costimulatory signaling results in a state of T cell unresponsiveness or anergy [50]. Cbl-b displays a high level of expression in murine and human $CD4^+$ and $CD8^+$ T cells, and functions as a gatekeeper which prohibits excessive T cell activation [51]. Loss of Cbl-b uncouples the requirement for CD28 costimulation for T cell proliferation and IL-2 production [52, 53], suggesting that Cbl-b is involved in the CD28 costimulatory signaling pathway. This notion is supported by the fact that CD28 costimulation potentiates Cbl-b ubiquitination and degradation induced by TCR stimulation [39].

We and others have previously shown that Vav-1, a key guanine nucleotide exchange factor in lymphoid cells for the Rho family of GTP-binding proteins (Rac1, Rac2 and Cdc42), is involved in the CD28 costimulatory signaling pathway [54, 55]. In support of a critical role of Cbl-b in the CD28 costimulatory signaling pathway, there is a selective enhancement of phosphorylation and activity of Vav-1 in T cells lacking Cbl-b upon TCR stimulation and uncouples the requirement for CD28 costimulation [53]. These studies also indicate that Cbl-b is a negative regulator of Vav-1. Vav-1 can link TCR signaling to actin cytoskeletal reorganization and valid T cell activation via Rac-1/CDC42/WASP [56]. Consistent with this, Cbl-b has been shown to inhibit the extent of TCR clustering and T cell proliferation through a CDC42/WASP pathway-dependent mechanism downstream of Vav-1 [57]. Thereby, these studies demonstrate that Cbl-b inhibits the Vav-1/Rac-1/CDC42/WASP pathway downstream of CD28. In support of this notion, introducing Cbl-b deficiency into a $Vav1^{-/-}$ background relieves the functional defects of $Vav1^{-/-}$ T cells and leads to the development of spontaneous autoimmunity [56].

PI3K, which converts phosphoatidylinositol-4,5-bisphosphate (PIP_2) to phosphoatidylinositol-3,4,5-bisphosphate (PIP_3) at the plasma membrane, provides docking sites for signaling proteins with pleckstrin homology (PH) domains, which includes Vav-1, and elevates Vav-1 nucleotide exchange activity [58]. Therefore, the down-regulation of Vav-1 activation by Cbl-b may result from Cbl-b-mediated-ubiquitination of PI3K, one of the upstream regulators of Vav-1 [25]. Cbl-b binds to p85, the regulatory subunit of PI3K, and targets p85 for ubiquitination [25]. Ubiquitinated p85 fails to associate with TCRζ and CD28, thus inhibiting T cell activation [59]. However, since PTEN, a negative regulator of the PI3K signaling pathway by dephosphorylating PIP_3 [60], also associates with both TCRζ and CD28, the contribution of p85 in the CD28 costimulatory signaling pathway remains to be further defined. Indeed, our studies indicate that Cbl-b does not directly modulate PI3K activity in T cells upon TCR stimulation, but impedes the binding of PTEN to Nedd4 which targets PTEN K13 for K63-linked ubiquitination, thus inactivating PTEN [61]. Therefore, Cbl-b exerts its inhibitory effect on T cell activation via inhibiting PTEN inactivation independently of its ubiquitin ligase activity [62]. Our group also further characterized the signaling pathway(s) downstream of PTEN in T cells. We showed that Cbl-

b associates with PKC-θ upon TCR stimulation and regulates TCR-induced PKC-θ activation via Vav-1, which couples PKC-θ to PI3-K and allows it to be phosphorylated. PKC-θ then couples IκB kinases (IKKs) to the CARMA1/Bcl-10/MALT1 complex, resulting in activation of the IKK complex [62]. Therefore, down-regulation of TCR-induced NF-κB activation by Cbl-b is coordinately mediated by both Akt- and PKC-θ-dependent signaling pathways in primary T cells (Fig. 3).

Since Vav-1 is required for the activation of Rho GTPase family members such as Rap-1, which has been shown to be crucial for the inside-out signaling pathway during T cell activation [63], the regulation of inside-out signaling by Cbl-b was of interest. Crk-L, an adaptor protein, exerts an important role in Rap-1 activation [64]. It was reported that Cbl-b acts as a negative regulator of the activation of the small GTPase Rap1 and the integrin LFA-1, and that it is mediated by the Crk-L-C3G pathway in response to TCR stimulation [64]. Cbl-b appears to function as an E3 Ub ligase that promotes the ubiquitination of Crk-L, which inhibits the binding of Crk-L to C3G, thus down-regulating Rap-1 activity and then inside-out signaling [65].

