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Targeting CBLB as a Potential Therapeutic Approach for Disseminated Candidiasis

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Abstract

Disseminated candidiasis has become one of the leading causes of hospital-acquired blood stream infections with high mobility and mortality. However, the molecular basis of host defense against disseminated candidiasis remains elusive, and treatment options are limited. Here, we report that the E3 ubiquitin ligase CBLB directs polyubiquitination of dectin-1 and -2, two key pattern recognition receptors for sensing *Candida albicans*, and their downstream kinase SYK, thus inhibiting dectin-1/2-mediated innate immune responses. CBLB deficiency or inactivation protects

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Authors Contributions

Y.X. performed most of experiments and analyzed the data; J.T., H.G., Y.Z., R.T., S.O., Q.Z., and B.T.L. performed some *in vitro* and *in vivo* experiments; C.R. helped design the research, analyzed and interpreted the data, and edited the manuscript; M.V.S.R. performed experiments using human macrophages; L.S.S., M.V.S.R., and J.Z. designed human macrophage experiments and edited the manuscript; L.T. helped design kidney experiments and data analysis; G.D.B. provided *Clec7a*$^{-/-}$ mice; W.Y.L. provided *Cblb*^{C373A} knockin mice and edited the manuscript; J.Z. conceived and planned the research, analyzed data and wrote the manuscript.

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mice from systemic infection with a lethal dose of *Candida albicans*, and deficiency of dectin-1, -2, or both, in *Cblb*$^{-/-}$ mice abrogates this protection. Importantly, silencing the *Cblb* gene *in vivo* protects mice from lethal systemic *Candida albicans* infection. Our data reveal that CBLB is crucial for homeostatic control of innate immune responses mediated by dectin-1 and -2. Our data also indicate that CBLB represents a potential therapeutic target for protection from disseminated candidiasis.

Introduction

Candida albicans (C. albicans) infection is the most common cause of fungal infections in humans and has become one of the leading causes of hospital-acquired blood stream infections. Despite the availability of several anti-fungal drugs, invasive candidiasis still has a high mortality rate ranging from 45 to 75% 1. The high morbidity and mortality associated with disseminated candidiasis are mainly due to the lack of early and accurate diagnostic tools, limited anti-fungal drugs, and emergence of drug resistance. These factors highlight the need to further understand host-pathogen interactions and the mechanisms of immune resistance to fungal spread, and to develop immune-based strategies to combat candidemia.

The fungi-responsive C-type lectin receptors (CLRs) play a central role in the detection of Candida during bloodstream infection. In normal hosts, *C. albicans* is controlled by activation of innate immune cells via cell surface pattern recognition receptors (PRRs) such as Toll-like receptor 2 (TLR2) and CLRs that detect the infecting fungus. The CLRs dectin-1 and -2 recognize *C. albicans* yeast cells and hyphae by binding to surface β-glucans and α-mannans on the two fungal forms, respectively 2–4. Recognition of these molecules results in the release of inflammatory cytokines from innate immune cells, which is critical for anti-fungal immunity 5. However, the regulation of dectin-mediated signaling pathways, including SYK, that control the pro-inflammatory response to fungal infection, is completely unknown.

Casitas B lymphoma-b (CBLB), a member of the RING finger type E3 ubiquitin ligases that directs the ubiquitination of an array of signaling proteins 6. We and others have shown a crucial role for CBLB in T cell activation, tolerance induction, and $T_H2/9$ cell differentiation 7–14**,** but its role in innate immune responses is unclear. In this study, we report that CBLB functions as a negative regulator of fungal recognition during systemic *C. albicans* infection by targeting dectin-1, -2, and SYK for K48-linked polyubiquitination. Negative regulation by CBLB of dectin-1- and -2-mediated signaling is crucial for restraining the magnitude of innate immune responses against *C. albicans* infection, but leads to suboptimal protection of the host. Systemic *in vivo* delivery of *Cblb* siRNA protects C57BL/6 mice from systemic *C.albicans* infection. Therefore, our data suggest that CBLB is a potential drug target for systemic candidiasis.

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Results

CBLB inhibits signaling via Dectin receptors

To determine the role of CBLB in innate immune responses we stimulated WT and *Cblb*$^{-/-}$ bone marrow-derived macrophages (BMDMs) and BM-derived dendritic cells (BMDCs) with TLR 1-9 ligands or zymosan (a ligand for TLR2 and dectin-1). We found that whereas TLR ligand-induced production of TNF-α and IL-6 was comparable between WT and *Cblb*$^{-/-}$ BMDMs and BMDCs, zymosan-induced TNF-α and IL-6 production was significantly higher in *Cblb*$^{-/-}$ than WT BMDMs and BMDCs (Supplementary Fig. 1a, b). Given that zymosan activates both TLR2 and dectin-1 15, this result suggests that CBLB could regulate the dectin-1 signaling pathway. To directly test this we stimulated WT and *Cblb*$^{-/-}$ BMDMs and BMDCs with curdlan, a purified β-glucan which specifically activates dectin-1 16. Curdlan stimulation induced a significantly higher level of TNF-α and IL-6 in *Cblb*$^{-/-}$ than WT BMDMs and BMDCs (Supplementary Fig. 1a,b).

To confirm this observation, and to determine whether CBLB regulates other Dectin family members, we infected BMDMs, BMDCs and BM neutrophils from WT and *Cblb*$^{-/-}$ mice with a *C. albicans* yeast-only mutant (*cap1*; hereafter referred to as yeast), in which the adenylate cyclase-associated protein-1 gene was disrupted, causing the failure of yeast-hypha transition due to lack of cAMP 17. Dectin-1 and dectin-2 recognize the yeast and hyphal forms of *C. albicans*, respectively, by binding to the surface β-glucans (dectin-1) and α-mannans (dectin-2) of the two fungal forms 2–4. As shown in Fig. 1a and Supplementary Fig. 2a, CBLB deficiency resulted in increased production of TNF-α and IL-6 by BMDMs and BMDCs in response to signaling via both the yeast and hyphal forms of *C. albicans* infection. In contrast, *Cblb*$^{-/-}$ neutrophils produced comparable amounts of TNF-α and IL-6 compared to WT neutrophils, except for the 3 h time point after infection (Supplementary Fig. 2b), suggesting that CBLB may have a limited role in affecting the inflammatory response of neutrophils against *C. albicans* infection. *Cblb*$^{-/-}$ BMDMs also produced more TNF-α and IL-6 than WT BMDMs infected with *A. fumigatus* conidia (Fig. 1b), a prevalent fungus that causes potentially lethal infections in immunosuppressed patients 18. This finding is notable since dectin-1 is a major PRR recognizing *A. fumigatus* 19–21. Therefore, CBLB has the potential to regulate the dectin family of CLRs in response to some fungal pathogens. Since several studies indicate that either the NLRP3 inflammasome or a non-canonical, caspase-8-mediated inflammasome participates in host defense against *C. albicans* infection 22, 23, we measured IL-1β production by WT and *Cblb*$^{-/-}$ BMDMs upon *C. albicans* yeast and hyphal infection. Both WT and *Cblb*$^{-/-}$ BMDMs produced comparable levels of IL-1β (Fig. 1a), suggesting that CBLB does not regulate the inflammasome activation mediated by dectin-1 or -2.

A recent report showed that β-glucan of *C. albicans* induces a strong IL-1RA response in human peripheral blood mononuclear cells (PBMC), which is independent of dectin-1 and CR3 24. To test whether CBLB affects the release of anti-inflammatory stimuli such as IL-1RA, we measured the production of IL-1RA in BMDMs of WT and *Cblb*$^{-/-}$ mice upon infection with live *C. albicans* yeast and hyphae. Our data showed that there was no significant difference in IL-1RA release between WT and *Cblb*$^{-/-}$ BMDMs infected with

both forms of *C. albicans* (Fig. 1c). These data suggest that CBLB does not modulate the release of IL-1RA.

To determine whether CBLB has a similar effect on human macrophages upon *C. albicans* infection, human monocyte-derived macrophages (MDMs) were generated 25, 26, and transfected with *Cblb* siRNA or scrambled siRNA. Consistent with the mouse results, we found that silencing *Cblb* in MDMs resulted in significantly increased production of TNF-α and IL-6 upon infection with *C. albicans* yeast and hyphae, with IL-6 production being the more profound (Supplementary Fig. 3a, b). These results also correlated with impaired down-modulation of dectin-1 and -2 expression (Supplementary Fig. 3d), thus indicating that our observations in mouse macrophages can be recapitulated in human macrophages.

CBLB associates with dectin-1 and -2 in macrophages upon infection with *C. albicans* yeast and hyphal forms

Dectin family CLRs play a major role in fungal recognition and host innate responses against fungal infection 15, 27, 28. Dectin-1's cytoplasmic tail contains an ITAM motif that can be phosphorylated by Src family kinases. Phosphorylated dectin-1 in turn, recruits and activates SYK, thereby initiating downstream signaling via the CARD9/BCL10/MALT1 complex 15, 28. Since dectin-2 lacks this ITAM-like motif it binds FcR-γ 3 which contains ITAMs 29 that recruit SYK and transduce dectin-2 signaling 30–32. We sought to determine whether and how CBLB regulates signaling via dectin-1 and -2 during *C. albicans* infection. First, we determined whether CBLB physically interacts with dectin receptors or their signaling intermediates, and if so, how this occurs. To this end, we infected WT BMDMs with *C. albicans* yeast or hyphae for different times. We found that CBLB was inducibly associated by co-immunoprecipitation with dectin-1, dectin-2, SYK and CARD9 upon infection with *C. albicans* yeasts or hyphae (Fig. 2a, b).

It has previously been shown that CBLB binds to SYK in B cells upon BCR stimulation 33, or CARMA1 (CARD11), a homologue of CARD9, in NK T cells 34. To determine whether SYK and CARD9 are potential binding partners of CBLB in the signaling pathways derived from dectin-1 and -2, we silenced *Syk* gene expression in WT BMDMs by *Syk* siRNA. We found that knocking down *Syk* expression did not affect the association of CBLB with either dectin-1 or dectin-2 (Fig. 2c). Similarly, CARD9 deficiency also did not affect CBLB-dectin-1 or CBLB-dectin-2 association (Fig. 2d). Next we wanted to determine whether phosphorylation of the ITAM within dectin-1 and the ITAMs within FcR-γ is required for CBLB association in macrophages upon *C. albicans* infection (yeasts and hyphae). To accomplish this, we mutated the tyrosine (Y) of the hemi-ITAM to phenylalanine (F) in dectin-1's cytoplasmic tail (Y15F), and the tyrosines within the ITAMs of the FcR-γ to F (FcR-$\gamma^{Y65F,Y76F}$), then reconstituted *Clec7a*$^{-/-}$ BMDMs and *Fcerg1*$^{-/-}$ BMDMs with these mutants, and infected them with *C. albicans* yeast and hyphae, respectively. Mutation of dectin-1 at Y15 or FcR-γ at Y65 and Y76 completely abrogated the binding of CBLB to dectin-1 or dectin-2 (Fig. 2e, f), indicating that phospho-Y15 of dectin-1 or phospho-Y65 and Y76 of FcR-γ is critical for their binding to CBLB. Indeed, CBLB bound to FcR-γ in WT BMDMs upon *C. albicans* hyphal infection (Fig. 2g).

