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BLNK negatively regulates innate antifungal immunity through inhibiting c-Cbl-mediated macrophage migration

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B cell linker protein (BLNK) is crucial for orchestrating B cell receptor-associated spleen tyrosine kinase (Syk) signaling. However, the role of BLNK in Syk-coupled C-type lectin receptor (CLR) signaling in macrophages remains unclear. Here, we delineate that CLRs govern the Syk-mediated activation of BLNK, thereby impeding macrophage migration by disrupting podosome ring formation upon stimulation with fungal β-glucans or α-mannans. Mechanistically, BLNK instigates its association with casitas B-lineage lymphoma (c-Cbl), competitively impeding the interaction between c-Cbl and Src-family kinase Fyn. This interference disrupts Fyn-mediated phosphorylation of c-Cbl and subsequent c-Cbl-associated F-actin assembly. Consequently, BLNK deficiency intensifies CLR-mediated recruitment of the c-Cbl/phosphatidylinositol 3-kinase complex to the F-actin cytoskeleton, thereby enhancing macrophage migration. Notably, mice with monocyte-specific BLNK deficiency exhibit heightened resistance to infection with *Candida albicans*, a prominent human fungal pathogen. This resistance is attributed to the increased infiltration of Ly6C$^+$ macrophages into renal tissue. These findings unveil a previously unrecognized role of BLNK for the negative regulation of macrophage migration through inhibiting CLR-mediated podosome ring formation during fungal infections.

B cell linker protein | C-type lectin receptors | antifungal immunity | macrophage migration | negative regulation

Significance

B cell linker protein (BLNK) has been widely recognized for its critical role in orchestrating B cell receptor (BCR)-associated spleen tyrosine kinase (Syk) signaling. Here, we elucidate that C-type lectin receptors (CLRs) govern the Syk-mediated activation of BLNK, leading to the inhibition of macrophage migration by disrupting podosome ring formation upon stimulation with fungal β-glucans or α-mannans. This insight expands our understanding of BLNK's functional repertoire, highlighting its significance beyond the confines of BCR signaling. Our findings not only unveil a previously unrecognized facet of BLNK's activity, but also pave the way for the development of immunotherapeutic strategies targeting BLNK for enhancing macrophage migration against fungal infections.

B cell linker protein (BLNK), also referred to as SLP-65 (SH2 domain-containing leukocyte protein of 65 kDa) or BASH (B cell adaptor containing Src homolog 2 domain), is a crucial cytoplasmic adaptor protein involved in the signaling pathways of B cell receptor (BCR) (1). BLNK encompasses distinct domains, namely a proline-rich region, a central domain, and multiple tyrosine phosphorylation sites. Upon activation of the BCR, BLNK undergoes phosphorylation by the spleen tyrosine kinase (Syk) (2). Phosphorylated BLNK then serves as a scaffold to recruit and activate downstream signaling molecules such as Btk, PLCγ, and Vav, leading to the activation of multiple pathways, including the mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) pathways (3–5). Ultimately, these pathways culminate in B cell activation, proliferation, differentiation, and antibody production (6). Mutations or deficiencies in the gene encoding either BLNK or Syk can result in impaired B cell signaling, leading to various B cell immunodeficiency disorders, including X-linked agammaglobulinemia (XLA) (7, 8). Conversely, overactive Syk and BLNK signaling pathways can contribute to the onset or progression of autoimmune diseases and B cell lymphomas (9). In essence, BLNK and Syk have a closely intertwined relationship in B cell signaling, with BLNK serving as an adaptor protein and Syk acting as a vital kinase in the BCR signaling cascade.

C-type lectin receptors (CLRs) are a family of pattern recognition receptors (PRRs) expressed on various innate immune cells, including dendritic cells (DCs), macrophages, and neutrophils (10). CLRs play a crucial role in the detection and subsequent response to infections caused by *Candida albicans*, a prevalent fungal pathogen known to induce severe infections in humans. In these processes, Syk serves as an important intracellular signaling molecule that is recruited by CLRs through their intracellular immunoreceptor tyrosine-based activation motif (ITAM) (11). Two well-characterized CLRs, Dectin-1 and Dectin-2, are involved in the recognition of β-glucans or α-mannans from *C. albicans*, respectively. Upon recognition, Dectin-1 and Dectin-2 activate Syk through phosphorylation, leading to the activation of various transcription factors such as NF-κB and AP-1. These transcription factors regulate the production of reactive oxygen species (ROS), as well as the release of cytokines and chemokines, which contribute to the effective immune

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defense against *C. albicans* infections (12, 13). It has been observed that defects in the Dectin-1/Dectin-2-Syk pathway increase susceptibility to fungal infections, underscoring the critical role of this pathway in antifungal immunity (14, 15). However, it completely remains unclear whether BLNK, another signaling molecule, is involved in Syk-coupled CLR signaling in innate immune cells for the regulation of antifungal immunity against *C. albicans* infection.

Macrophages play a crucial role in maintaining tissue homeostasis and defending the host against pathogens (16). One of their key functions is to migrate to different locations in the body in response to chemotactic signals (17). Macrophage migration is a complex process that relies on the coordinated regulation of various cytoskeletal events, including the dynamics of F-actin (18). F-actin, a component of the cytoskeleton, forms filamentous structures that provide mechanical support, enable changes in cell shape, and facilitate macrophage adhesion to and movement through the extracellular matrix (19). The protein casitas B-lineage lymphoma (c-Cbl) is a cytoplasmic regulator that influences several intracellular signaling pathways, including F-actin dynamics and macrophage migration (20). The interaction between c-Cbl and F-actin is believed to play a role in regulating macrophage migration, potentially by recruiting phosphatidylinositol 3-kinase (PI3K) and inhibiting its enzymatic activity (21). The interaction between BLNK and c-Cbl has been extensively studied in the context of BCR signaling. Studies have demonstrated that BLNK can recruit c-Cbl to the BCR complex, subsequently inhibiting PLCγ2 activation (22). Our previous study shows that c-Cbl plays a role downstream of CLRs in facilitating the ubiquitination and degradation of the noncanonical NF-κB subunit RelB in DCs (23). However, the precise involvement of the interaction between BLNK and c-Cbl in the regulation of CLR-mediated macrophage migration remains to be fully elucidated.

In the present study, we showed that the activation of Dectin-1 and Dectin-2 by their respective ligands resulted in the phosphorylation of BLNK. This phosphorylated BLNK then interacted with c-Cbl in a Syk-dependent manner. This interaction served to negatively regulate macrophage migration by inhibiting the recruitment of the c-Cbl/PI3K complex to the cytoskeleton, which was mediated by CLR signaling. Interestingly, we observed that mice with a specific deficiency of BLNK in monocytes exhibited increased infiltration of Ly6C$^+$ macrophages into the kidneys and displayed heightened resistance to *C. albicans* infection. These findings highlight the crucial role of BLNK in the negative regulation of macrophage migration and suggest potential implications for the development of immunotherapeutic strategies targeting macrophage migration against fungal infections.

Results

CLRs Mediated the Phosphorylation of BLNK in Macrophages upon *C. albicans* Stimulation. BLNK is primarily expressed in B cells (1). Moreover, it has been observed to be present in a variety of innate immune cell types, including macrophages and DCs (24, 25). We observed a significantly higher expression level of BLNK in macrophages compared to DCs and neutrophils (Fig. 1*A* and *SI Appendix*, Fig. S1*A*). Previous studies have shown that BLNK in B cells is phosphorylated by Syk upon BCR activation (26). Here, we found that the activation of bone marrow–derived macrophages (BMDMs) by *C. albicans* yeasts or hyphae led to the tyrosine phosphorylation of BLNK (specifically pTyr^{84}) (Fig. 1*B* and *SI Appendix*, Fig. S1*B*). Similarly, stimulation with β-glucan-containing curdlan or α-mannans also induced BLNK phosphorylation in BMDMs (Fig. 1*C* and *SI Appendix*, Fig. S1*C*). Notably, the absence of Syk in BMDMs entirely hindered BLNK phosphorylation (Fig. 1*D* and *SI Appendix*, Fig. S1*D*), suggesting that BLNK acted downstream of Syk in macrophages in response to fungal infections.

To further explore whether BLNK participated in the Syk-coupled CLR signaling pathway, we stimulated BMDMs lacking Dectin-1, Dectin-2, or Mincle, alongside their downstream mediators Fc-receptor gamma-chain (FcRγ) or caspase recruitment domain family member 9 (CARD9). We found that the deficiency of CLRs such as Dectin-1, Dectin-2, or Mincle in BMDMs notably impeded BLNK phosphorylation after stimulation with their respective ligands including β-glucans, α-mannans, and TDM (Fig. 1 *E* and *F* and *SI Appendix*, Fig. S1 *E*, *F*, and *H*). Furthermore, the absence of FcRγ in BMDMs significantly hindered the α-mannan-induced phosphorylation of BLNK, while CARD9 deficiency did not affect BLNK phosphorylation induced by β-glucans or α-mannans (Fig. 1*G* and *SI Appendix*, Fig. S1 *G* and *I*). Taken together, these data strongly indicated that BLNK operated downstream of the Syk-coupled CLR signaling in macrophages during fungal infections.