4.1b. Cbl-b in T cell tolerance

Cbl-b in T cell anergy induction: The tolerance of T cells begins as soon as a TCR is formed and expressed on the cell surface of a T cell progenitor in the thymus. Self-reactive T cells are eliminated in the thymus by negative selection. However, not all antigens that T cells need to be tolerant of are expressed in the thymus, and thus negative selection mechanisms alone are insufficient [66]. The potential self-reactive T cells in the periphery are eliminated by apoptosis or inactivated by anergy, or actively suppressed by regulatory T cells (Tregs) [67]. T cell anergy is considered to be one of the major peripheral T cell tolerance mechanisms [50]. It has been shown that induction of T cell anergy in vivo requires CTLA-4 engagement [68], but the molecular mechanism for the role of CTLA-4 in T cell anergy induction was unknown. We have shown that CTLA-4-B7 interaction is crucial for inducing Cbl-b expression [40] (Fig. 3), suggesting that Cbl-b is involved in CTLA-4-mediated T cell anergy induction. In support of this notion, loss of Cbl-b in mice results in impaired induction of T cell tolerance both in vitro and in vivo [69, 70]. Anergic T cells express heightened levels of Cbl-b. Importantly, rechallenge of *Cblb*$^{-/-}$ mice with the tolerizing antigen results in massive lethality [70]. Moreover, ablation of Cbl-b results in exacerbated autoimmunity [70]. Mechanistically, Cbl-b may target PLC-γ1 and PKC-θ for ubiquitination, thus regulating the activation of PLC-γ1 and PKC-θ in anergic T cells [69, 70] However, whether the severe autoimmunity observed in *Cblb*$^{-/-}$ mice is T cell-intrinsic or extrinsic remains to be further investigated using *Cblb* conditional knockout (KO) mouse strains.

Cbl-b in Treg development: CD4^{+}CD25^{+} Tregs are essential for keeping self-reactive T cells in check, and prevent the development of autoimmunity in both mice and humans [71]. The master transcription factor that regulates Treg development is Foxp3 [72, 73]. Earlier studies revealed that CD4^{+}CD25^{-} effector T cells (Teffs) of *Cblb*$^{-/-}$ mice are resistant to suppression by both wild-type (WT) and *Cblb*$^{-/-}$ Tregs, or TGF-β [74]. The resistance of *Cblb*$^{-/-}$ Teffs to suppression by Tregs and TGF-β may contribute to the high susceptibility

of *Cblb*$^{-/-}$ mice to autoimmunity. Further studies showed that Cbl-b promotes the conversion of naive CD4^{+}CD25^{-} T cells into inducible CD4^{+}CD25^{+}Foxp3^{+} T cells (iTregs) [75], and that the induction of Foxp3 in iTregs depends on the transcription factors Foxo1 and Foxo3a which are phosphorylated and inactivated by Akt [76, 77]. Interestingly, we found that Cbl-b mediates the development of iTregs and peripherally converted Tregs (pTreg) via Akt-2 but not Akt-1 by controlling the threshold for T cell activation [75] (Fig. 4). Furthermore, Cbl-b together with Stub1 targets Foxp3 for ubiquitination, thus controlling the development of thymic-derived Tregs and maintaining Foxp3 expression at a steady state [77] (Fig. 4). However, these data need to be further validated by a *Foxp3 Cre-Cblb*$^{f/f}$ mouse strain. Taking together, Cbl-b modulates T cell tolerance by multiple mechanisms.

4.2. Cbl-b in T cell differentiation

Naïve T helper (Th) cells differentiate in response to antigen stimulation into either Th1, Th2, or Th17 effector cells, which are characterized by the secretion of different sets of cytokines [78]. Since *Cblb*$^{-/-}$ mice are highly susceptible to experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA), which are believed to be mediated by the pathogenic Th17 response, it was assumed that Cbl-b may inhibit Th17 cell differentiation and EAE. Surprisingly, the loss of Cbl-b facilitates Th2 and Th9 cell differentiation in vitro and Th2/Th9-mediated allergic airway inflammation [80]. Mechanistically, Cbl-b selectively associates with Stat-6 and targets Stat-6 for ubiquitination and degradation [79] (Fig. 4). In order to dissect the role of Cbl-b in adaptive and innate immunity, we have generated *Cblb* conditional KO strains. Our recent data indicate that loss of Cbl-b in the myeloid cell lineage is responsible for the heightened Th17 response and EAE development (our unpublished data).

4.3. Cbl-b in CD8^{+} T cell functions

4.3a. Cbl-b in CD8^{+} T cell responses in infections—The CD8^{+} T cell response is crucial for controlling viral infections [80, 81]. Although loss of Cbl-b does not impact the clonal expansion of viral specific CD8^{+} T cells in vivo, using an acute lymphocytic choriomeningitis virus (LCMV) infection model, Cbl-b deficiency was shown to lead to the impairment of antigen-induced TCR down-regulation by effector CD8^{+} T cells, resulting in enhanced IFN-γ production [82]. However, the functional affinity of effector CD8^{+} T cells is not affected by Cbl-b ablation [82]. This study provides evidence for understanding the regulation of CD8^{+} T cell responses by Cbl-b during acute viral infection. Although Cbl-b deficiency enhances CD8^{+} T cell response during chronic LCMV infection, *Cblb*$^{-/-}$ mice succumb to fatal immunopathologic condition possibly due to cytokine storm [83]. Hence, caution should be taken in the future when targeting Cbl-b by potential Cbl-b inhibitors to treat chronic viral infections. In addition, a recent study demonstrates that the absence of Cbl-b augments inactivated vaccines and immunity against systemic fungal infections by enhancing CD8^{+} T cell response [84]. Therefore, inactivation of Cbl-b may have therapeutic potential to treat acute viral infections or as an adjuvant to boost CD8^{+} T cell responses to fungal vaccination.

