

E3 ubiquitin ligase Cbl-b negatively regulates C-type lectin receptor-mediated antifungal innate immunity

Le-Le Zhu,^{1,2*} Tian-Ming Luo,^{1*} Xia Xu,² Ya-Hui Guo,¹ Xue-Qiang Zhao,¹ Ting-Ting Wang,⁴ Bing Tang,⁵ Yuan-Ying Jiang,⁶ Jin-Fu Xu,³ Xin Lin,^{1,4} and Xin-Ming Jia²

¹Institute for Immunology, Department of Basic Medical Sciences, Tsinghua University School of Medicine, Beijing 100084, China

²Clinical Translational Research Center and ³Department of Respiratory Medicine, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai 200433, China

⁴Department of Molecular and Cellular Oncology, University of Texas MD Anderson Cancer Center, Houston, TX 77030

⁵Department of Burns, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou 510080, China

⁶School of Pharmacy, Second Military Medical University, Shanghai 200433, China

Activation of various C-type lectin receptors (CLRs) initiates potent proinflammatory responses against various microbial infections. However, how activated CLRs are negatively regulated remains unknown. In this study, we report that activation of CLRs Dectin-2 and Dectin-3 by fungi infections triggers them for ubiquitination and degradation in a Syk-dependent manner. Furthermore, we found that E3 ubiquitin ligase Casitas B-lineage lymphoma protein b (Cbl-b) mediates the ubiquitination of these activated CLRs through associating with each other via adapter protein FcR- γ and tyrosine kinase Syk, and then the ubiquitinated CLRs are sorted into lysosomes for degradation by an endosomal sorting complex required for transport (ESCRT) system. Therefore, the deficiency of either Cbl-b or ESCRT subunits significantly decreases the degradation of activated CLRs, thereby resulting in the higher expression of proinflammatory cytokines and inflammation. Consistently, Cbl-b-deficient mice are more resistant to fungi infections compared with wild-type controls. Together, our study indicates that Cbl-b negatively regulates CLR-mediated antifungal innate immunity, which provides molecular insight for designing antifungal therapeutic agents.

C-type lectin receptors (CLRs) including Dectin-2 and Dectin-3 (also called CLEC4E, MCL [macrophage C-type lectin], or *Clec4d*) have been shown to play critical roles as pattern recognition receptors for both antifungal and antimycobacterial immunity (Ishikawa et al., 2009; Robinson et al., 2009; Saijo et al., 2010; Zhu et al., 2013; Zhao et al., 2014). Dectin-2 recognizes α -mannans from *Candida albicans* hyphae and mannose-capped lipoarabinomannan (Man-LAM) from *Mycobacterium tuberculosis* (Saijo et al., 2010; Yonekawa et al., 2014). Dectin-3 can recognize α -mannans from *C. albicans* hyphae and trehalose 6,6'-dimycolate (TDM), a cell wall component from *M. tuberculosis* (Ishikawa et al., 2009; Zhu et al., 2013; Zhao et al., 2014). Our previous study shows that Dectin-2 and Dectin-3 can form heterodimers to enhance sensitivities for binding α -mannans, which suggests that CLR collaboration provides various diversities for a host immune system to sense microbial infections (Zhu et al., 2013).

After engagement by α -mannans, Dectin-2 and Dectin-3 recruit the tyrosine kinase Syk through the immunoreceptor tyrosine-based activation motif (ITAM)-containing adapter FcR- γ to form the CLR complex (Sato et al., 2006; Graham et al., 2012). Syk contains tandem N-terminal Src homology 2 (SH2) and C-terminal SH2 domains followed by a C-terminal kinase domain. Structural and biochemical analyses suggest that the SH2 domains must bind to the phosphorylated Tyr-X-X-Ile/Leu (X indicates any amino acid) sequences within an ITAM to activate Syk through an SH2 domain-containing protein-tyrosine phosphatase-2 (SHP-2; Mócsai et al., 2010; Deng et al., 2015). Once the CLR complex is formed, Syk becomes phosphorylated and activated through an intermolecular autophosphorylation mechanism (Mócsai et al., 2010). The activated Syk further activates phospholipase C- γ 2 (PLC- γ 2) and protein kinase C- δ (PKC- δ), which phosphorylates the adapter caspase recruitment domain containing protein 9 (CARD9; Gorjestani et al., 2011; Strasser et al., 2012) and results in assembly of the complex of CARD9, B cell leukemia-lymphoma 10 (Bcl10), and mucosa-associated lymphoid tissue 1 (Malt1; Gross et al., 2006; Hara and Saito, 2009). The CARD9-Bcl10-Malt1 complex is responsible for activation of the canonical pathway of TAK1-IKK-NF- κ B (Bi et al., 2010; Gorjestani et al., 2012), which

*L.-L. Zhu and T.-M. Luo contributed equally to this paper.

Correspondence to Xin-Ming Jia: jiaxm@tongji.edu.cn; or Xin Lin: xlin@mdanderson.org

Abbreviations used: CARD9, caspase recruitment domain containing protein 9; Cbl-b, Casitas B-lineage lymphoma protein b; CLR, C-type lectin receptor; dKO, double KO; ESCRT, endosomal sorting complex required for transport; iBMDM, immortalized BMDM; ITAM, immunoreceptor tyrosine-based activation motif; Man-LAM, mannose-capped lipoarabinomannan; MOI, multiplicity of infection; MVB, multivesicular body; sgRNA, small guide RNA; SH2, Src homology 2; STAM, signal-transducing adapter molecule; TDM, trehalose 6,6'-dimycolate.

© 2016 Zhu et al. This article is distributed under the terms of an Attribution-Noncommercial-Share Alike-No Mirror Sites license for the first six months after the publication date (see <http://www.rupress.org/terms>). After six months it is available under a Creative Commons License (Attribution-Noncommercial-Share Alike 3.0 Unported license, as described at <http://creativecommons.org/licenses/by-nc-sa/3.0/>).

induces the expression of inflammatory cytokines, including IL-1 β , IL-6, IL-23, IL-12, and TNF- α and chemokines including CXCL1, CXCL2, and CCL3 (Gross et al., 2006; Sato et al., 2006; Robinson et al., 2009; Saijo et al., 2010; Zhu et al., 2013). Although many studies have been focusing on characterizing the signaling induced by different CLRs (Sancho and Reis e Sousa, 2012), how CLR signaling is negatively regulated remains to be determined.

Accumulating evidence suggests that E3 ubiquitin protein ligases are crucial regulators in innate and adaptive immunity (Qingjun et al., 2014; Lutz-Nicoladoni et al., 2015). Among E3 ligases, Casitas B-lineage lymphoma protein b (Cbl-b) is ubiquitously expressed in all leukocyte subsets and negatively regulates several activation signaling pathways derived from TCRs (Naramura et al., 2002; Shamim et al., 2007), BCRs (Sohn et al., 2003), CD28 (co-stimulation molecule; Chiang et al., 2000), TLR4 (Han et al., 2010), Fc ϵ R1 (high-affinity Ig ϵ receptor; Zhang et al., 2004), and epidermal growth factor receptors (Ettenberg et al., 1999). Cbl-b can specifically bind to proteins containing specific phosphorylated tyrosine-containing motifs, such as Zap-70 and Syk, for ubiquitin conjugation (Elly et al., 1999; Zhang et al., 1999; Sohn et al., 2003). After ubiquitin activation, Cbl-b transfers activated ubiquitin to the ϵ amino group of a lysine (K) residue on its protein substrates, which regulates their functions and fates.

Generally, proteins that are polyubiquitinated through Lys48 (K48) linkage are degraded in the 26S proteasome, whereas protein monoubiquitination (or multiubiquitination) serves as a sorting signal targeting membrane proteins for the internalization, endosome to lysosome trafficking, and subsequent degradation in lysosomes. The internalized protein can either recycle to the plasma membrane or sort into the multivesicular body (MVB) within endosomes, which ultimately fuse with lysosomes for protein degradation. These two different fates are dependent on which route the ubiquitinated proteins utilize to enter into the cell. One of the ubiquitin-dependent down-regulation events of receptor signaling is through the endosomal sorting complex required for transport (ESCRT) machinery (Wegner et al., 2011), which comprises four main distinct complexes (ESCRT-0, -I, -II, and -III) and several accessory components recognizing and delivering ubiquitinated membrane proteins into the MVB (Wegner et al., 2011). The upstream complexes of ESCRT-0, -I, and -II contain ubiquitin-binding domains that are responsible for interactions with ubiquitinated proteins and membrane budding into the lumen of the MVB. Therefore, a major consequence of the internalization of activated receptors is their trafficking to lysosomes where they undergo degradation. This provides a mechanism of signal termination that is essential for regulating signaling outputs from activated receptors under physiological conditions (Wegner et al., 2011).

Here, we find that Cbl-b facilitates the ubiquitination of Dectin-2 or Dectin-3 after activation by α -mannans on the surfaces of *C. albicans* hyphae. Furthermore, these

activated CLRs are sorted into lysosomes for degradation through the ESCRT system. Our study indicates that CLRs are negatively regulated through ubiquitination-lysosome-mediated degradation by both Cbl-b and ESCRTs, which down-regulate innate immune and inflammatory responses against fungi infections.

RESULTS

Activated Dectin-2 and Dectin-3 by α -mannan or *C. albicans* hyphae are ubiquitinated and degraded

Earlier studies showed that cross-linking of either Dectin-2 or Dectin-3 by their ligands leads to a rapid internalization, suggesting that both Dectin-2 and Dectin-3 function as endocytic CLRs (Arce et al., 2004; Robinson et al., 2009). However, little is known about the mechanism controlling Dectin-2 and Dectin-3 protein dynamics in the cell. Ubiquitination of cell surface receptors in eukaryotic cells has been shown to control receptor internalization (Wegner et al., 2011). To establish whether the activation-induced endocytosis of Dectin-2 and Dectin-3 was a result of signal-induced ubiquitination, mouse BMDMs were challenged with *C. albicans* hyphae for different times. The lysates from these cells were immunoprecipitated with either anti-Dectin-2 or -Dectin-3, and immunoprecipitates were probed with ubiquitin antibodies that recognize monoubiquitin and several forms of polyubiquitin chains. A high molecular weight smear, typical of ubiquitinated proteins, was specifically observed from ~55 to 170 kD in mouse BMDMs upon hyphae stimulation (Fig. 1 A). We next examined whether mouse Dectin-2 and Dectin-3 would be degraded after ligand-induced internalization and found that the amount of Dectin-2 and Dectin-3 were greatly diminished relative to untreated BMDMs in a time-dependent manner (Fig. 1 B). Consistently, the highest levels of human Dectin-2 or Dectin-3 ubiquitination were observed in RAW264.7 cells overexpressing Dectin-2 or Dectin-3, respectively, upon stimulation with α -mannan (Fig. 1, C and D). A comparable high molecular weight smear was observed when probed with a K48-linked polyubiquitin chain-specific antibody, indicating that both Dectin-2 and Dectin-3 were conjugated with K48 polyubiquitin chains in vivo (Fig. 1 E). In contrast, Dectin-2 or Dectin-3 immunoprecipitates showed no signal when using a K63-linked polyubiquitin chain-specific antibody (not depicted), indicating that both Dectin-2 and Dectin-3 are not conjugated with K63-linked polyubiquitin chains.