Lyz2$^+$ Myeloid Cell-Specific Deficiency of BLNK Improved Survival in *C. albicans*-Infected Mice. In order to elucidate the involvement of myeloid BLNK in the context of antifungal infections, we employed a mouse model in which BLNK was specifically depleted in myeloid cells ($Lyz2^{Cre/+}Blnk^{fl/fl}$). This was accomplished through the creation of $Blnk^{fl/fl}$ mice and subsequent crossing with $Lyz2^{Cre/+}$ mice (*SI Appendix*, Fig. S2 *A* and *B*). Intravenous injections of *C. albicans* standard strain SC5314 (2×10^5 fungal cells per mouse) were administered via the tail vein to both inbred $Blnk^{fl/fl}$ mice and $Lyz2^{Cre/+}Blnk^{fl/fl}$ mice. Our observations revealed a significantly lower mortality rate and fungal burden in the kidneys of $Lyz2^{Cre/+}Blnk^{fl/fl}$ mice in comparison to the $Blnk^{fl/fl}$ mice (Fig. 2 *A* and *B*). Histological examination of the kidneys in $Lyz2^{Cre/+}Blnk^{fl/fl}$ mice revealed a reduction in fungal cells, as evidenced by Periodic acid–Schiff (PAS) staining, but no changes of kidney inflammation, as indicated by hematoxylin and eosin (H&E) staining, following a 4-d infection period (Fig. 2*C*). Moreover, BLNK deficiency did not have any impact on the production of TNF-α, IL-6, IL-1β, and IL-12p40 as well as IFN-γ and IL-17A (Fig. 2*D*). Notably, we observed a higher number and frequency of F4/80$^+$Ly6C$^+$ infiltrating macrophages in the kidneys of $Lyz2^{Cre/+}Blnk^{fl/fl}$ mice in comparison to $Blnk^{fl/fl}$ mice on Day 1 and Day 2 postinfection with *C. albicans* (Fig. 2*E* and *SI Appendix*, Fig. S2*C*). This suggests that monocyte-specific BLNK deficiency promoted the infiltration of Ly6C$^+$ macrophages into the kidneys during *C. albicans* infections. In contrast, BLNK deficiency appeared to have no effect on the frequency and number of F4/80$^+$Ly6C$^-$ kidney-resident macrophages during *C. albicans* infections (Fig. 2*E* and *SI Appendix*, Fig. S2*C*). Moreover, BLNK deficiency did not affect the expression levels of C-C chemokine receptor type 2 (CCR2), an essential receptor for macrophage recruitment to inflammation sites, on F4/80$^+$Ly6C$^+$ infiltrating macrophages (*SI Appendix*, Fig. S2*D*), indicating that BLNK did not affect the capability of macrophage migration in response to chemotaxis. Furthermore, we observed a heightened number and percentage of CD11b$^+$Ly6G$^+$ neutrophils and a reduced frequency of CD11c$^+$MHCII$^+$ dendritic cells (DCs) in the kidneys of $Lyz2^{Cre/+}Blnk^{fl/fl}$ mice when compared to their $Blnk^{fl/fl}$ counterparts on Day 2 following *C. albicans* infection (*SI Appendix*, Fig. S2 *F* and *G*). Altogether, these observations suggested that Lyz2$^+$ myeloid cell-specific BLNK deficiency improved the survival of mice infected with *C. albicans*, possibly attributed to the increased infiltration of Ly6C+ macrophages into renal tissue with monocyte-specific

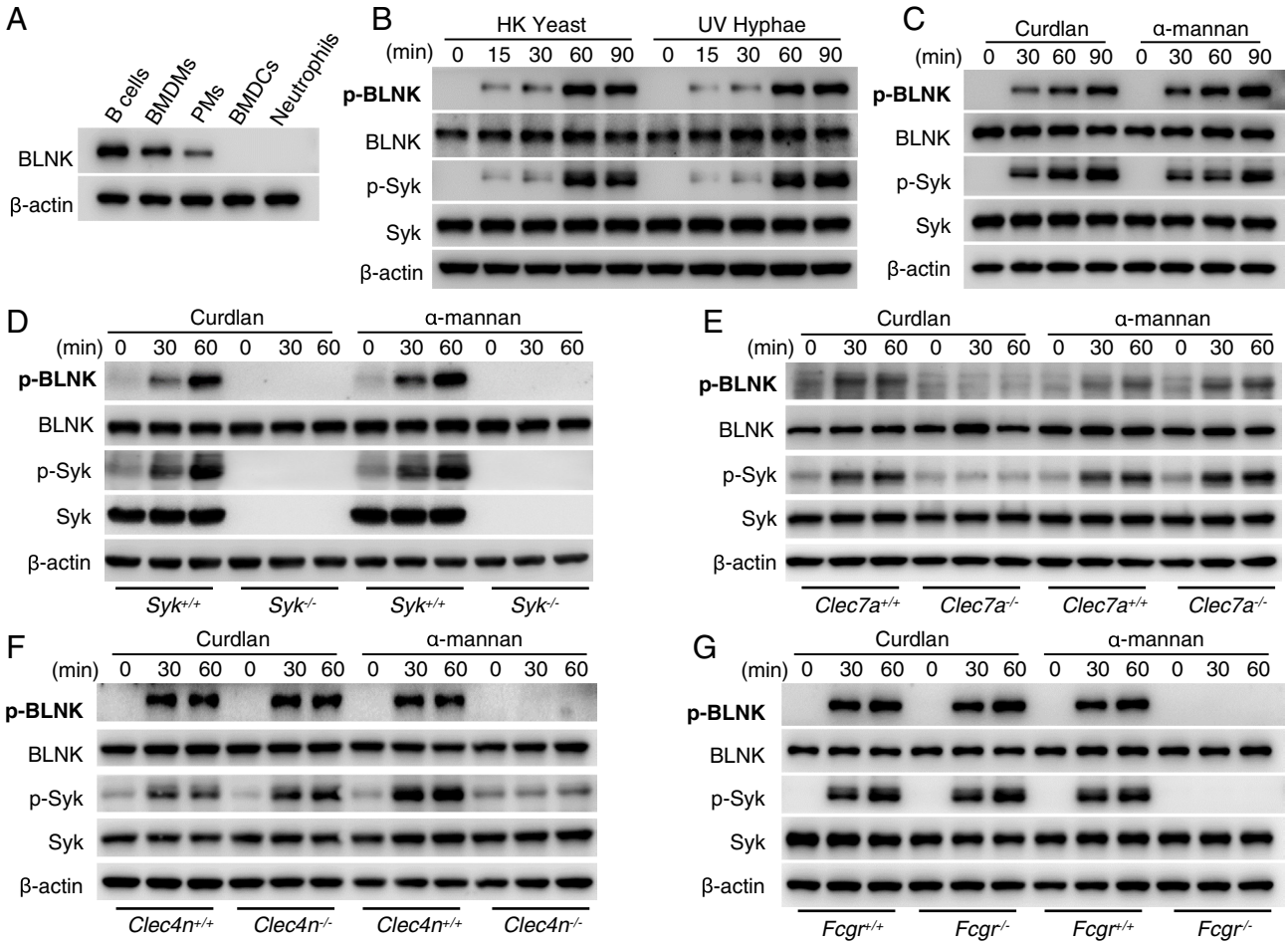

Fig. 1. CLRs mediated the phosphorylation of BLNK in macrophages upon *C. albicans* stimulation. (*A*) The protein level of BLNK was detected by immunoblotting in splenic B cells, BMDMs, PMs, BMDCs, and neutrophils. (*B* and *C*) Immunoblot analysis was performed to assess the phosphorylation of the indicated protein in wild-type BMDMs, which were stimulated with *C. albicans* heat-killed yeast (MOI = 5) or UV-inactivated hyphae (MOI = 0.1) (*B*), or plate-coated curdlan (50 μg/mL) or α-mannans (250 μg/mL) (*C*) for the indicated time. (*D*–*G*) Immunoblot analysis was conducted to examine the phosphorylation of the indicated protein in wild-type or Syk deficient ($Syk^{-/-}$, *D*) or Dectin-1 deficient ($Clec7a^{-/-}$, *E*) or Dectin-2 deficient ($Clec4n^{-/-}$, *F*) or FcRγ deficient ($Fcgr^{-/-}$, *G*) BMDMs, which were stimulated with plate-coated curdlan (50 μg/mL) or α-mannans (250 μg/mL) for the indicated time. Data presented are representative of three independent experiments. Source data are provided in Dataset S1.

BLNK deficiency. These results implied that BLNK in myeloid cells exerted an inhibitory influence on the regulation of antifungal immunity in response to *C. albicans* infection.

BLNK Inhibited the Migration of F4/80$^+$Ly6C$^+$ Infiltrating Macrophages into the Kidney during *C. albicans* Infection. To investigate the potential role of BLNK in the regulation of macrophage migration during *C. albicans* infection, we employed an intraperitoneal injection of thioglycollate to induce macrophage recruitment in mice. Prior to the injection, we found no significant differences in the numbers and proportions of peritoneal macrophages (PMs) and neutrophils between $Blnk^{fl/fl}$ mice and $Lyz2^{Cre/+}Blnk^{fl/fl}$ mice (Fig. 3*A* and *SI Appendix*, Fig. S2 *A*–*D*). However, following thioglycollate treatment, intraperitoneal injection of fungi-derived α-mannans in the mice resulted in notable differences in our observations (Fig. 3*A*). Specifically, on Days 1 and 3 after injection, $Lyz2^{Cre/+}Blnk^{fl/fl}$ mice exhibited a heightened number of elicited macrophages compared to $Blnk^{fl/fl}$ mice, while neutrophil numbers remained largely unchanged (Fig. 3*A* and *SI Appendix*, Fig. S3 *C* and *D*). Furthermore, following administration of α-mannans alone, $Lyz2^{Cre/+}Blnk^{fl/fl}$ mice also exhibited a significant increase of macrophage counts and proportions on both Days 1 and 3 relative to $Blnk^{fl/fl}$ mice (Fig. 3*A* and *SI Appendix*, Fig. S3*C*). In comparison, no notable changes in macrophage and neutrophil counts and proportions were noted between the two groups of mice following thioglycollate injection alone (Fig. 3*A* and *SI Appendix*, Fig. S3 *C* and *D*). These findings suggested that BLNK functioned to inhibit α-mannan-induced macrophage migration.

