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Akt-2 Is a Potential Therapeutic Target for Disseminated Candidiasis

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Abstract

Akt-1 and Akt-2 are the major isoforms of the serine/threonine Akt family that play a key role in controlling immune responses. However, the involvement of Akt-1 and Akt-2 isoforms in anti-fungal innate immunity is completely unknown. Here we show that $Akt2^{-/-}$, but not $Akt1^{-/-}$, mice are protected from lethal *C. albicans* infection. Loss of Akt-2 facilitates the recruitment of neutrophils and macrophages to the spleen, and increases ROS expression in these cells. Treating C57BL/6 mice with a specific inhibitor for Akt-2, but not Akt-1, provides protection from lethal *C. albicans* infection. Our data demonstrate that Akt-2 inhibits anti-fungal innate immunity by hampering neutrophil and macrophage recruitment to spleens and suppressing oxidative burst, myeloperoxidase activity and NETosis. We thus describe a novel role for Akt-2 in the regulation of anti-fungal innate immunity and unveil Akt-2 as a potential target for the treatment of fungal sepsis.

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AUTHORS CONTRIBUTIONS

L. Huang performed most of experiments and analyzed the data; Y. Ma, H. Guo, N. Tang, P. Nuro-Gyina, M.C. O'Brien, and S. Ouyang performed some in vitro and in vivo experiments; L. Tao helped data analysis; Y. Liu helped with experimental design and data analysis; J. Zhang conceived and supervised the research, and analyzed data; L. Huang, Y. Ma, W.Y. Langdon, and J. Zhang wrote the manuscript.

COMPETING FINANCIAL INTERESTS STATEMENT

The authors declare no competing financial interests.

Introduction

Acute disseminated candidiasis or invasive candidiasis is a lethal fungal infection of *Candida* spp. It is usually present as candidemia and progresses to several viscera. Invasive fungal infections with *Candida albicans* (*C. albicans*) kill more than 1.5 million people worldwide annually (1). Moreover, other identified pathogenic fungi such as *Aspergillus fumigatus* (*A. Fumigatus*), *Candida auris* (*C. auris*), and *Cryptococcus gattii* (*C. gattii*) also pose a great threat to public health (2–4). Toxicity and resistance to the limited number of anti-fungal agents that are currently available contributes to high morbidity and mortality associated with invasive fungal infections. Therefore, there is an urgent need to better understand the host-pathogen interaction during fungal infection and develop new immuno-therapeutic approaches to fight invasive candidiasis.

C-type lectin receptors (CLRs) are most important pattern recognition receptor (PRR) family for the detection of fungi. Dectin-1, −2, and −3 are the major CLRs involved in anti-fungal immune responses (5–7). These CLRs share a similar molecular structure consisting of a carbohydrate-recognition domain (CRD), a stalk region, a transmembrane domain, and a cytoplasmic domain (5, 6, 8). Dectin-1 and Dectin-2/3 recognize both the yeast and hyphae forms of *C. albicans* by binding to surface β-glucans and α-mannans on the two fungal forms, respectively, leading to the activation of PKC-δ, and subsequent formation of CARD9/Bcl-10/MALT1 complexes which elicits the activation of NF-κB and MAPKs (5, 9–11). Recognition of these molecules by the innate immune cells results in production of inflammatory cytokines and the generation of reactive oxygen species (ROS), which are critical for anti-fungal immunity (12). However, the cell signaling mechanisms of how the signaling pathways that control the inflammatory responses and ROS generation during fungal infection remain to be elucidated.

Akt, also known as protein kinase B (PKB), is crucial for many cellular processes (13, 14). The Akt family is comprised of three highly homologous isoforms Akt-1, Akt-2, and Akt-3, which all possess a catalytic domain, a pleckstrin homology (PH) domain, and a regulatory domain (15). Akt-1 has a wide tissue distribution and is implicated in cell growth and survival, whereas Akt-2 is highly expressed in muscle and adipocytes and contributes to the maintenance of glucose homeostasis (14, 15). Akt-3 is most highly expressed in the brain and testis, and plays a crucial role in brain development (15). Previously, we have shown that Akt-1 and Akt-2 isoforms differentially regulate inducible regulatory T cell (Treg) development, and thymus-derived Treg (tTreg) proliferation (16, 17). Our studies indicate that although structurally similar, Akt-1 and Akt-2 have distinct functions in Treg biology. However, whether Akt-1 and Akt-2 isoforms play differential roles in innate immunity, in particular anti-fungal innate immune response, is completely unknown.

