Targeting CBLB as a Potential Therapeutic Approach for Disseminated Candidiasis

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Running Title: CBLB regulates CLR-mediated innate responses

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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 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.

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**

194 **panel**). To determine whether ubiquitination of dectin-1 or -2 is K48 or K63-linked,
195 we utilized anti-K48 ubiquitin or anti-K63 ubiquitin antibodies. We confirmed that
196 both dectin-1 and -2 underwent K48-linked polyubiquitination, and this K48-linked
197 polyubiquitination of dectin-1- and -2 was abrogated in BMDMs expressing the
198 CBLB C373A mutation or lacking CBLB (**Fig. 3c, d, lower panel;**
199 **Supplementary Fig. 4a, b, lower panel**; **data not shown**).

200

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

212

213 To examine the functional relevance of CBLB-mediated ubiquitination of dectin-1
214 and -2 we generated single and triple K to R mutations of dectin-1^{K2R}, dectin-
215 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

238 vesicle sorting to the lysosome. To determine whether CBLB is critical for this
239 process, the cell surface and internalized expression levels of dectin-1 and
240 dectin-2 in BMDMs from WT and *Cblb*$^{-/-}$ mice was investigated. We found a
241 minimal level of intracellular dectin-1 or -2 in *Cblb*$^{-/-}$ BMDMs (**Fig. 4a,b**),
242 suggesting that CBLB promotes internalization of dectin-1 or dectin-2 after
243 infection with *C. albicans* yeast or hyphae.
244

245 We next investigated whether retention of ligand-engaged dectin-1 or -2 in *Cblb*$^{-}$
246 $^{/-}$ BMDMs is due to impaired sorting of endosomal vesicles to lysosomes. We
247 compared the subcellular localization of ligand-engaged dectin-1 or -2 in WT and
248 *Cblb*$^{-/-}$ BMDMs by confocal microscopy. In support of impaired lysosomal
249 degradation of dectin-1 and -2 in BMDMs lacking CBLB, intracellular trafficking of
250 internalized dectin-1 or -2 to the lysosome was significantly reduced in the
251 absence of CBLB (**Fig. 4c, d**).
252

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

255 Neutrophils and macrophages are professional phagocytes of the innate immune
256 system that are essential in controlling bacterial and fungal infections by
257 phagocytosis and killing mechanisms [40]. The production of highly reactive oxygen
258 species (ROS) is one of the primary effector mechanisms used by phagocytes to
259 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

283 *Cblb*^{C373A} mice, lower fungal burden in the kidney, lung, spleen, and liver, and
284 decreased *C. albicans* hyphae in the kidney on day 2 as assessed by PAS
285 staining (**Fig. 5b-d; Supplementary Fig. 6a**). We also observed multifocal
286 tubulointerstitial nephritis in WT mice infected with *C. albicans*, which was
287 ameliorated in mice lacking CBLB or expressing the CBLB C373A mutation (**Fig.**
288 **5c**). This observation is consistent with fact that more immune cells traffic to the
289 kidneys in WT than *Cblb*^{C373A} mice including macrophages, dendritic cells (DCs),
290 and neutrophils (**Supplementary Fig. 6b**). Improved survival rate was also
291 observed in *Rag1*$^{-/-}$*Cblb*$^{-/-}$ mice that lack functional adaptive immune cells (**Fig.**
292 **5e**), supporting a critical role of CBLB in down-regulating innate immune
293 responses.
294
295 To further determine whether monocytes, macrophages and neutrophils have a
296 greater capacity to kill *C. albicans* during systemic infection, we monitored fungal
297 burden in the blood of WT and *Cblb*^{C373A} mice at 2 and 6 h after infection. We
298 found that fungal burden in the blood of *Cblb*^{C373A} mice was significantly lower
299 than that of WT mice at 2 and 6 h after infection (**Supplementary Fig. 7a**). The
300 lower fungal burden in the blood of *Cblb*^{C373A} mice correlated with enhanced
301 fungal killing activity by PBMCs, but not by neutrophils of *Cblb*^{C373A} mice
302 (**Supplementary Fig. 7a)**. Increased fungal killing was also observed in
303 monocytes from the spleen of *Cblb*^{C373A} mice **(Supplementary Fig. 7b).** We also
304 monitored ROS activity in monocytes, macrophages and neutrophils from WT
305 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.

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AUTHORS CONTRIBUTIONS

Y.Xiao performed most of experiments and analyzed the data; J.Tang, H.Guo, Y.Zhao, R.Tang, S.Ouyang, Q.Zeng, and B.T.Li performed some in vitro and in vivo experiments; C.Rappleye helped design the research, analyzed and interpreted the data, and edited the manuscript; M.V.S.Rajaram performed experiments using human macrophages; L.S.Schlesinger, M.V.S.Rajaram, and J.Zhang designed human macrophage experiments and edited the manuscript; L.Tao helped design kidney experiments and data analysis; G.D.Brown provided *Clec7a*$^{-/-}$ mice; W.Y.Langdon provided *Cblb*^{C373A} knockin mice and edited the manuscript; J.Zhang conceived and planned the research, analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS STATEMENT

The authors declare no competing financial interests.