During systemic *C. albicans* infection, the kidney serves as a primary target immune organ in mice (27). To assess whether BLNK activation hinders renal macrophage infiltration, we isolated BMDMs from wild-type mice and BLNK-deficient mice, labeled with CFSE and eFluor670, respectively. These labeled cells were uniformly mixed and subsequently intravenously injected into *C. albicans*-infected wild-type mice (Fig. 3*B* and *SI Appendix*, Fig. S3*E*). Surprisingly, following *C. albicans* infection, the proportion and quantity of eFluor670$^+$ BLNK-deficient macrophages in the kidneys were higher than CFSE$^+$ wild-type macrophages (Fig. 3*C*). However, there was no difference in the proportion and quantity of these macrophage populations in peripheral blood, regardless of infection status (Fig. 3 *C* and *D*). These findings suggested that BLNK inhibited macrophage mobilization and subsequent migration to the kidneys during *C. albicans* infection.

To validate the functional significance of BLNK in macrophage renal infiltration during *C. albicans* infection, we employed CCR2-[RA], a specific allosteric antagonist of CCR2, in $Blnk^{fl/fl}$

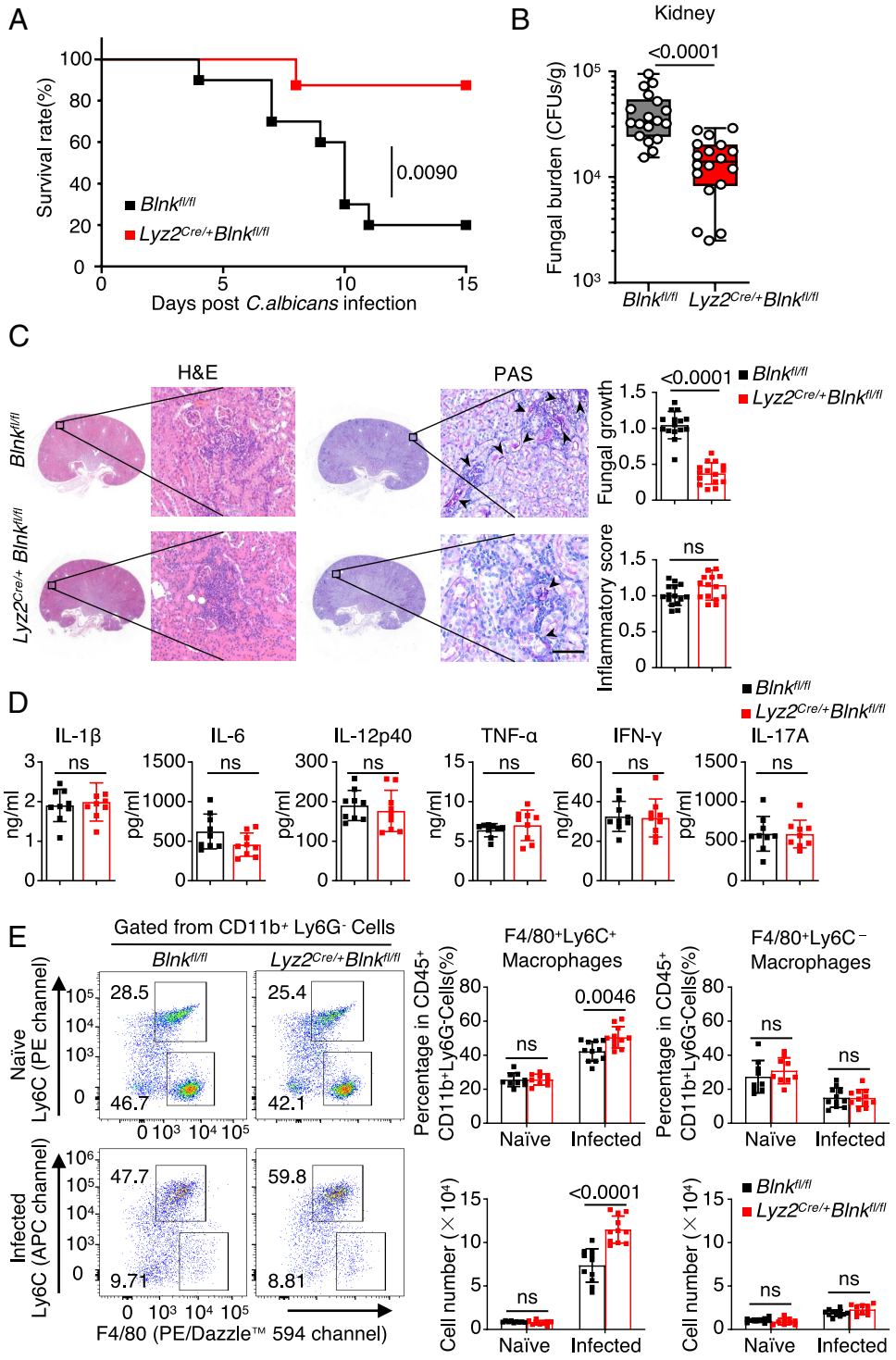

Fig. 2. Lyz2$^+$ myeloid cell-specific deficiency of BLNK improved survival in *C. albicans*-infected mice. *Blnk*$^{fl/fl}$ and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$mice received intravenous injection of *C. albicans* SC5314 (2 × 10^5 CFU/mouse). (*A*) Survival rates of *Blnk*$^{fl/fl}$(n = 10) and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$(n = 8, respectively) mice following infection. (*B*) Kidney fungal load (n = 18) and (*C*) representative histological images stained with H&E or periodic acid–Schiff (PAS) on Day 4 postinfection (n = 3, respectively). Five regions were randomly selected from kidney sections of each mouse for analysis of fungal hyphal growth and inflammation scores using ImageJ software. *Insets* depicted regions of fungal inflammation and fungal growth respectively at higher-magnification (×160) views, with the scale bar representing 100 μm. The arrows indicated the hyphae of *C. albicans*. (*D*) ELISA of indicated cytokines in homogenized kidneys of *C. albicans*-infected *Blnk*$^{fl/fl}$and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$mice (n = 9, respectively). (*E*) Flow cytometry analysis of macrophage infiltration in the kidneys of *Blnk*$^{fl/fl}$and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$mice on Day 2 postinfection with *C. albicans* (naïve group, n = 9, infected group, n = 11). For (*A*), the log-rank test was performed; for (*B–E*), data are shown as means ± SD, and the two-tailed unpaired *t* test was performed. Data were integrated from three independent experiments.

and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$ mice. Treatment with CCR2-[RA] led to a reduction in the recruitment of F4/80$^+$Ly6C$^+$ macrophages to the kidneys in both groups of mice, but did not reduce F4/80$^+$Ly6C$^-$ macrophages, eliminating the disparity in fungal load clearance between *Blnk*$^{fl/fl}$ and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$ mice (Fig. 3 *E* and *F* and *SI Appendix*, Fig. S3*F*). We found that treatment with CCR2-[RA] resulted in significantly reduced survival rates and increased fungal burden in *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$ mice (Fig. 3 *E* and *F*). In addition, in *Blnk*$^{fl/fl}$and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$ mice, we observed that JMS-17-2, a specific inhibitor of CX3CR1, effectively hindered the migration