In this study, we report that the loss of Akt-2, but not Akt-1, protect mice from lethal systemic *C. albicans* infection, and that this protection appears to be due to enhanced fungal killing due to increased ROS production in neutrophils and macrophages. Interestingly, pro-inflammatory cytokine production by neutrophils and macrophages are defective in *Akt2*$^{-/-}$ mice upon both *C. albicans* yeast and hyphal infections. Moreover, treating C57BL/6 mice with an Akt-2 inhibitor, but not an Akt-1 inhibitor, protects these mice from systemic *C.*

albicans infection. Therefore, our data suggest that Akt-2 is a potential drug target for disseminated candidiasis.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 (B6), *Akt1*$^{-/-}$, *Akt2*$^{-/-}$, *Rag-1*$^{-/-}$, *LysM* Cre mice and *Akt2*$^{f/f}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *Rag-1*$^{-/-}$ mice (B6 background) were crossed with *Akt2*$^{-/-}$mice to generate *Rag-1*$^{-/-}$*Akt-2*$^{-/-}$ mice which only lack Akt-2 in innate immune cells and non-immune cells such as stromal cells. We also crossed *Akt2*$^{f/f}$ mice to *LysM* Cre mice to generate *Akt2*$^{\Delta M}$ mice which lack Akt-2 in the myeloid specific cells. All experimental protocols were approved by the Institutional Animal Care and Use Committees of The Ohio State University and the University of Iowa. All mice were used for experiments at ages of 8 to 12 weeks and both male and female mice were used in this study.

Reagents

Phospho-Abs against p44/42 MAPK (Thr202/Tyr204; #9101), p38 MAPK (Thr180/Tyr182; #4631), JNK (Thr183/Tyr185; #9251S), Foxo-1(T24)/3a (T32) (#9464), p40phox (Thr154; #4311), NF-κB p65 (Ser276; #3034) and were purchased from Cell Signaling Technology (Danvers, MA). Abs against Akt-1 (G-5; sc-55523) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt-2 (07–372) was purchased from EMD Millipore (Billerica, MA). Anti-MPO (#AF3667) was purchased from R&D Systems. Anti-neutrophil elastase (ab21595) was purchased from Abcam. Anti-actin (AC-15; A-1978) and Akt-1 inhibitor (A-674563) was purchased from Sigma-Aldrich (St. Louis, MO). 124029 Akt Inhibitor XII (Akt-2 inhibitor) was purchased from Calbiochem (San Diego, CA). The following Abs and ELISA kits were purchased from BioLegend (San Diego, CA): Pacific Blue conjugated anti-mouse CD45.2 antibody (#109820), PE conjugated anti-mouse CD11b (#101208), FITC-conjugated anti-mouse CD11b (#101205), APC-Cy7 conjugated anti-mouse F4/80 (#123118), PE-Cy7 conjugated anti-mouse CD11c (#117318), PerCP conjugated anti-mouse Ly6C (#128012), Brilliant Violet 605 conjugated anti-mouse Ly6G (#127639), FITC-conjugated anti-mouse CXCR2 (#149309), ELISA kits for mouse IL-6 (#431304), IL-1β (#432604), CXCL1 (#447507) and TNF-α (#430904). Mouse neutrophil isolation kit (#130097658) was purchased from Miltenyi Biotec (San Diego, CA). CellRox Deep Red (C10422) was purchased from ThermoFisher Scientific (Waltham, MA). The validation of the antibodies used is provided on the manufacturers' websites. The NETosis assay kit was purchased from Cayman Chemical (Cat # 601010; Ann Arbor, MI). The MPO colorimetric activity assay kit was obtained from BioVsion Inc. (K744–100; Milpitas, CA).