To identify ubiquitination sites in Dectin-2 and Dectin-3, we performed Blast analysis and found that the intracellular domain of Dectin-2 and Dectin-3 from the human, mouse, and rat contains a highly conserved lysine residue (not depicted), which is supposed to be a critical site for ubiquitin modification. To examine the biological role of human Dectin-2 K12 and Dectin-3 K9 ubiquitination, we stably transfected RAW264.7 cells with WT Dectin-2 and Dectin-3 or their mutants, in which residue K12 in Dectin-2 and K9 in Dectin-3 were substituted with a nonubiquitinable Ala res-

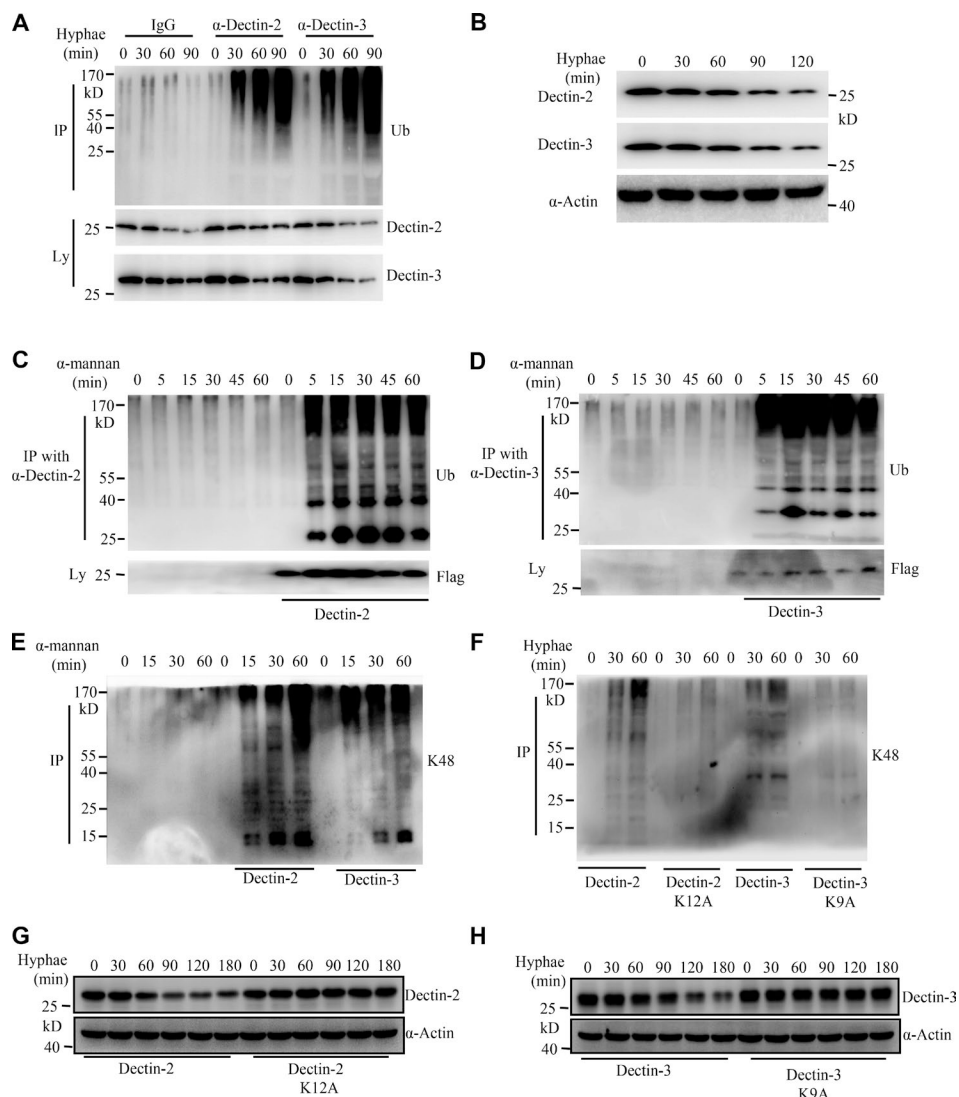


Figure 1. Activated Dectin-2 and Dectin-3 can trigger themselves for ubiquitination and degradation. (A and B) Hyphae-induced ubiquitination and degradation of Dectin-2 and Dectin-3 in mouse BMDMs. (C and D) α -Mannan-induced ubiquitination of Dectin-2 and Dectin-3 in RAW264.7 cells overexpressing Dectin-2 or Dectin-3. (E) α -Mannan-induced K48 ubiquitination of Dectin-2 and Dectin-3 in RAW264.7 cells overexpressing Dectin-2 and Dectin-3. (F) Hyphae-induced K48 ubiquitination of Dectin-2 and Dectin-3 in RAW264.7 cells overexpressing mutant Dectin-2_{K12A} and Dectin-3_{K9A}. Immunoprecipitation was performed using anti-Dectin-2 or -Dectin-3 antibodies on solubilized protein extracts from BMDMs or RAW264.7 cells and subjected to immunoblotting with the indicated antibodies. (G and H) Hyphae-induced degradation levels in RAW264.7 cells expressing WT or mutant Dectin-2 (G) and Dectin-3 (H), which were pretreated with 10 μ g/ml cycloheximide for 15 min and then stimulated with hyphae (multiplicity of infection [MOI] = 1) for the indicated times. The cell lysates were subjected to immunoblotting using the indicated antibodies. The data shown are representative of three independent and reproducible experiments. IP, immunoprecipitation; Ly, lysis; Ub, ubiquitination.

idue (Dectin-2_{K12A} and Dectin-3_{K9A}, respectively). In contrast to what is observed for the WT form of Dectin-2 and Dectin-3, Dectin-2_{K12A} and Dectin-3_{K9A} showed significantly reduced ubiquitination after hyphae stimulation (Fig. 1 F). Furthermore, stimulation of RAW264.7 cells expressing WT Dectin-2 or Dectin-3 with *C. albicans* hyphae triggered a significant degradation of Dectin-2 or Dectin-3, respectively (Fig. 1, G and H). But the K12A mutant of Dectin-2 and the K9A mutant of Dectin-3 were more resistant to the hyphae-induced degradation of Dectin-2 or Dectin-3 in these

cells (Fig. 1, G and H). These data suggest that the intracellular lysine residue of Dectin-2 and Dectin-3 is a major ubiquitination site. Together, these data suggest that Dectin-2 and Dectin-3 are activated by their ligands, which trigger themselves for ubiquitination and degradation.

Cbl-b mediates the ubiquitination and degradation of Dectin-2 and Dectin-3

We next focused on the possible molecular mediators of Dectin-2 and Dectin-3 ubiquitination. Extensive work shows that

the tyrosine kinase Syk is activated and recruited to Dectin-2 and Dectin-3 through the ITAM-containing adapter FcR- γ after ligand engagement (Sato et al., 2006; Lobato-Pascual et al., 2013). Because the E3 ubiquitin ligase Cbl-b can be specifically recruited to receptor proteins containing specific phosphorylated tyrosine-containing motifs, such as Syk, for ubiquitin conjugation (Elly et al., 1999; Zhang et al., 1999; Sohn et al., 2003), we hypothesized that Cbl-b may be involved in Dectin-2 and Dectin-3 ubiquitination and degradation. To test this hypothesis, we first examined whether Dectin-2 or Dectin-3 is associated with Cbl-b in mouse BMDMs and found that both Dectin-2 and Dectin-3 could inducibly associate with Cbl-b after stimulation with *C. albicans* hyphae (Fig. 2, A and B). We then examined the effect of Cbl-b deficiency on Dectin-2 and Dectin-3 ubiquitination and found that Cbl-b deficiency significantly impaired hyphae-induced ubiquitination of Dectin-2 and Dectin-3 (Fig. 2, C and D). Consistently, the signal-induced degradation of Dectin-2 and Dectin-3 was almost completely blocked in Cbl-b-deficient cells (Fig. 2 E). However, no significant degradation of Mincle, Syk, and SHP-2 was observed in both WT and Cbl-b-deficient BMDMs after stimulation with *C. albicans* hyphae (Fig. 2 F).

To further confirm that hyphae-induced degradation of Dectin-2 and Dectin-3 is in a Cbl-b-dependent manner, we examined the surface expressions of Dectin-2 and Dectin-3 on innate cells under nonendocytic conditions after hyphae stimulations as reported previously by us (Zhu et al., 2013). We found that both Dectin-2 and Dectin-3 appeared on the cell surface of untreated WT BMDMs under nonendocytic conditions at 4°C after incubation with anti-Dectin-2 or -Dectin-3 monoclonal antibody (Fig. 2, G and H). However, stimulation with *C. albicans* hyphae induced a significant reduction of surface Dectin-2 or Dectin-3 in WT BMDMs, and Cbl-b deficiency completely blocked the hyphae-induced reduction of surface Dectin-2 or Dectin-3 at the time points 60 and 120 min (Fig. 2, G and H). Together, these data indicate that Cbl-b plays a critical role in the signal-induced ubiquitination and degradation of Dectin-2 and Dectin-3.

Syk, but not CARD9, is required for Cbl-b-mediated ubiquitination and degradation of Dectin-2 and Dectin-3

We next investigated the molecular mechanisms by which Cbl-b drives Dectin-2 and Dectin-3 ubiquitination and degradation. Several CLRs, including Dectin-2 and Dectin-3, are coupled to Syk kinase, which allows these receptors to signal via CARD9, leading to NF- κ B and MAPK activation (Gross et al., 2006; Zhu et al., 2013). The activation of these downstream signaling effectors contributes to the induction of both innate and adaptive immunity against fungal infections. To determine whether Syk is required for Cbl-b-dependent ubiquitination and degradation of Dectin-2 and Dectin-3, WT mouse BMDMs were treated with or without the Syk inhibitor piceatannol. We found that Cbl-b was inducibly phosphorylated, whereas piceatannol treatment abolished this phosphorylation (Fig. 3 A). Furthermore, Cbl-b was inducibly associated with Syk after hyphae stimulation, and this associa-

tion was dependent on its kinase activity because piceatannol treatment also blocked this association (Fig. 3 B). Interestingly, piceatannol treatment not only inhibited the association of Dectin-2 and Dectin-3 with Cbl-b and Syk (Fig. 3, C and D), but also blocked the signal-induced polyubiquitination of Dectin-2 and Dectin-3 (Fig. 3, C–E). Together, these results indicate that the hyphae-induced phosphorylation of Syk plays a direct role in Cbl-b-mediated polyubiquitination and degradation of Dectin-2 and Dectin-3.

Interestingly, we found that Cbl-b could not directly associate with Dectin-2 and Dectin-3 or FcR- γ , an ITAM-containing adapter protein, when they were coexpressed in HEK293 T cells (Fig. 3 F, first three lanes). But Cbl-b could associate with Syk when they were coexpressed in these cells (Fig. 3 F, fourth lane). However, Cbl-b could associate with FcR- γ in the presence of Syk (Fig. 3 F, seventh lane). Furthermore, Cbl-b could associate with Dectin-2 or Dectin-3 when both Syk and FcR- γ were coexpressed in these cells (Fig. 3 F, eighth and ninth lanes). These results suggest that Dectin-2/Dectin-3 forms a complex with Cbl-b via FcR- γ and Syk.

However, we found that CARD9 deficiency in BMDMs had no effects on hyphae-induced polyubiquitination and degradation of Dectin-2 and Dectin-3 compared with those in WT BMDMs (Fig. 3, G and H). These data suggest that CARD9 is not required for the down-regulation of Dectin-2 and Dectin-3.

Cbl-b negatively regulates CLR-mediated innate immune responses triggered by α -mannans or *C. albicans* hyphae

To investigate the functional role of Cbl-b in CLR-induced signaling pathways and cytokine production, we challenged BMDMs from WT (Cbl-b^{+/+}) or Cbl-b-deficient (Cbl-b^{-/-}) mice with α -mannans, the ligand of Dectin-2 and Dectin-3, and found that Cbl-b deficiency significantly increased the nuclear translocation of NF- κ B (p65 subunit) after α -mannan stimulation (Fig. 4, A and B). Furthermore, we found that Cbl-b deficiency in BMDMs significantly elicited a higher amount of TNF secretion after stimulation with α -mannans at the indicated concentrations (Fig. 4 C). Similarly, Cbl-b deficiency in BMDMs significantly increased α -mannan-induced production of different proinflammatory cytokines including IL-6, IL-1 β , and IL-12p40 compared with that in WT BMDMs ($P < 0.05$; Fig. 4 D). When stimulated with Man-LAM, another ligand of Dectin-2, or TDM, another ligand of Dectin-3, Cbl-b deficiency in BMDMs also significantly increased TNF and IL-6 secretions (Fig. 4, E and F). Collectively, these data suggest that Cbl-b also negatively regulated innate immune and inflammatory responses induced by other CLRs such as Mincle.