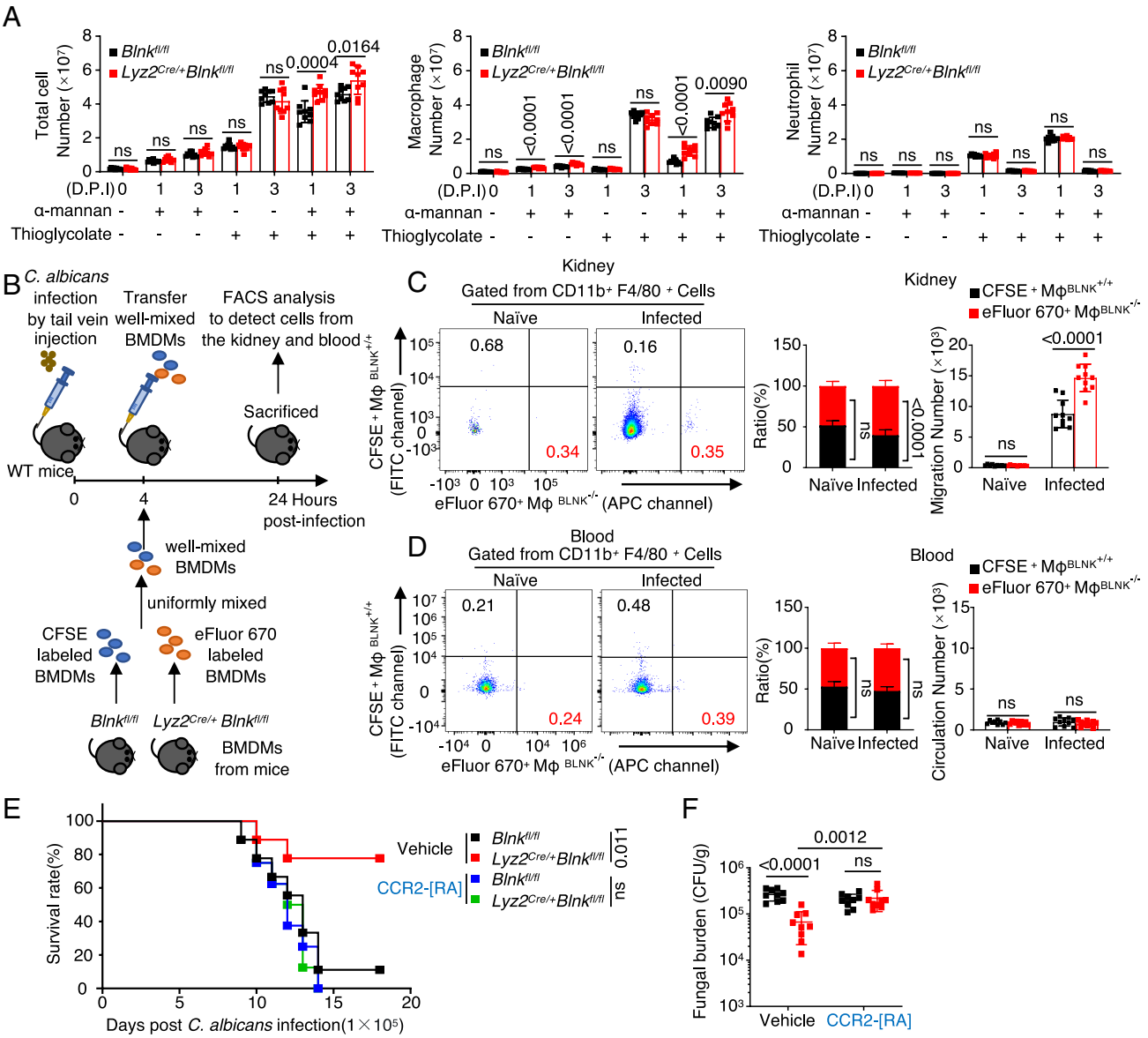

Fig. 3. BLNK inhibited macrophage migration during *C. albicans* infection. (*A*) Recruitment of total cells (*Left*), macrophages (*Middle*), or neutrophils (*Right*) in the peritoneal cavity of $Blnk^{fl/fl}$ and $Lyz2^{Cre/+}Blnk^{fl/fl}$ mice was analyzed by flow cytometry after intraperitoneal injection with *C. albicans*-derived α-mannans (1 mg) or thioglycollate with α-mannans (1 mg) on the indicated days (n = 9, respectively). (*B*) Schematic diagram of in vivo assay strategy. (*C* and *D*) Flow cytometry analysis (*Left*) of macrophages migration in the kidney (*C*) and blood (*D*) 24 h after 2 × 10^5 CFU of *C. albicans* infection. The relative migration ratio was calculated as [$CFSE^+$BMDMs/($CFSE^+$BMDMs+ $eFluor670^+$ BMDMs)] (*Middle*), and migration number (*Right*) of macrophages from $Blnk^{fl/fl}$ or $Lyz2^{Cre/+}Blnk^{fl/fl}$ mice were determined (naïve group, n = 9, infected group, n = 10). (*E* and *F*) $Blnk^{fl/fl}$ and $Lyz2^{Cre/+}Blnk^{fl/fl}$ mice (n = 9, respectively) were intraperitoneally injected with CCR2-[RA] (30 mg/kg/day) or vehicle. Survival rate was evaluated after 1 × 10^5 CFU *C. albicans* infection (*E*), and kidney fungal burden was assayed (*F*) 2 d after intravenous injection with 2 × 10^5 CFU of *C. albicans*. The log-rank test was performed for (*E*). In (*A*), (*C*), (*D*), (*F*), the data were shown as means ± SD and the two-tailed unpaired *t* test was performed. Data were integrated from three independent experiments.

of $F4/80^+Ly6C^+$ macrophages derived from monocytes, into the kidneys and reduced the tissue-resident $F4/80^+Ly6C$ macrophages. Following JMS-17-2 treatment, both $Blnk^{fl/fl}$ and $Lyz2^{Cre/+}Blnk^{fl/fl}$ mice exhibited a significant increase in fungal burden (*SI Appendix*, Fig. S3 *G* and *H*). These results indicated that BLNK inhibited the migration of $F4/80^+Ly6C^+$ infiltrating macrophages into the kidney, thereby influencing fungal clearance of mice.

BLNK Inhibited Macrophage Migration through Impairing the Formation of Podosome Ring upon Infection with *C. albicans*. To elucidate the underlying mechanisms that BLNK regulated macrophage migration, we conducted comprehensive analyses using wild-type and BLNK-deficient BMDMs stimulated with *C. albicans* hyphae, followed by microarray analysis. A total of 936 differentially expressed genes (DEGs) associated with BLNK deficiency and hyphae stimulation were identified. Subsequent Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses highlighted the high relevance of these DEGs to cell mobilization and monocyte chemotaxis (*SI Appendix*, Fig. S4 *A–C*). To investigate the role of BLNK in macrophage migration, we performed trans-well migration assays and found that, upon α-mannan stimulation, BMDMs from $Lyz2^{Cre/+}Blnk^{fl/fl}$ mice exhibited significantly increased transmembrane migration compared to $Blnk^{fl/fl}$ mice (Fig. 4*A*). Additionally, 3D cell spheroid and invasion assays were employed to assess the mobilization and invasion abilities of cells in the matrix gel (Fig. 4*B*). BMDMs from $Lyz2^{Cre/+}Blnk^{fl/fl}$ mice also showed significantly increased 3D migration under α-mannan stimulation compared to $Blnk^{fl/fl}$ mice (Fig. 4*B*). Besides, scratch

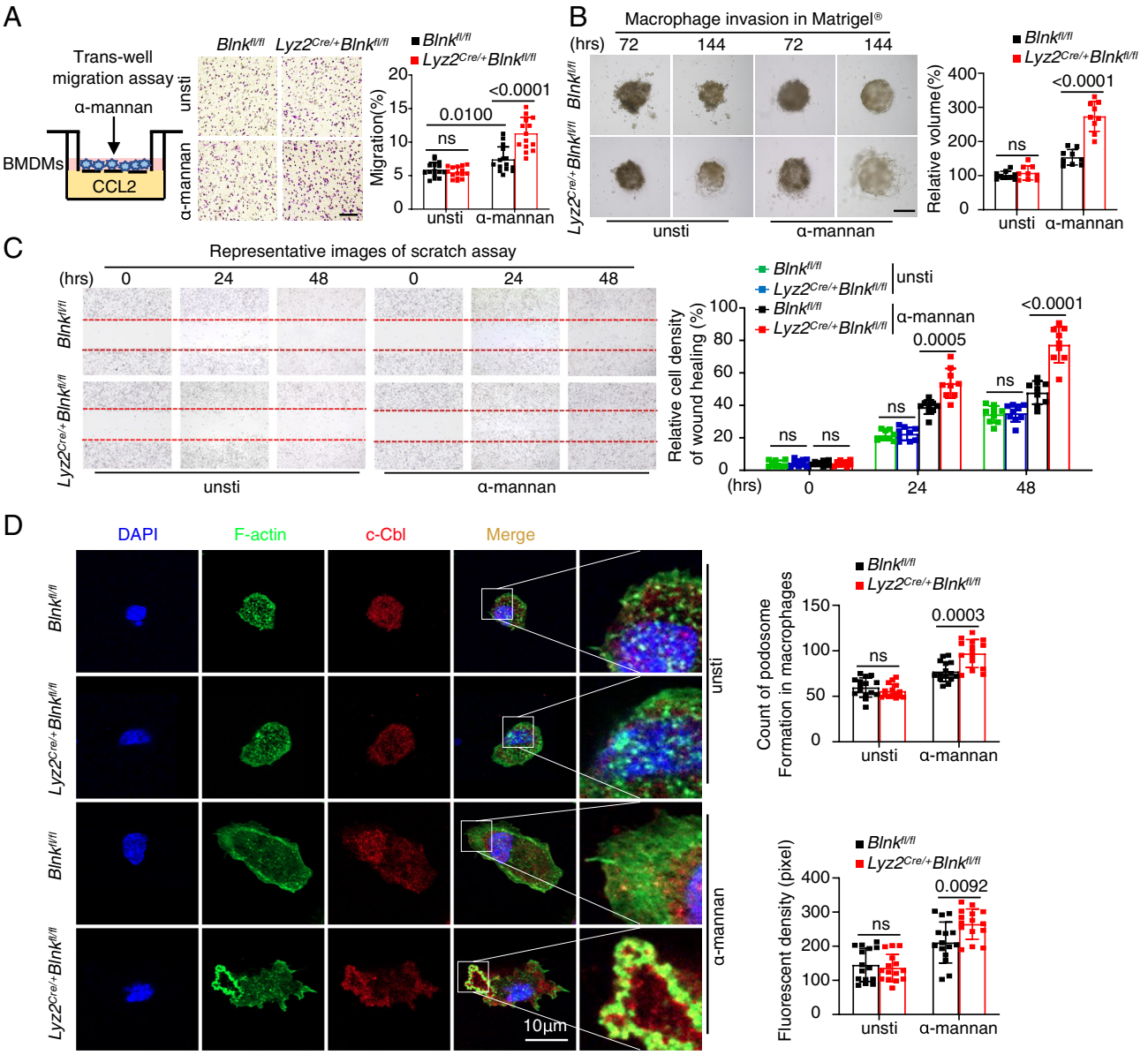

Fig. 4. BLNK impaired macrophage migration by disrupting podosome formation. (*A*) Trans-well migration assay was performed in BMDMs from *Blnk*$^{fl/fl}$ and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$mice induced by α-mannans (250 μg/mL in upper chamber) and CCL2 (40 ng/mL in bottom chamber) (*Left*). Migration ratio was calculated from five random of view (*Right*). Scale bar represents 1 mm. (*B*) 3D invasion assay was performed in BMDMs from *Blnk*$^{fl/fl}$ and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$mice in Matrigel ® after stimulation by α-mannans and CCL2 (*Left*) and invasion ratio was measured (*Right*). Scale bar represents 1 mm. (*C*) Wound healing assays were performed in BMDMs (5 × 10^6 cells) from *Blnk*$^{fl/fl}$ and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$mice induced with α-mannans (250 μg/mL). Relative cell densities of wound healing were calculated from 5 random sights (*Right*). (*D*) Typical macrophage cellular images acquired using immunofluorescence confocal laser scanning microscopy (CLSM) under α-mannan stimulation (Imaging performed with a total magnification of 400×, and the image on the *Right* is further magnified 16 times). The number of podosome ring formation in macrophages was compared in BMDMs from *Blnk*$^{fl/fl}$ and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$ mice using immunofluorescence CLMS imaging. The average fluorescence density (in pixels) of the colocalization of c-Cbl (red) and F-actin (green) was observed. Immunofluorescence staining was performed using anti-c-Cbl (red), phalloidin (green), and DNA-binding dye DAPI (blue). The scale bar represents 10 μm. The analysis was conducted using ImageJ software, n = 5 per group, with three random fields selected from BMDMs of each mouse. Data represented means ± SD from three independent experiments, analyzed using the two-tailed unpaired *t* test.

assays demonstrated that BLNK-deficient BMDMs displayed a higher ability for wound healing in response to α-mannan stimulation (Fig. 4*C*). These findings implied that BLNK was involved in the negative regulation of macrophage migration during *C. albicans* infection.