4.3b. Cbl-b in CD8^{+} T cell responses in tumors—CD8^{+} T cell responses also play a crucial role in eradicating tumors. Mice lacking Cbl-b reject spontaneous tumor cells that

express human papilloma virus antigens or ultraviolet B (UVB)-induced skin tumors [84], or T cell lymphoma in ataxia telangiectasia mutated–deficient (*Atm*$^{-/-}$) mice [84]. *Cblb*$^{-/-}$ mice also reject transplanted tumors. The rejection of spontaneous tumors or transplanted tumors is mediated by CD8$^+$ T cells [85–87]. Interestingly, *Cblb*$^{-/-}$ CD8$^+$ T cells are resistant to suppression by CD4$^+$CD25$^+$ Tregs, TGF-β, or PD-L1 [84, 85, 87]. Taken together, these studies indicate that inhibition of Cbl-b by small molecule inhibitors or *Cblb* siRNA may represent an effective strategy to treat patients with tumors in combination with the current immune check-point blockers such anti-PD-1 or anti-PD-L1.

5. Regulation of B cell responses by Cbl-b

5.1. Cbl-b in B cell activation and B cell tolerance

Earlier studies showed that Cbl-b positively regulates BCR-induced Ca^{2+} signaling in an immature DT40 chicken B cell line via facilitating the assembly of the Btk/BLNK/PLC-γ2 complex [88]. However, mature B cells deficient for Cbl-b showed enhanced proliferative responses upon stimulation through the BCR [51]. Loss of Cbl-b in B cells leads to sustained phosphorylation of Igα, Syk, and phospholipase C (PLC)-γ2, resulting in prolonged Ca^{2+} mobilization, and increases in ERK and JNK activation [89]. Syk ubiquitination is reduced in B cells lacking Cbl-b in response to BCR crosslinking, suggesting that Cbl-b might be the E3 Ub ligase for Syk. Mice with B cell-specific c-Cbl deficiency and systemic loss of Cbl-b result in manifestation of lupus-like autoimmune disease, displaying an increase in marginal zone (MZ) and B1 B cells [12]. Interestingly, B cells are not hyper-responsive in terms of proliferation and antibody production upon BCR stimulation [12]. Impaired B cell anergy is observed in B cell-specific *c-Cbl*$^{-/-}$*Cblb*$^{-/-}$mice, indicating that Cbl proteins affect B cell intrinsic checkpoint of tolerance induction [89]. The proximal BCR signaling including phosphorylation of Syk, PLC-γ2, and Vav-2 is heightened in B cells lacking both c-Cbl and Cbl-b upon BCR stimulation. Both Ig-α and Syk ubiquitination are markedly compromised in *c-Cbl*$^{-/-}$*Cblb*$^{-/-}$ B cells [12]. However, it remains unclear whether these Cbl E3 Ub ligases target Igα and Syk directly or indirectly. This could be confirmed by using an E3 ligase inactive form of Cbl-b or c-Cbl in B cells.

5.2. Cbl-b in CD40-mediated signaling in B cells

CD40 is a member of the TNF receptor superfamily, which plays central roles in the homeostatic regulation of B cell functions. The interaction of CD40 on B cells with the CD40 ligand (CD40L) on activated CD4$^+$ T cells represents the key event in the initiation of B cell proliferation, differentiation, isotype switching, up-regulation of surface molecules contributing to antigen presentation, germinal center (GC) development, and the humoral memory response to thymus-dependent (TD) antigens [90]. It was reported that defective Ig class switching and GC formation in *Vav1*$^{-/-}$ mice during a vesicular stomatitis Indiana virus (VSV) challenge are rescued by Cbl-b deficiency [91]. Intriguingly, loss of Cbl-b also restores defective Ig class switching and VSV-specific antibody production [91]. These results reveal that Cbl-b functions as an important modulator in GC formation mediated by CD40 signaling. In support of this notion, CD40-induced B cell proliferation is significantly increased in mice lacking Cbl-b. Furthermore, mice deficient for Cbl-b display enhanced TD antibody responses and GC formation, whereas introduction of CD40 deficiency abolishes

these effects [91]. Hyper TD humoral response in *Cblb*$^{-/-}$ mice is partially due to an intrinsic defect in B cells. At the molecular level, Cbl-b specifically inhibits CD40-induced activation of NF-κB and JNK. Cbl-b binds with TNF receptor-associated factor 2 (TRAF-2) upon CD40 ligation, and suppresses the recruitment of TRAF-2 to CD40 [92]. Therefore, Cbl-b attenuates CD40-mediated NF-κB and JNK activation, thereby inhibiting B cell responses. These data also suggested a potential role of Cbl-b in T follicular helper cell (Tfh) development.