To determine whether Cbl-b negatively regulates innate immune responses through activated CLR signaling, we blocked the functions of Dectin-2 or Dectin-3 using their specific monoclonal antibodies described previously (Zhu et al., 2013) in WT and Cbl-b-deficient BMDMs. We found

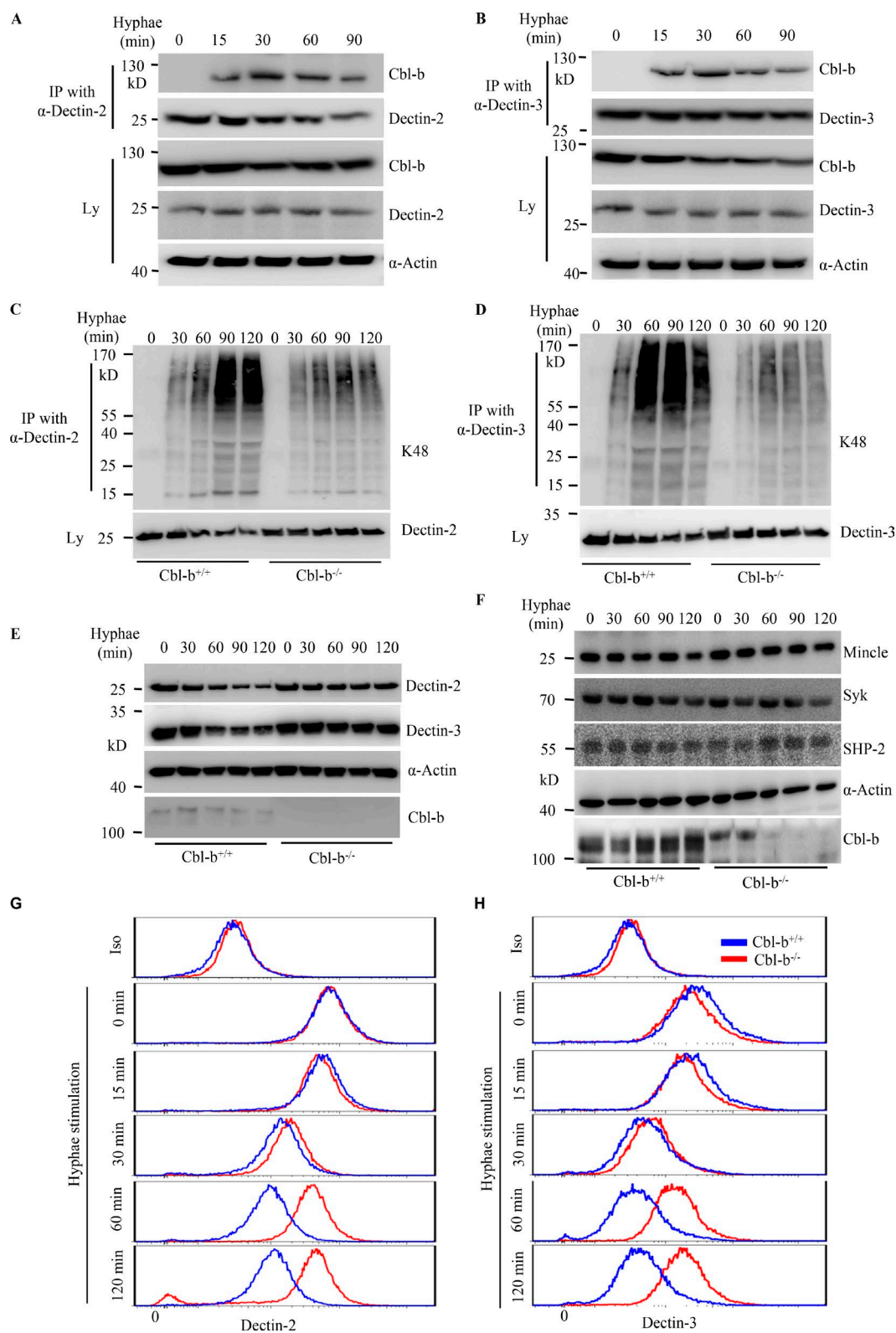


Figure 2. Cbl-b mediates ubiquitination and degradation of Dectin-2 and Dectin-3. (A and B) Association analyses of Cbl-b with Dectin-2 or Dectin-3 in mouse BMDMs, which were stimulated with *C. albicans* hyphae for the indicated times. (C and D) Hyphae-induced ubiquitination of Dectin-2 and Dectin-3 in WT and Cbl-b-deficient BMDMs. (E) Hyphae-induced degradation of Dectin-2 and Dectin-3 in WT and Cbl-b-deficient BMDMs.

that blockade of either Dectin-2 or Dectin-3 significantly impaired *C. albicans* hyphae-induced TNF and IL-6 secretions in WT BMDMs as well as Cbl-b-deficient BMDMs compared with their controls (Fig. 4 G). Because CARD9 is a critical adapter protein that functions downstream of several ITAM-associated CLRs, including Dectin-2 and Dectin-3 (Bi et al., 2010; Graham et al., 2012; Zhao et al., 2014), we bred CARD9 and Cbl-b double-deficient mice (double KO [dKO]) and then challenged BMDMs from CARD9 KO, Cbl-b KO, or CARD9/Cbl-b dKO mice with *C. albicans* hyphae. As expected, Cbl-b deficiency in WT BMDMs significantly induced higher levels of TNF and IL-6 secretion after the stimulation, whereas CARD9 KO in both the WT and Cbl-b KO background completely blocked hyphae-induced TNF and IL-6 production (Fig. 4 H). Together, these data suggest that Cbl-b mediated negative regulation of CLR signaling functions upstream of CARD9-dependent proinflammatory responses.

Activated Dectin-2 and Dectin-3 are sorted into lysosomes by the ESCRT system for degradation

Because the ubiquitinated receptors can be internalized and then degraded through either proteasomes or lysosomes, we decided to determine which organelle is responsible for the degradation of the ubiquitinated Dectin-2 and Dectin-3. We pretreated BMDMs from WT mice with the proteasome inhibitor MG132 or lysosome inhibitor chloroquine and then stimulated the pretreated cells with *C. albicans* hyphae (Fig. 5 A). We found that chloroquine treatment completely inhibited the degradation of activated Dectin-2 and Dectin-3, but the MG132 treatment had no influences on their degradations compared with their unpretreated controls (Fig. 5 A), suggesting that the activated Dectin-2 and Dectin-3 are degraded through a lysosome-mediated pathway.

To further investigate the molecular mechanism by which the ubiquitinated Dectin-2 and Dectin-3 are targeted to lysosome-mediated degradation, we tested whether the ESCRT system is involved in the trafficking of the activated Dectin-2 and Dectin-3 from endosomes to lysosomes. First, we knocked down the expression of ESCRT-0 subunits STAM (signal-transducing adapter molecule) and HRS (hepatocyte growth factor-regulated tyrosine kinase substrate) and ESCRT-I subunits Vps28 and Tsg101 in immortalized BMDM (iBMDM) cells using siRNA (not depicted). Because all of them only partially suppressed the expression of these genes, we decided to use a mixture of these siRNA against STAM, HRS, VPS28, and Tsg101 to knock down

their expression in iBMDMs at the same time and then stimulated these cells with *C. albicans* hyphae (Fig. 5, B–D). We found that knockdown expression of the ESCRT-0 and -1 subunits significantly reduced the degradation of hyphae-activated Dectin-2 and Dectin-3 compared with their mock controls at the later time points 4 and 6 h (Fig. 5 B), suggesting that the ESCRT system is responsible for the trafficking of the ubiquitinated Dectin-2 and Dectin-3 from endosomes to lysosomes. Consequently, knockdown of these genes significantly increased the hyphae-induced levels of the NF- κ B subunit (p65) nuclear translocation (Fig. 5 C) and proinflammation cytokines TNF, IL-6, and IL-12p40 for 6 h compared with their controls (Fig. 5 D). Together, these data suggest that the ESCRT system is critical for the degradation of the activated Dectin-2 and Dectin-3.

STAM negatively regulates CLR-mediated innate immune responses triggered by their ligands

To further confirm the role of the ESCRT system in the trafficking of ubiquitinated CLRs from endosomes to lysosomes using genetic methods, we disrupted the ESCRT-0 subunit STAM gene in iBMDM cells using the CRISPR/Cas9 system (Shalem et al., 2014), and STAM protein was almost invisible in STAM-deficient iBMDMs (Fig. 5 E). We then stimulated these cells with α -mannan, Man-LAM, and TDM, the ligands for Dectin-2/Dectin-3, Dectin-2, and Dectin-3. We found that STAM deficiency maintained a higher level of p65 nuclear translocation induced by α -mannan, Man-LAM, or TDM (Fig. 5, F–H) and proinflammation cytokines such as TNF compared with their controls (Fig. 5 I). These data suggest that STAM plays a critical role for the degradation of the down-regulation of activated Dectin-2 and Dectin-3. In addition, we also used the same approach to disrupt the ESCRT-0 subunit HRS and ESCRT-I subunit Vps28 genes in iBMDM cells. Similar to STAM deficiency, HRS and Vps28 deficiency in iBMDMs also significantly increased α -mannan-, Man-LAM-, or TDM-induced TNF expression compared with their controls (Fig. 5 I). Collectively, these data suggest that the ESCRT system plays a critical role for the degradation of the activated CLRs, thereby negatively regulating the CLR-mediated innate immune response.

Cbl-b negatively regulates in vivo host immune defense against *C. albicans* and *Aspergillus fumigatus* infections

To examine the effect of Cbl-b-mediated immune responses on the host's ability to clear systemic *C. albicans* infections, we challenged WT or Cbl-b-deficient mice with a sublethal dose

(F) Hyphae-induced degradation of Mincle, Syk, and SHP-2 in WT and Cbl-b-deficient BMDMs. Cell lysates were immunoprecipitated with anti-Dectin-2 or -Dectin-3 antibodies as indicated. The cell lysate and immunoprecipitates were subjected to immunoblotting using the indicated antibodies. (G and H) Surface expression levels of Dectin-2 (G) or Dectin-3 (H) in WT and Cbl-b-deficient BMDMs, which were stimulated with *C. albicans* hyphae (MOI = 1) for the indicated times. BMDMs were incubated with anti-Dectin-2, anti-Dectin-3, or isotype control IgG for 30 min at 4°C and then stained with FITC-labeled goat anti-mouse secondary antibodies. Samples were then examined for FITC intensity by flow cytometry. The data shown are representative of three independent and reproducible experiments. IP, immunoprecipitation; Iso, isotype; Ly, lysis.

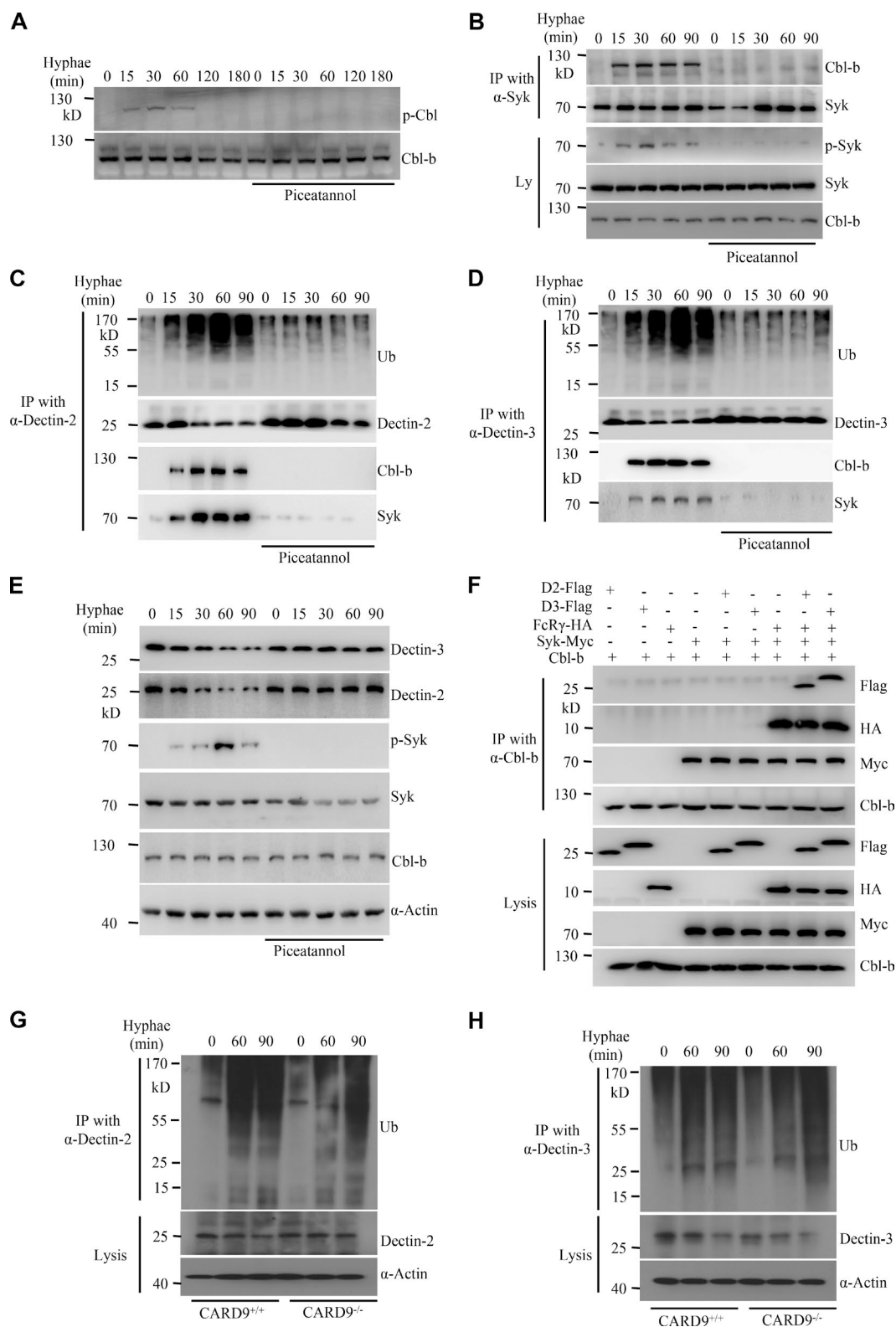


Figure 3. Syk is required for Cbl-b-mediated ubiquitination and degradation of Dectin-2 and Dectin-3. (A) Hyphae-induced phosphorylation of Cbl-b in mouse BMDMs, which were pretreated with or without Syk inhibitor (picetannol) for 30 min. p-Cbl, phosphorylated Cbl. (B) Hyphae-induced association analyses of Cbl-b with Dectin-2 or Dectin-3 in mouse BMDMs, which were pretreated with or without Syk inhibitor (picetannol) for 30 min. (C and D) Hyphae-induced ubiquitination of Dectin-2 and Dectin-3 in mouse BMDMs, which were pretreated with or without Syk inhibitor (picetannol)