We further investigated the role of BLNK on the formation of podosomes, which are the dynamic F-actin-rich structures and critical for macrophage migration. Immunofluorescence staining revealed a significant increase in podosome ring formation in BLNK-deficient macrophages compared to wild-type BMDMs following α-mannan stimulation (Fig. 4*D* and *SI Appendix*, Fig. S4*D*). Interestingly, in BLNK-deficient macrophages, we observed a significant elevation in the colocalization fluorescence intensity of the podosome F-actin with the protein Casitas B-lineage Lymphoma (c-Cbl) (Fig. 4*D* and *SI Appendix*, Fig. S4*D*). Moreover, the expression of c-Cbl exhibited a robust correlation with F-actin after α-mannan stimulation, as assessed by Pearson correlation coefficient analysis (*SI Appendix*, Fig. S4*D*). Although the role of c-Cbl in macrophage migration is well characterized, its involvement in antifungal immunity remained unclear. To address this, we employed a systemic *C. albicans* infection model in *c-Cbl*$^{fl/fl}$ and *Lyz2*$^{Cre/+}$*c-Cbl*$^{fl/fl}$ mice. We found that *Lyz2*$^{Cre/+}$*c-Cbl*$^{fl/fl}$ mice exhibited significantly increased susceptibility to *C. albicans* infection compared to

c-Cbl$^{fl/fl}$ mice, accompanied by reduced infiltration of Ly6C^{+} macrophage in the kidneys (*SI Appendix*, Fig. S4 *E–G*). To further evaluate the role of c-Cbl in macrophage migration in the kidney, we isolated BMDMs from wild-type mice and c-Cbl-deficient mice. Using the method established in Fig. 3*B*, these BMDMs were injected into wild-type mice infected with *C. albicans*. We found that c-Cbl is indispensable for macrophage migration to the kidney during *C. albicans* infection (*SI Appendix*, Fig. S4*H*). In trans-well migration assays, we also confirmed that c-Cbl was crucial for macrophage migration during *C. albicans* infection (*SI Appendix*, Fig. S4*I*). These findings suggested that BLNK might impede macrophage migration by inhibiting podosome ring formation, negatively regulating c-Cbl-mediated macrophage migration in response to *C. albicans* infection.

CLRs Mediated the Phosphorylation of BLNK to Elicit Its Impact on c-Cbl. To explore the underlying mechanisms of c-Cbl-mediated macrophage migration regulated by BLNK, we stimulated BMDMs with *C. albicans* hyphae and found that the deficiency of BLNK did not impact the phosphorylation levels of established CLR-downstream mediators, including Syk, PLCγ2, p65, ERK, JNK, and p38 (*SI Appendix*, Fig. S5*A*). Additionally, a protein–protein interaction (PPI) assay revealed a potential interaction between BLNK and c-Cbl (*SI Appendix*, Fig. S5 *B* and *C*). c-Cbl has been implicated in the PI3K-dependent migration of macrophages (21). The known association between BLNK and c-Cbl in the context of BCR signaling (22) prompted us to investigate this interaction in BMDMs. Notably, following α-mannan stimulation, we observed a direct association of BLNK with c-Cbl in BMDMs, accompanied by a remarkable increase in the accumulation of endogenous BLNK-associated c-Cbl protein (Fig. 5*A* and *SI Appendix*, Fig. S5*D*). Immunofluorescence staining confirmed a significant increase in colocalization of BLNK with c-Cbl in wild-type macrophages after α-mannan stimulation (Fig. 5*B*). Importantly, the deficiency of Dectin-1/Dectin-2 or treatment with a Syk inhibitor in BMDMs completely abolished the association between BLNK and c-Cbl (Fig. 5 *C–E* and *SI Appendix*, Fig. S5 *E–G*). However, the lack of CARD9 had no discernible effect on this association (Fig. 5*F* and *SI Appendix*, Fig. S5*H*). These findings suggested that CLRs, including Dectin-1 and Dectin-2, governed the Syk-mediated phosphorylation of BLNK, leading to its association with c-Cbl for the negative regulation of macrophage migration.

Further investigations were conducted by reconstituting the Dectin-1/Syk signaling pathway in 293T cells to elucidate their necessity for their association between BLNK and c-Cbl (Fig. 5*G* and *SI Appendix*, Fig. S5*I*). Stimulation with heat-killed *C. albicans* yeast induced a significant association between c-Cbl and BLNK in 293T cells expressing Dectin-1 and Syk (Fig. 5*G* and *SI Appendix*, Fig. S5*I*). However, the absence of either Dectin-1 or Syk hindered the formation of the BLNK/c-Cbl complex (Fig. 5*G* and *SI Appendix*, Fig. S5*I*). To assess the requirement of tyrosine phosphorylation of BLNK for its impact on c-Cbl, we generated BLNK mutants with amino acid residues Y72/84/96/178/189 substituted with a nonphosphorylated PHE (F) residue (Fig. 5*H* and *SI Appendix*, Fig. S5*J*). Expression levels of BLNK mutants, including BLNK^{Y84F}, BLNK^{Y96F}, and BLNK^{Y189F}, were comparable to those of wild-type BLNK when transfected into 293T cells (Fig. 5*H* and *SI Appendix*, Fig. S5*J*). However, the association of c-Cbl with these BLNK mutants was significantly reduced (Fig. 5*H* and *SI Appendix*, Fig. S5*J*), indicating that tyrosine phosphorylation of BLNK at Y84/96/189 was crucial for its CLR/Syk-mediated interaction with c-Cbl.

BLNK Inhibited the Fyn-Mediated Phosphorylation of c-Cbl through Blocking Their Interactions. The proto-oncogenic protein c-Cbl has been demonstrated to undergo tyrosine phosphorylation in response to TCR/CD3 or BCR ligation stimulation (28). We found that stimulation with β-glucans or α-mannans induced the tyrosine phosphorylation of c-Cbl at amino acid residues Tyr(Y)700 and Y737 in BMDMs. Notably, the deficiency of BLNK significantly enhanced the tyrosine phosphorylation of c-Cbl at Y737, rather than Y700 (Fig. 6*A* and *SI Appendix*, Fig. S6*A*). We further examined whether tyrosine phosphorylation of BLNK at Y84/96/189 was required for the inhibition of the tyrosine phosphorylation of c-Cbl at Y737 in BMDMs. BLNK-deficient BMDMs expressing BLNK$^{Y84/96/189F}$ mutant displayed a higher level of tyrosine phosphorylation of c-Cbl at Y737 compared to those expressing wild-type BLNK upon stimulation with heat-killed *C. albicans* yeast (Fig. 6*B* and *SI Appendix*, Fig. S6*B*). These findings indicated that CLRs, including Dectin-1 and Dectin-2, mediated the tyrosine phosphorylation of BLNK at Y84/96/189, initiating its association with c-Cbl, which in turn inhibits the tyrosine phosphorylation of c-Cbl at Y737 in BMDMs.

Previous studies have established that Fyn can associate with c-Cbl to phosphorylate tyrosine 731 in c-Cbl (corresponding to Y737 in mice), a binding site for PI3K. We found that BLNK-deficient BMDMs exhibited a higher level of endogenous c-Cbl-associated Fyn protein accumulation compared to wild-type BMDMs (Fig. 6*C* and *SI Appendix*, Fig. S6*C*), indicating that BLNK presence hindered the interaction between c-Cbl and Fyn. Interestingly, c-Cbl was constitutively associated with BLNK or Fyn when they were coexpressed in 293T cells (Fig. 6*D* and *SI Appendix*, Fig. S6*D*). However, the association of c-Cbl with Fyn was completely impaired when all three (c-Cbl, BLNK, and Fyn) were coexpressed in 293T cells (Fig. 6*D* and *SI Appendix*, Fig. S6*D*). Additionally, the expression of BLNK in 293T cells dose-dependently reduced the accumulation of c-Cbl-associated Fyn protein as well as the tyrosine phosphorylation level of c-Cbl at Y737 (Fig. 6*E* and *SI Appendix*, Fig. S6*E*). Furthermore, the presence of BLNK significantly reduced the accumulation of c-Cbl-associated Src protein and the tyrosine phosphorylation level of c-Cbl at Y737 (*SI Appendix*, Fig. S6*F*). However, the presence of BLNK had no impact on the association of c-Cbl with Syk or the Src-family kinase Lyn (*SI Appendix*, Fig. S6 *G* and *H*). Consequently, knocking down of endogenous Fyn in BMDMs completely inhibited the tyrosine phosphorylation of c-Cbl at Y737 and Y700 following heat-killed *C. albicans* yeast stimulation (Fig. 6*F* and *SI Appendix*, Fig. S6*I*). These data implied that CLRs promoted the Syk-mediated phosphorylation of BLNK, leading to the inhibition of the Fyn-mediated phosphorylation of c-Cbl by disrupting their interactions.