Dectin-1, dectin-2, and SYK are targets of CBLB

To determine whether dectin-1 and dectin-2, or the downstream signaling molecules are the targets of CBLB, we first examined protein stability of dectin-1, dectin-2, SYK and CARD9 in macrophages infected with *C. albicans* yeast or hyphae. Interestingly, dectin-1 and -2, but not SYK or CARD9, underwent degradation in WT BMDMs upon infection with *C. albicans* yeasts and hyphae, but not in BMDMs lacking CBLB (Fig. 3a). These findings suggest that dectin receptors are the likely targets of CBLB. Furthermore, dectin-1 and -2 degradation was completely abrogated by pretreatment with E-64, a lysosome inhibitor, but not with MG-132, a proteasome inhibitor (Fig. 3b), suggesting that dectin-1 and -2 undergo lysosome-mediated degradation.

To further determine whether CBLB is the E3 ubiquitin ligase for dectin-1 or dectin-2, BMDMs generated from WT, *Cblb*$^{-/-}$ or mice expressing an E3 ligase dead mutation (C373A) (*Cblb*^{C373A}) 35 were infected with *C. albicans* yeast or hyphae. The CBLB C373A mutation or deficiency abrogated ubiquitination of dectin-1 and -2 (Fig. 3c,d, upper panel; Supplementary Fig. 4a, b, upper panel). To determine whether ubiquitination of dectin-1 or -2 is K48 or K63-linked, we utilized anti-K48 ubiquitin or anti-K63 ubiquitin antibodies. We confirmed that both dectin-1 and -2 underwent K48-linked polyubiquitination, and this K48-linked polyubiquitination of dectin-1- and -2 was abrogated in BMDMs expressing the CBLB C373A mutation or lacking CBLB (Fig. 3c, d, lower panel; Supplementary Fig. 4a, b, lower panel; data not shown).

It was previously shown that CBLB targets SYK for polyubiquitination but not degradation in B cells 33. To determine whether SYK is also a potential target of CBLB in macrophages triggered by dectin-1 or -2 receptor-ligand interactions, we examined SYK ubiquitination in WT and *Cblb*^{C373A} BMDMs upon infection with *C. albicans* yeast or hyphae. Indeed, SYK underwent K48-linked polyubiquitination upon infection with both *C. albicans* yeast and hyphae, but this ubiquitination was greatly reduced in BMDMs expressing C373A CBLB (Supplementary Fig. 4c, d). Therefore, our data suggest that dectin-1/2 and SYK are targets of CBLB, and that CBLB keeps the expression of these CLRs in check. Consistent with these data, SYK and NF-κB were highly activated in BMDMs lacking CBLB upon *C. albicans* yeast and hyphal infection (Supplementary Fig. 4e).

To examine the functional relevance of CBLB-mediated ubiquitination of dectin-1 and -2 we generated single and triple K to R mutations of dectin-1^{K2R}, dectin-1^{K27R}, dectin-1^{K34R}, and dectin-1^{K2R,K27R,K34R} and dectin-2^{K10R} by site-directed mutagenesis. We reconstituted BMDMs lacking dectin-1 (from *Clec7a*$^{-/-}$ mice) with WT dectin-1 or dectin-1 K/R mutants and BMDMs lacking dectin-2 (from *Clec4n*$^{-/-}$ mice) with WT dectin-2 or dectin-2^{K10R} mutant by Lipofectamine transfection, and infected them with *C. albicans* yeast or hyphae. Reconstituting *Clec7a*$^{-/-}$ BMDMs with WT dectin-1 or dectin-1^{K2R,K27R,K34R} completely or partially restored Dectin-1 ubiquitination, whereas dectin-1^{K2R,K27R,K34R} mutants were not ubiquitinated (Fig. 3e). As expected, *Clec4n*$^{-/-}$ BMDMs reconstituted with WT dectin-2 but not dectin-2^{K10R} mutant restored ubiquitination of dectin-2 (Fig. 3f). These data indicate that dectin-1 K2, K27, and K34, and dectin-2 K10 are the sites of ubiquitination of dectin-1 and -2, respectively. Consistent with these data, *Clec7a*$^{-/-}$ BMDMs reconstituted with dectin-1^{K2R,K27R,K34R}, or *Clec4n*$^{-/-}$ BMDMs reconstituted with dectin-2^{K10R}, produced

significantly higher amounts of TNF-α and IL-6 upon infection with *C. albicans* yeast or hyphae (Fig. 3g, h).

CBLB regulates the internalization of dectin-1 and -2, and their trafficking to the lysosome

Cell surface receptor internalization can occur when receptors are mono- or poly-ubiquitinated following ligand-induced activation, and subsequently sorted into endocytic vesicles for delivery to the lysosome for degradation 36–38. Internalization of dectin-1 has been shown to terminate inflammatory responses in order to keep inflammation in check 39. Thus, impaired down-modulation of dectin-1 and -2 could be due to a lack of internalization or a block in intracellular vesicle sorting to the lysosome. To determine whether CBLB is critical for this process, the cell surface and internalized expression levels of dectin-1 and dectin-2 in BMDMs from WT and *Cblb*$^{-/-}$ mice was investigated. We found a minimal level of intracellular dectin-1 or -2 in *Cblb*$^{-/-}$ BMDMs (Fig. 4a,b), suggesting that CBLB promotes internalization of dectin-1 or dectin-2 after infection with *C. albicans* yeast or hyphae.

We next investigated whether retention of ligand-engaged dectin-1 or -2 in *Cblb*$^{-/-}$ BMDMs is due to impaired sorting of endosomal vesicles to lysosomes. We compared the subcellular localization of ligand-engaged dectin-1 or -2 in WT and *Cblb*$^{-/-}$ BMDMs by confocal microscopy. In support of impaired lysosomal degradation of dectin-1 and -2 in BMDMs lacking CBLB, intracellular trafficking of internalized dectin-1 or -2 to the lysosome was significantly reduced in the absence of CBLB (Fig. 4c, d).

CBLB negatively regulates ROS production and fungal killing but not phagocytosis of *C. albicans*

Neutrophils and macrophages are professional phagocytes of the innate immune system that are essential in controlling bacterial and fungal infections by phagocytosis and killing mechanisms 40. The production of highly reactive oxygen species (ROS) is one of the primary effector mechanisms used by phagocytes to control or clear microbial infections. ROS plays an important role in the initial step of fungal killing in phagosomes 41 and can be potentiated by dectin signaling. We measured ROS production by co-culturing the *C. albicans* yeast *cap1/cap1* mutant or hyphae with WT or *Cblb*$^{-/-}$ BMDMs. We found that *Cblb*$^{-/-}$ and *Cblb*^{C373A} BMDMs produced more ROS than WT controls at MOIs of 5:1 and 2:1 (Supplementary Fig. 5a). Enhanced ROS activity in *Cblb*$^{-/-}$ BMDMs correlated with an increase in their fungal killing potency (Supplementary Fig. 5b). Consistent with a limited role of CBLB in pro-inflammatory cytokine production by neutrophils, we did not observe a significant increase in ROS activity and fungal killing in neutrophils isolated from the BM of *Cblb*$^{-/-}$ or *Cblb*^{C373A} mice compared to WT controls (Supplementary Fig. 5c). However, phagocytosis of *C. albicans* by *Cblb*$^{-/-}$ BMDMs was not increased compared to WT BMDMs (Supplementary Fig. 5d).

CBLB inhibits innate immune responses against systemic *C. albicans* infection mediated by the dectin family of CLRs

The recognition of β-glucans and α-mannans by dectin-1 and dectin-2 respectively is thought to trigger immune responses that are primarily designed for the control of fungal

pathogens 2–4. To assess the role of CBLB in anti-fungal immunity we infected WT, *Cblb*$^{-/-}$, and *Cblb*^{C373A} mice with a lethal dose of *C. albicans* to monitor survival, and a sub-lethal dose to measure serum cytokines and fungal burden. We found that most *Cblb*$^{-/-}$ and *Cblb*^{C373A} mice were protected from lethal systemic infection with *C. albicans* (Fig. 5a), which correlated with heightened levels of TNF-α and IL-6 in the sera of *Cblb*$^{-/-}$ and *Cblb*^{C373A} mice, lower fungal burden in the kidney, lung, spleen, and liver, and decreased *C. albicans* hyphae in the kidney on day 2 as assessed by PAS staining (Fig. 5b-d; Supplementary Fig. 6a). We also observed multifocal tubulointerstitial nephritis in WT mice infected with *C. albicans*, which was ameliorated in mice lacking CBLB or expressing the CBLB C373A mutation (Fig. 5c). This observation is consistent with fact that more immune cells traffic to the kidneys in WT than *Cblb*^{C373A} mice including macrophages, dendritic cells (DCs), and neutrophils (Supplementary Fig. 6b). Improved survival rate was also observed in *Rag1*$^{-/-}$*Cblb*$^{-/-}$ mice that lack functional adaptive immune cells (Fig. 5e), supporting a critical role of CBLB in down-regulating innate immune responses.

To further determine whether monocytes, macrophages and neutrophils have a greater capacity to kill *C. albicans* during systemic infection, we monitored fungal burden in the blood of WT and *Cblb*^{C373A} mice at 2 and 6 h after infection. We found that fungal burden in the blood of *Cblb*^{C373A} mice was significantly lower than that of WT mice at 2 and 6 h after infection (Supplementary Fig. 7a). The lower fungal burden in the blood of *Cblb*^{C373A} mice correlated with enhanced fungal killing activity by PBMCs, but not by neutrophils of *Cblb*^{C373A} mice (Supplementary Fig. 7a). Increased fungal killing was also observed in monocytes from the spleen of *Cblb*^{C373A} mice (Supplementary Fig. 7b). We also monitored ROS activity in monocytes, macrophages and neutrophils from WT and *Cblb*^{C373A} spleens and kidneys by CellRox dye. As shown in Supplementary Figure 7c, monocytes and macrophages, but not neutrophils, displayed augmented ROS expression in *C. albicans*-infected *Cblb*^{C373A} mice when they were infected *in vitro* with *C. albicans*. Consistent with the lower fungal burden and less inflammation in *Cblb*^{C373A} kidneys, trafficking of CD45.2^{+} leukocytes, including macrophages, DCs and neutrophils to *Cblb*^{C373A} kidneys were significantly reduced (Supplementary Fig. 6b). Even with decreased myeloid cells in *Cblb*^{C373A} kidneys upon infection with *C. albicans*, we observed an increase in ROS expression in monocytes and macrophages, and fungal killing using CD45^{+} cells isolated from *Cblb*^{C373A} kidneys (Supplementary Fig. 7d), and increased TNF-α and IL-6 in the kidney homogenates of *Cblb*^{C373A} mice (Supplementary Fig. 7e).

To further determine whether heightened inflammatory responses caused by CBLB deficiency are mediated by dectin-1 and -2, we generated *Cblb*$^{-/-}$*Clec7a*$^{-/-}$, *Cblb*$^{-/-}$*Clec4n*$^{-/-}$, and *Cblb*$^{-/-}$*Clec7a*$^{-/-}$*Clec4n*$^{-/-}$ mice. We infected WT, *Cblb*$^{-/-}$, *Clec7a*$^{-/-}$, *Cblb*$^{-/-}$*Clec7a*$^{-/-}$, *Clec4n*$^{-/-}$, *Cblb*$^{-/-}$*Clec4n*$^{-/-}$, and *Cblb*$^{-/-}$*Clec7a*$^{-/-}$*Clec4n*$^{-/-}$ mice with *C. albicans*. Dectin-1 or dectin-2 single deficiency rendered *Cblb*$^{-/-}$ mice susceptible to *C. albicans* infection, and dectin-1 and dectin-2 double deficiency greatly increased the sensitivity of *Cblb*$^{-/-}$ mice to systemic *C. albicans* infection. All of the triple knockout mice died within four days after infection at a dose at which all *Cblb*$^{-/-}$ mice survived (Fig. 5f), which correlated with significantly lower levels of TNF-α and IL-6 in their sera and fungal burden in the kidneys (Supplementary Fig. 8a,b). Therefore, our results suggest that CBLB negatively regulates both dectin-1 and -2, and that CBLB dampens

inflammatory responses mediated by dectin-1 and -2 during systemic fungal infection. Notably, *Cblb*$^{-/-}$ or *Cblb*^{C373A} mice at 8-12 weeks of age did not display signs of autoimmunity as revealed by comparable anti-dsDNA and anti-ssDNA antibody titers and IL-17/IFN-γ in the sera of WT and *Cblb*$^{-/-}$ or *Cblb*^{C373A} mice, and no elevated IL-17 and IFN-γ in the kidneys of *Cblb*$^{-/-}$ or *Cblb*^{C373A} mice compared to WT mice (Supplementary Fig. 9a-d). These data suggest that a pre-existing autoimmunity in *Cblb*$^{-/-}$ or *Cblb*^{C373A} mice does not account for differences relative to WT mice after fungal infection.