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650 **Figure legends**

651 **Figure 1.** CBLB inhibits pro-inflammatory cytokine production by macrophages
652 upon infection with *C. albicans* yeast or hyphae and *A. fumigatus* conidia**.** (**a**)
653 ELISA of TNF-α, IL-6, and IL-1β production in the supernatants collected from
654 BMDMs of WT and *Cblb*$^{-/-}$ mice infected with *C. albicans* yeast *cap1* mutant
655 (thereafter yeast) and hyphal forms (WT strain SC5314) (MOI: 1:1) for 1 and 3 h.
656 For preparation of hyphae, washed yeast cells were counted, re-suspended in
657 RPMI-1640 medium, grown in 12-well plates at 37 °C for 3 h, and washed three
658 times with PBS. (**b**) ELISA of TNF-α and IL-6 production in the supernatants
659 collected from BMDMs of WT and *Cblb*$^{-/-}$ mice infected with swollen *A.*
660 *Fumigatus* conidia (AF293) (MOI = 1:1) for 2 and 4 h. (**c**) ELISA of IL-1RA
661 production in the supernatants collected from BMDMs of WT and *Cblb*$^{-/-}$ mice
662 infected with *C. albicans* yeast and hyphal forms. For all ELISA experiments
663 data are representative of three independent experiments (biological replicates).
664 Error bars are mean ± s.d. **P* < 0.05, ***P* < 0.01; unpaired two-tailed Student's *t*
665 test. *n* = 3 per group, each with three repeated wells.

666

667 **Figure 2.** CBLB associates with dectin-1 and dectin-2 in macrophages upon *C.*
668 *albicans* yeast and hyphal infection**.** (**a,b**) Immunoblot analysis of dectin-1 or
669 dectin-2, SYK, and CARD9 after immunoprecipitation (IP) with CBLB antibodies
670 from lysates of BMDMs uninfected or infected with *C. albicans* yeast or hyphae.
671 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 =

694 1:1) with antibodies against dectin-1, dectin-2, SYK, CBLB, and ACTIN,
695 respectively. Images are representative of five independent experiments
696 (biological replicates). (**b**) Immunoblot analysis of WT BMDMs pretreated with E-
697 64 (10 μM), MG-132 (5 μM), or both for 30 min, then infected with *C. albicans*
698 yeast or hyphae (MOI = 1:1), with antibodies against to dectin-1 and dectin-2,
699 respectively. Images are representative of three independent experiments
700 (biological replicates). (**c, d**) Immunoblot analysis of dectin-1 and dectin-2
701 ubiquitination of dectin-1 or dectin-2 immunoprecipitates isolated from BMDMs
702 from WT and *Cblb*^{C373A} mice infected with *C. albicans* yeast and hyphae,
703 respectively, by anti-ubiquitin and anti-K48 ubiquitin antibodies. Images are
704 representative of four independent experiments (biological replicates), and each
705 IP was blotted separately. (**e**) Ubiquitination of dectin-1 in *Clec7a*$^{-/-}$ BMDMs
706 reconstituted with WT dectin-1 or dectin-1^{K2R}, dectin-1^{K27R}, dectin-1^{K34R} mutant,
707 or dectin-1$^{K2R, K27R, K34R}$ triple mutant infected with *C. albicans* yeast. Images are
708 representative of three independent experiments (biological replicates), and each
709 IP was blotted separately. (**f**) Ubiquitination of dectin-2 in *Clec4n*$^{-/-}$ BMDMs
710 reconstituted with WT dectin-2 or dectin-2^{K10R} infected with *C. albicans* hyphae.
711 Images are representative of three independent experiments (biological
712 replicates), and each IP was blotted separately. (**g, h**) ELISA of TNF-α and IL-6
713 production by *Clec7a*$^{-/-}$ BMDMs reconstituted with WT dectin-1 or dectin-
714 1^{K2R,K27R,K34R} infected with *C. albicans* yeast (**g**), or by *Clec4n*$^{-/-}$ BMDMs
715 reconstituted with WT dectin-2 or dectin-2^{K10R} infected with *C. albicans* hyphae
716 (**h**). Data are representative of three independent experiments (biological

717 replicates). Error bars are mean ± s.d. *P < 0.05, **P < 0.01; unpaired two-tailed
718 Student's t test. n = 3 per group, each with three repeated wells.