In vitro infection of macrophages and neutrophils with *C. albicans* yeasts and hyphae

Wild type *C. albicans* strain SC5314 was grown in yeast peptone dextrose (YPD) medium overnight at 30 °C with shaking. The cells were washed twice with sterile PBS and adjusted to the required cell density before use as live yeasts. The *cap1* yeast-only mutant, described previously (18), was obtained from Dr. Paula R. Sundstrom (Dartmouth University). For the hyphal forms, the washed yeasts were resuspended at 10^7 cells/ml in RPMI 1640 with

10% FCS and grown for 3 h at 37 °C under constant stirring of 200 rpm/min. Subsequently the hyphae were washed in sterile PBS and adjusted to the required cell density before use as live stimulations. For analysis of cytokine production, 10^6 marrow-derived macrophages (BMDMs) or BM neutrophils (BMNs) from WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice were cultured overnight, and infected with live *C. albicans cap1* mutant or hyphae at an MOI of 1 for times indicated. The cytokine production in the supernatants was measured by sandwich ELISA.

Generation of BMDMs and isolation of mouse BMNs

Bone marrow (BM) cells were harvested from the femurs and tibias of mice, and were cultured in DMEM containing 10% FBS and 30% conditioned medium from L929 cells expressing M-CSF (19). After one week of culture, nonadherent cells were removed, and adherent cells were 80–90% F4/80$^+$CD11b$^+$ as determined by flow cytometric analysis. For isolation of BM neutrophils, total BM cells were recovered from the femurs and tibias by flushing with RPMI medium with an 18-gauge needle; erythrocytes were lysed with red blood cells (RBC) lysis buffer (eBioscience) and BM neutrophils were isolated by neutrophil isolation kit (Miltenyl), and neutrophil purity (>98%) was confirmed by flow cytometry.

ROS assay

ROS assay was described previously (20, 21). In brief, WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice were infected with *C. albicans* by tail vein injection at a dose of 0.25×10^6 CFU. 48 h later, mice were sacrificed, and leukocytes from spleens and kidneys were infected with *C. albicans* for 30 min, and stained with CellRox and cell surface markers to determine ROS expression in monocytes (Kidney: CD45.2$^+$CD11b$^+$Ly6C^{hi}Ly6G$^-$; Spleen: CD11b$^+$Ly6C$^+$Ly6G$^-$), macrophages (Kidney:CD45.2$^+$CD11b$^+$F4/80$^+$ Spleen: CD11b$^+$F4/80$^+$), and neutrophils (Kidney: CD45.2$^+$CD11b$^+$Ly6G$^+$; Spleen: CD11b$^+$Ly6G$^+$) (21).

Myeloperoxidase (MPO) activity assay and NETosis assay

To detect myeloperoxidase (MPO) activity, BMNs (1×10^5) from *LysM Cre* and *Akt2*$^{\Delta M}$ mice were infected with *C. albicans* yeasts (MOI:1:1) and hyphae (MOI:1:1) for 1 h, and lysed. The MPO activity in BMN lysates of *LysM Cre* and *Akt2*$^{\Delta M}$ mice will be measured by an MPO colorimetric activity assay kit (BioVision Inc.) according to manufacture's instructions.

Detection of NETosis in BMNs of *LysM Cre* and *Akt2*$^{\Delta M}$ mice was performed using a NETosis assay kit based on the manufacture's instructions (Cayman Chemical). In brief, BMNs (2×10^6) from *LysM Cre* and *Akt2*$^{\Delta M}$ mice were suspended in 1 ml pre-warmed NET assay buffer, and then infected with *C. albicans* yeasts (MOI:1:1) and hyphae (MOI:1:1) for 1 and 2 h. Following digestion of NET DNA by S7 nuclease, the supernatants containing neutrophil elastase were added to a substrate, which is selectively cleaved by neutrophil elastase to yield a 4-nitroaniline product that absorbs light at 405 nm.

Fungal killing assay

For in vitro fungal killing assay, BMDMs or BMNs from WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice were plated in replicates at a density of 1×10^6 cells/well in 48-well plates. Cells were incubated with *C. albican* yeast or hyphae (MOI = 1:100) for 24 h. After co-culture, a

100-μl suspension 1: 10^2, 10^3, or 10^4 dilution was spread on YPD plates. The plates were incubated at 37 °C for 24 h, killing was determined by counting the Candida colonies, with and without the indicated cells (22)