We also observed that *Clec7a*$^{-/-}$ and *Clec4n*$^{-/-}$ mice die at a similar rate upon systemic *C. albicans* infection, suggesting that both dectin-1 and dectin-2 are equally important for fungal recognition (Fig. 5f). Since *Cblb*$^{-/-}$*Clec7a*$^{-/-}$, *Cblb*$^{-/-}$ *Clec4n*$^{-/-}$, and *Cblb*$^{-/-}$*Clec7a*$^{-/-}$*Clec4n*$^{-/-}$ mice did not die at the same rate after infection as did *Clec7a*$^{-/-}$, *Clec4n*$^{-/-}$, or *Clec7a*$^{-/-}$*Clec4n*$^{-/-}$ mice (Fig. 5f), these results suggest that CBLB may regulate an additional CLR(s) such as the mannose receptor (MR), dectin-3 or Mincle which have been shown to be involved in host defense against *C. albicans* infection 4, 42–44. Indeed, loss of CBLB appeared to stabilize the protein expression of dectin-3, but not MR, Mincle and DC-SIGN (Supplementary Fig. 10).

CBLB is a potential therapeutic target for anti-fungal infection

Since CBLB down-regulates dectin family CLR signaling and host innate immune responses, decreasing CBLB expression may enhance phagocyte anti-fungal responses providing evidence for a new therapeutic approach. We performed experiments using *in vivo* delivery of *Cblb* siRNA to knock down *Cblb*. We first infected WT mice with *C. albicans* by i.v. injection, and 24 h later we injected *Cblb* siRNA or a nonsense siRNA via the tail vein. Mortality of the mice was monitored for 7 days. While all WT mice treated with nonsense siRNA died within 7 days after infection, 7 out of 9 WT mice treated with *Cblb* siRNA survived. There was a significantly higher fungal burden in the kidneys of WT mice receiving the nonsense siRNA compared to those receiving *Cblb* siRNA (Fig. 6). These data indicate that CBLB may serve as a potent therapeutic target for enhancing host defense against fungal infections.

Discussion

The fungal cell wall consists mainly of carbohydrates, including mannose-based structures (the mannoproteins), β-glucan, and chitin. Recognition of β-glucans and α-mannans by dectin-1 and -2 is essential for anti-fungal immunity 27. However, the regulation of dectin family receptors is unknown. Here we show that CBLB functions as a negative regulator of dectin-1 and -2 CLRs which initiate innate immune responses to fungal pathogens in human and mouse macrophages. CBLB targets dectin-1 and -2, and SYK for K48-linked polyubiquitination, which inhibits dectin-1/2-mediated signaling pathways. CBLB deficiency or inactivation leads to increased pro-inflammatory responses that decrease dissemination of *C. albicans* and bolster host defense.

To our knowledge, our findings are the first to identify a negative regulator of dectin receptor-mediated innate immune responses. We show that dectin-1$^{K2R, K27R, K34R}$ and dectin-2^{K10R} mutations, which abrogate their ubiquitination, result in increased production

of TNF-α and IL-6 by macrophages infected with *C. albicans* yeast or hyphae (Fig. 3g,h), thus mirroring the data obtained from *Cblb*$^{-/-}$ and *Cblb*^{C373A} mice. Our data therefore provide evidence that ubiquitination of dectin-1 and -2 is a key mechanism for terminating innate immune responses during fungal infection, thus avoiding excessive inflammation and subsequent tissue damage while at the same time damping optimal host defense properties.

Phagocytosis is a key cellular process, both during homeostasis and upon infection or tissue damage, and dectin-1 has been shown to be a phagocytic receptor 45. ROS production by phagocytes is associated with pathogen killing 46 and it was reported that dectin-1 activates SYK in macrophages and is important for dectin-1-stimulated ROS production, but not for phagocytosis 47. Consistent with this report, our data show that CBLB regulates both dectin-1 and -2 expression and ROS production by macrophages, but does not affect fungal phagocytosis (Supplementary Fig. 5). Our data suggest that additional receptor(s) such as Fcγ receptor family or DC-SIGN 44, 45, independent of regulation by CBLB, may be involved in controlling fungal phagocytosis.

Since CBLB is critical for T cell activation, tolerance induction and $T_H2/9$ cell differentiation 6, it is possible that the enhanced anti-fungal immunity in the absence of CBLB may result in heightened adaptive T cell responses. However, this possibility is excluded by the fact that the phenotype of *Cblb*$^{-/-}$*Rag1*$^{-/-}$ mice upon *C. albicans* infection, which do not have T and B cells, phenocopies that of *Cblb*$^{-/-}$ mice (Fig. 5e), supporting the notion that CBLB is crucial for controlling innate immune responses against systemic *C. albicans* infection. We also further demonstrate that the heightened innate immune responses observed during systemic *C. albicans* infection is mediated by dectin-1 and -2 because introducing dectin-1 or -2 deficiency, or both into *Cblb*$^{-/-}$ mice abrogates these heightened responses, and renders *Cblb*$^{-/-}$ mice susceptible to *C. albicans* infection (Fig. 5f; Supplementary Fig. 8a). More importantly, systemic *in vivo* delivery of *Cblb* siRNA to C57BL/6 mice protects them from lethal systemic *C. albicans* infection (Fig. 6). These data suggest that CBLB is a potential therapeutic target for controlling disseminated candidiasis. Of note, inhibition of CBLB may have detrimental effects due to unchecked inflammation, particularly on patients in intensive care. However, inhibition of *Cblb* by siRNA *in vivo* has a limited half-life, and dosages could be modulated to minimize the degree of inflammation. In addition, no signs of autoimmunity were observed in *Cblb*$^{-/-}$ or *Cblb*^{C373A} mice (Supplementary Fig. 9). However, given that we have shown that *Cblb*$^{-/-}$ mice develop severe airway inflammation, and an aberrant T_H2 response using ovalbumin-induced asthma model 14, it would be interesting to test whether mice deficient for CBLB or expressing the CBLB C373A mutation are susceptible to allergic bronchopulmonary aspergillosis in the future.

In summary, our data provide the first evidence that CBLB plays an essential role in regulating dectin-mediated innate immune responses to fungal pathogens following inflammatory responses to fungi in immunocompetent hosts. One consequence of this dampening of inflammatory responses is the creation of a less than optimal host defense program. Targeting CBLB may therefore serve as a new therapeutic strategy in fighting fungal infections.

Online Methods

Mice

C57BL/6 mice and *Rag*$^{-/-}$ were purchased from the Jackson Laboratory. *Fcer1g*$^{-/-}$ mice were purchased from Taconic (Hudson, NY). *Cblb*$^{-/-}$ mice 7 were kindly provided by Dr. Josef M. Penninger (University of Toronto; Toronto, ON, Canada). *Cblb*^{C373A} mice and *Clec7a*$^{-/-}$ were described previously 2, 35. *Clec4n*$^{-/-}$ mice described previously 3 were provided by Dr. Yoichiro Iwakura (Tokyo University of Science; Chiba, Japan). *Cblb*$^{-/-}$ mice on a C57BL/6 background were crossed onto *Clec7a*$^{-/-}$ or *Clec4n*$^{-/-}$ mice to generate *Cblb*$^{-/-}$*Clec7a*$^{-/-}$ and *Cblb*$^{-/-}$*Clec4n*$^{-/-}$ mice, or *Cblb*$^{-/-}$*Clec7a*$^{-/-}$*Clec4n*$^{-/-}$ mice. *Cblb*$^{-/-}$ mice were also crossed onto *Rag1*$^{-/-}$ strain to generate *Cblb*$^{-/-}$*Rag1*$^{-/-}$ mice. The mice were used at 8-12 weeks of age, and both male and female were used in this study. The use of animals was approved by the Institutional Animal Care and Use Committees (IACUCs) of the Ohio State University and Xiangya School of Medicine, Central South University.

Reagents

Antibodies against CBLB (G-1), SYK (N-19), CARD9 (H-90), DC-SIGN (T-13), CD206 (H-300), and Ubiquitin (P4D1), were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-dectin-1 (GE2; Ab82888) was obtained from Abcam (Cambridge, MA). Anti-dectin-2 (217611) and mouse IL-1RA/IL-1F3 Quantikine ELISA Kit (MRA00) was purchased from R&D System (Minneapolis, MN). The following items were purchased from BioLegend (San Diego, CA): PE-conjugated anti-dectin-1 (144204), FITC-conjugated anti-LAMP-1 (1D4B), anti-mouse CD45.2 antibody (104), anti-mouse CD8 (53-6.7), anti-mouse CD11b (M1/70), anti-mouse F4/80 (BM8), anti-mouse CD11c (N418), anti-mouse I-A/I-E (M5/114.15.2), anti-mouse Ly6C (HK1.4), anti-mouse Ly6G (1A8), ELISA kits for mouse IL-17A (432504), IFN-γ (430805), IL-6 (431304) and TNF-α (430904). PE-conjugated anti-dectin-2 (MCA2415PE) was obtained from AbD Serotec (Raleigh, NC). Anti-Mincle (D292-3) was purchased from MBL Life Science (Woburn, MA). ELISA kits for mouse IgG (88-50400) and IgE (88-50460) were purchased from eBioscience (San Diego, CA). ELISA kits for anti-ssDNA (5310) and anti-dsDNA (total (A+G+M) (5110) were purchased from Alpha Diagnostic International Inc (San Antonio, TX). The plasmids encoding *Clec7a* (dectin-1) and *Clec4n* (dectin-2) (pCMV2-Flag) were purchased from Sino Biologicals, Inc. (Beijing, P.R. China). Anti-K48-linkage specific polyubiquitin (4289), anti-K63-linkage specific polyubiquitin (D7A11), anti-phospho-SYK (Y525/526; #2711) and anti-phospho-NF-κB p65 (S536; #3031), and anti-phospho-IκBα (Ser32/36) (5A5; #9246) were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-dectin-3 was kindly provided by Dr. Xin Lin at MD Anderson Cancer Center (Houston, TX, USA). Mouse neutrophil isolation kit, monocyte isolation kit, and CD45 microbeads (mouse) were purchased from Miltenyi Biotec (San Diego, CA). Histopaque 1119 (Sigma 11191), Histopaque 1077 (Sigma 10771), and anti-Flag (M2) were obtained from Sigma-Aldrich (St. Louis, MO). Collagenase type IV (02195110) was purchased from MP Biomedicals (Santa Ana, CA). CellRox Deep Red (C10422) was purchased from ThermoFisher Scientific (Waltham, MA). The validation of the antibodies used is provided on the manufacturers' websites.