BLNK Inhibited the c-Cbl-Associated F-Actin Assembly Induced by β-Glucans and α-Mannans. c-Cbl has been implicated in the PI3K-dependent migration of macrophages through regulating actin cytoskeletal rearrangements (21). Upon stimulation with α-mannans, we observed a significant reduction in the levels of PI3K-associated β-actin protein in c-Cbl-deficient BMDMs (Fig. 7*A* and *SI Appendix*, Fig. S7*A*). Conversely, BLNK deficiency resulted in a notable increase in the accumulation of β-actin protein associated with PI3K or c-Cbl (Fig. 7 *B* and *C* and *SI Appendix*, Fig. S7 *B* and *C*). To investigate the interaction between c-Cbl, PI3K, and the cytoskeleton, we employed a Triton X-100 extraction technique, as the cytoskeleton is detergent-insoluble, and Triton X-100 is commonly used to expose the cytoskeleton for immunological assays. Our findings revealed that BLNK deficiency led to a

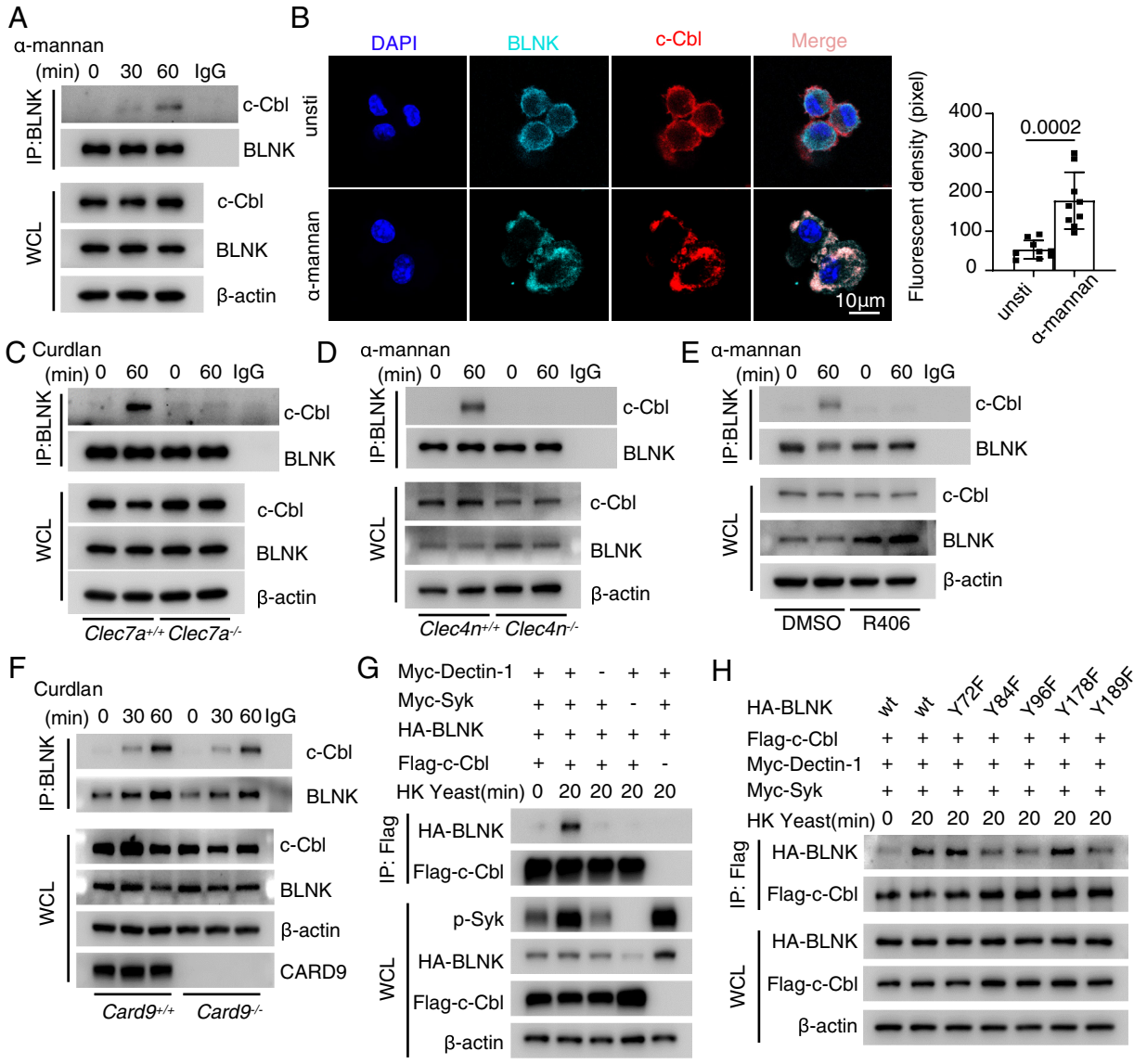

Fig. 5. CLR-mediated BLNK phosphorylation facilitates its impact on c-Cbl. (*A*) Wild-type BMDMs were stimulated with α-mannan (250 μg/mL) for a specific duration. Subsequent immunoprecipitation using IgG or anti-BLNK was conducted, followed by immunoblot analysis to examine the interaction between c-Cbl and BLNK. (*B*) Following stimulation of wild-type BMDMs with α-mannan, CLMS images were examined to detect the average fluorescence intensity (in pixels) of colocalized c-Cbl and BLNK. Immunostaining was performed using anti-c-Cbl (red), anti-BLNK (cyan), and DNA-binding dye DAPI (blue). The scale bar represents 10 μm. Image analysis was carried out using ImageJ software (Imaging performed with a total magnification of 100×). Each column and bar in the graph represent the means ± SD, with n = 3 for each group, and three random fields of BMDMs were selected from each mouse for analysis. (*C*–*F*): Immunoblotting analysis of the interaction between c-Cbl and BLNK in Dectin-1 deficient (*Clec7a*$^{-/-}$, *C*) or Dectin-2 deficient (*Clec4n*$^{-/-}$, *D*) or Syk inhibitor R406 (5μM) pretreated wild type (*E*) or CARD9 deficient (*Card9*$^{-/-}$, *F*) BMDMs, which were immunoprecipitated with IgG or anti-BLNK after the stimulation of plate-coated curdlan (50μg/mL) or α-mannans (250μg/mL) for the indicated time. (*G* and *H*) Immunoblotting analysis was conducted to evaluate the interaction between c-Cbl and BLNK overexpressed in 293T cells as the indicated combinations, which were immunoprecipitated with anti-Flag after stimulation with heat-killed *C. albicans* yeast (MOI = 5). Data were expressed as means ± SD and analyzed using two-tailed unpaired *t* tests. All data were representative of three independent experiments. Source data are provided in Dataset S2.

significant increase in the accumulation of either PI3K or c-Cbl in the Triton X-100-insoluble fraction upon stimulation with α-mannans (*SI Appendix*, Fig. S7*D*). Moreover, it is noteworthy that BLNK did not directly interact with PI3K (*SI Appendix*, Fig. S7*E*). Intriguingly, the association between BLNK and PI3K was markedly enhanced when all three proteins (BLNK, c-Cbl, and PI3K) were coexpressed in 293T cells (*SI Appendix*, Fig. S7*F*). These observations suggested a complex interplay between c-Cbl, BLNK, and PI3K in regulating macrophage migration, with BLNK influencing the cytoskeletal association of c-Cbl and PI3K.

To assess the requirement of tyrosine phosphorylation of either c-Cbl or BLNK for c-Cbl-associated F-actin assembly, we engineered mutants c-Cbl^{Y737F} and BLNK$^{Y84/96/189F}$ (Fig. 7 *D* and *E* and *SI Appendix*, Fig. S7 *G* and *H*). Upon transfection into BMDMs, the expression levels of c-Cbl^{Y737F} and BLNK$^{Y84/96/189F}$ were comparable to their respective wild-type counterparts (Fig. 7 *D* and *E*). In addition, the introduction of wild-type c-Cbl into c-Cbl-deficient BMDMs significantly elevated the accumulation of c-Cbl-associated β-actin, along with associated proteins such as WAVE2 and ARP2/3 (Fig. 7*D* and *SI Appendix*, Fig. S7*G*). Conversely, expression of the c-Cbl^{Y737F} mutant in c-Cbl-deficient BMDMs was only enhanced the interaction between c-Cbl and Fyn upon stimulation with heat-killed *C. albicans* yeast (Fig. 7*D* and *SI Appendix*, Fig. S7*G*). Furthermore, expression of wild-type BLNK in BLNK-deficient BMDMs notably reduced the accumulation of c-Cbl-associated Fyn, β-actin, and associated proteins WAVE2 and ARP2/3 (Fig. 7*E* and *SI Appendix*, Fig. S7*H*). Conversely, expression of the BLNK$^{Y84/96/189F}$ mutant in BLNK-deficient BMDMs failed to impede these interactions upon stimulation with *C. albicans* yeast (Fig. 7*E* and *SI Appendix*, Fig. S7*H*). Knocking down of