719

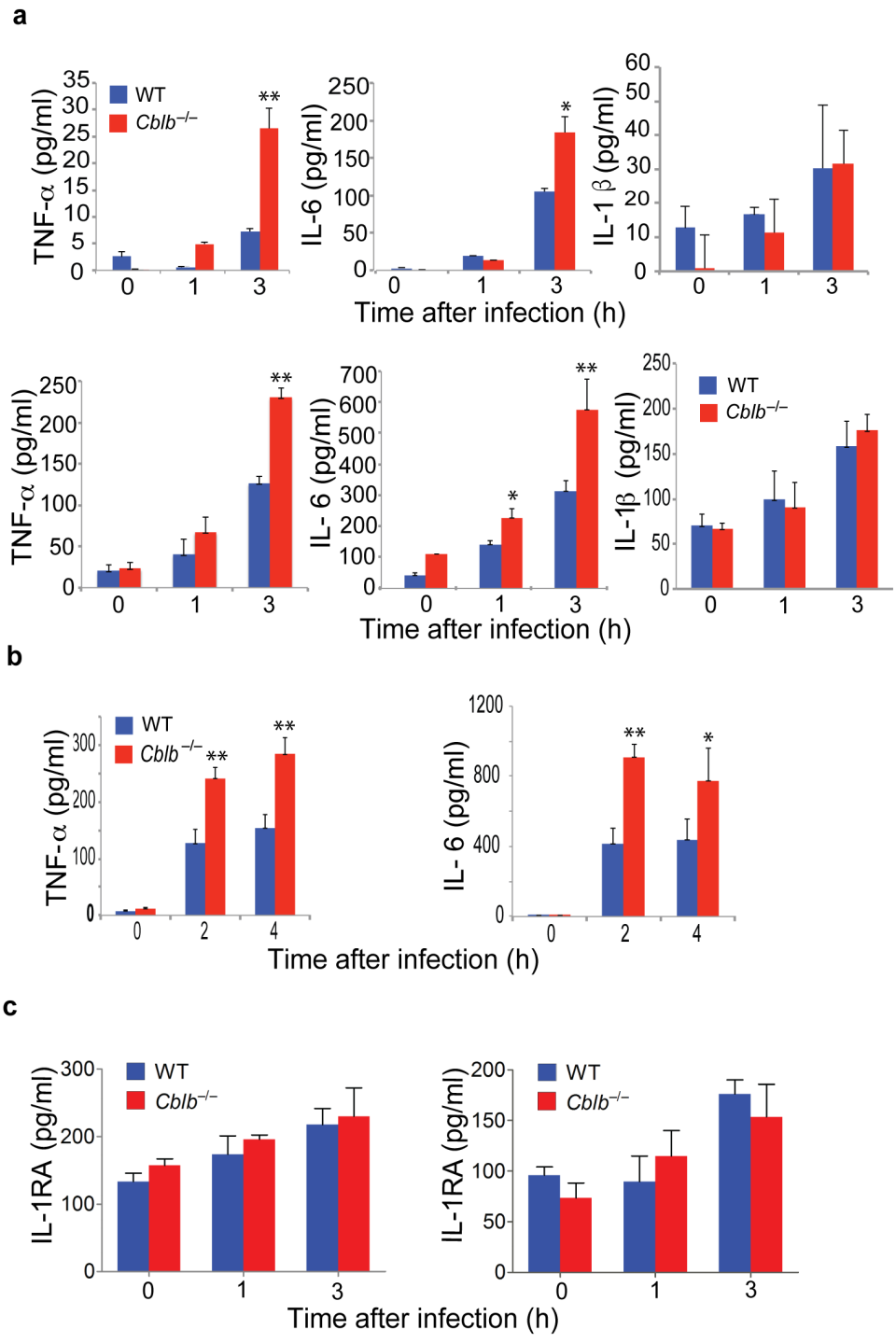
720 **Figure 4.** Loss of CBLB impairs dectin-1 and dectin-2 internalization and their
721 down-regulation at the cell surface**.** (**a**,**b**) Cell surface and intracellular expression
722 of dectin-1 and dectin-2 of WT and *Cblb*$^{-/-}$ BMDMs infected with *C. albicans*
723 yeast or hyphae (MOI = 1:1) for times indicated by flow cytometry. For
724 internalization of dectin-1 and dectin-2, WT and *Cblb*$^{-/-}$ BMDMs were treated with
725 acid buffer to strip the antibodies remaining at the cell surface after infection at
726 each time-point. Data are representative of three independent experiments
727 (biological replicates). Error bars are mean ± s.d. *P < 0.05; unpaired two-tailed
728 Student's t test. n = 3 per group, each with three repeated wells. (**c,d**) Confocal
729 image of dectin-1 and dectin-2 internalization and lysosome sorting of WT and
730 *Cblb*$^{-/-}$ BMDMs infected or uninfected with *C. albicans* yeast (**c**) or hyphae (**d**)
731 (MOI = 1:1) for 30 min. Images are representative of five independent
732 experiments (biological replicates). n = 3 per group, each with three repeated
733 wells. Scale bar, 5 μm.