Site-directed mutagenesis

Single and triple K to R mutations of dectin-1^{K2R}, dectin-1^{K27R}, $dectin^{K34R}$, and $dectin^{K2R,K27R,K34R}$, dectin-2^{K10R}, dectin-1^{Y15F}, and FcR-$\gamma^{Y65F,Y76F}$ were generated by site-directed mutagenesis at Mutagenex Inc. (Piscataway, NJ).

Generation of BMDMs and BMDCs and isolation of mouse BM neutrophils

BM cells were harvested from the femurs and tibias of mice. Cells were cultured in DMEM containing 10% FBS and 30% conditioned medium from L929 cells expressing M-CSF. After one week of culture, nonadherent cells were removed, and adherent cells were 80-90% $F4/80^{+}CD11b^{+}$ as determined by flow cytometric analysis. Mouse BMDCs were generated using GM-CSF and purified from bulk cultures by magnetic selection with anti-CD11c microbeads. This routinely gave purities of >98%. For isolation of BM neutrophils, total BM cells were recovered from the femurs and tibias by flushing with RPMI medium with an 18-gauge needle; erythrocytes were lysed with red blood cells (RBC) lysis buffer (eBioscience) and BM neutrophils were isolated by neutrophil isolation kit (Miltenyl), and neutrophil purity (>98%) was confirmed by flow cytometry.

Isolation of mouse PBMCs and neutrophils from blood, and splenic monocytes, neutrophils, and kidney CD 45^{+} cells

WT and *Cblb*^{C373A} mice were anesthetized, and blood was collected from tail vein. The RBC were lysed using RBC lysis buffer (eBioscience). PBMCs and neutrophils were isolated by gradient centrifugation over Histopaque 1119 (density, 1.119 g/ml) and Histopaque 1077 (density, 1.077 g/ml) according to the manufacturer's instructions at 400 × g for 30 min at 25 °C 48. PBMCs were collected from the interface between the plasma and Histopaque 1077. Neutrophils were recovered at the interface of the interface of the Histopaque 1119 and Histopaque 1077 layers, and were 80–90% pure and >95% viable as determined by flow cytometry. PBMCs and neutrophils were washed twice and were resuspended in RPMI 1640 medium supplemented with 10% FBS. Splenic monocytes and neutrophils of WT and *Cblb*^{C373A} mice were isolated by monocyte isolation and neutrophil isolation kits (Miltenyl), and monocyte and neutrophil purity (>98%) were confirmed by flow cytometry.

WT and *Cblb*^{C373A} mice were sacrificed at 48 h after infection with *C. albicans* by tail vein injection at a dose of 1×10^{6} CFU. Kidneys were perfused, minced, and placed in 2 ml of Hank's balanced salt solution (HBSS) (50 mM HEPES, 12 mM Dextrose, 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, PH to 7.05) containing 2 mg/ml collagenase IV, and incubated at 37 °C for 30 min with gentle agitation. Digested kidney tissues were passed through a 40 μm Falcon™ cell strainer using the rubber end of a 1 ml syringe plunger, and a cell suspension was obtained via centrifugation at 1200 rpm for 10 min. The $CD45^{+}$ cells were purified by CD45 MicroBeads (Miltenyl). The purity (~90%) was determined by flow cytometry.

In vitro infection of macrophages, dendritic cells, and neutrophils with *C. albicans* yeast and hyphal forms

A single colony of *C. albicans* strain SC5314 was grown overnight at 30 °C in yeast peptone dextrose media. The cells were washed twice with PBS before use as live yeasts. The *cap1* yeast-only mutant described previously 17 was obtained from Dr. Paula Sundstrom (Dartmouth University). For the hyphal forms, the washed yeasts were resuspended at 10^7 cells/ml in RPMI 1640 with 10% FCS and grown 3 h at 37 °C. After washing in PBS, the hyphae were used for live stimulations. For analysis of cytokine production, 10^5 BMDMs, BMDCs, or neutrophils were cultured overnight in a 96-well U-bottomed plate with live *C. albicans cap1* mutant or hyphae at MOI of 1 for times indicated, cytokine levels in the supernatant were measured by sandwich ELISA.

BMDM reconstitution

Clec7a$^{-/-}$ BMDMs were transfected with Flag-tagged dectin-1 or dectin-1^{K2R}, dectin-1^{K27R}, dectin^{K34R}, or dectin-1^{K2R,K27R,K34R}, or dectin-1^{Y15F} by Lipofectamine 2000. *Clec4n*$^{-/-}$ or *Fcerg1*$^{-/-}$ BMDMs were transfected with Flag-tagged dectin-2, dectin-2^{K10R}, FcR-γ, and FcR-γ^{Y65F,Y76F}, respectively.

ROS assay, phagocytosis of *C. albicans* and fungal killing assay

For ROS production assay, 2×10^5 WT or BMDMs were washed with PBS twice, and replated in PBS containing 100 mM luminol and 5 units of horseradish peroxidase. The cells were incubated at 37 °C for 30 min, and then infected with *C. albicans* at MOI = 5:1 and 2:1, respectively. The relative amount of ROS generated by neutrophils was detected at regular intervals over 75 min by measuring the luminescence. Relative light units (RLU) were plotted as a function of time to evaluate chemiluminescence (CL) rate. To measure ROS expression in monocytes, macrophages and neutrophils in spleens and kidneys, WT and *Cblb*$^{-/-}$ mice were infected with *C. albicans* by tail vein injection at a dose of 1×10^6 CFU. 48 h later mice were sacrificed, and leukocytes from spleens and kidneys were infected with *C. albicans* for 30 min, and stained with CellRox and cell surface markers to determine ROS expression in monocytes (Kidney: CD45.2$^+$CD11b$^+$ Ly6C^{hi}Ly6G$^-$; Spleen: CD11b$^+$Ly6C$^+$Ly6G$^-$), macrophages (Kidney: CD45.2$^+$ CD11b$^+$F4/80$^+$Ly6C^{lo}CD11c$^-$; Spleen: CD11b$^+$F4/80$^+$Ly6C^{lo}Ly6G$^-$), and neutrophils (Kidney: CD45.2$^+$CD11b$^+$ Ly6C^{lo} Ly6G$^+$; Spleen: CD11b$^+$Ly6G$^+$Ly6C$^-$).

For phagocytosis *of C. albicans*, *C. albicans* yeast were labeled with Alexa Fluor 488 (Invitrogen) in 100 mM HEPES buffer (pH 7.5)(diluted to 1:500), and then co-cultured with WT or *Cblb*$^{-/-}$ BMDMs for 45 min at 37 °C. Adherent fungal cells were quenched with trypan blue, and the rate of phagocytosis was determined by flow cytometry 49.

For *in vitro* fungal killing assay, WT or *Cblb*$^{-/-}$ BMDMs (1×10^5/well) were incubated with *C.albicans* at MOI 1:500 for 24 h. To determine the fungal killing capacity of PBMCs, blood neutrophils, splenic monocytes and neutrophils, and kidney CD45$^+$ cells, WT and *Cblb*^{C373A} mice were infected with *C. albicans* by tail vein injection (1×10^6 CFU). PBMCs, blood neutrophils, and splenic monocytes, neutrophils and kidney CD45$^+$ cells were co-cultured with *C.albicans* form at MOI 1:10 for 24 h. After co-culture, a 100 μl suspension was spread

($1:10^4$ dilution) on YPD plates. After incubation at 37 °C for 36 h, killing was determined by counting the Candida colonies with and without indicated cells 49.

Immunopreciptation and Western blotting

For co-immunoprecipitation, WT BMDMs were infected with *C. albicans* yeast or hyphae (MOI = 1:1) for various times, and lysed in 0.5% NP40 lysis buffer. The cell lysates were immunoprecipitated with anti-CBLB (1:100) and blotted with anti-dectin-1 (1:1000) or anti-dectin-2 (1:5000), anti-SYK (1:1000), and anti-CARD9 (1:1000). For detection of dectin-1 or dectin-2 ubiquitination, BMDMs from WT, and *Cblb*$^{-/-}$ or *Cblb*^{C373A} mice were infected with *C. albicans* yeast *cap1* mutant or hyphae (MOI = 1:1) for various times, and lysed in RIPA buffer containing 2% SDS, and diluted to 0.5% of SDS. The cell lysates were immunoprecipitated with anti-dectin-1 (1:100) or anti-dectin-2 (1:100), and blotted with anti-ubiquitin (1:1000), anti-K48- or anti-K63-specific ubiquitin antibodies (1:1000). To assess the protein stability of dectin-1, dectin-2, dectin-3, MR, Mincle, DC-SIGN, SYK and CARD9, BMDMs from WT and *Cblb*$^{-/-}$ mice were infected with *C. albicans* yeast or hyphae (MOI = 1:1) at indicated times and lysed for immunoblotting with antibodies against dectin-1 (1:1000), dectin-2 (1:1000), dectin-3 (1:1000), MR (1:1000), Mincle (1:1000), DC-SIGN (1:1000), SYK, and CARD9, respectively. To determine whether dectin-1 and dectin-2 undergo proteasome or lysosome-mediated degradation, WT BMDMs were pretreated with MG-132 (5 μM) or E64 (10 μM) for 30 min, and then infected with *C. albicans* yeast *cap1* mutant or hyphae (MOI = 1:1) for various times, and lysed. The cell lysates were blotted with anti-dectin-1 or anti-dectin-2.

Detection of serum and kidney cytokines, serum IgG and IgE, and autoantibodies by ELISA

For detection of TNF-α, IL-6, IL-1β, and IL-1RA in macrophage culture supernatants, 10^5 BMDMs from WT, *Cblb*$^{-/-}$ or *Cblb*^{C373A} mice were infected with live *C. albicans cap1* mutant or hyphae at MOI 1:1 for the times indicated, and cytokine production in the supernatant was measured by ELISA. WT and *Cblb*$^{-/-}$ BMDMs were also infected with *A. fumigatus* conidia (MOI = 1:1) for the times indicated, TNF-α and IL-6 in the supernatant were measured by ELISA.

For detection of serum IL-17, IFN-γ, IL-6, TNF-α and IL-1β, WT, *Cblb*$^{-/-}$ or *Cblb*^{C373A} mice were infected with *C. albicans* (5×10^4, or 1×10^6 CFU for some experiments), sera were collected at different time-points, and subjected for ELISA analysis. The kidneys harvested at 48 h after infection were homogenized, and the supernatant was recovered following centrifugation at 15000 g for 20 min at 4 °C. The cytokines including IL-17, IFN-γ, and IL-6 in the kidney homogenates were determined by using were collected by ELISA kits according to the manufacturer's instructions. The ELISA results were expressed as pg/g of kidney. For detection of serum IgG, IgE, and anti-ssDNA, anti-dsDNA, sera were collected from WT, *Cblb*$^{-/-}$ or *Cblb*^{C373A} mice before *C. albicans* infection and 48 h after infection, and subjected to ELISA analysis.

Internalization of dectin-1 and dectin-2 in macrophages upon infection with *C. albicans* yeast and hyphae

WT and *Cblb*$^{-/-}$ BMDMs were infected with *C. albicans* yeast *cap1* mutant (MOI: 1:1) for times indicated. The flow cytometry was then used to determine the surface expression of dectin-1 and dectin-2. For dectin-1 internalization, BMDMs from WT and *Cblb*$^{-/-}$ mice were labeled with PE-conjugated anti-dectin-1 (1:200) or anti-dectin-2 (1:200). Cells were then incubated at 37 °C for 5, 15, and 30 min. To remove uninternalized dectin-1- or dectin-2 coupled antibodies from the cell surface, half the cells from each time point were treated briefly with ice-cold acidic buffer (1% BSA at pH 3.0) and immediately neutralized in PBS containing 1% BSA and 0.5% NaN_3. Both treated and untreated cells were stained with anti-F4/80 and anti-CD11b. Dectin-1 internalization was calculated with gated F4/80 and CD11b-positive cells using the formula: % of dectin-1 or dectin-2 internalization = 100 × [MFI of acid-resistant PE fluorescence (at time t)-MFI of acid-resistant PE fluorescence (at time 0)/MFI of total PE fluorescence of untreated cells].