(10^5) of *C. albicans* strain SC5314 and found that all Cbl-b-deficient mice survived for 15 d after infection, whereas only 30% of WT mice survived at that time ($P < 0.001$; Fig. 6 A). Consistently, the fungal burdens in the kidneys of Cbl-b-deficient mice were significantly lower than those in WT mice after infection for 24 h ($P < 0.01$; Fig. 6 B). In contrast, Cbl-b-deficient mice had significantly higher levels of proinflammation cytokines TNF and IL-6 as well as chemokines CXCL1, CXCL2, and CCL3 in their kidneys after infection for 24 h (Fig. 6, C and D). These data suggest that Cbl-b negatively regulates host immune responses for the clearance of *C. albicans* infections.

Previous studies show that infection of *A. fumigatus*, another opportunistic fungus pathogen, induces CARD9-dependent innate immune response (Jhingran et al., 2012), and human plasmacytoid DCs can directly recognize *A. fumigatus* hyphae via Dectin-2 to elicit cytokine release and antifungal activity (Loures et al., 2015). Therefore, we examined the effect of Cbl-b deficiency on the host's ability to clear pulmonary *A. fumigatus* infection and found that the fungal burdens in lungs of Cbl-b-deficient mice were significantly lower than those in WT mice after infection for 24 h ($P < 0.001$; Fig. 6 E). In contrast, Cbl-b-deficient mice had significantly higher levels of proinflammation cytokine TNFs in their lungs after infection (Fig. 6 F). These data indicate that Cbl-b also negatively regulates host innate immune responses for the clearance of *A. fumigatus* infection.

Cbl-b regulates CLR/CARD9-mediated antifungal innate immune regulation

To determine whether Cbl-b negatively regulates host immune responses through activated CLR/CARD9 signaling, we bred Dectin-3 and Cbl-b dKO mice and then challenged Dectin-3 KO, Cbl-b KO, or Dectin-3/Cbl-b dKO mice with a sublethal dose (10^5) of *C. albicans* strain SC5314. Interestingly, Dectin-3 deficiency in Cbl-b KO mice significantly increased their survival compared with those of Dectin-3 KO mice after fungal infection ($P < 0.001$; Fig. 7 A). Consistently, the fungal burden in kidneys of Dectin-3/Cbl-b dKO mice was significantly lower than those in Dectin-3 KO mice after infection for 24 h ($P < 0.001$; Fig. 7 A). In contrast, Dectin-3/Cbl-b dKO mice had significantly higher levels of proinflammation cytokines TNF and IL-6 in their kidneys compared with those of Dectin-3 KO mice ($P < 0.001$; Fig. 7 B). These data suggest that the Cbl-b-mediated antifungal effect is only partially through Dectin-3 and that other CLRs may also contribute to this antifungal response.

Because CARD9 is involved in mediating innate immune and inflammatory responses induced by multiple CLRs, including Dectin-2 and Dectin-3, we generated Cbl-b and CARD9 dKO mice. Similarly, we then challenged CARD9 KO, Cbl-b KO, or CARD9/Cbl-b dKO mice with a sublethal dose (10^5) of *C. albicans* strain. As expected, we found that CARD9 deficiency in both the WT and Cbl-b KO background significantly reduced their survival compared with their respective parental strains after infection with *C. albicans* ($P < 0.001$; Fig. 7 C), and fungal burdens in the kidneys of CARD9-deficient and CARD9/Cbl-b double-deficient mice are much higher than those of their respective parental mice when challenged with *C. albicans* for 24 h ($P < 0.001$; Fig. 7 D). Consistently, CARD9 deficiency in both the WT and Cbl-b-deficient background failed to elicit enough proinflammation cytokines TNF, IL-6, IL-1 β , and IL-12p40 to combat *C. albicans* infections (Fig. 7 E). These data demonstrate that CARD9-mediated signaling is critical for activating Cbl-b-mediated regulation of host immune responses against *C. albicans* infections.

DISCUSSION

CLRs, including Dectin-2 and Dectin-3, have been recently characterized as pattern recognition receptors for sensing both fungal and mycobacteria infections (Matsunaga and Moody, 2009; Robinson et al., 2009; Saijo et al., 2010; Zhu et al., 2013; Zhao et al., 2014). In the current study, we found that activation of Dectin-2 and Dectin-3 by their ligand α -mannans from *C. albicans* hyphae can induce their ubiquitination and subsequent degradation in a Syk-dependent manner. Furthermore, we found that Cbl-b, an E3 ubiquitin ligase, mediates the ubiquitination and degradation of the activated Dectin-2 and Dectin-3 to negatively regulate CLR-mediated innate immune responses against fungal infections. Finally, we found that the ESCRT system transports the ligand-activated Dectin-2 and Dectin-3 into lysosomes for degradation, thereby negatively regulating the CLR-mediated innate immune responses. Together, our results indicate that both Cbl-b and ESCRTs are negative regulators of CLR-mediated signals by inducing the ubiquitin-lysosome-mediated degradation of activated CLRs.

Accumulating evidence suggests that Cbl-b-mediated protein ubiquitination and degradation is a crucial regulation mechanism in innate and adaptive cells (Bhoj and Chen, 2009). Our study shows that the activation of Dectin-2 and Dectin-3 by their ligands triggers them for ubiquitination

for 30 min. (E) Hyphae-induced degradation of Dectin-2 and Dectin-3 in mouse BMDMs, which were pretreated with or without Syk inhibitor (piceatanol) for 30 min. (C–E) Cell lysates were immunoprecipitated with the indicated antibodies. (F) HEK293 T cells were transfected with expression vectors encoding Flag–Dectin-2 (D2-Flag), Flag–Dectin-3 (D3-Flag), FcR- γ -HA, Myc-Syk, and Cbl-b in different combinations. Cell lysates were subjected to immunoprecipitation with anti-Myc antibody. The cell lysate and immunoprecipitates were subjected to immunoblots using the indicated antibodies. (G and H) Hyphae-induced ubiquitination of Dectin-2 and Dectin-3 in WT and CARD9-deficient BMDMs. Cell lysates were immunoprecipitated with anti-Dectin-2 or –Dectin-3 antibodies as indicated. The cell lysate and immunoprecipitates were subjected to immunoblotting using the indicated antibodies. The data shown are representative of three independent and reproducible experiments. IP, immunoprecipitation; Ly, lysis; p-Syk, phosphorylated Syk; Ub, ubiquitination.

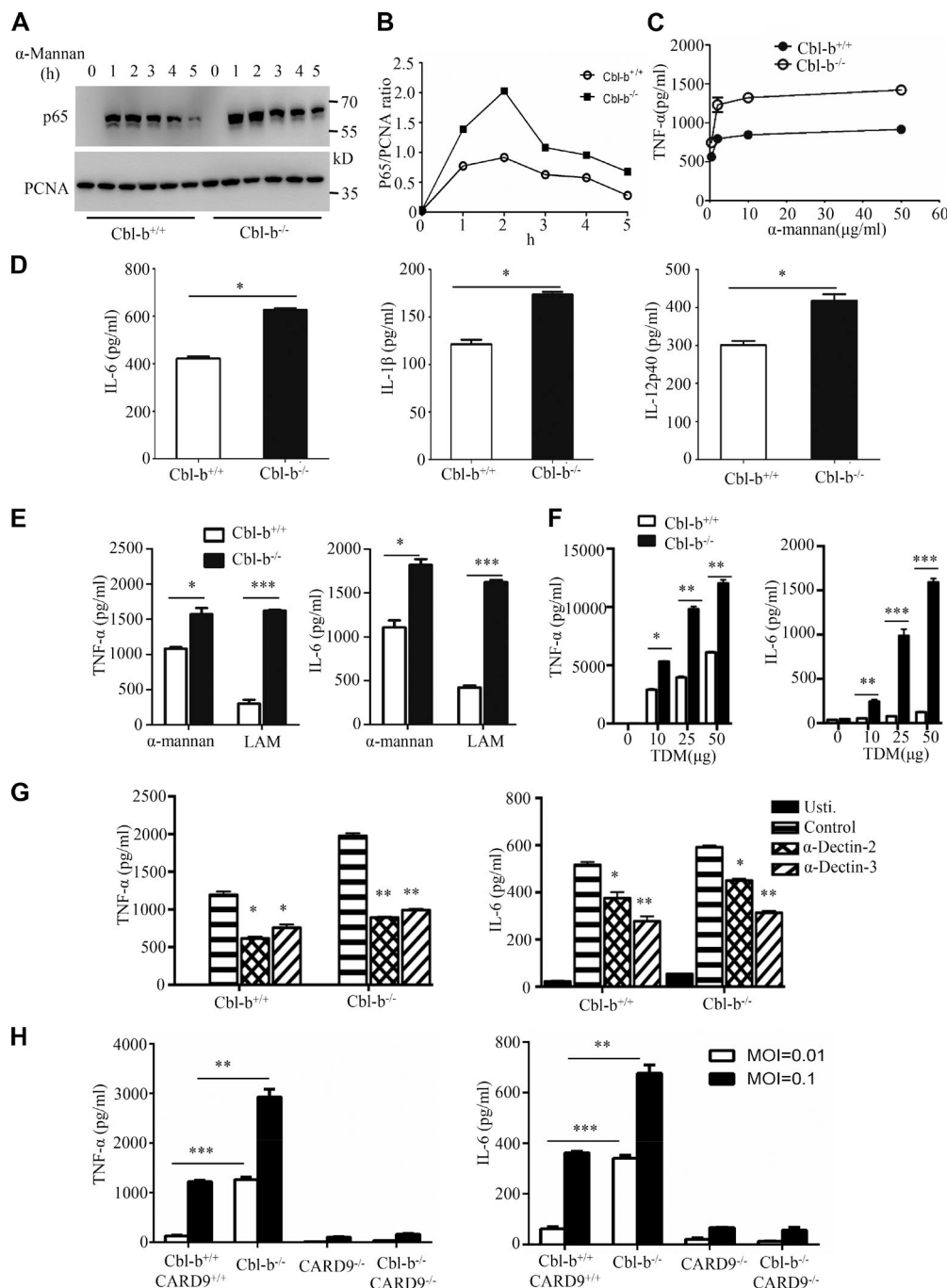


Figure 4. Cbl-b negatively regulates in vitro CLR-mediated innate immune responses. (A) Nuclear p65 in WT and Cbl-b-deficient BMDMs that were stimulated with 40 μg/ml of pre-coated α-mannans for different times. The data shown are representative of three independent and reproducible experiments. (B) Quantification grayscale analysis of p65 versus internal control proliferating cell nuclear antigens (PCNA) in A. (C) ELISA results for TNF in supernatants of WT and Cbl-b-deficient BMDMs, which were stimulated with pre-coated α-mannans at the indicated concentrations for 12 h. (D) ELISA results for IL-6, IL-1β, and IL-12p40 in supernatants of WT and Cbl-b-deficient BMDMs, which were stimulated with 40 μg/ml of pre-coated α-mannans for 12 h. (E and F) ELISA results for TNF and IL-6 in supernatants of WT and Cbl-b-deficient BMDMs, which were stimulated with 40 μg/ml of pre-coated LAM (E) or TDM (F) at the indicated concentrations for 12 h. (G) ELISA results for TNF and IL-6 in supernatants of WT and Cbl-b-deficient BMDMs, which were pretreated with or without anti-Dectin-3 antibody (α-Dectin-3), α-Dectin-2, or an isotype-matched control IgG for 30 min before stimulation with *C. albicans* hyphae (MOI = 0.1) for 12 h. Usti., unstimulated. (H) ELISA results for TNF and IL-6 in supernatants of WT, Cbl-b KO, CARD9 KO, or Cbl-b/CARD9 dKO BMDMs, which were stimulated with *C. albicans* hyphae (MOI = 0.01 or 0.1) for 12 h. Data are means ± SD of triplicate samples and are representative of three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Student's *t* test or ANOVA).