A recent study led by Dr. Gu showed that the absence of c-Cbl and Cbl-b (Cbls) in GC B cells leads to the early exit of high-affinity antigen-specific B cells from the GC reaction, thereby resulting in defective clonal expansion of high-affinity B cells [93]. IRF-4, a transcription factor facilitating fate choice of plasma cells, is ubiquitinated and degraded by c-Cbl and Cbl-b in GC LZ B cells. Similar to CD28 costimulation-induced Cbl-b degradation in T cells [39], costimulation of B cells with CD40 and BCR induces the degradation of both c-Cbl and Cbl-b, resulting in increased IRF-4 expression and exiting from GC affinity selection [93]. A caveat of this study is the many functions of Cbl E3 Ub ligases in T cells. As such these findings should be confirmed in mice lacking Cbl-b and c-Cbl only in B cells, or even more specifically in GC B cells.

6. Regulation of innate immune cell responses by Cbl-b

During the last decade, accumulating evidence indicates that Cbl-b also plays a critical role in the regulation of innate immune responses involving natural killer (NK) cells and macrophages.

6.1. Cbl-b in NK cell responses

NK cells have properties of both innate and adaptive immune responses by the cytokines they produce, and are involved in host-rejection of both tumor cells and virally infected cells. NK cell activation is controlled by a dynamic balance between activating and antagonistic pathways, conducted by a number of activating and inhibitory receptors expressed on the NK cell surface [94]. Although loss or inactivation of Cbl-b exerts no obvious effects on NK cell development, NK cells from mice lacking Cbl-b or expressing the Cbl-b C373A mutation, an E3 ligase dead mutation, display markedly augmented proliferation and interferon (IFN)-γ production when activated *in vitro*, and are more efficient in killing tumor cell lines in vivo [95]. Consistent with this, mice deficient for Cbl-b or expressing the Cbl-b C373A mutation spontaneously reject various metastatic tumors in an NK cell-dependent manner [88, 95]. The anti-tumor effect of NK cells lacking Cbl-b is mediated by the TAM receptor kinases (Tyro-3, Axl and Me), which negatively regulate NK cell activity including proliferation and IFN-γ production when stimulated with GAS6, a known ligand for TAM receptors [95]. Cbl-b targets TAM receptors for ubiquitination in NK cells induced by GAS6 stimulation [95]. Treating wild-type NK cells with a newly-developed small molecule TAM inhibitor significantly increases anti-metastatic NK cell activity in vivo. Furthermore, administration of this inhibitor orally or intraperitoneally significantly inhibits tumor growth and metastases [95], indicating that targeting the Cbl-

b/TAM inhibitory pathway may represent a novel strategy to develop drugs that activate the innate immune system to eradicate cancer metastases.

6.2. Cbl-b in C-type lectin receptor (CLR)-mediated innate immune responses in macrophages

Although Cbl-b deficiency does not appear to affect the phenotypes of macrophages and dendritic cells [51], several studies showed that *Cblb*$^{-/-}$ mice are highly susceptible to lipopolysaccharide (LPS)-induced septic shock, and that TLR4 is suggested to be the target of Cbl-b [96]. However, a detailed molecular mechanism was not provided in this study. Furthermore, LPS-induced septic shock has been shown to be dependent on caspase-11, a newly-identified cytosolic receptor for LPS [97], and can be independent of TLR4 [98,99]. Cbl-b has also been suggested to be the E3 Ub ligase for the adaptor protein MyD88 [100]. However, whether this is the case in primary mouse macrophages is unknown since no data were provided using macrophages derived from mice lacking Cbl-b or expressing the Cbl-b C373A mutation. Several recent studies using *Cblb*$^{-/-}$ macrophages clearly indicate that Cbl-b does not regulate TLR signaling, but inhibits signaling from the Dectin-1, −2, and −3 CLRs [14, 15, 101]. Dectin-1, 2, and −3 are the major fungal recognition receptors that sense β-glucans and α-mannans on the fungal cell wall [102]. Activation of Dectin-1, −2, and −3 leads to the phosphorylation and activation of SHP-2, which is able to recruit Syk to Dectin-1 or to the adaptor FcR, thus resulting in the activation of Syk and the induction of anti-fungal innate immune responses and Th17 responses [102]. Loss of Cbl-b renders mice resistant to systemic *C. albicans* infection, which is consistent with the hyper-production of pro-inflammatory cytokines TNF-α and IL-6, robust release of reactive oxygen species (ROS), and enhanced fungal killing [14, 15]. Mechanistically, Cbl-b targets Dectin-1, −2, −3, and Syk for K48-linked polyubiquitination and proteasome-mediated degradation, which then inhibits the inflammatory response [14, 15, 103]. Intriguingly, inhibition of Cbl-b by Cbl-b small inhibitory peptides or *Cblb*-specific siRNA provides protective efficacy against disseminated candidiasis [14, 15] (Fig. 5). Therefore, targeting Cbl-b may be a potential therapeutic approach for disseminated candidiasis.