Confocal microscopy

WT and *Cblb*$^{-/-}$ BMDMs were attached to poly(L)lysine-coated coverslips, surface-labeled PE-conjugated anti-dectin-1 or anti-dectin-2 on ice. Labeled cells were infected with *C. albicans* yeast *cap1* mutant or hyphae for 30 min at 37 °C to allow dectin-1 or dectin-2 internalization to occur. The cells were fixed in 1% paraformaldehyde, permeabilized in 0.05% saponin and stained with FITC-conjugated anti-LAMP-1. Imaging was performed on a Leica TCS-SP2 confocal microscope (1:100). Imaging was performed on a laser scanning confocal microscope (Flowview 1000, Olympus).

Systemic *C. albicans* dissemination

For survival analysis, mice were infected with *C. albicans* i.v. at 1-5 × 10^5 CFU, and monitored daily. After infection, mice were weighed and monitored daily. Mice were euthanized if they lost > 20% of their body weight. In a separate group, the kidneys were harvested 2 days after infection. The left kidneys were photographed and homogenized for enumeration of fungal burden. The right kidneys were fixed for histological analysis. The fungal burden in the kidneys, spleens, livers, and lungs was determined by CFU in kidney, spleen, liver, and lung homogenates. The fungal burden in the blood at 2 and 6 h after infection was also determined. Mice were allocated to experimental groups based upon their genotypes and randomized within their sex and age matched groups. No blinding was done in this study.

Generation of human monocyte-derived macrophages (MDM) and silencing of the *Cblb* gene

Human MDMs were generated as previously described 25, 26. In brief, peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated from heparinized blood on Ficoll-sodium diatrizoate gradients and then cultured for 5 days in RPMI containing 20% autologous serum (2.0 × 10^6 mononuclear cells/ml) at 37 °C. On Day 5 human MDMs were transfected with control siRNA or *Cblb* siRNA (100 or 200 nM) by using Lonza nucleofector reagent and plated in RPMI 1640 containing 20% autologous serum. Peripheral

blood mononuclear cells (PBMCs) from healthy donors were isolated from heparinized blood on Ficoll-sodium diatrizoate gradients and then cultured for 5 days in RPMI containing 20% autologous serum (2.0×10^6 mononuclear cells/ml) at 37 °C. On Day 5 human MDMs were transfected with control siRNA or *Cblb* siRNA (100 or 200 nM; Dharmacon RNA Technologies) by using Lonza nucleofector reagent and plated in RPMI 1640 containing 20% autologous serum. After 36 h, the MDMs were washed and infected with yeast or hyphae of *C.albicans*. The protocol was approved by The Ohio State University Institutional Review Board.

In vivo delivery of *Cblb* siRNA

WT mice were injected with treated with *C. albicans* i.v. at 5×10^5 CFU, and 24 h later were treated with in vivo grade *Cblb* siRNA (5'-AAAUUCUCGAAGUAUGCUCUU-3') or a non-sense siRNA (2 mg/kg/mouse) (Dharmacon RNA Technologies) in *In vivo*-jetPEI®-FluoF (Polyplus-transfection, Inc.; New York, NY) via tail vein injection. Three days later, the spleen cells are collected, and lysed in RIPA buffer. The cell lysates were blotted with anti-CBLB and anti-ACTIN, respectively.

Data analysis and statistic analysis

Differences in concentrations of cytokines and fungal burden were analyzed using the Student's *t* test. Survival data were analyzed using the Kaplan-Meyer log rank test. Differences were considered significant at a *P* value of < 0.05. No animals were excluded from the analysis. Mice were allocated to experimental groups based upon their genotypes and randomized within their sex and age matched groups. No statistical method was used to predetermine sample size. It was assumed that normal variance occurs between experimental groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Brown GD, et al. Hidden killers: human fungal infections. Sci Transl Med. 2012; 4:165rv113.
2. Taylor PR, et al. Dectin-1 is required for beta-glucan recognition and control of fungal infection. Nat Immunol. 2007; 8:31–38. [PubMed: 17159984]
3. Saijo S, et al. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against Candida albicans. Immunity. 2010; 32:681–691. [PubMed: 20493731]

4. Zhu LL, et al. C-type lectin receptors Dectin-3 and Dectin-2 form a heterodimeric pattern-recognition receptor for host defense against fungal infection. Immunity. 2013; 39:324–334. [PubMed: 23911656]
5. Hernandez-Santos N, Gaffen SL. Th17 cells in immunity to Candida albicans. Cell Host Microbe. 2012; 11:425–435. [PubMed: 22607796]
6. Liu Q, Zhou H, Langdon WY, Zhang J. E3 ubiquitin ligase Cbl-b in innate and adaptive immunity. Cell Cycle. 2014; 13:1875–1884. [PubMed: 24875217]
7. Bachmaier K, et al. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. Nature. 2000; 403:211–216. [PubMed: 10646608]
8. Jeon MS, et al. Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction. Immunity. 2004; 21:167–177. [PubMed: 15308098]
9. Qiao G, et al. T-cell receptor-induced NF-kappaB activation is negatively regulated by E3 ubiquitin ligase Cbl-b. Mol Cell Biol. 2008; 28:2470–2480. [PubMed: 18227156]
10. Li D, et al. Cutting edge: Cbl-b: one of the key molecules tuning CD28- and CTLA-4-mediated T cell costimulation. J Immunol. 2004; 173:7135–7139. [PubMed: 15585834]
11. Zhang J, et al. Cutting edge: regulation of T cell activation threshold by CD28 costimulation through targeting Cbl-b for ubiquitination. J Immunol. 2002; 169:2236–2240. [PubMed: 12193687]
12. Guo H, et al. E3 ubiquitin ligase Cbl-b regulates Pten via Nedd4 in T cells independently of its ubiquitin ligase activity. Cell Rep. 2012; 1:472–482. [PubMed: 22763434]
13. Qiao G, et al. T cell activation threshold regulated by E3 ubiquitin ligase Cbl-b determines fate of inducible regulatory T cells. J Immunol. 2013; 191:632–639. [PubMed: 23749633]
14. Qiao G, et al. E3 Ubiquitin Ligase Cbl-b suppresses proallergic T cell development and allergic airway inflammation. Cell Rep. 2014; 6:709–723. [PubMed: 24508458]
15. Brown GD. Dectin-1: a signalling non-TLR pattern-recognition receptor. Nat Rev Immunol. 2006; 6:33–43. [PubMed: 16341139]
16. Yoshitomi H, et al. A role for fungal beta-glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. J Exp Med. 2005; 201:949–960. [PubMed: 15781585]
17. Bahn YS, Sundstrom P. CAP1, an adenylate cyclase-associated protein gene, regulates bud-hypha transitions, filamentous growth, and cyclic AMP levels and is required for virulence of Candida albicans. J Bacteriol. 2001; 183:3211–3223. [PubMed: 11325951]
18. Hohl TM, Feldmesser M. Aspergillus fumigatus: principles of pathogenesis and host defense. Eukaryot Cell. 2007; 6:1953–1963. [PubMed: 17890370]
19. Steele C, et al. The beta-glucan receptor dectin-1 recognizes specific morphologies of Aspergillus fumigatus. PLoS Pathog. 2005; 1:e42. [PubMed: 16344862]
20. Gersuk GM, Underhill DM, Zhu L, Marr KA. Dectin-1 and TLRs permit macrophages to distinguish between different Aspergillus fumigatus cellular states. J Immunol. 2006; 176:3717–3724. [PubMed: 16517740]
21. Rivera A, et al. Dectin-1 diversifies Aspergillus fumigatus-specific T cell responses by inhibiting T helper type 1 CD4 T cell differentiation. J Exp Med. 2011; 208:369–381. [PubMed: 21242294]
22. Hise AG, et al. An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen Candida albicans. Cell Host Microbe. 2009; 5:487–497. [PubMed: 19454352]
23. Gringhuis SI, et al. Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1beta via a noncanonical caspase-8 inflammasome. Nat Immunol. 2012; 13:246–254. [PubMed: 22267217]
24. Smeekens SP, et al. An anti-inflammatory property of Candida albicans beta-glucan: Induction of high levels of interleukin-1 receptor antagonist via a Dectin-1/CR3 independent mechanism. Cytokine. 2015; 71:215–222. [PubMed: 25461401]
25. Kang PB, et al. The human macrophage mannose receptor directs Mycobacterium tuberculosis lipoarabinomannan-mediated phagosome biogenesis. J Exp Med. 2005; 202:987–999. [PubMed: 16203868]

26. Rajaram MV, et al. Mycobacterium tuberculosis lipomannan blocks TNF biosynthesis by regulating macrophage MAPK-activated protein kinase 2 (MK2) and microRNA miR-125b. Proc Natl Acad Sci U S A. 2011; 108:17408–17413. [PubMed: 21969554]
27. Brown GD. Innate antifungal immunity: the key role of phagocytes. Annu Rev Immunol. 2011; 29:1–21. [PubMed: 20936972]
28. Hardison SE, Brown GD. C-type lectin receptors orchestrate antifungal immunity. Nat Immunol. 2012; 13:817–822. [PubMed: 22910394]
29. Osorio F, Reis e Sousa C. Myeloid C-type lectin receptors in pathogen recognition and host defense. Immunity. 2011; 34:651–664. [PubMed: 21616435]
30. Kerscher B, Willment JA, Brown GD. The Dectin-2 family of C-type lectin-like receptors: an update. Int Immunol. 2013; 25:271–277. [PubMed: 23606632]
31. Miyake Y, et al. C-type lectin MCL is an FcRgamma-coupled receptor that mediates the adjuvanticity of mycobacterial cord factor. Immunity. 2013; 38:1050–1062. [PubMed: 23602766]
32. Sato K, et al. Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses. J Biol Chem. 2006; 281:38854–38866. [PubMed: 17050534]
33. Sohn HW, Gu H, Pierce SK. Cbl-b negatively regulates B cell antigen receptor signaling in mature B cells through ubiquitination of the tyrosine kinase Syk. J Exp Med. 2003; 197:1511–1524. [PubMed: 12771181]
34. Kojo S, et al. Mechanisms of NKT cell anergy induction involve Cbl-b-promoted monoubiquitination of CARMA1. Proc Natl Acad Sci U S A. 2009; 106:17847–17851. [PubMed: 19815501]
35. Oksvold MP, Dagger SA, Thien CB, Langdon WY. The Cbl-b RING finger domain has a limited role in regulating inflammatory cytokine production by IgE-activated mast cells. Mol Immunol. 2008; 45:925–936. [PubMed: 17868870]
36. Sorkin A, Von Zastrow M. Signal transduction and endocytosis: close encounters of many kinds. Nat Rev Mol Cell Biol. 2002; 3:600–614. [PubMed: 12154371]
37. Haglund K, et al. Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nat Cell Biol. 2003; 5:461–466. [PubMed: 12717448]
38. Lin Q, et al. HECT E3 ubiquitin ligase Nedd4-1 ubiquitinates ACK and regulates epidermal growth factor (EGF)-induced degradation of EGF receptor and ACK. Mol Cell Biol. 2010; 30:1541–1554. [PubMed: 20086093]
39. Hernanz-Falcon P, Joffre O, Williams DL, Reis e Sousa C. Internalization of Dectin-1 terminates induction of inflammatory responses. Eur J Immunol. 2009; 39:507–513. [PubMed: 19130473]
40. Nicola AM, Casadevall A, Goldman DL. Fungal killing by mammalian phagocytic cells. Curr Opin Microbiol. 2008; 11:313–317. [PubMed: 18573683]
41. Brown AJ, Haynes K, Quinn J. Nitrosative and oxidative stress responses in fungal pathogenicity. Curr Opin Microbiol. 2009; 12:384–391. [PubMed: 19616469]
42. Wells CA, et al. The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to Candida albicans. J Immunol. 2008; 180:7404–7413. [PubMed: 18490740]
43. van de Veerdonk FL, et al. The macrophage mannose receptor induces IL-17 in response to Candida albicans. Cell Host Microbe. 2009; 5:329–340. [PubMed: 19380112]
44. Cambi A, et al. The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for Candida albicans on dendritic cells. Eur J Immunol. 2003; 33:532–538. [PubMed: 12645952]
45. Goodridge HS, Underhill DM, Touret N. Mechanisms of Fc receptor and dectin-1 activation for phagocytosis. Traffic. 2012; 13:1062–1071. [PubMed: 22624959]
46. Dupre-Crochet S, Erard M, Nubetae O. ROS production in phagocytes: why, when, and where? J Leukoc Biol. 2013; 94:657–670. [PubMed: 23610146]
47. Underhill DM, Rossnagle E, Lowell CA, Simmons RM. Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. Blood. 2005; 106:2543–2550. [PubMed: 15956283]