734

735 **Figure 5.** Introducing dectin-1 and dectin-2 deficiency, or double deficiency into
736 *Cblb*$^{-/-}$ mice renders *Cblb*$^{-/-}$ mice susceptible to systemic *C. albicans* infection.
737 (**a**) Kaplan-Meier Survival curve of WT, *Cblb*$^{-/-}$, and *Cblb*^{C373A} mice (n = 10 per
738 group) infected with 5 x 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 x 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 x 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 x 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 x 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.

762 **Figure 6.** Systemic *in vivo* delivery of *Cblb* siRNA into C57BL/6 mice protects
763 them from lethal disseminated candidiasis. (**a**) Survival of C57BL/6 mice treated
764 with *in vivo* grade *Cblb* siRNA (5'-AAAUUCUCGAAGUAUGCUCUU-3') or a non-
765 sense siRNA (2 mg/kg/mouse) via tail vein injection 24 h after infection with *C.*
766 *albicans* (5 x 10^5 CFU). Data are representative of three independent
767 experiments (biological replicates). **P* < 0.05, Log-rank test. *n* = 9 per group. (**b**)
768 Fungal burden in the kidneys on day 2 after infection. Data are representative of
769 three independent experiments (biological replicates). Error bars are mean ± s.d.
770 **P* < 0.05; unpaired two-tailed Student's *t* test. *n* = 9 per group. (**c)** Immunoblot
771 analysis of spleen cells from control siRNA or *Cblb* siRNA-treated C57BL/6 mice
772 with anti-CBLB and anti-actin, respectively. Data are representative of four
773 independent experiments (biological replicates). *n* = 3 per group.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WT
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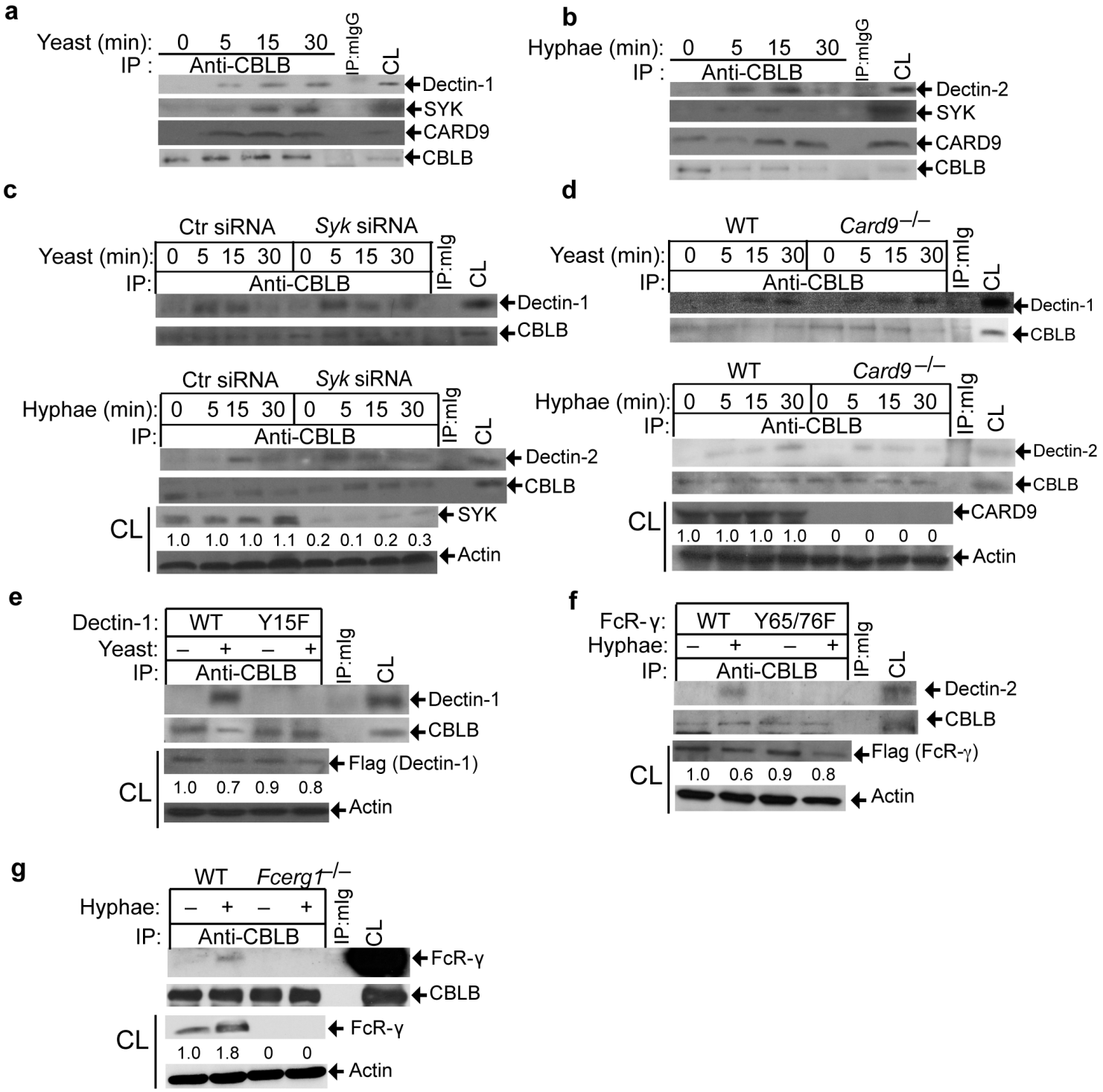
Time after infection (h)
TNF-α (pg/ml)
IL- 6 (pg/ml)
IL-1β (pg/ml)
WT
Cblb−/−
Time after infection (h)
b
TNF-α (pg/ml)
IL- 6 (pg/ml)
WT
Cblb −/−
Time after infection (h)
c
IL-1RA (pg/ml)
IL-1RA (pg/ml)
WT
Cblb−/−
Time after infection (h)