7. Conclusion and Perspective

Accumulating studies collectively unveil the important role of Cbl-b in adaptive immunity including T-cell activation, differentiation and tolerance. Yet, the involvement of Cbl-b in innate immunity has only been revealed recently. However, the molecular mechanisms of immune response regulated by Cbl-b and the potential crosstalk between innate and adaptive immunities still needs further study. Additionally, small molecule inhibitors of Cbl-b or *Cblb*-specific siRNA offer a promising therapeutic target for the treatment of immune-related disorders including autoimmune diseases, disseminated candidiasis, and tumors. Thus, the immunological gate keeper Cbl-b may provide new target to treat a range of immune related disorders.

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Reference

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Highlights

- Cbl-b is a member of RING finger family E3 ubiquitin ligases
- By targeting different substrates in innate and adaptive immune cells, Cbl-b acts as a gate keeper in immune system
- Targeting Cbl-b may represent a potential therapeutic strategy for immune-related disorders

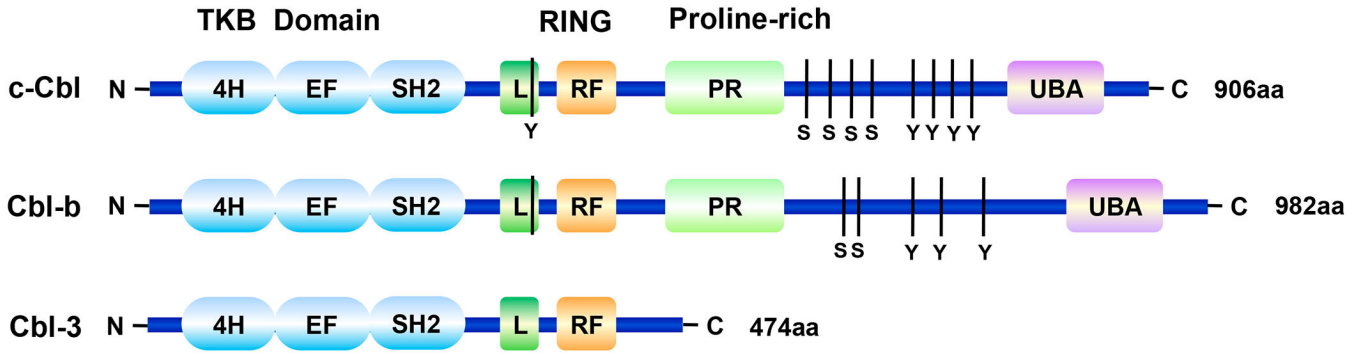

Figure 1. Functional domain structure of Cbl family proteins in mammals.

All three Cbl proteins share an N-terminal tyrosine kinase binding (TKB) region, composed of a four helix (4H) bundle, an EF Hand and an SH2 domain. The TKB domain is connected through a conserved helical linker (L) to a RING finger (RF) domain, which contributes to the E3 ligase activity. The C-terminal region includes proline-rich (PR) motifs, multiple serine and tyrosine phosphorylation sites, and leucine zipper (LZ)/ubiquitin association (UBA) domain. Cbl-3 lacks most of the C-terminal domains of c-Cbl and Cbl-b.

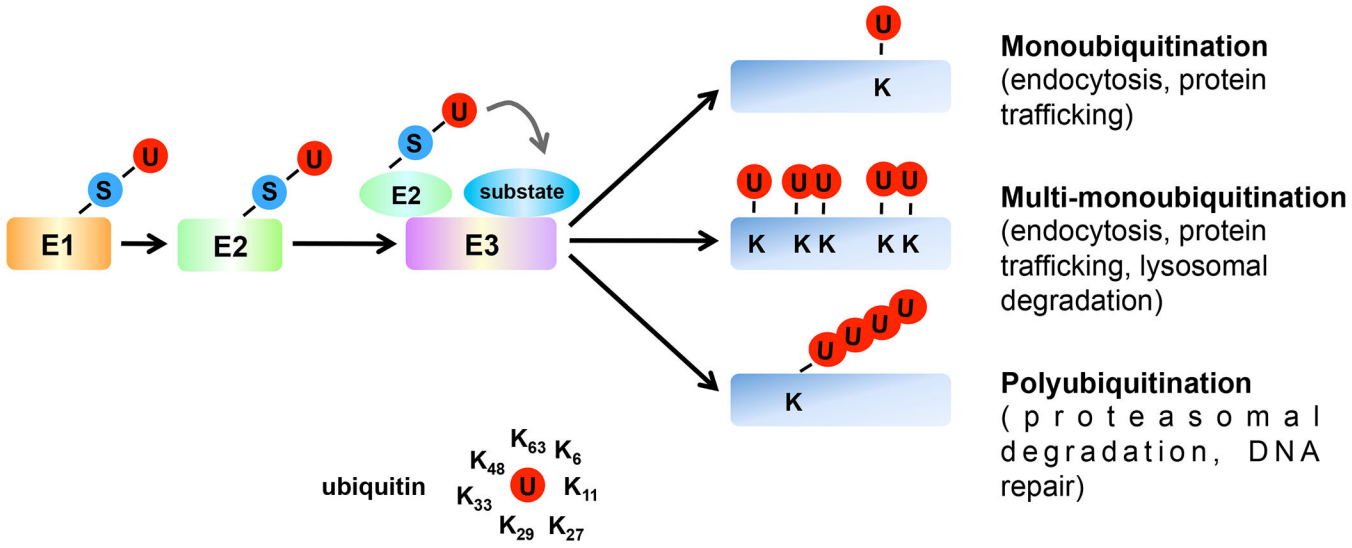

Figure 2. Ubiquitination pathway related to E3 ubiquitin ligases.

Ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-protein ligase (E3) enzymes are necessary for substrate ubiquitination. The process of ubiquitination is performed through an E1–E2–E3 cascade. The substrate protein can be ubiquitinated on a single or multiple lysine residues, resulting in monoubiquitination or multi-monoubiquitination respectively.

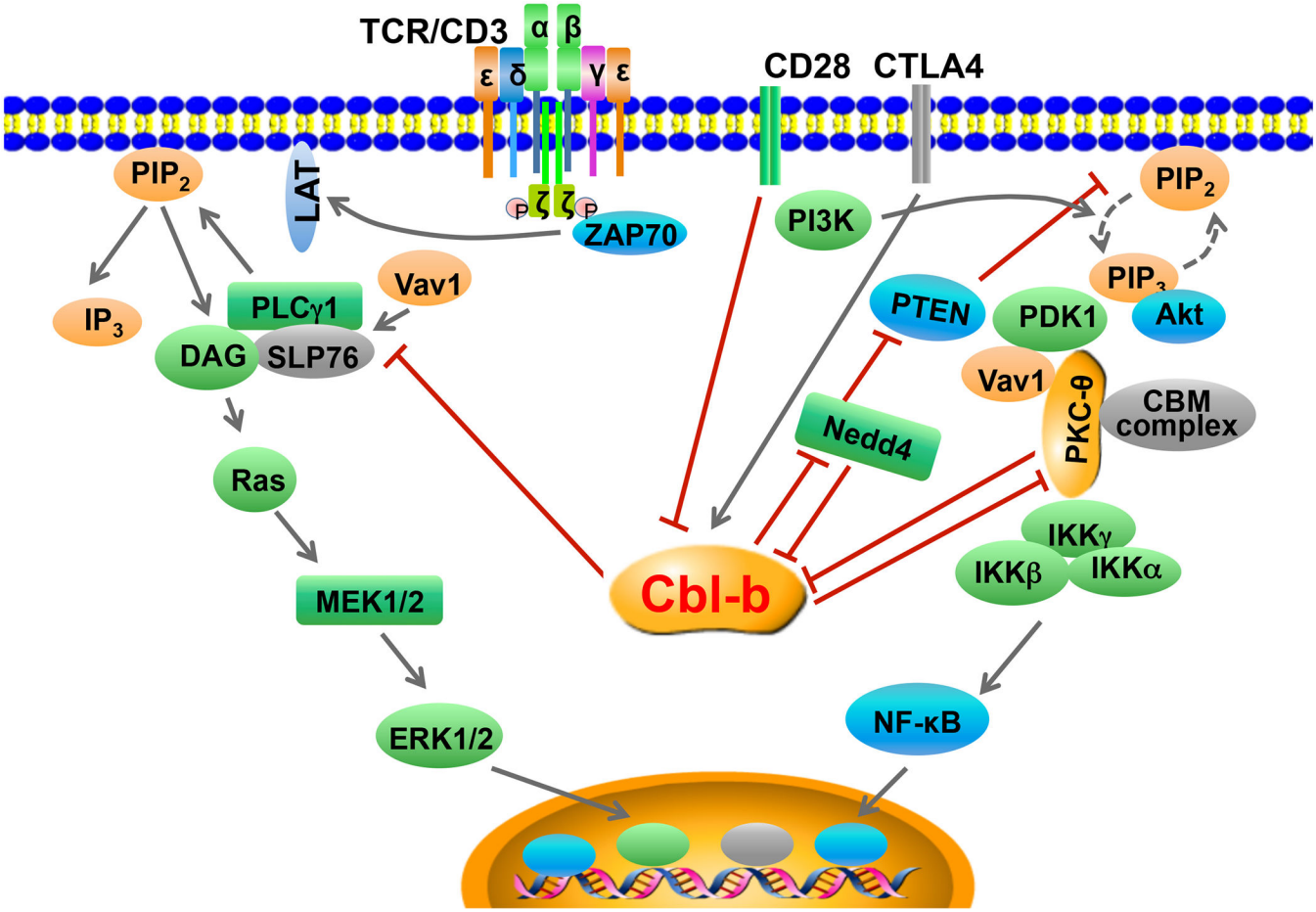

Figure 3. Cbl-b in T-cell activation and tolerance.