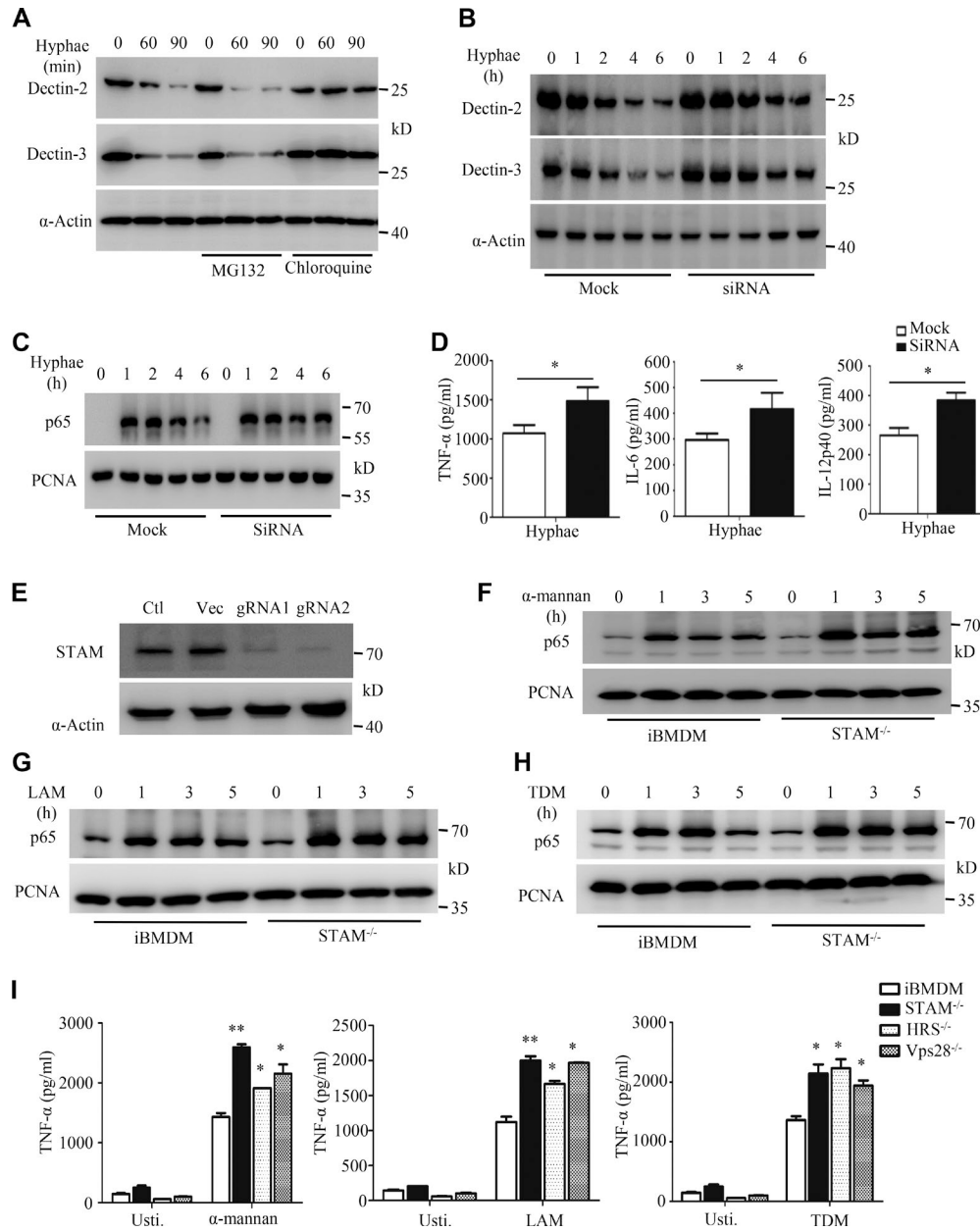


Figure 5. Activated Dectin-2 and Dectin-3 are sorted into lysosomes by the ESCRT system for degradation. (A) Hyphae-induced degradation of Dectin-2 and Dectin-3 in mouse BMDMs, which were pretreated with or without proteasome inhibitor (MG132) or lysosome inhibitor (chloroquine). Cell lysates were probed with the indicated antibodies. (B) Hyphae-induced degradation of Dectin-2 and Dectin-3 in iBMDMs, which were transfected with siRNA against ESCRT-0 subunits (STAM and HRS), ESCRT-I subunits (Vps28 and Tsg101), and nontargeting control siRNA using Trans-IT-TKO transfection reagent (Mirus). (C) Nuclear p65 in iBMDMs, which were transfected with siRNA against ESCRT-0 subunits (STAM and HRS), ESCRT-I subunits (Vps28 and Tsg101), and nontargeting control siRNA before stimulation with *C. albicans* hyphae (MOI = 0.1) for the indicated times. (D) ELISA results for TNF, IL-6, and IL-12p40 in supernatants of iBMDMs, which were transfected with siRNA against ESCRT-0 subunits (STAM and HRS), ESCRT-I subunits (Vps28 and Tsg101), and nontargeting control siRNA before stimulation with *C. albicans* hyphae (MOI = 0.1) for 12 h. (E) STAM protein amounts in WT and STAM-deficient iBMDMs. The ESCRT-0 subunit STAM gene was disrupted in iBMDM cells using the CRISPR/Cas9 system. (F–H) Nuclear p65 in WT and STAM-deficient iBMDMs upon stimulation with 40 μg/ml of precoated α-mannans (F), 40 μg/ml LAM (G), or 50 μg/ml TDM (H) for the indicated times. (I) ELISA results for TNF in supernatants of WT and STAM^{-/-}, HRS^{-/-}, and Vps28^{-/-} iBMDMs upon stimulation with 40 μg/ml of precoated α-mannans, 40 μg/ml LAM, or 50 μg/ml TDM for 12 h. (A–C and E–H) The data shown are representative of three independent and reproducible experiments. (D and I) Data are means ± SD of triplicate samples and are representative of three independent experiments. *, P < 0.05; **, P < 0.01 (Student's t test or ANOVA). Ctl, control; PCNA, proliferating cell nuclear antigen; Usti., unstimulated; Vec, vector.

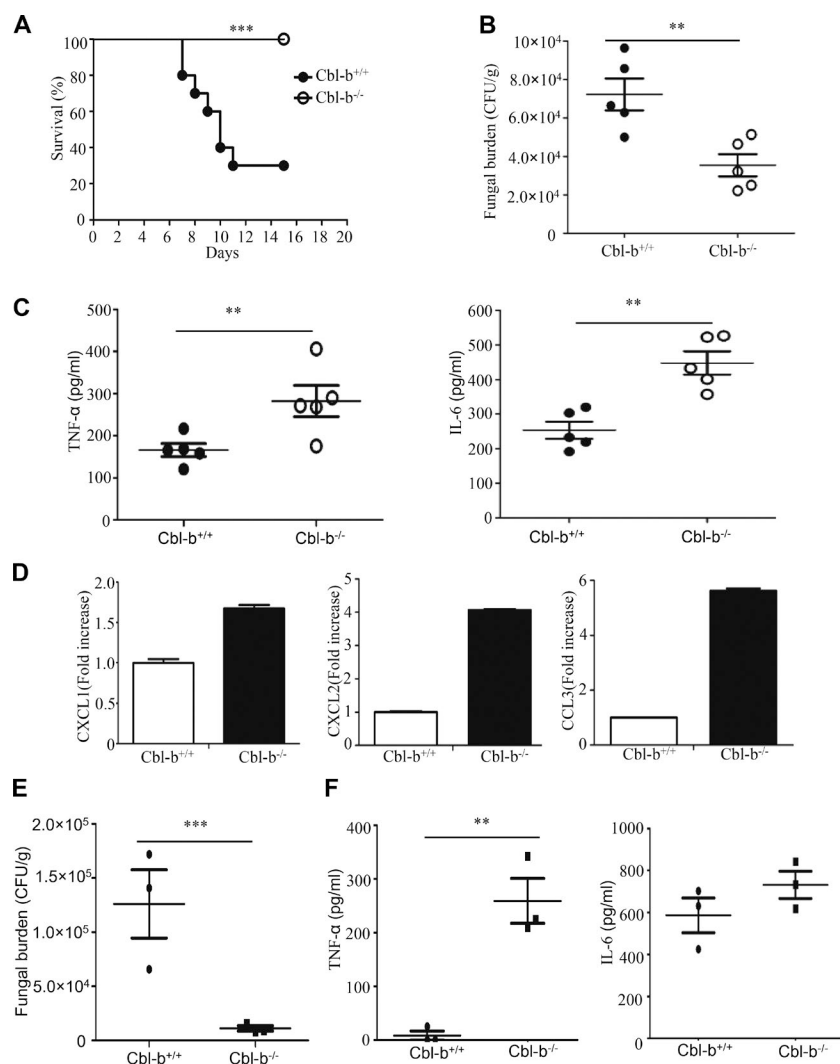


Figure 6. Cbl-b negatively regulates in vivo CLR-mediated antifungal immunity. (A) Survival of WT and Cbl-b KO mice ($n = 10$ per group) infected intravenously with 10⁵ CFU of *C. albicans* (SC5314). (B) Kidney CFU assay of WT and Cbl-b KO mice ($n = 5$ per group) infected intravenously with 10⁵ CFU of *C. albicans* (SC5314) for 24 h. (C) Amounts of TNF and IL-6 in kidneys of infected WT and Cbl-b KO mice after infection with 10⁵ CFU of *C. albicans* (SC5314) for 24 h. (D) Quantitative real-time PCR assay of chemokines including CXCL1, CXCL2, and CCL3 in kidneys of WT and Cbl-b KO mice after infection with 10⁵ CFU of *C. albicans* (SC5314) for 24 h. (E) Lung CFU assay of WT and Cbl-b KO mice ($n = 3$ per group) after trans-nasal infection with 10⁶ CFU of *A. fumigatus* conidia for 24 h. The data shown are representative of three independent and reproducible experiments. (F) Amounts of TNF and IL-6 in lungs of WT and Cbl-b KO mice after trans-nasal infection with 10⁶ CFU of *A. fumigatus* conidia for 24 h. (C, D, and F) Data are means \pm SD of triplicate samples and are representative of three independent experiments. **, $P < 0.01$; ***, $P < 0.001$ (Student's *t* test).

and degradation in macrophages. Mutations of the intracellular highly conserved K residue in Dectin-2 or Dectin-3 completely block their ubiquitination and degradation. Furthermore, Cbl-b mediates ubiquitination of the activated Dectin-2 and Dectin-3 to negatively regulate the CLR-mediated innate immune responses against fungal infections both in vitro and in vivo. Mechanistically, activation of Dectin-2 and Dectin-3 induces phosphorylation of Syk, which recruits Cbl-b to the association with Dectin-2 and Dectin-3 through an ITAM-containing adapter, FcR-γ. Then, Cbl-b facilitates ubiquitination of Dectin-2 and Dectin-3 by K48 polyubiquitin chains. Consistent with the conclusion in the previous paragraph, our data show that Cbl-b deficiency in mouse BMDMs blocks the ligand-induced ubiquitination and degradation of Dectin-2 and Dectin-3, and inhibition of Syk in BMDMs also completely blocks Cbl-b-mediated ubiquitination and degradation of Dectin-2 and Dectin-3. Furthermore, CARD9 deficiency in BMDMs has no effects on hyphae-induced polyubiquitination and degradation of Dectin-2 and Dectin-3, which suggests that CARD9

is not required for hyphae-induced polyubiquitination and degradation of Dectin-2 and Dectin-3. In terms of Cbl-b-mediated physiological effects, we found that Cbl-b deficiency in mouse BMDMs significantly increases CLR-mediated NF-κB activation and proinflammation cytokine secretions after challenge with CLR ligands including α-mannans, Man-LAM, and TDM, and CLR blockade or CARD9 deficiency in Cbl-b-deficient BMDMs could significantly reduce the ligand-induced TNF and IL-6 production. Consistently, we have found that Cbl-b-deficient mice elicited significantly higher levels of proinflammation cytokine in their kidneys to combat fungal infections, and CARD9 deficiency in Cbl-b-deficient mice failed to elicit enough proinflammation cytokine to combat fungal infections. Collectively, our data suggests that Cbl-b negatively regulates CLR-induced innate immune responses and that activated CLR/Syk signaling is critical for activating Cbl-b-mediated regulation of the innate immune responses.