a
Yeast (min): 0 5 15 30
IP : Anti-CBLB
IP:mIgG
CL
Dectin-1
SYK
CARD9
CBLB
b
Hyphae (min): 0 5 15 30
IP : Anti-CBLB
IP:mIgG
CL
Dectin-2
SYK
CARD9
CBLB
c
Ctr siRNA
Syk siRNA
Yeast (min): 0 5 15 30 0 5 15 30
IP: Anti-CBLB
IP:mIg
CL
Dectin-1
CBLB
Hyphae (min): 0 5 15 30 0 5 15 30
IP: Anti-CBLB
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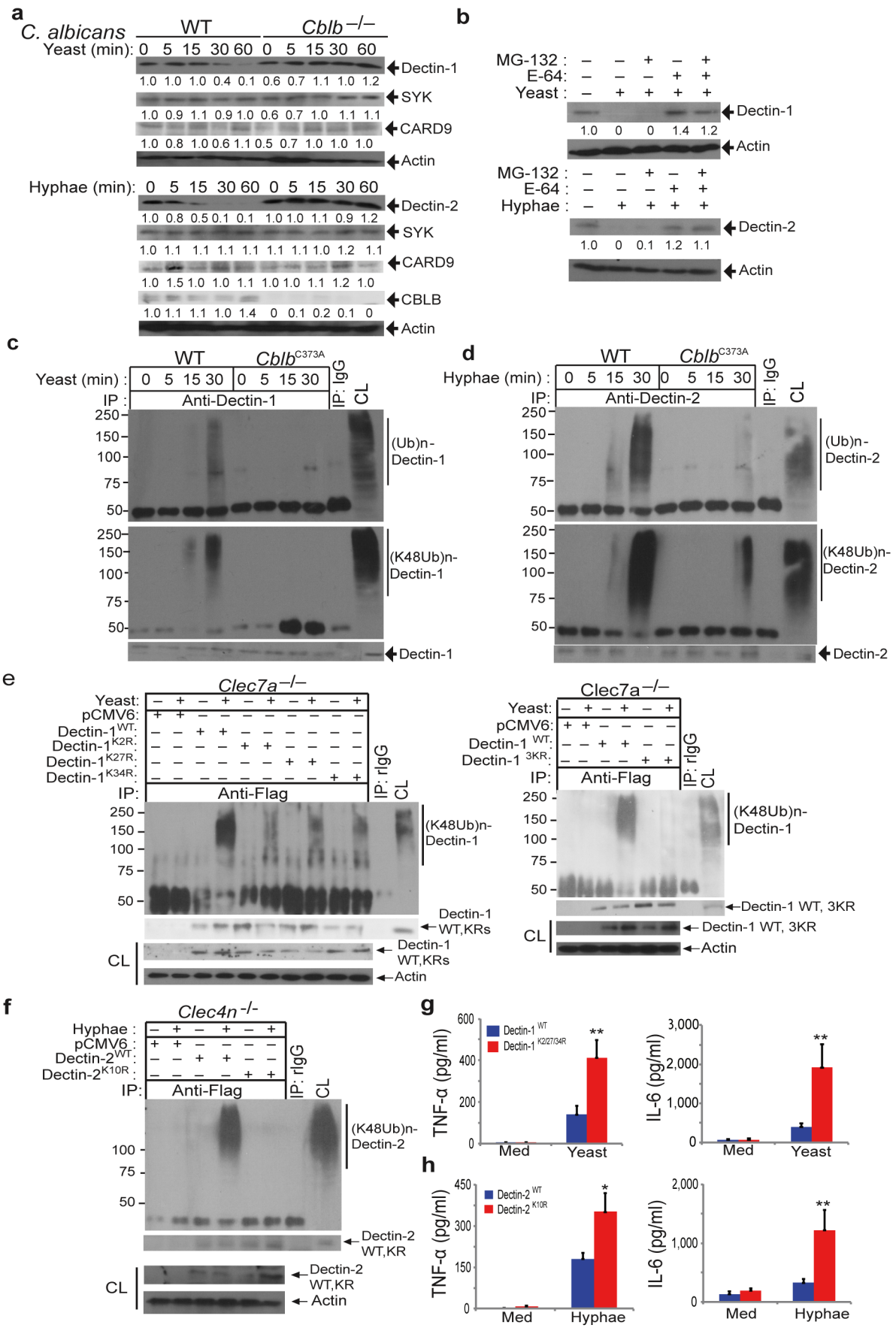
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Yeast (min): 0 5 15 30 60 0 5 15 30 60
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CARD9
1.0 0.8 1.0 0.6 1.1 0.5 0.7 1.0 1.0 1.0
Actin
Hyphae (min): 0 5 15 30 60 0 5 15 30 60
Dectin-2
1.0 0.8 0.5 0.1 0.1 1.0 1.0 1.1 0.9 1.2
SYK
1.0 1.1 1.1 1.1 1.1 1.1 1.1 1.0 1.2 1.1
CARD9
1.0 1.5 1.0 1.0 1.1 1.0 1.0 1.1 1.2 1.0
CBLB