PTEN is inactivated by Nedd4, which targets PTEN for K63-linked polyubiquitination in T cells upon TCR stimulation, and this process can be suppressed by Cbl-b. Nedd4 also ubiquitinates Cbl-b upon TCR stimulation. CD28-mediated inactivation of PTEN increases PIP_3, which recruits Akt, PDK-1 and Vav-1 to the plasma membrane through its association with the PH domains of these molecules. Activated Akt promotes the formation of CARMA1/Bcl-10/MALT1 (CBM) complex via phosphorylating CARMA1. Furthermore, Vav-1 links PKC-θ to PDK-1, then coupling IKKs to the CBM complex. Thus Cbl-b inhibits NF-κB activation through Akt and PKC-θ. In anergic T cells, Cbl-b induces ubiquitination of PLC-γ1 and PKC-θ, thereby inhibiting T cell anergy induction. Cbl-b expression in T cells is regulated via multiple mechanisms, CTLA-4-B7 interaction facilitates Cbl-b expression, while CD28 costimulation potentiates Cbl-b ubiquitination and proteasomal degradation, which is possibly mediated by Nedd4 and PKC-θ, and by auto-ubiquitination.

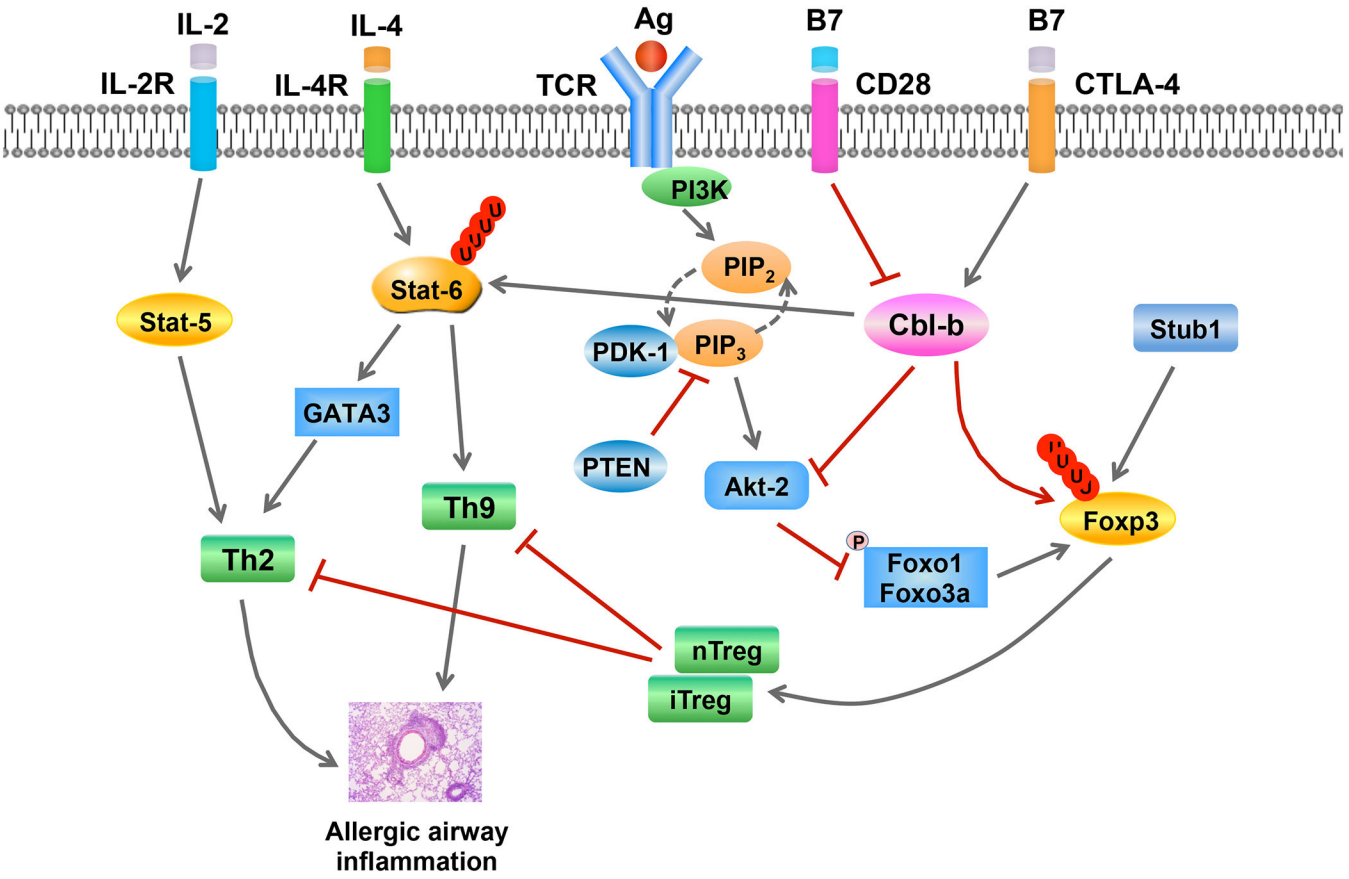

Figure 4. Cbl-b in Th2/9 differentiation, iTreg development, and allergic airway inflammation.