Previous studies show that Cbl-b negatively regulates the signaling pathways of macrophages, DCs, NK cells, NKT

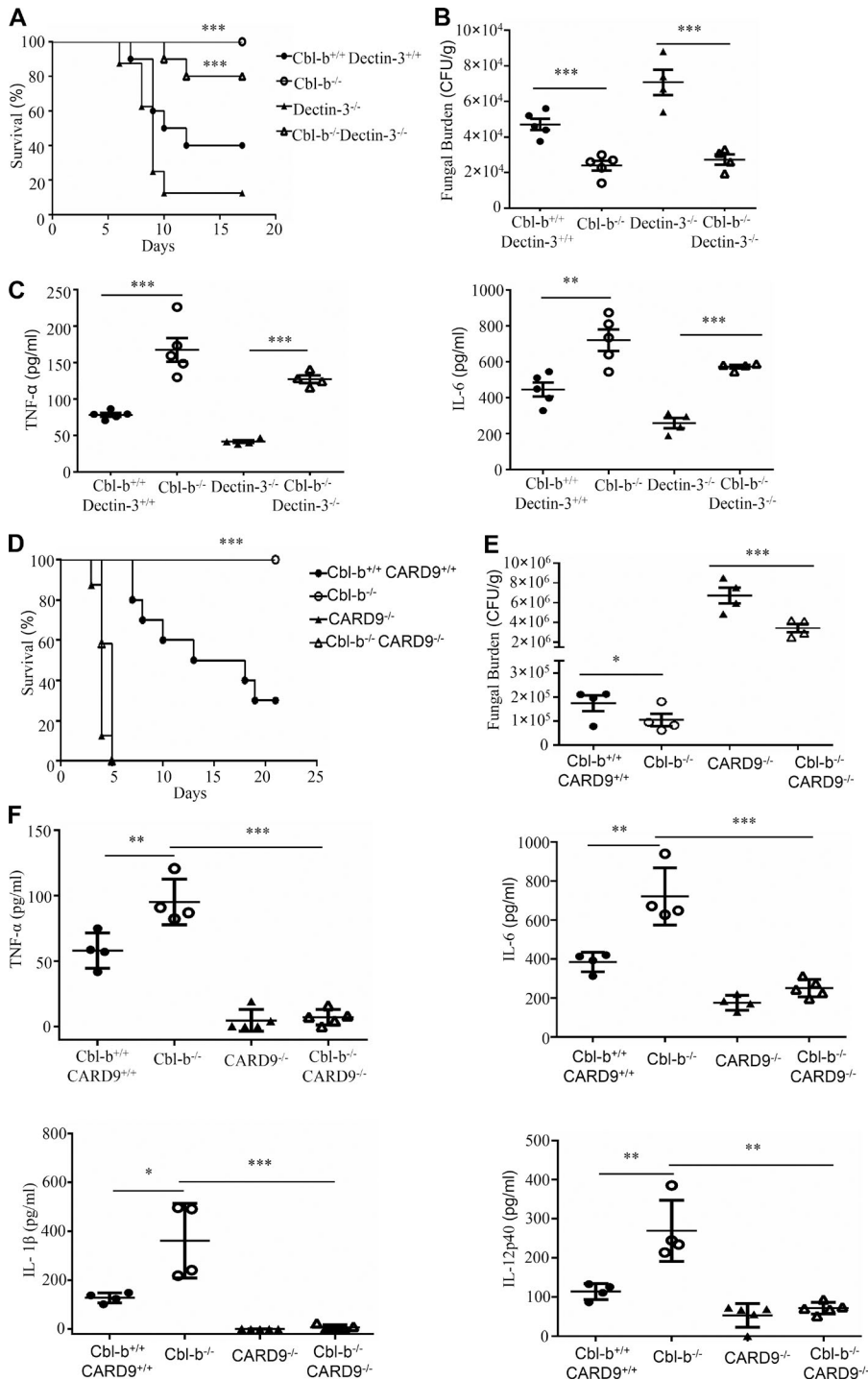


Figure 7. CLR/CARD9 cascade is required for host Cbl-b-mediated antifungal immune regulation. (A) Survival of WT, Cbl-b KO, Dectin-3 KO, or Cbl-b/Dectin-3 dKO mice ($n = 10$ per group) infected intravenously with 10^5 CFU of *C. albicans* (SC5314). (B) Kidney CFU assay of WT, Cbl-b KO, Dectin-3 KO, or Cbl-b/Dectin-3 dKO mice ($n = 15$ per group) infected intravenously with 10^5 CFU of *C. albicans* (SC5314) for 24 h. The data shown are representative of three independent and reproducible experiments. (C) Amounts of TNF and IL-6 in kidneys of WT, Cbl-b KO, Dectin-3 KO, or Cbl-b/Dectin-3 dKO mice after infection with 10^5 CFU of *C. albicans* (SC5314) for 24 h. Data are means \pm SD of triplicate samples and are representative of three independent experiments. (D) Survival of WT, Cbl-b KO, CARD9 KO, or Cbl-b/CARD9 dKO mice ($n = 10$ per group) infected intravenously with 10^5 CFU of *C. albicans* (SC5314). (E) Kidney CFU assay of WT, Cbl-b KO, CARD9 KO, or Cbl-b/CARD9 dKO mice ($n = 5$ per group) after intravenous infection with 10^5 CFU of *C. albicans* (SC5314) for 24 h. The data shown are representative of three independent and reproducible experiments. (F) Amounts of TNF, IL-6, IL-1 β , and IL-12p40 in kidneys of WT, Cbl-b KO, CARD9 KO, or Cbl-b/CARD9 dKO mice after infection with 10^5 CFU of *C. albicans* (SC5314) for 24 h. Data are means \pm SD of triplicate samples and are representative of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (ANOVA).

cells, and mast cells in innate immunity (Qingjun et al., 2014; Lutz-Nicoladoni et al., 2015). Cbl-b plays an important role in integrin signal transduction, in which Cbl-b deficiency facilitates activation of $\beta 2$ -integrin leukocyte function-associated antigen-1 (LFA-1) and LFA-1-mediated inflammatory cell recruitment (Choi et al., 2008). Cbl-b is also implicated in the negative regulation of the integrin $\alpha(M)$ (CD11b)

pathway, which is activated by TLR-triggered phosphatidylinositol 3-OH kinase (PI(3)K; Han et al., 2010). Subsequently, Cbl-b was shown to target MyD88 and TRIF (Toll/IL-1 receptor domain-containing adapter inducing IFN- β ; Han et al., 2010), which is potentiated by activating the tyrosine kinases Src and Syk in macrophages upon TLR stimulation. Furthermore, a recent study showed that Cbl-b-deficient

BMDCs upon TLR stimulation can produce higher levels of proinflammatory cytokines including IL-1 α , IL-6, and TNF and exhibit a slightly higher level of FITC-dextran uptake (Wallner et al., 2013). It has been reported that Cbl-b targets CARMA1, a critical signaling molecule for NF- κ B activation, for monoubiquitination in NKT cells (Kojo et al., 2009) and regulates NK cell functions via TAM receptors (Paolino et al., 2014). Furthermore, Cbl-b negatively regulates IgE-mediated activation of mast cells as well as the activation and tissue infiltration of macrophages (Qu et al., 2004; Hirasaka et al., 2007). Because of the diversities of substrates of Cbl-b in different cell types, it appears that Cbl-b regulates various signaling pathways in a cell type-dependent manner. Our study shows that Cbl-b mediates the ubiquitination and degradation of activated Dectin-2 and Dectin-3 to negatively regulate the CLR-mediated signaling pathways.

Cbl-b also plays an important negative regulatory role in T and B cells with multiple signaling pathways. In naive T cells, Cbl-b promotes the ubiquitination of p85, the regulatory subunit of PI3K, to inhibit its binding with TCR- ζ and CD28, thus restraining the TCR-mediated NF- κ B signaling pathway and preventing excessive activation of T cells (Fang et al., 2001). In anergic T cells, Cbl-b also promotes the ubiquitination of PLC- γ 1 and PKC- θ to inhibit calcium mobilization and the activation of NFAT that lead to IL-2 production (Gruber et al., 2009). In B cells, Cbl-b induces the ubiquitination and proteasomal degradation of Syk and Ig- α to suppress BCR-mediated activation of MAPK and NF- κ B signaling pathways during the normal response course (Sohn et al., 2003). However, our study shows that activation of Dectin-2 and Dectin-3 by *C. albicans* hyphae cannot promote the degradation of Syk in macrophages, indicating that CLR-induced activity of Syk may be negatively regulated by other unknown mechanisms, but not Cbl-b-mediated ubiquitination and degradation systems in macrophages. Cbl-b also associates with TRAF-2 to inhibit its recruitment to CD40 and subsequently attenuates CD40-mediated NF- κ B and JNK activation, thereby suppressing excessive activation of B cells (Qiao et al., 2007). It has been shown that growth factor receptor-bound protein 2 (Grb2) is degraded in a Cbl-b-dependent manner, and the Cbl-b-Grb2 signaling pathway might play an important role in germinal center formation in the spleen (Jang et al., 2011). Thus, Cbl-b functions in multiple pathways and coordinates signaling to TCR and BCR stimulation.

Cellular proteins are degraded in either proteasomes or lysosomes depending on the types of ubiquitin chains that covalently modify them (Ciechanover, 2005). Generally, it is thought that K48-linked polyubiquitin chains target proteins to proteasomal degradation, whereas monoubiquitination targets membrane proteins to lysosomal degradation (Nathan et al., 2013). Our study showed that the activated Dectin-2 and Dectin-3 were conjugated with K48 polyubiquitin chains in a Syk- and Cbl-b-dependent manner. Unexpectedly, we found that Dectin-2 and Dectin-3 polyubiquitinated by K48-

linked chains are degraded in lysosomes because inhibition of lysosomes with chloroquine could completely block the degradation of the activated Dectin-2 and Dectin-3. A recent study also shows that canonical Wnt signaling translocates some K48-linked polyubiquitinated proteins together with GSK3 and Axin into MVBs for lysosomal degradation (Kim et al., 2015).

It is well known that chloroquine can cause lysosome alkalinization and inhibition of acidic hydrolases downstream of MVB formation (Dobrowolski et al., 2012). The ESCRT system is well established to mediate the endosomal sorting of ubiquitinated membrane proteins into MVBs (Wegner et al., 2011). In this study, we found that the ESCRT system is responsible for the trafficking of ubiquitinated Dectin-2 and Dectin-3 into lysosomes for degradation because simultaneous knockdown of ESCRT-0 subunits STAM and HRS and ESCRT-I subunits Vps28 and Tsg101 by siRNA could significantly block the degradation of the activated Dectin-2 and Dectin-3 but significantly increase CLR-mediated NF- κ B activation and subsequent proinflammation cytokine secretions. More importantly, the individual KO of ESCRT subunits STAM, HRS, or Vps28 also significantly increased CLR-mediated NF- κ B activation and subsequent proinflammation cytokine secretions when stimulated with their ligands, including α -mannans, Man-LAM, and TDM. Together, our data indicate that the ESCRT system negatively regulates CLR-induced innate immune responses by transporting the ubiquitinated CLR into lysosomes for degradation. However, the molecular mechanism underlying lysosomal degradation of CLRs remains to be elucidated.

In summary, our study shows that the E3 ligase Cbl-b facilitates the ubiquitination of the activated Dectin-2 and Dectin-3, and the ubiquitinated Dectin-2 and Dectin-3 are targeted for lysosome-mediated degradation by the ESCRT system. Our study reveals that both Cbl-b and the ESCRT system are negative regulators of the CLR-mediated antifungal innate immune responses, which provides molecular insight for the development of an antifungal therapeutic strategy.