48. Swamydas M, Luo Y, Dorf ME, Lionakis MS. Isolation of Mouse Neutrophils. Curr Protoc Immunol. 2015; 110 3 20 21-23 20 15.

49. Wirnsberger G, et al. Jagunal homolog 1 is a critical regulator of neutrophil function in fungal host defense. Nat Genet. 2014; 46:1028–1033. [PubMed: 25129145]

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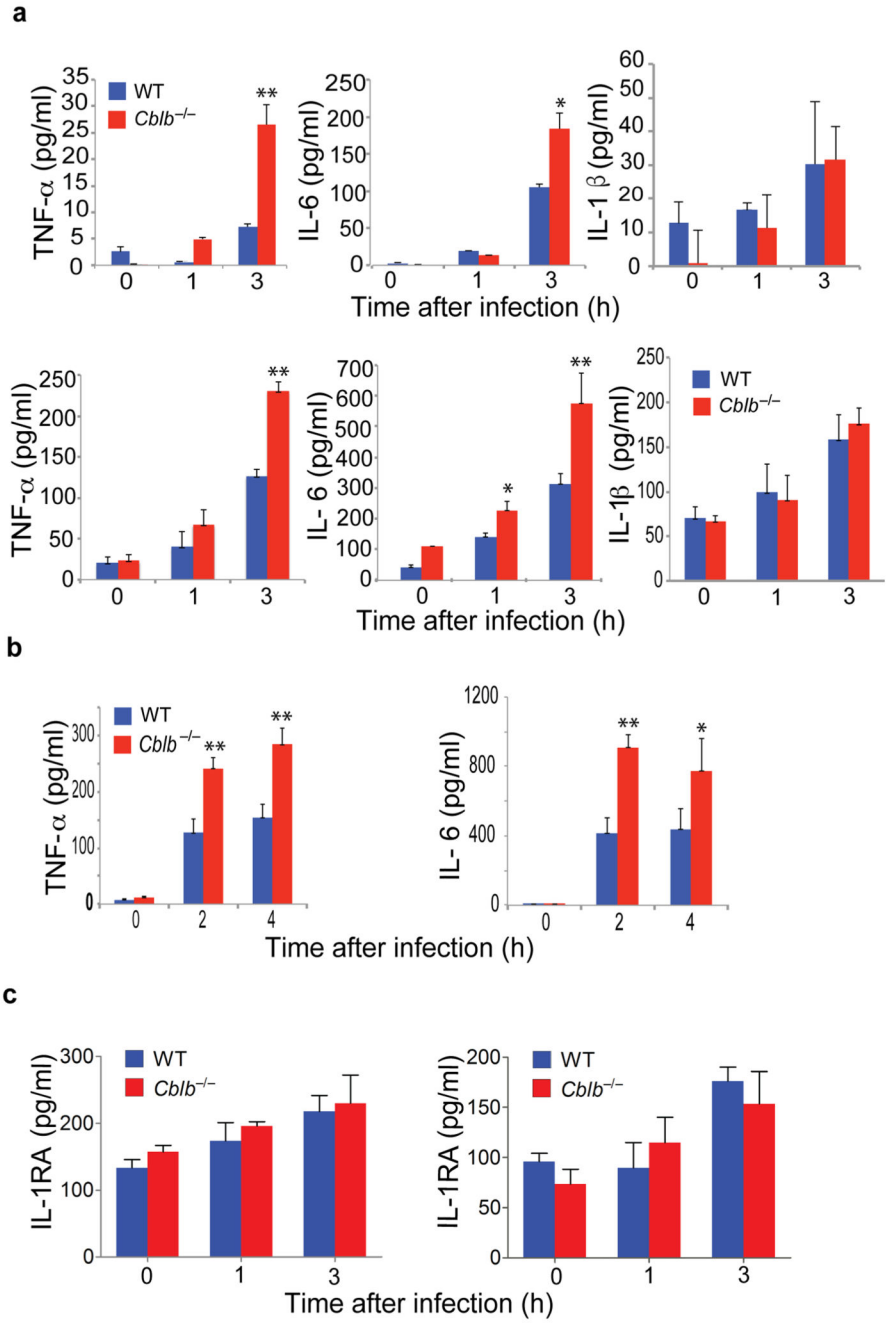

Figure 1.

CBLB inhibits pro-inflammatory cytokine production by macrophages upon infection with *C. albicans* yeast or hyphae and *A. fumigatus* conidia. (**a**) ELISA of TNF-α, IL-6, and IL-1β production in the supernatants collected from BMDMs of WT and *Cblb*$^{-/-}$ mice infected with *C. albicans* yeast *cap1* mutant (thereafter yeast) and hyphal forms (WT strain SC5314) (MOI: 1:1) for 1 and 3 h. For preparation of hyphae, washed yeast cells were counted, re-suspended in RPMI-1640 medium, grown in 12-well plates at 37 °C for 3 h, and washed three times with PBS. (**b**) ELISA of TNF-α and IL-6 production in the supernatants

collected from BMDMs of WT and *Cblb*$^{-/-}$ mice infected with swollen *A. Fumigatus* conidia (AF293) (MOI = 1:1) for 2 and 4 h. (**c**) ELISA of IL-1RA production in the supernatants collected from BMDMs of WT and *Cblb*$^{-/-}$ mice infected with *C. albicans* yeast and hyphal forms. For all ELISA experiments data are representative of three independent experiments (biological replicates). Error bars are mean ± s.d. *$P < 0.05$, **$P < 0.01$; unpaired two-tailed Student's t test. $n = 3$ per group, each with three repeated wells.

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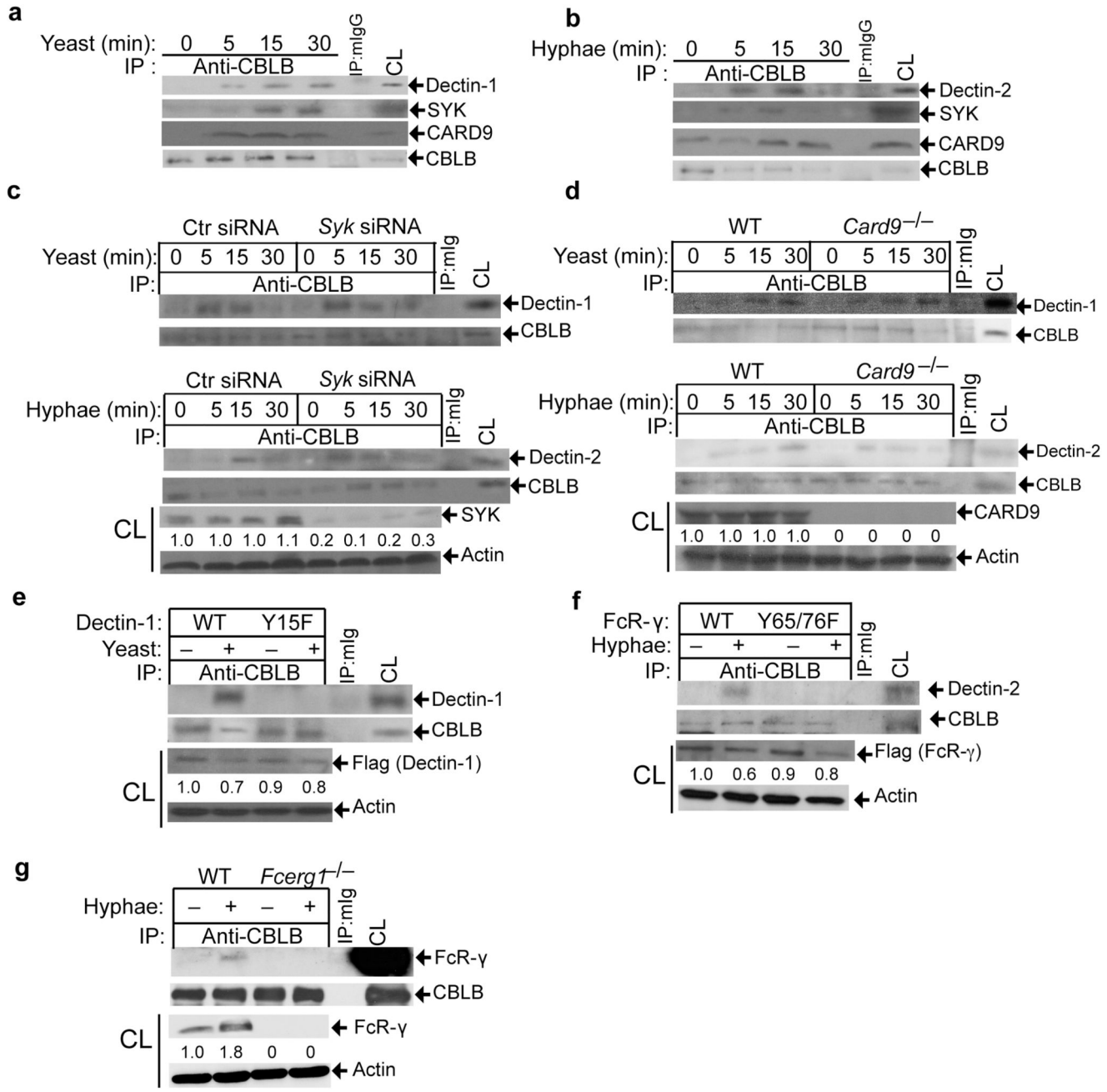

Figure 2.