1.0 1.1 1.1 1.0 1.4 0 0.1 0.2 0.1 0
Actin
b
MG-132 : – – + – +
E-64: – – – + +
Yeast : – + + + +
Dectin-1
1.0 0 0 1.4 1.2
Actin
MG-132 : – – + – +
E-64 : – – – + +
Hyphae : – + + + +
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Cblb C373A
Yeast (min) : 0 5 15 30 0 5 15 30
IP : Anti-Dectin-1
IP: IgG
CL
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(K48Ub)n-Dectin-1
Dectin-1
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Cblb C373A
Hyphae (min) : 0 5 15 30 0 5 15 30
IP: Anti-Dectin-2
IP: IgG
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250 150 100 75 50
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Clec7a −/−
Yeast:
pCMV6:
Dectin-1 WT:
Dectin-1 K2R:
Dectin-1 K27R:
Dectin-1 K34R:
IP: Anti-Flag
IP: rIgG
CL
250 150 100 75 50
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Dectin-1 WT,KRs
Actin
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Yeast:
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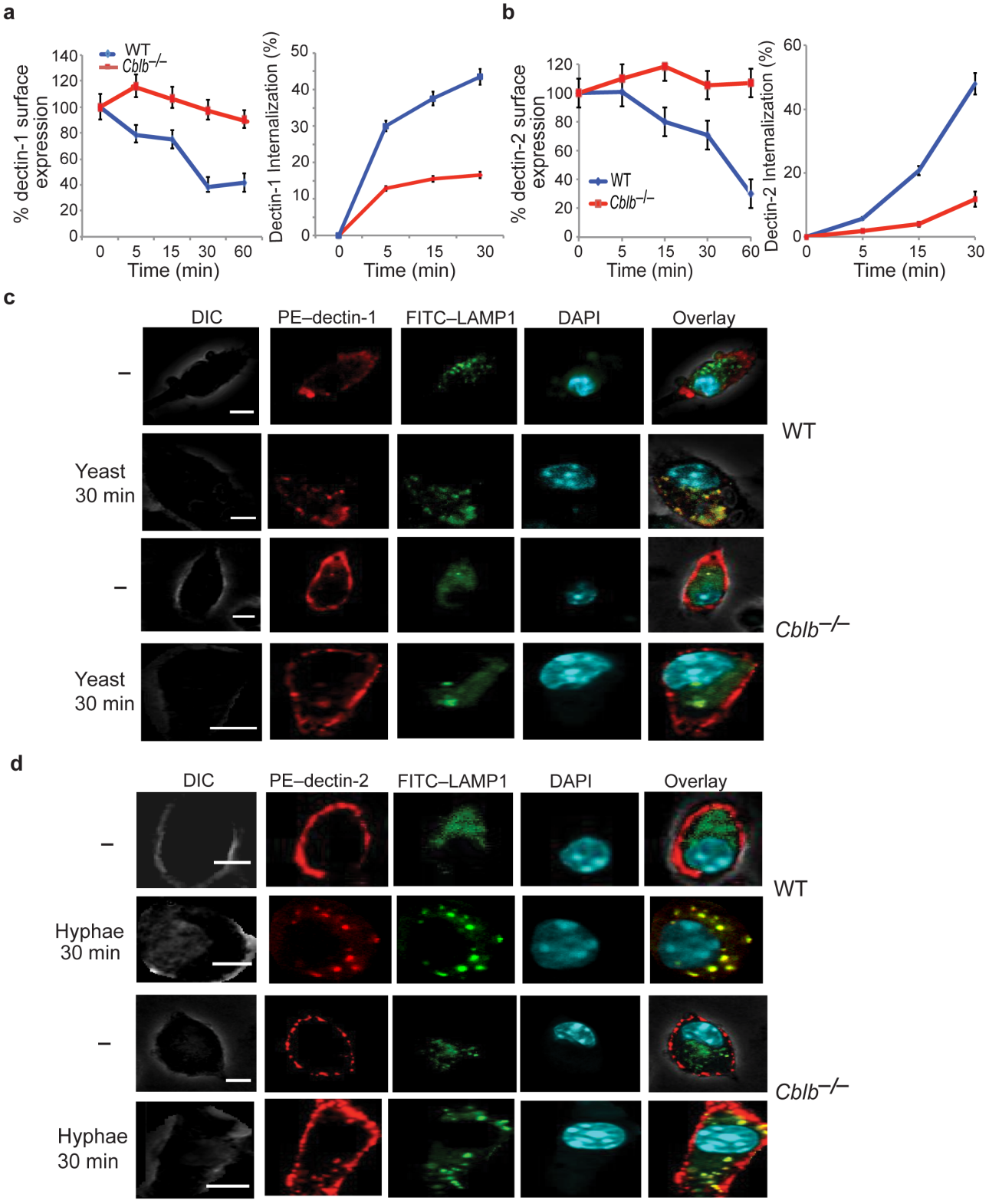
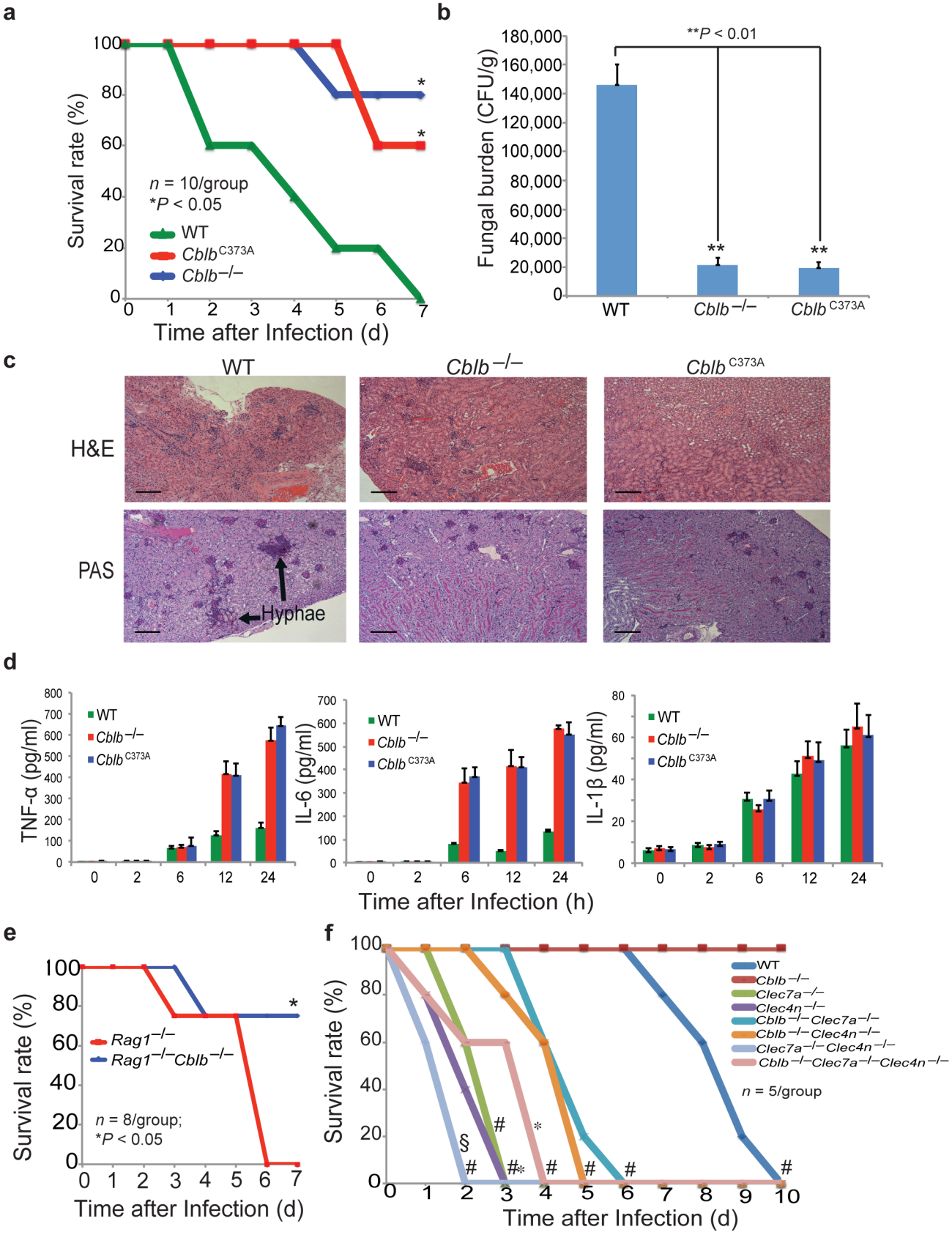
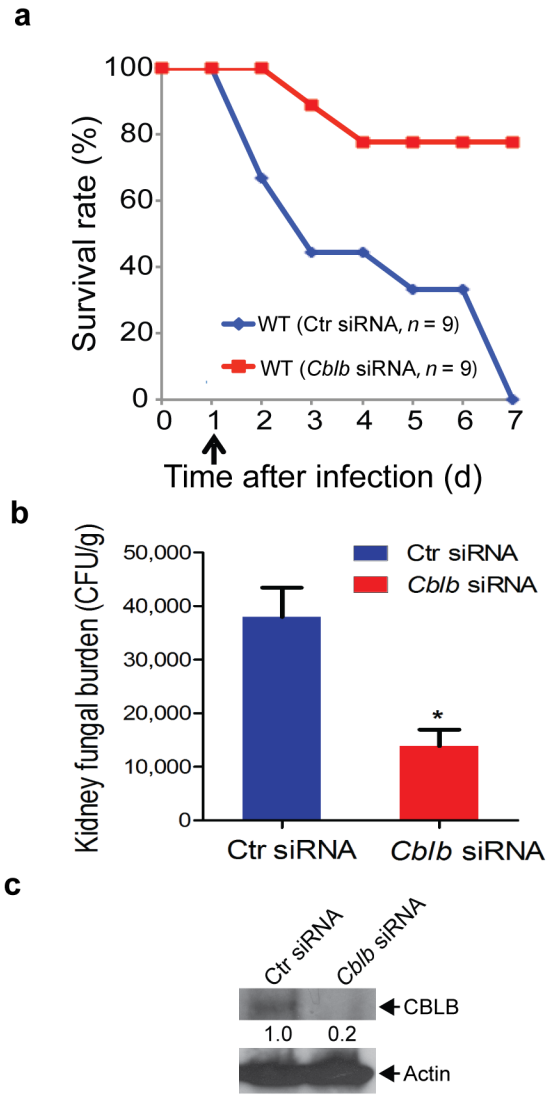
IP: Anti-Flag
IP: rIgG
CL
250 150 100 75 50
(K48Ub)n-Dectin-1
Dectin-1 WT, 3KR
CL
Dectin-1 WT, 3KR
Actin
f
Clec4n -/-
Hyphae :
pCMV6 :
Dectin-2 WT:
Dectin-2 K10R :
IP: Anti-Flag
IP: rIgG
CL
100 75 50
(K48Ub)n-Dectin-2
Dectin-2 WT,KR
CL
Dectin-2 WT,KR
Actin
g
TNF-α (pg/ml)
600 400 200 0
Dectin-1 WT
Dectin-1 K2/27/34R
**
Med Yeast
IL-6 (pg/ml)
3,000 2,000 1,000 0
**
Med Yeast
h
TNF-α (pg/ml)
450 300 150 0
Dectin-2 WT
Dectin-2 K10R
*
Med Hyphae
IL-6 (pg/ml)
2,000 1,000 0
**
Med Hyphae