MATERIALS AND METHODS

Plasmids, reagents, and antibodies. The open reading frames of human Dectin-2, Dectin-3, FcR- γ , and Syk were amplified by PCR with full-length cDNA of human peripheral blood cells as templates. Dectin-2 and Dectin-3 point mutants were generated using site-directed mutagenesis and confirmed by sequencing analysis. All PCR-amplifying fragments were inserted into lentiviral vector pRV3 or pcDNA3.1 (Flag, hemagglutinin, or Myc) plasmids as previously described by us (Zhu et al., 2013). The pCEFL-Cbl-b plasmid was provided by J. Zhang (Ohio State University, Columbus, OH). Antibodies against phosphoextracellular signal-regulated protein kinase (9101), phospho-IkBa (9246), phospho-Syk (2710), total Syk (2712), SHP-2 (3397), p65 (8242), K48 (8081), and K63 (5621) were purchased from Cell Signaling Technology; ubiquitin (sc-9133), Cbl-b (sc-1705), p-Cbl

(sc-377571), proliferating cell nuclear antigen (sc-56), and I κ B α (sc-371) were from Santa Cruz Biotechnology, Inc. α -Actin (M20010), Myc-Tag (M20002), HA-Tag (M20003), and DYKDDDDK-Tag (M20008) were from abmart. α -Mannans (M3640), Z-Leu-Leu-Leu-al (MG132; C2211), chloroquine sulfate (C6628), and cycloheximide (C7689) were from Sigma-Aldrich. Piceatannol (527948) was purchased from EMD Millipore. Dectin-2, Dectin-3, and Mincle monoclonal antibodies were generated by using their respective human recombinant extracellular domains as immunogens, which were previously described by us (Zhu et al., 2013; Zhao et al., 2014).

Mice. CARD9^{-/-} and Dectin-3^{-/-} mice were previously described by us (Wu et al., 2009; Zhu et al., 2013). Cbl-b^{-/-} mice in a C57BL/6 background were previously described (Fang and Liu, 2001) and provided by Y. Liu (La Jolla Institute of Allergy and Immunology, San Diego, CA) and H. Gu (Columbia University, New York, NY). All mice were housed under specific pathogen-free conditions in the Tsinghua University animal facility. All of the animal experiments were performed in compliance with the institutional guidelines and according to the protocol approved by the Institutional Animal Use and Care Committee of Tsinghua University.

C. albicans growth and hyphae preparation. *C. albicans* cells were cultured from a single colony of the standard strain SC5314, which was first grown overnight in yeast peptone dextrose medium for yeast-form growth at 30°C. For hyphae, washed yeast cells were counted, resuspended in RPMI-1640 medium, grown in 6-, 12-, or 48-well plates at 37°C for 3 h, and washed three times with phosphate-buffered saline.

BMDM preparation. Primary cultures of BMDMs from C57BL/6 mice were prepared as previously described (Bi et al., 2010). In brief, bone marrow cells were harvested from the femurs and tibias of mice. Erythrocytes were removed from cells by using a hypotonic solution. Cells were cultured for 7 d in DMEM containing 10% fetal bovine serum, 50 mM mercaptoethanol, 100 mg/ml streptomycin, 100 U/ml penicillin, and 30% conditioned medium from L929 cells expressing macrophage CSE. After culturing for 7 d, flow cytometry analysis indicated that the harvested cell population contained 86–95% CD11b⁺ F4/80⁺ cells.

Coimmunoprecipitation, immunoblot, and ubiquitination assays. For immunoprecipitation assay, cells were lysed in lysis buffer (50 mM Hepes, pH 7.4, 250 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM Na₃VO₄, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride) supplemented with protease inhibitor. The immunoprecipitates and lysates were analyzed by immunoblot using the indicated antibodies. For ubiquitination assays, cells were lysed in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 20 mM *N*-ethylmaleimide, and 1% Nonidet-P40). Lysates were immediately boiled for 5

min in the presence of 1% (vol/vol) SDS and then were diluted with lysis buffer until the concentration of SDS was decreased to 0.1%. Immunoprecipitates were analyzed by immunoblot with antiubiquitin antibody or K48-linked ubiquitin antibody.

siRNA interference. BMDMs were transfected with different concentrations of siRNA against mouse STAM, Hrs, Tsg101, and vps28. Trans-IT-TKO transfection reagent (Mirus) was used to transfect targeting siRNA and nontargeting control siRNA. Cells were lysed 48 h after transfection, and relative intensities of STAM, Hrs, Tsg101, and vps28 protein were determined by Western blotting.

KO of STAM, HRS, and Vps28 using the lenti-CRISPR/Cas9 system in iBMDMs. Three small guide RNAs (sgRNAs) targeting STAM, HRS, and Vps28 genes were designed. Guide RNAs were cloned into the lenti-CRISPR vector as previously described (Shalem et al., 2014). Each plasmid containing an inserted sgRNA sequence was verified by sequencing analysis. To make lentivirus, the constructed sgRNA-lenti-CRISPR plasmids were cotransfected into HEK293 T cells with packaging plasmids pMD2.G and psPAX2. Cell supernatant was collected every 24 h from 48 to 96 h after transfection, and cell debris was removed by centrifugation. The lentivirus was concentrated by ultracentrifugation for 2 h at 16,600 *g* and then resuspended in DMEM.

iBMDMs derived from C57BL/6 mice were previously described (Blasi et al., 1985) and provided by K.A. Fitzgerald and D. Wang (University of Massachusetts Medical School, Worcester, MA). iBMDMs were infected with lentivirus encoding targeting gRNAs and then selected with puromycin for 2 wk. The surviving cells were lysed to determine the KO efficiency by Western blotting.

Cytokine measurements. TNF, IL-10, IL-6, and IL-12p40 amounts in serum and extracts of kidney tissue from infected mice or cultures of infected BMDMs were measured with Ready-SET-GO ELISA kits (eBioscience). All samples were measured in triplicate according to the manufacturer's protocol.

Mouse systemic candidiasis model. For in vivo *C. albicans* infection, groups of mice (more than ~8–10 per test) were injected via lateral tail veins with 200 μ l of a suspension of *C. albicans* (SC5314) cells in sterile saline. Mouse survival rates were monitored for 3 wk after infection. Fungal burden was assessed by plating a series of diluted solutions of homogenized kidneys from mice. For cytokine measurement, extracts of kidney tissue from infected mice were detected by Ready-SET-GO ELISA kits.

Statistical analysis. At least two biological replicates were performed for all experiments unless indicated. Student's *t* tests for paired samples and ANOVA for multisamples were used for statistical analyses. Statistical significance was set at a *p*-value <0.05, 0.01, or 0.001.

ACKNOWLEDGMENTS

This work was partially supported by the National Natural Science Foundation of China (grants 81571611 to X.-M. Jia and 91542107 to X. Lin), National Basic Research Program of China (Program 973, 2013CB531602 to Y.-Y. Jiang), Fundamental Research Funds for the Central Universities (with grants from Tongji University to X.-M. Jia), and National Institutes of Health (grant AI116722 to X. Lin). X. Lin is a Changjiang lecture professor of the Ministry of Education of the People's Republic of China.

The authors declare no competing financial interests.

Submitted: 11 December 2015

Accepted: 15 June 2016

REFERENCES

- Arce, I., L. Martínez-Muñoz, P. Roda-Navarro, and E. Fernández-Ruiz. 2004. The human C-type lectin CLECSF8 is a novel monocyte/macrophage endocytic receptor. *Eur. J. Immunol.* 34:210–220. <http://dx.doi.org/10.1002/eji.200324230>
- Bhoj, V.G., and Z.J. Chen. 2009. Ubiquitylation in innate and adaptive immunity. *Nature*. 458:430–437. <http://dx.doi.org/10.1038/nature07959>
- Bi, L., S. Gojestani, W. Wu, Y.M.S. Hsu, J. Zhu, K. Ariizumi, and X. Lin. 2010. CARD9 mediates dectin-2-induced I κ B α kinase ubiquitination leading to activation of NF- κ B in response to stimulation by the hyphal form of *Candida albicans*. *J. Biol. Chem.* 285:25969–25977. <http://dx.doi.org/10.1074/jbc.M110.131300>
- Blasi, E., B.J. Mathieson, L. Varesio, J.L. Cleveland, P.A. Borchert, and U.R. Rapp. 1985. Selective immortalization of murine macrophages from fresh bone marrow by a raf/myc recombinant murine retrovirus. *Nature*. 318:667–670. <http://dx.doi.org/10.1038/318667a0>
- Chiang, Y.J., H.K. Koe, K. Brown, M. Naramura, S. Fukuhara, R.-J. Hu, I.K. Jang, J.S. Gutkind, E. Shevach, and H. Gu. 2000. Cbl-b regulates the CD28 dependence of T-cell activation. *Nature*. 403:216–220. <http://dx.doi.org/10.1038/35003235>
- Choi, E.Y., V.V. Orlova, S.C. Fagerholm, S.M. Nurmi, L. Zhang, C.M. Ballantyne, C.G. Gahmberg, and T. Chavakis. 2008. Regulation of LFA-1-dependent inflammatory cell recruitment by Cbl-b and 14-3-3 proteins. *Blood*. 111:3607–3614. <http://dx.doi.org/10.1182/blood-2007-07-103077>
- Ciechanover, A. 2005. Proteolysis: from the lysosome to ubiquitin and the proteasome. *Nat. Rev. Mol. Cell Biol.* 6:79–87. <http://dx.doi.org/10.1038/nrm1552>
- Deng, Z., S. Ma, H. Zhou, A. Zang, Y. Fang, T. Li, H. Shi, M. Liu, M. Du, P.R. Taylor, et al. 2015. Tyrosine phosphatase SHP-2 mediates C-type lectin receptor-induced activation of the kinase Syk and anti-fungal TH17 responses. *Nat. Immunol.* 16:642–652. <http://dx.doi.org/10.1038/ni.3155>
- Dobrowolski, R., P. Vick, D. Ploper, I. Gumper, H. Snitkin, D.D. Sabatini, and E.M. De Robertis. 2012. Presenilin deficiency or lysosomal inhibition enhances Wnt signaling through relocalization of GSK3 to the late-endosomal compartment. *Cell Reports*. 2:1316–1328. <http://dx.doi.org/10.1016/j.celrep.2012.09.026>
- Elly, C., S. Witte, Z. Zhang, O. Rosnet, S. Lipkowitz, A. Altman, and Y.C. Liu. 1999. Tyrosine phosphorylation and complex formation of Cbl-b upon T cell receptor stimulation. *Oncogene*. 18:1147–1156. <http://dx.doi.org/10.1038/sj.onc.1202411>
- Ettenberg, S.A., M.M. Keane, M.M. Nau, M. Frankel, L.M. Wang, J.H. Pierce, and S. Lipkowitz. 1999. cbl-b inhibits epidermal growth factor receptor signaling. *Oncogene*. 18:1855–1866. <http://dx.doi.org/10.1038/sj.onc.1202499>
- Fang, D., and Y.C. Liu. 2001. Proteolysis-independent regulation of PI3K by Cbl-b-mediated ubiquitination in T cells. *Nat. Immunol.* 2:870–875. <http://dx.doi.org/10.1038/ni0901-870>
- Fang, D., H.Y. Wang, N. Fang, Y. Altman, C. Elly, and Y.C. Liu. 2001. Cbl-b, a RING-type E3 ubiquitin ligase, targets phosphatidylinositol 3-kinase for ubiquitination in T cells. *J. Biol. Chem.* 276:4872–4878. <http://dx.doi.org/10.1074/jbc.M008901200>
- Gorjestani, S., M. Yu, B. Tang, D. Zhang, D. Wang, and X. Lin. 2011. Phospholipase C γ 2 (PLC γ 2) is key component in Dectin-2 signaling pathway, mediating anti-fungal innate immune responses. *J. Biol. Chem.* 286:43651–43659. <http://dx.doi.org/10.1074/jbc.M111.307389>
- Gorjestani, S., B.G. Darnay, and X. Lin. 2012. Tumor necrosis factor receptor-associated factor 6 (TRAF6) and TGF β -activated kinase 1 (TAK1) play essential roles in the C-type lectin receptor signaling in response to *Candida albicans* infection. *J. Biol. Chem.* 287:44143–44150. <http://dx.doi.org/10.1074/jbc.M112.414276>
- Graham, L.M., V. Gupta, G. Schafer, D.M. Reid, M. Kimberg, K.M. Dennehy, W.G. Hornsall, R. Guler, M.A. Campanero-Rhodes, A.S. Palma, et al. 2012. The C-type lectin receptor CLECSF8 (CLEC4D) is expressed by myeloid cells and triggers cellular activation through Syk kinase. *J. Biol. Chem.* 287:25964–25974. <http://dx.doi.org/10.1074/jbc.M112.384164>
- Gross, O., A. Gewies, K. Finger, M. Schäfer, T. Sparwasser, C. Peschel, I. Förster, and J. Ruland. 2006. Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature*. 442:651–656. <http://dx.doi.org/10.1038/nature04926>
- Gruber, T., N. Hermann-Kleiter, R. Hinterleitner, F. Fresser, R. Schneider, G. Gastl, J.M. Penninger, and G. Baier. 2009. PKC- θ modulates the strength of T cell responses by targeting Cbl-b for ubiquitination and degradation. *Sci. Signal.* 2:ra30. <http://dx.doi.org/10.1126/scisignal.2000046>
- Han, C., J. Jin, S. Xu, H. Liu, N. Li, and X. Cao. 2010. Integrin CD11b negatively regulates TLR-triggered inflammatory responses by activating Syk and promoting degradation of MyD88 and TRIF via Cbl-b. *Nat. Immunol.* 11:734–742. <http://dx.doi.org/10.1038/ni.1908>
- Hara, H., and T. Saito. 2009. CARD9 versus CARMA1 in innate and adaptive immunity. *Trends Immunol.* 30:234–242. <http://dx.doi.org/10.1016/j.it.2009.03.002>
- Hirasaka, K., S. Kohno, J. Goto, H. Furochi, K. Mawatari, N. Harada, T. Hosaka, Y. Nakaya, K. Ishidoh, T. Obata, et al. 2007. Deficiency of Cbl-b gene enhances infiltration and activation of macrophages in adipose tissue and causes peripheral insulin resistance in mice. *Diabetes*. 56:2511–2522. <http://dx.doi.org/10.2337/db06-1768>
- Ishikawa, E., T. Ishikawa, Y.S. Morita, K. Toyonaga, H. Yamada, O. Takeuchi, T. Kinoshita, S. Akira, Y. Yoshikai, and S. Yamasaki. 2009. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. *J. Exp. Med.* 206:2879–2888. <http://dx.doi.org/10.1084/jem.20091750>
- Jang, I.K., D.G. Cronshaw, L.K. Xie, G. Fang, J. Zhang, H. Oh, Y.X. Fu, H. Gu, and Y. Zou. 2011. Growth-factor receptor-bound protein-2 (Grb2) signaling in B cells controls lymphoid follicle organization and germinal center reaction. *Proc. Natl. Acad. Sci. USA*. 108:7926–7931. <http://dx.doi.org/10.1073/pnas.1016451108>
- Jhingran, A., K.B. Mar, D.K. Kumasaka, S.E. Knoblaugh, L.Y. Ngo, B.H. Segal, Y. Iwakura, C.A. Lowell, J.A. Hamerman, X. Lin, and T.M. Hohl. 2012. Tracing conidial fate and measuring host cell antifungal activity using a reporter of microbial viability in the lung. *Cell Reports*. 2:1762–1773. <http://dx.doi.org/10.1016/j.celrep.2012.10.026>
- Kim, H., P. Vick, J. Hedtke, D. Ploper, and E.M. De Robertis. 2015. Wnt signaling translocates Lys48-linked polyubiquitinated proteins to the lysosomal pathway. *Cell Reports*. 11:1151–1159. <http://dx.doi.org/10.1016/j.celrep.2015.04.048>
- Kojo, S., C. Elly, Y. Harada, W.Y. Langdon, M. Kronenberg, and Y.C. Liu. 2009. Mechanisms of NKT cell anergy induction involve Cbl-b-promoted monoubiquitination of CARMA1. *Proc. Natl. Acad. Sci. USA*. 106:17847–17851. <http://dx.doi.org/10.1073/pnas.0904078106>