CBLB associates with dectin-1 and dectin-2 in macrophages upon *C. albicans* yeast and hyphal infection. (**a,b**) Immunoblot analysis of dectin-1 or dectin-2, SYK, and CARD9 after immunoprecipitation (IP) with CBLB antibodies from lysates of BMDMs uninfected or infected with *C. albicans* yeast or hyphae. Images are representative of three independent experiments (biological replicates), and each IP was blotted separately. (**c,d**) Immunoblot analysis of dectin-1 or dectin-2, SYK and CARD9 after immunoprecipitation (IP) of proteins with CBLB antibodies from lysates of WT BMDMs with or without *Syk* gene silencing (**c**) or WT and *Card9*$^{-/-}$ BMDMs (**d**) uninfected or infected with *C. albicans* yeast

or hyphae. Images are representative of two independent experiments (biological replicates), and each IP was blotted separately. (**e**) Immunoblot analysis of dectin-1 after CBLB immunoprecipitation from lysates of *Clec7a*$^{-/-}$ BMDMs reconstituted with Flag-tagged dectin-1 or dectin-1^{Y15F} mutant, and infected with *C. albicans* yeast. Images are representative of three independent experiments (biological replicates), and each IP was blotted separately. (**f**) Immunoblot analysis of dectin-2 after CBLB immunoprecipitation from lysates of *Fcer1g*$^{-/-}$ BMDMs reconstituted with Flag-tagged FcR-γ or FcR-γ^{Y65F,Y76F} mutant, and infected with *C. albicans* hyphae. Images are representative of two independent experiments (biological replicates), and each IP was blotted separately. (**g**) Immunoblot analysis of FcR-γ after CBLB immunoprecipitation from lysates of WT and *Fcer1g*$^{-/-}$ BMDMs infected with *C. albicans* hyphae. Images are representative of three independent experiments (biological replicates), and each IP was blotted separately.

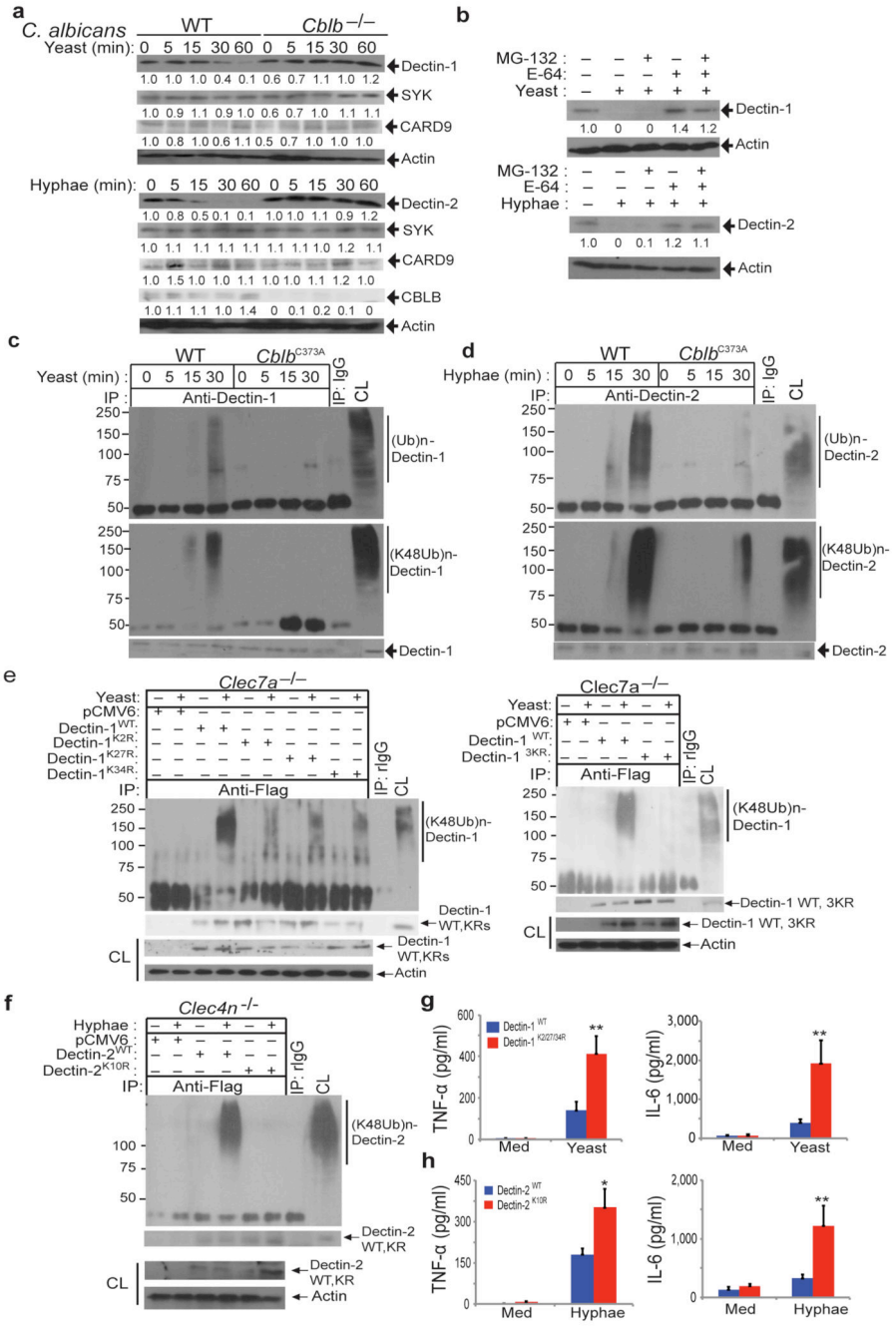

Figure 3.

CBLB targets dectin-1 and dectin-2 for polyubiquitination and subsequent degradation in the lysosome. **(a)** Immunoblot analysis of lysates of WT and *Cblb*$^{-/-}$ BMDMs infected with *C. albicans* yeast and hyphal forms (MOI = 1:1) with antibodies against dectin-1, dectin-2, SYK, CBLB, and ACTIN, respectively. Images are representative of five independent experiments (biological replicates). (**b**) Immunoblot analysis of WT BMDMs pretreated with E-64 (10 μM), MG-132 (5 μM), or both for 30 min, then infected with *C. albicans* yeast or hyphae (MOI = 1:1), with antibodies against to dectin-1 and dectin-2, respectively.

Images are representative of three independent experiments (biological replicates). (**c, d**) Immunoblot analysis of dectin-1 and dectin-2 ubiquitination of dectin-1 or dectin-2 immunoprecipitates isolated from BMDMs from WT and *Cblb*^{C373A} mice infected with *C. albicans* yeast and hyphae, respectively, by anti-ubiquitin and anti-K48 ubiquitin antibodies. Images are representative of four independent experiments (biological replicates), and each IP was blotted separately. (**e**) Ubiquitination of dectin-1 in *Clec7a*$^{-/-}$ BMDMs reconstituted with WT dectin-1 or dectin-1^{K2R}, dectin-1^{K27R}, dectin-1^{K34R} mutant, or dectin-1$^{K2R, K27R, K34R}$ triple mutant infected with *C. albicans* yeast. Images are representative of three independent experiments (biological replicates), and each IP was blotted separately. (**f**) Ubiquitination of dectin-2 in *Clec4n*$^{-/-}$ BMDMs reconstituted with WT dectin-2 or dectin-2^{K10R} infected with *C. albicans* hyphae. Images are representative of three independent experiments (biological replicates), and each IP was blotted separately. (**g, h**) ELISA of TNF-α and IL-6 production by *Clec7a*$^{-/-}$ BMDMs reconstituted with WT dectin-1 or dectin-1^{K2R,K27R,K34R} infected with *C. albicans* yeast (**g**), or by *Clec4n*$^{-/-}$ BMDMs reconstituted with WT dectin-2 or dectin-2^{K10R} infected with *C. albicans* hyphae (**h**). Data are representative of three independent experiments (biological replicates). Error bars are mean ± s.d. $*P < 0.05$, $**P < 0.01$; unpaired two-tailed Student's *t* test. $n = 3$ per group, each with three repeated wells.

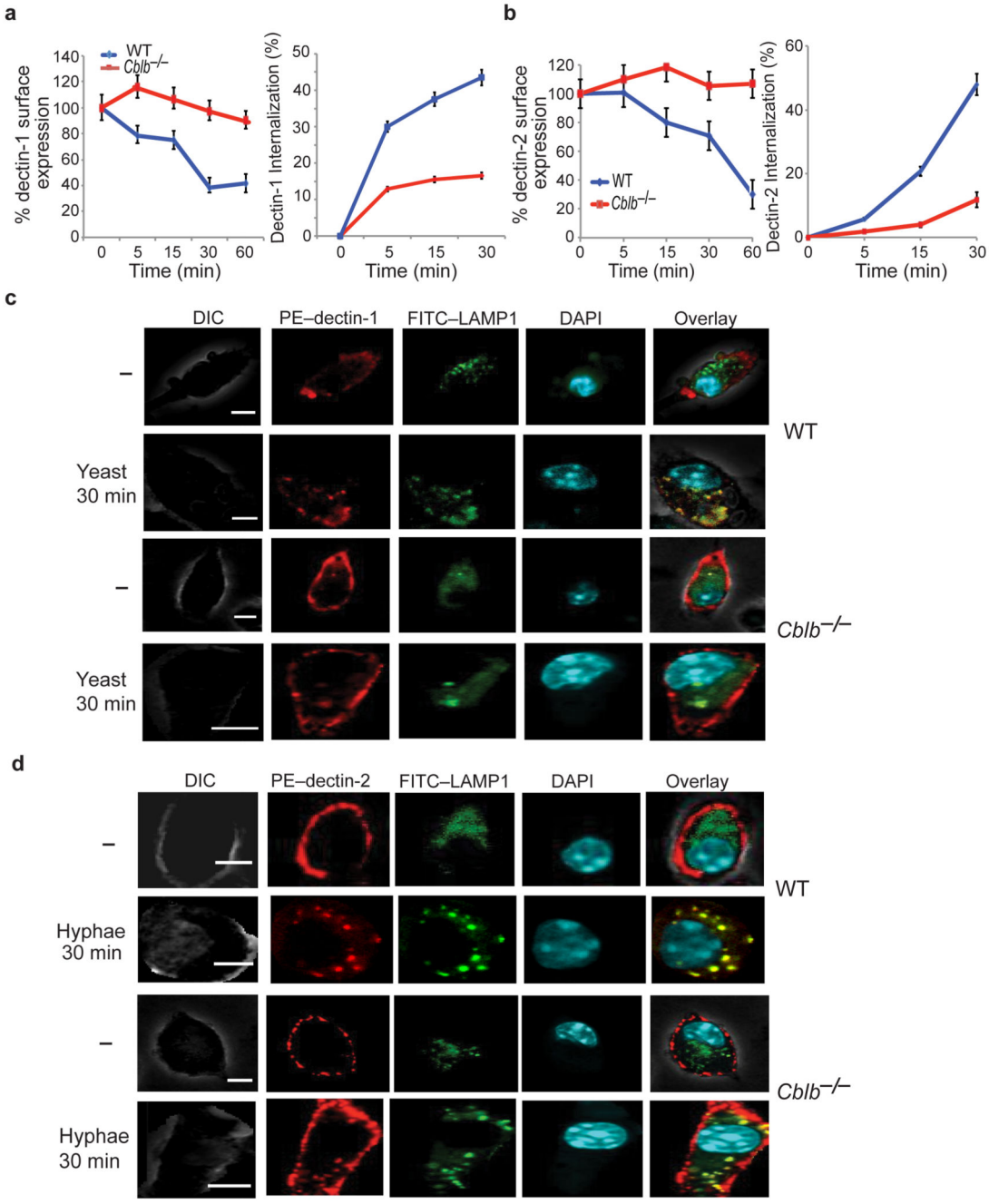

Figure 4.