Cbl-b selectively associates with Stat-6 and targets Stat-6 for ubiquitination and degradation upon TCR/CD28 and IL-4 stimulation. The negative regulation of Stat-6 by Cbl-b leads to inhibition of Th2/9 responses and allergic airway inflammation. Stub1 initiates Foxp3 ubiquitination, which in turn allows the recruitment of Cbl-b through its UBA domain, thus enhancing Foxp3 ubiquitination. Moreover, Cbl-b inhibits the activation of Akt-2, which phosphorylates Foxo1/Foxo3a, resulting in the exclusion of Foxo1/Foxo3a from the nuclei, whereas Foxo1/Foxo3a is required for the induction of Foxp3. The absence of Cbl-b impairs iTreg development, leading to aberrant Th2/9 response and severe allergic airway inflammation.

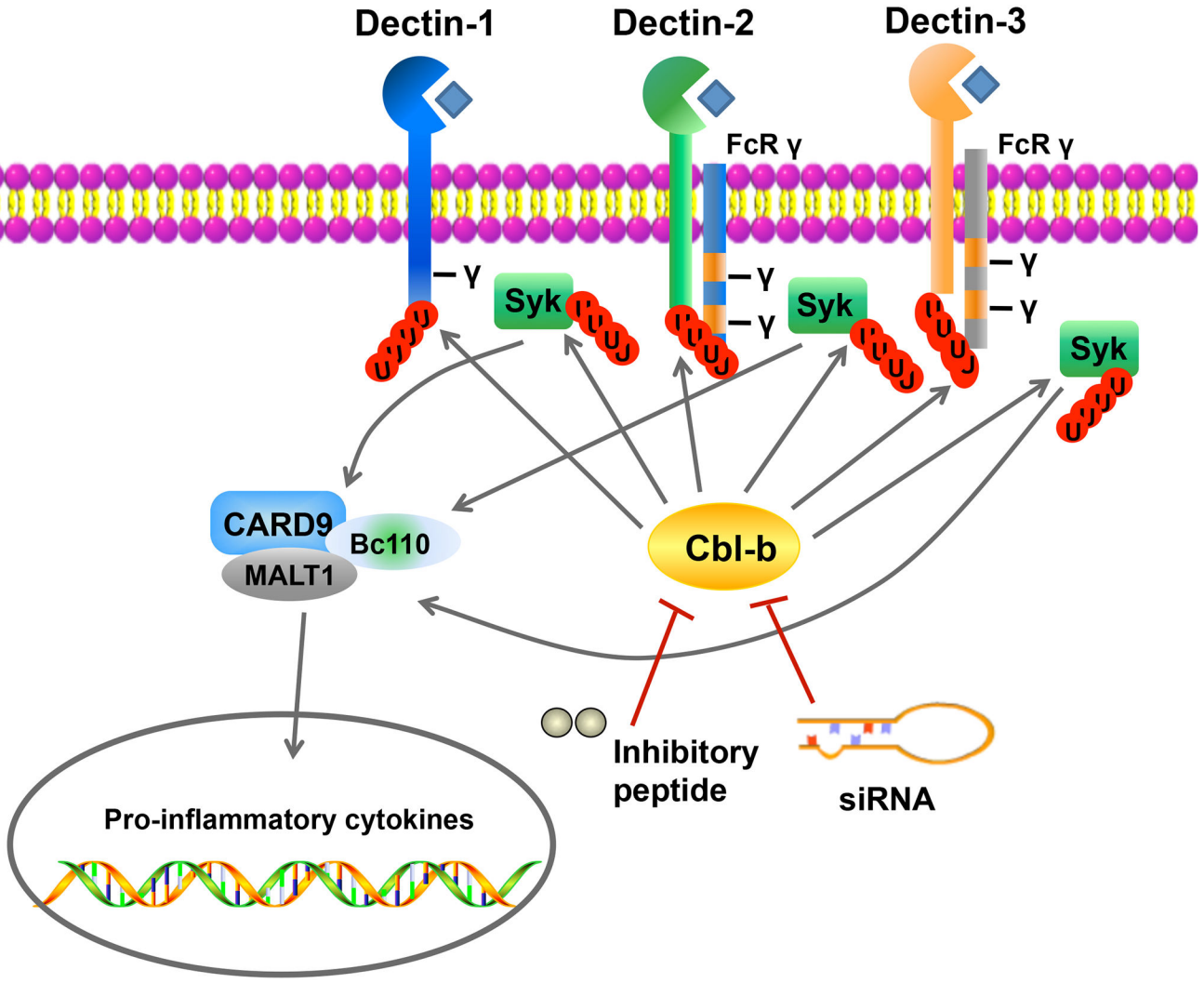

Figure 5. Cbl-b in CLR signaling.

Cbl-b targets CLRs Dectin-1, Dectin-2, Dectin-3, and Syk for K48-linked polyubiquitination, leading to degradation of these molecules. Therefore, the downstream signaling mediated by CARD9/Bcl-10/MALT1 complex is inhibited, which results in the suppression of inflammatory responses against fungal pathogens in the presence of Cbl-b. Moreover, inhibition of Cbl-b by small inhibitory peptides or *Cblb*-specific siRNA can enhance innate antifungal immunity, thus providing a potential therapeutic strategy for fungal infections.