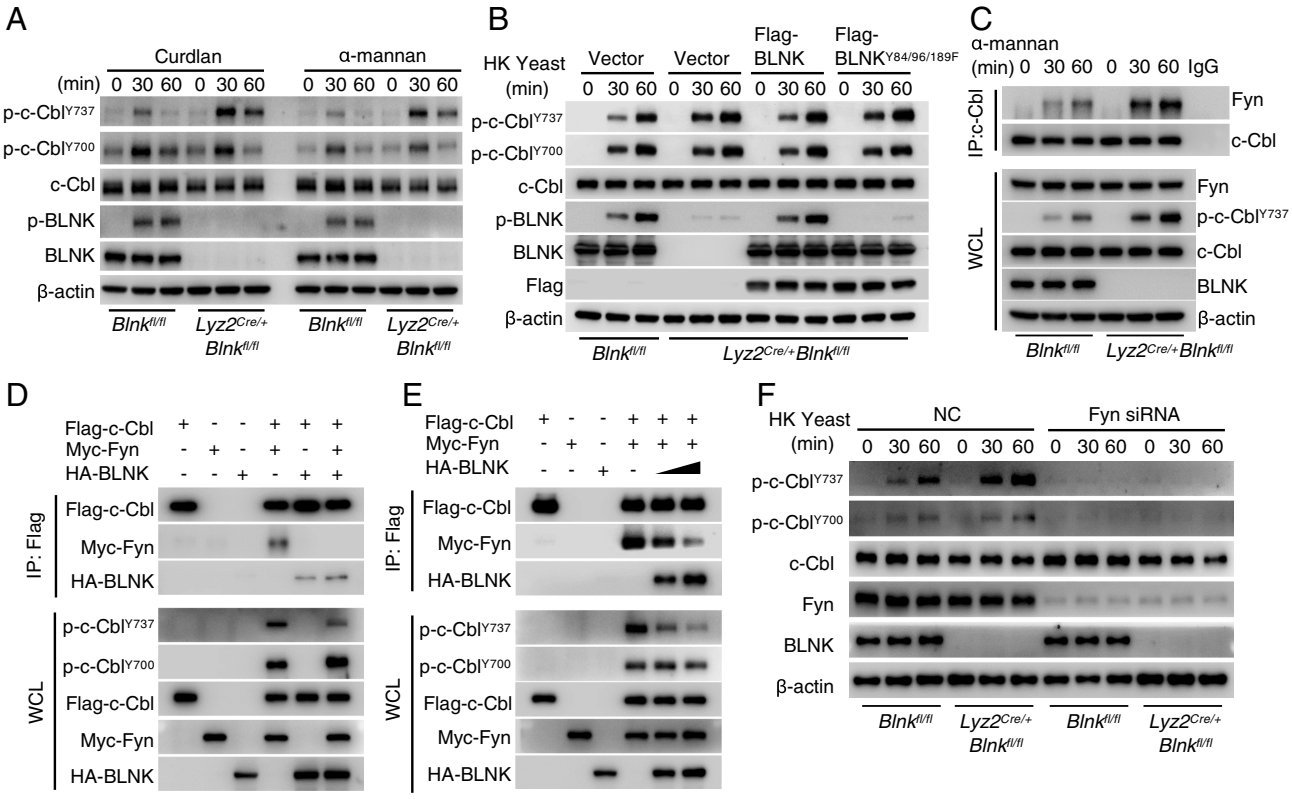

Fig. 6. BLNK dampened c-Cbl phosphorylation through disrupting the interaction of c-Cbl and Fyn. (*A*) Immunoblotting analysis of the indicated protein phosphorylation in BMDMs from *Blnk*$^{fl/fl}$ and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$mice, which were stimulated with plate-coated curdlan (50 μg/mL) or α-mannans (250 μg/mL) for the indicated time. (*B*) Immunoblotting analysis of the indicated protein phosphorylation in BMDMs from *Blnk*$^{fl/fl}$ and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$mice, which were transfected with indicated lentivirus and then stimulated with heat-killed *C. albicans* yeast (MOI = 5) for the indicated time. (*C*) Immunoblotting analysis of the interaction between c-Cbl and Fyn in BMDMs from *Blnk*$^{fl/fl}$ and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$mice, which were immunoprecipitated with IgG or anti-c-Cbl after the stimulation of α-mannans (250 μg/mL) for the indicated time. (*D* and *E*) Immunoblotting analysis of interaction between c-Cbl and Fyn overexpressed in 293T cells with the indicated combinations by immunoprecipitation with anti-Flag. (*F*) Immunoblotting analysis of the indicated protein phosphorylation in BMDMs from *Blnk*$^{fl/fl}$ and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$mice, which were transfected with the small interference RNA (siRNA) targeting *Fyn* or control siRNA (NC) and then stimulated with heat-killed *C. albicans* yeast (MOI = 5) for the indicated time. Data were shown as means ± SD, and the two-tailed unpaired *t* test was performed. Data were representative of three independent experiments. Source data are provided in Dataset S3.

endogenous Fyn in BLNK-deficient BMDMs effectively inhibited c-Cbl-associated F-actin assembly upon stimulation with *C. albicans* yeast (Fig. 7*F* and *SI Appendix*, Fig. S7*I*). Together, these data indicated that BLNK functioned to inhibit c-Cbl-associated F-actin assembly induced by β-glucans and α-mannans.

Discussion

BLNK, serving as an adaptor, plays a crucial role downstream of the Syk-coupled BCR signaling pathway (2). In this study, we propose a model demonstrating that BLNK exerts a negative regulatory influence on antifungal immunity by impeding CLR-mediated macrophage migration. We identify that, in response to *C. albicans* infection, BLNK in macrophages is phosphorylated by Syk in a CLR-dependent manner. Additionally, we observe that phosphorylated BLNK interacts with c-Cbl. Notably, the interaction between BLNK and c-Cbl competitively inhibits the binding of Fyn to c-Cbl, thereby impairing Fyn-mediated phosphorylation of c-Cbl. Furthermore, we elucidate that BLNK, acting as a suppressor factor, negatively regulates the assembly of F-actin cytoskeleton and impedes podosome ring formation by inhibiting c-Cbl phosphorylation. Consequently, BLNK-deficient macrophages exhibit an enhanced capacity for mobilization and migration in response to *C. albicans* infection. This augmented mobilization and migration ability bestows a survival advantage upon mice with Lyz2^{+} myeloid-specific BLNK deficiency, as evidenced by increased macrophage infiltration in the kidney during *C. albicans* infection. In summary, our study uncovers a previously unrecognized role of BLNK in macrophages, thereby enriching our comprehension of the regulatory network governing host immune defense against *C. albicans*.

It has been observed that upon BCR ligation, BLNK, as the directed substrate of Syk, undergoes immediate tyrosine phosphorylation, facilitating the provision of docking sites for various proteins (26). This process triggers PLCγ2-mediated calcium signaling, subsequently activating MAPK and NF-κB pathways (5). While BLNK and its homologue, Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76), are expressed in macrophages and undergo tyrosine phosphorylation following FcγR ligation, the absence of BLNK does not result in a discernible phenotype (24). In human plasmacytoid DCs, BLNK phosphorylation occurs upon CD303 triggering (25). In our study, we have identified BLNK's involvement in the CLR signaling pathway activated by *C. albicans*, with phosphorylation occurring in a Syk-dependent manner. Notably, BLNK deficiency does not affect the activation of MAPK or NF-κB but leads to an elevation in the phosphorylation of c-Cbl. Previous research has shown that, in B cells, c-Cbl interacts with BLNK to negatively regulate PLCγ2 activation (22). Our findings reveal that, in macrophages responding to *C. albicans* infection, BLNK interacts with c-Cbl in a CLR-mediated tyrosine phosphorylation-dependent manner. Importantly, this interaction competitively inhibits the binding between the Src-family kinase