a
% dectin-1 surface expression
WT
Cblb−/−
Time (min)
Dectin-1 Internalization (%)
Time (min)
b
% dectin-2 surface expression
WT
Cblb−/−
Time (min)
Dectin-2 Internalization (%)
Time (min)
c
DIC
PE–dectin-1
FITC–LAMP1
DAPI
Overlay
–
Yeast 30 min
WT
–
Cblb−/−
Yeast 30 min
d
DIC
PE–dectin-2
FITC–LAMP1
DAPI
Overlay
–
Hyphae 30 min
WT
–
Cblb−/−
Hyphae 30 min


a
Survival rate (%)
Time after Infection (d)
n = 10/group
*P < 0.05
WT
Cblb C373A
Cblb −/−
b
Fungal burden (CFU/g)
**P < 0.01
WT
Cblb −/−
Cblb C373A
c
WT
Cblb −/−
Cblb C373A
H&E
PAS
Hyphae
d
TNF-α (pg/ml)
IL-6 (pg/ml)
IL-1β (pg/ml)
Time after Infection (h)
e
Survival rate (%)
Rag1−/−
Rag1−/−Cblb−/−
n = 8/group;
*P < 0.05
Time after Infection (d)
f
Survival rate (%)
WT
Cblb −/−
Clec7a −/−
Clec4n −/−
Cblb −/− Clec7a −/−
Cblb −/− Clec4n −/−
Clec7a −/− Clec4n −/−
Cblb −/− Clec7a −/− Clec4n −/−
n = 5/group
Time after Infection (d)


a
100
80
60
40
20
0
Survival rate (%)
WT (Ctr siRNA, n = 9)
WT (Cblb siRNA, n = 9)
0 1 2 3 4 5 6 7
Time after infection (d)
b
Kidney fungal burden (CFU/g)
50,000
40,000
30,000
20,000
10,000
0
Ctr siRNA
Cblb siRNA
*
Ctr siRNA
Cblb siRNA
c
Ctr siRNA
Cblb siRNA
CBLB
1.0
0.2
Actin