- Lobato-Pascual, A., P.C. Saether, S. Fossum, E. Dissen, and M.R. Daws. 2013. Mincle, the receptor for mycobacterial cord factor, forms a functional receptor complex with MCL and Fc ϵ RI- γ . *Eur. J. Immunol.* 43:3167–3174. <http://dx.doi.org/10.1002/eji.201343752>
- Loures, F.V., M. Röhm, C.K. Lee, E. Santos, J.P. Wang, C.A. Specht, V.L. Calich, C.F. Urban, and S.M. Levitz. 2015. Recognition of *Aspergillus fumigatus* hyphae by human plasmacytoid dendritic cells is mediated by dectin-2 and results in formation of extracellular traps. *PLoS Pathog.* 11:e1004643. <http://dx.doi.org/10.1371/journal.ppat.1004643>
- Lutz-Nicoladoni, C., D. Wolf, and S. Sopfer. 2015. Modulation of immune cell functions by the E3 ligase Cbl-b. *Front. Oncol.* 5:58. <http://dx.doi.org/10.3389/fonc.2015.00058>
- Matsunaga, I., and D.B. Moody. 2009. Mincle is a long sought receptor for mycobacterial cord factor. *J. Exp. Med.* 206:2865–2868. <http://dx.doi.org/10.1084/jem.20092533>
- Mócsai, A., J. Ruland, and V.L. Tybulewicz. 2010. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat. Rev. Immunol.* 10:387–402. <http://dx.doi.org/10.1038/nri2765>
- Naramura, M., I.K. Jang, H. Kole, F. Huang, D. Haines, and H. Gu. 2002. c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation. *Nat. Immunol.* 3:1192–1199. <http://dx.doi.org/10.1038/ni855>
- Nathan, J.A., H.T. Kim, L. Ting, S.P. Gygi, and A.L. Goldberg. 2013. Why do cellular proteins linked to K63-polyubiquitin chains not associate with proteasomes? *EMBO J.* 32:552–565. <http://dx.doi.org/10.1038/emboj.2012.354>
- Paolino, M., A. Choidas, S. Wallner, B. Pranjic, I. Uribealago, S. Loeser, A.M. Jamieson, W.Y. Langdon, F. Ikeda, J.P. Fededa, et al. 2014. The E3 ligase Cbl-b and TAM receptors regulate cancer metastasis via natural killer cells. *Nature.* 507:508–512. <http://dx.doi.org/10.1038/nature12998>
- Qiao, G., M. Lei, Z. Li, Y. Sun, A. Minto, Y.X. Fu, H. Ying, R.J. Quigg, and J. Zhang. 2007. Negative regulation of CD40-mediated B cell responses by E3 ubiquitin ligase Casitas-B-lineage lymphoma protein-B. *J. Immunol.* 179:4473–4479. <http://dx.doi.org/10.4049/jimmunol.179.7.4473>
- Qingjun, L., H. Zhou, W.Y. Langdon, and J. Zhang. 2014. E3 ubiquitin ligase Cbl-b in innate and adaptive immunity. *Cell Cycle.* 13:1875–1884. <http://dx.doi.org/10.4161/cc.29213>
- Qu, X., K. Sada, S. Kyo, K. Maeno, S.M.S. Miah, and H. Yamamura. 2004. Negative regulation of Fc ϵ RI-mediated mast cell activation by a ubiquitin-protein ligase Cbl-b. *Blood.* 103:1779–1786. <http://dx.doi.org/10.1182/blood-2003-07-2260>
- Robinson, M.J., F. Osorio, M. Rosas, R.P. Freitas, E. Schweighoffer, O. Gross, J.S. Verbeek, J. Ruland, V. Tybulewicz, G.D. Brown, et al. 2009. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J. Exp. Med.* 206:2037–2051. <http://dx.doi.org/10.1084/jem.20082818>
- Saijo, S., S. Ikeda, K. Yamabe, S. Kakuta, H. Ishigame, A. Akitsu, N. Fujikado, T. Kusaka, S. Kubo, S.H. Chung, et al. 2010. Dectin-2 recognition of α -mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. *Immunity.* 32:681–691. <http://dx.doi.org/10.1016/j.immuni.2010.05.001>
- Sancho, D., and C. Reis e Sousa. 2012. Signaling by myeloid C-type lectin receptors in immunity and homeostasis. *Annu. Rev. Immunol.* 30:491–529. <http://dx.doi.org/10.1146/annurev-immunol-031210-101352>
- Sato, K., X.L. Yang, T. Yudate, J.S. Chung, J. Wu, K. Luby-Phelps, R.P. Kimberly, D. Underhill, P.D. Cruz Jr., and K. Ariizumi. 2006. Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor γ chain to induce innate immune responses. *J. Biol. Chem.* 281:38854–38866. <http://dx.doi.org/10.1074/jbc.M606542200>
- Shalem, O., N.E. Sanjana, E. Hartenian, X. Shi, D.A. Scott, T.S. Mikkelsen, D. Heckl, B.L. Ebert, D.E. Root, J.G. Doench, and F. Zhang. 2014. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science.* 343:84–87. <http://dx.doi.org/10.1126/science.1247005>
- Shamim, M., S.G. Nanjappa, A. Singh, E.H. Plisch, S.E. LeBlanc, J. Walent, J. Svaren, C. Seroogy, and M. Suresh. 2007. Cbl-b regulates antigen-induced TCR down-regulation and IFN- γ production by effector CD8 T cells without affecting functional avidity. *J. Immunol.* 179:7233–7243. <http://dx.doi.org/10.4049/jimmunol.179.11.7233>
- Sohn, H.W., H. Gu, and S.K. Pierce. 2003. Cbl-b negatively regulates B cell antigen receptor signaling in mature B cells through ubiquitination of the tyrosine kinase Syk. *J. Exp. Med.* 197:1511–1524. <http://dx.doi.org/10.1084/jem.20021686>
- Strasser, D., K. Neumann, H. Bergmann, M.J. Marakalala, R. Guler, A. Rojowska, K.P. Hopfner, F. Brombacher, H. Urlaub, G. Baier, et al. 2012. Syk kinase-coupled C-type lectin receptors engage protein kinase C- σ to elicit Card9 adaptor-mediated innate immunity. *Immunity.* 36:32–42. <http://dx.doi.org/10.1016/j.immuni.2011.11.015>
- Wallner, S., C. Lutz-Nicoladoni, C.H. Tripp, G. Gastl, G. Baier, J.M. Penninger, P. Stoitzner, and D. Wolf. 2013. The role of the e3 ligase cbl-B in murine dendritic cells. *PLoS One.* 8:e65178. <http://dx.doi.org/10.1371/journal.pone.0065178>
- Wegner, C.S., L.M. Rodahl, and H. Stenmark. 2011. ESCRT proteins and cell signalling. *Traffic.* 12:1291–1297. <http://dx.doi.org/10.1111/j.1600-0854.2011.01210.x>
- Wu, W., Y.M. Hsu, L. Bi, Z. Songyang, and X. Lin. 2009. CARD9 facilitates microbe-elicited production of reactive oxygen species by regulating the LyGDI-Rac1 complex. *Nat. Immunol.* 10:1208–1214. <http://dx.doi.org/10.1038/ni.1788>
- Yonekawa, A., S. Saijo, Y. Hoshino, Y. Miyake, E. Ishikawa, M. Suzukawa, H. Inoue, M. Tanaka, M. Yoneyama, M. Oh-Hora, et al. 2014. Dectin-2 is a direct receptor for mannose-capped lipoarabinomannan of mycobacteria. *Immunity.* 41:402–413. <http://dx.doi.org/10.1016/j.immuni.2014.08.005>
- Zhang, J., Y.J. Chiang, R.J. Hodes, and R.P. Siraganian. 2004. Inactivation of c-Cbl or Cbl-b differentially affects signaling from the high affinity IgE receptor. *J. Immunol.* 173:1811–1818. <http://dx.doi.org/10.4049/jimmunol.173.3.1811>
- Zhang, Z., C. Elly, L. Qiu, A. Altman, and Y.-C. Liu. 1999. A direct interaction between the adaptor protein Cbl-b and the kinase Zap-70 induces a positive signal in T cells. *Curr. Biol.* 9:203–210. [http://dx.doi.org/10.1016/S0960-9822\(99\)80090-6](http://dx.doi.org/10.1016/S0960-9822(99)80090-6)
- Zhao, X.Q., L.L. Zhu, Q. Chang, C. Jiang, Y. You, T. Luo, X.M. Jia, and X. Lin. 2014. C-type lectin receptor dectin-3 mediates trehalose 6,6'-dimycolate (TDM)-induced Mincle expression through CARD9/Bcl10/MALT1-dependent nuclear factor (NF)- κ B activation. *J. Biol. Chem.* 289:30052–30062. <http://dx.doi.org/10.1074/jbc.M114.588574>
- Zhu, L.-L., X.-Q. Zhao, C. Jiang, Y. You, X.-P. Chen, Y.-Y. Jiang, X.-M. Jia, and X. Lin. 2013. C-type lectin receptors Dectin-3 and Dectin-2 form a heterodimeric pattern-recognition receptor for host defense against fungal infection. *Immunity.* 39:324–334. <http://dx.doi.org/10.1016/j.immuni.2013.05.017>