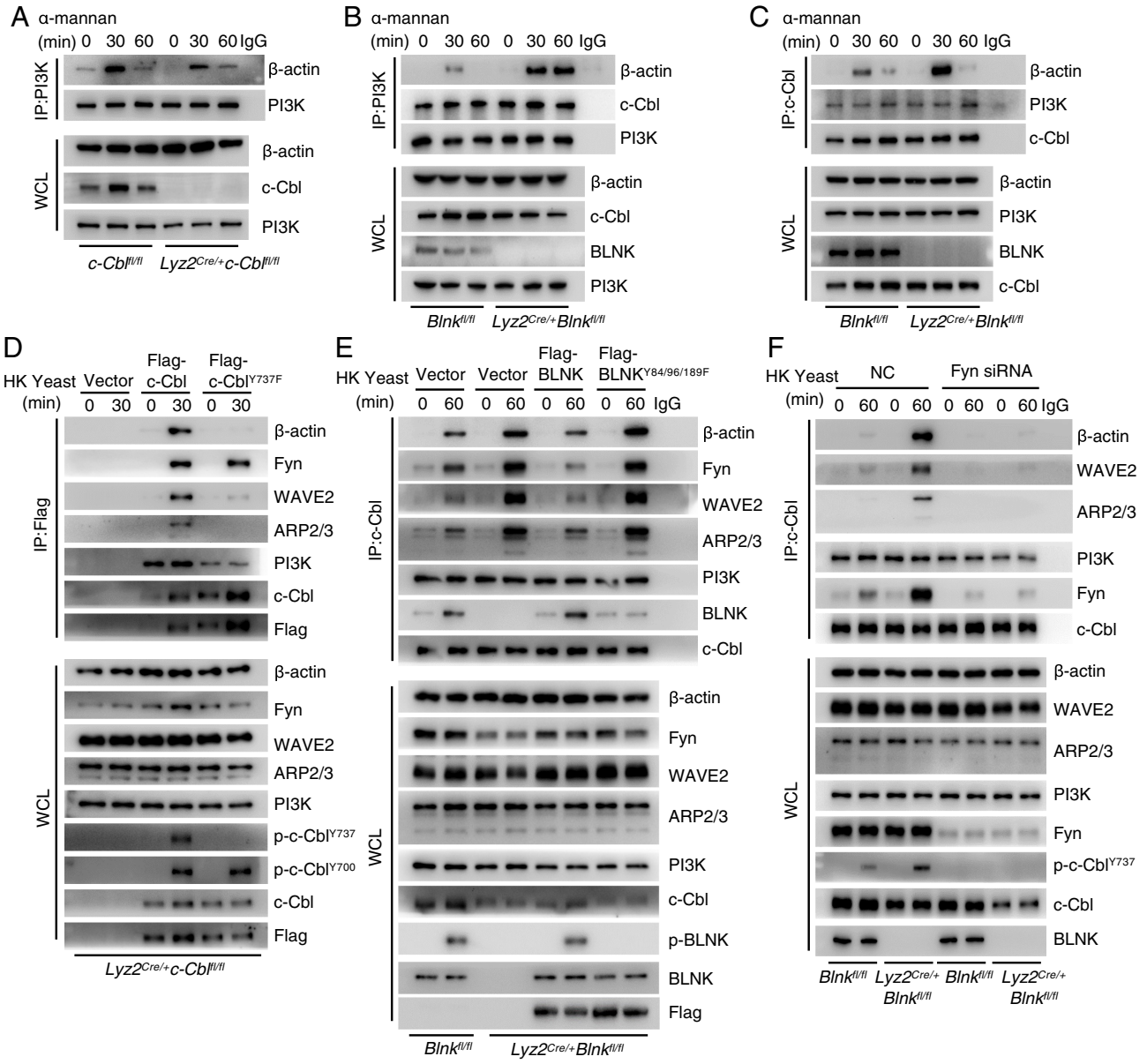

Fig. 7. BLNK inhibited c-Cbl mediated F-actin assembly. (*A*) Immunoblotting analysis of the interaction between PI3K and β-actin in BMDMs from *c-Cbl*$^{fl/fl}$and *Lyz2*$^{Cre/+}$*c-Cbl*$^{fl/fl}$ mice by immunoprecipitation with IgG or anti-PI3K p85 after the stimulation of α-mannans (250 μg/mL) for the indicated time. (*B* and *C*) Immunoblotting analysis of the interaction between PI3K and β-actin (*B*) or the interaction between c-Cbl and β-actin (*C*) in BMDMs from *Blnk*$^{fl/fl}$ and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$mice, which were immunoprecipitated with IgG or anti-PI3K or anti-c-Cbl after stimulation with α-mannans (250 μg/mL) for the indicated time. (*D*) Immunoblotting analysis of the interaction between c-Cbl and actin-associated proteins in c-Cbl-deficient BMDMs, which were transfected with the indicated lentivirus and then immunoprecipitated with anti-Flag after the stimulation of heat-killed *C. albicans* yeast (MOI = 5) for the indicated time. (*E*) Immunoblotting analysis of the interaction between c-Cbl and actin-associated proteins in BMDMs from *Blnk*$^{fl/fl}$ and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$mice which were transfected with the indicated lentivirus by immunoprecipitation with IgG or anti-c-Cbl after stimulation with heat-killed *C. albicans* yeast (MOI = 5) for the indicated time. (*F*) Immunoblotting analysis of the interaction between c-Cbl and actin-associated proteins in BMDMs from *Blnk*$^{fl/fl}$ and *Lyz2*$^{Cre/+}$*Blnk*$^{fl/fl}$mice, which were transfected with the small interference RNA (siRNA) targeting *Fyn* or control siRNA (NC) and then immunoprecipitated with IgG or anti-c-Cbl after stimulation with heat-killed *C. albicans* yeast (MOI = 5) for the indicated time. Data were representative of three independent experiments. Source data are provided in Dataset S4.

Fyn and c-Cbl, thereby impairing Fyn-mediated phosphorylation of c-Cbl at Y737. No significant differences were observed in the phosphorylation of c-Cbl at Y700 between BLNK-deficient and wild-type BMDMs. These results indicate that, in contrast to B cells, in macrophages, Dectin-1 and Dectin-2, mediate *C. albicans*-induced BLNK activation, which specifically inhibits Fyn-mediated phosphorylation of c-Cbl.

The migration and adhesion of macrophages into tissues are essential processes for eliminating multiple pathogens and orchestrating the pathogenesis of inflammation (17). Podosomes, highly dynamic F-actin-rich organelles in myeloid cells, play a crucial role in macrophage migration (29). The regulation of podosome formation primarily depends on G-protein-coupled receptors, selectin, and integrin-mediated signaling. Upon binding of ligands such as chemokines, PSGL1, and ICAM1, rearrangement of the F-actin cytoskeleton occurs, initiating podosome formation (17, 19, 30). In our current investigation, we identified that during *C. albicans* infection, BLNK inhibits macrophage migration by impairing CLR-mediated podosome formation. BLNK-deficient macrophages displayed increased trans-membrane migration, wound healing, and 3D invasion in response to α-mannans stimulation. Actin nucleation and polymerization represent fundamental steps in podosome formation (31). Members of the Rho-GTPase family, Rac and Cdc42, initiate actin nucleation,

and actin-associated proteins such as WASP/N-WASP, WAVE2, Profilin, and the ARP2/3 complex facilitate actin filament extension and branching (32). In this process, PI3K activation promotes podosome formation, while c-Cbl mediates the colocalization of PI3K and the F-actin cytoskeleton (33). Our findings reveal that the interaction between PI3K and β-actin significantly decreases in c-Cbl-deficient macrophages upon α-mannan stimulation. Furthermore, c-Cbl-deficient macrophages exhibit significantly decreased migration during *C. albicans* infection both in vivo and in vitro. These observations align with previous reports indicating that c-Cbl downstream of the β-integrin signaling pathway is essential for macrophage migration (21). Interestingly, we found that BLNK deficiency enhances the interaction between the c-Cbl/PI3K complex and β-actin, WAVE2, and ARP2/3 induced by β-glucans and mannans. This increased interaction is dependent on the phosphorylation of c-Cbl at Y737 in BMDMs. Our data correspond with previous findings indicating that phosphorylation of c-Cbl at Y737 serves as the binding site for the PI3K p85 subunit (34). In conclusion, our study suggests that BLNK negatively regulates podosome formation and subsequent macrophage migration by inhibiting the colocalization of the c-Cbl/PI3K complex and the F-actin complex.

C. albicans, a prominent human fungal pathogen, poses significant health risks, especially to immunocompromised individuals (35). Efficiently recruitment of innate immune cells, including macrophages and neutrophils, to the sites of infection is crucial for the host's defense against *C. albicans*. Notably, the kidneys serve as the primary target organ for *C. albicans* colonization (16). Though macrophages are pivotal in clearing *C. albicans* and maintaining homeostasis, the regulatory mechanisms governing macrophage migration during *C. albicans* infection remain unclear. In this study, we present findings indicating that BLNK negatively regulates the migration of monocyte-derived Ly6C$^+$ macrophages during *C. albicans* infection, thereby suppressing antifungal immunity. While CLRs coordinately regulate *C. albicans*-induced cytokines and chemokines in monocytes and macrophages, mediating the protective effect of monocytes (36), our research reveals that BLNK activation does not influence chemokine production. Recent studies have shown that in tissue-resident macrophages, *C. albicans* components induce CCL2 production in a Dectin-2-dependent manner (37). It has been identified that CCR2-mediated macrophage migration to the kidneys is crucial in antifungal immunity (38). In our present study, we demonstrate that targeting CCR2$^+$ macrophages eliminates the tolerance observed in BLNK conditional knockout mice to systemic *C. albicans* infection. Importantly, BLNK deficiency does not affect CCR2 expression but enhances c-Cbl-mediated F-actin assembly and podosome formation. Furthermore, our investigation reveals that due to decreased macrophage infiltration in the kidneys, mice with myeloid cell-specific c-Cbl deficiency exhibit significantly increased sensitivity to systemic *C. albicans* infection, underscoring the critical role of macrophage migration in *C. albicans* infection. In summary, our data collectively indicate that BLNK negatively regulates antifungal immunity by inhibiting c-Cbl-mediated macrophage migration, shedding light on a previously unrecognized mechanism in the host response to *C. albicans* infection.

Over the last two decades, BLNK has been recognized as a pivotal adapter protein in the BCR signaling pathway. However, our current research reveals a role for BLNK, indicating its involvement downstream of the CLR signaling pathway. Specifically, our findings demonstrate that BLNK serves as a negative regulator of antifungal immunity by inhibiting c-Cbl-mediated macrophage migration. This finding expands our understanding of BLNK's functional repertoire, highlighting its significance beyond the confines of BCR signaling. The identified role in regulating macrophage migration presents a potential target for immunotherapeutic interventions aimed at enhancing the body's defense mechanisms against infectious diseases. Our work not only unveils a previously unrecognized facet of BLNK's activity but also paves the way for the development of innovative immunotherapeutic strategies focused on modulating macrophage migration, a crucial component in the body's response to combatting a spectrum of infectious diseases.

Materials and Methods

Our study complied with ethical standards for animal experiments as approved by the Animal Ethics Committee at Tongji University School of Medicine. We utilized Lyz2$^+$ Myeloid Cell-specific BLNK-deficient mice and various knockout strains to assess immune responses, alongside regular and gene-modified mice strains. Antibodies and plasmids were used to study protein interactions and expressions, validated through stringent DNA sequencing, while chemical reagents facilitated various assays. Cell cultures, including 293T and BMDMs, were grown under controlled conditions to study cellular responses to different ligands including curdlan, α-mannans, or *C. albicans* yeast and hyphae. Methodologies employed included immunoblotting, immunoprecipitation, RNA sequencing, and various cellular assays to evaluate the impacts of genetic modifications and treatments on cellular behavior and immune responses. For details on materials and methods, see *SI Appendix, Materials and Methods*.

Data, Materials, and Software Availability. The Transcriptome data of RNA sequence analysis of BMDMs in this study has been submitted to GEO DataSets (GSE246891) available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE246891 (39). All other data are included in the article and/or supporting information.

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