Loss of CBLB impairs dectin-1 and dectin-2 internalization and their down-regulation at the cell surface. (**a**,**b**) Cell surface and intracellular expression of dectin-1 and dectin-2 of WT and *Cblb*$^{-/-}$ BMDMs infected with *C. albicans* yeast or hyphae (MOI = 1:1) for times indicated by flow cytometry. For internalization of dectin-1 and dectin-2, WT and *Cblb*$^{-/-}$ BMDMs were treated with acid buffer to strip the antibodies remaining at the cell surface after infection at each time-point. Data are representative of three independent experiments (biological replicates). Error bars are mean ± s.d. **P* < 0.05; unpaired two-tailed Student's *t*

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test. $n = 3$ per group, each with three repeated wells. (**c,d**) Confocal image of dectin-1 and dectin-2 internalization and lysosome sorting of WT and *Cblb*$^{-/-}$ BMDMs infected or uninfected with *C. albicans* yeast (**c**) or hyphae (**d**) (MOI = 1:1) for 30 min. Images are representative of five independent experiments (biological replicates). $n = 3$ per group, each with three repeated wells. Scale bar, 5 μm.

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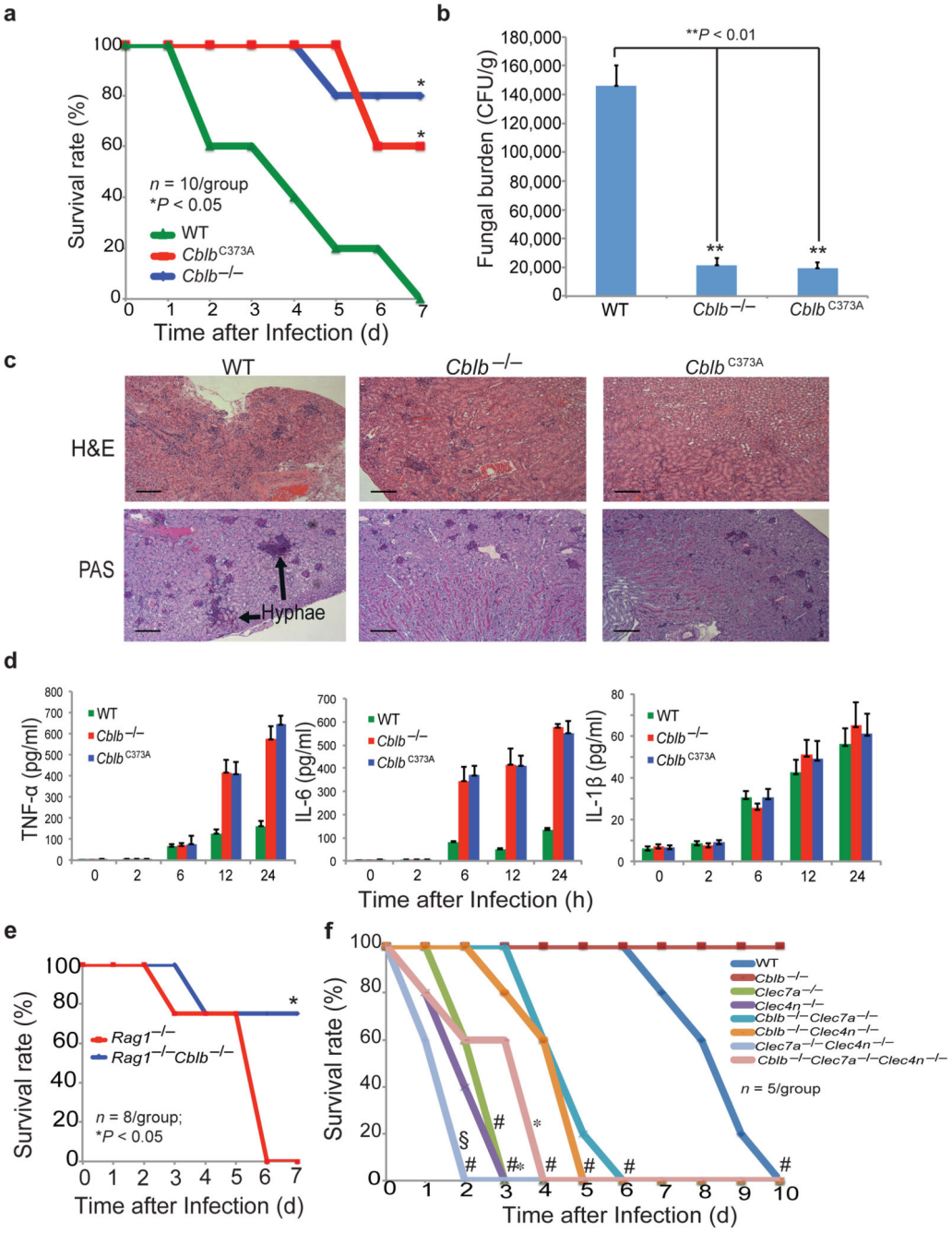

Figure 5.

Introducing dectin-1 and dectin-2 deficiency, or double deficiency into *Cblb*$^{-/-}$ mice renders *Cblb*$^{-/-}$ mice susceptible to systemic *C. albicans* infection. (**a**) Kaplan-Meier Survival curve of WT, *Cblb*$^{-/-}$, and *Cblb*^{C373A} mice ($n = 10$ per group) infected with 5×10^5 CFU of *C. albicans* (SC5314), and monitored for 7 days for survival. Data are representative of three independent experiments (biological replicates). *$P < 0.05$; Log-rank test. (**b**) CFU assay of paired kidneys of WT, *Cblb*$^{-/-}$ and *Cblb*^{C373A} mice ($n = 10$ per group) infected with 1×10^5 CFU of *C. albicans* performed at day 2 after infection. Data are representative of three

independent experiments (biological replicates). $^{**}P < 0.01$; unpaired two-tailed Student's *t* test. (**c**) Kidney histopathology analysis by H&E and PAS staining. Fungal burden (hyphae) in the kidneys visualized by PAS staining**.** Images are representative of two independent experiments (biological replicates). $n = 10$ per group. Scale bar, 200 μm. (**d**) ELISA of serum TNF-α, IL-6, and IL-1β levels of WT and *Cblb*$^{-/-}$ mice ($n = 10$ per group) infected with 1×10^5 CFU of *C. albicans* at 2, 6, 12, and 24 h after infection. Data are representative of three independent experiments (biological replicates). Error bars are mean ± s.d. $^{*}P < 0.05$, $^{**}P < 0.01$; unpaired two-tailed Student's *t* test. Each with three repeated wells. (**e**) Survival rate of *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Cblb*$^{-/-}$ mice ($n = 8$). infected with 1×10^5 CFU of *C. albicans*. Data are representative of three independent experiments (biological replicates). $^{*}P < 0.05$, Log-rank test. (**f**) Survival rate of WT, *Cblb*$^{-/-}$, *Clec7a*$^{-/-}$, *Clec4n*$^{-/-}$, *Cblb*$^{-/-}$*Clec7a*$^{-/-}$, *Cblb*$^{-/-}$*Clec4n*$^{-/-}$, and *Cblb*$^{-/-}$*Clec7a*$^{-/-}$*Clec4n*$^{-/-}$ mice ($n = 5$ per group) infected with *C. albicans* (3.5×10^5 CFU) by i.v. injection. Data are representative of three independent experiments (biological replicates). $^{\#}P < 0.01$, *Cblb*$^{-/-}$ vs. all other groups; $^{*}P < 0.05$, *Cblb*$^{-/-}$*Clec7a*$^{-/-}$ vs. *Clec7a*$^{-/-}$ or *Cblb*$^{-/-}$*Clec4n*$^{-/-}$ vs. *Clec4n*$^{-/-}$; and $^{\S}P < 0.05$, *Cblb*$^{-/-}$ *Clec7a*$^{-/-}$ *Clec4n*$^{-/-}$ vs *Clec7a*$^{-/-}$ *Clec4n*$^{-/-}$; Log-rank test.

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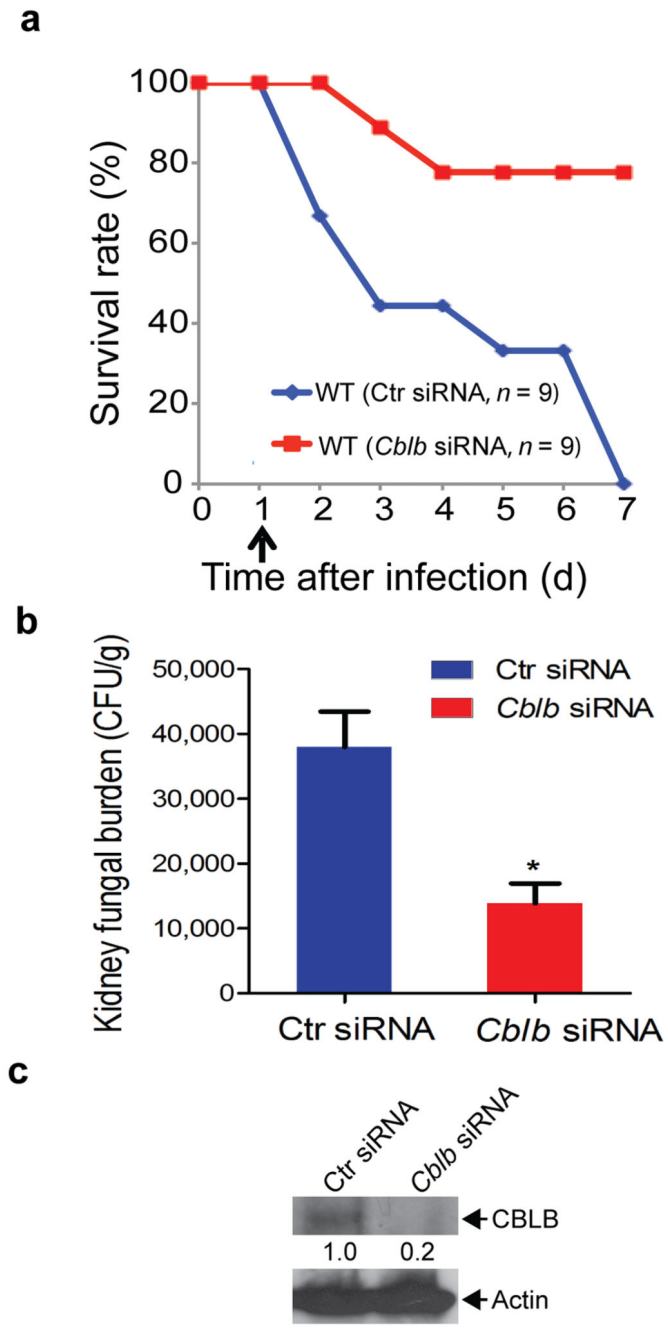

Figure 6.

Systemic *in vivo* delivery of *Cblb* siRNA into C57BL/6 mice protects them from lethal disseminated candidiasis. (**a**) Survival of C57BL/6 mice treated with *in vivo* grade *Cblb* siRNA (5'-AAAUUCUCGAAGUAUGCUCUU-3') or a non-sense siRNA (2 mg/kg/mouse) via tail vein injection 24 h after infection with *C. albicans* (5×10^5 CFU). Data are representative of three independent experiments (biological replicates). $^*P < 0.05$, Log-rank test. $n = 9$ per group. (**b**) Fungal burden in the kidneys on day 2 after infection. Data are representative of three independent experiments (biological replicates). Error bars are mean

± s.d. *$P < 0.05$; unpaired two-tailed Student's t test. $n = 9$ per group. **(c)** Immunoblot analysis of spleen cells from control siRNA or *Cblb* siRNA-treated C57BL/6 mice with anti-CBLB and anti-actin, respectively. Data are representative of four independent experiments (biological replicates). $n = 3$ per group.

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