Western blotting

WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ BMNs were infected with *C. albicans* yeast or hyphae (MOI = 1:1) at the indicated times, and lysed in 1% Triton X-100 lysis buffer as previously described (23). The cell lysates were blotted with anti-phospho-Foxo1 (1:1000), anti-phospho-ERK (1:1000), anti-phospho-JNK (1:1000), anti-phospho-p38 (1:1000), anti-phospho-NF-κB p65 (1:1000), and anti-phospho-p40^{phox} (1:1000), respectively. The membranes were stripped and reprobed with anti-Akt-1 (1:1000), anti-Akt-2 (1:1000), and anti-actin (1:8000). Additionally, WT and *Akt2*$^{-/-}$ BMNs were stimulated with curdlan, a Dectin-1 specific ligand, for 15 and 30 min. The cell lysates were blotted with anti-phospho-p40^{phox}. To detect Foxo1 expression in the cytosolic vs. nuclear fractions, *LysM Cre* and *Akt2*$^{\Delta M}$ BMNs were infected with *C. albicans* yeasts for 15 and 30 min, and cytosolic vs. nuclear fractions were purified as described (24). The expression Foxo1 in the cytosols and nuclear fractions was determined by immunoblotting. The purity of the cytosolic and nuclear fractions was determined by immunoblotting with anti-G6DPH and anti-YY1, respectively.

Detection of CXCR2 mRNA expression by RT-PCR

BMNs (2 ×10^6) from WT and *Akt2*$^{-/-}$ mice were infected with *C. albicans* yeasts (MOI:1:1) and hyphae (MOI:1:1) for 1 h or 3 h, respectively. The cells were washed with ice-cold 1xPBS and lysed in TRizol (Invitrogen). The purified mRNA (1 μg) was reverse transcribed into cDNA using a high capacity cDNA reverse transcription kit (4368814,applied biosystems). The transcription (Ct value) of CXCR2 and actin mRNA were tested using SYBR green master mix (A25742, Applied Biosystems). The relative expression of CXCR2 mRNA relative to non-infection was calculated as fold changes (2-△△Ct).

Primers for CXCR2:

Forward (5’−3’):GCTCACAAACAGCGTCGTAG

Reverse (5’−3’):CCACCTTGAATTCTCCCATC

Primer for actin:

Forward (5’−3’): TTGCTGACAGGATGCAGAAG

Reverse (5’−3’): GTACTTGCGCTCAGGAGGAG

Detection of cytokines in serum and culture supernatants and CXCL1 expression in the homogenates of spleens and kidneys by ELISA

For detection of TNF-α, IL-6, and IL-1β in BMDMs or BMNs culture supernatants, 10^6 BMDMs or BMNs from WT, *Akt1*$^{-/-}$ or *Akt2*$^{-/-}$ mice were infected with live *C. albicans cap1* mutant or hyphae at MOI 1:1 for the times indicated, and cytokine production in the

supernatant was measured by ELISA. For detection of serum IL-6, TNF-α and IL-1β, WT, *Akt1*$^{-/-}$ or *Akt2*$^{-/-}$ mice were infected with 2.5 × 10^5 CFU *C. albicans*, serum was collected at different time-points, and subjected for ELISA analysis according to the manufacturer's instructions.

To measure CXCL1 expression, WT and *Akt2*$^{-/-}$ mice were i.v. infected with *C. albicans* (0.25 ×10^6) and euthanized 48 h later. The spleen and kidney were homogenized and centrifuged. The concentration of CXCL1 in the supernatant was tested using a CXCL1 ELISA kit (BioLegend) according to the manufacture's instructions.

Systemic *C. albicans* dissemination

For survival analysis, WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$, *Akt*$^{f/f}$ and *Akt2*$^{\Delta M}$, or *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Akt2*$^{-/-}$ mice were infected with *C. albicans* iv at 4 × 10^5 CFU, and monitored daily. After infection, mice were weighed and monitored daily. Mice were euthanized if they lost > 20% of their body weight. In a separate group, the kidneys were harvested 2 days after infection. The left kidneys were photographed and homogenized for enumeration of fungal burden. The right kidneys were fixed in 4% paraformaldehyde for histological analysis. The fungal burden was determined by CFU in kidney and spleen homogenates. To assess whether inhibition of Akt-2 represents a potential therapeutic approach, WT mice were first infected with *C. albicans* i.v. at 4 × 10^5 CFU. Twenty-four h later, we treated the mice with A-674563, a selective Akt-1 inhibitor, or 124029 Akt Inhibitor XII, a selective Akt-2 inhibitor, at a dose of 15 mg/kg by i.p injection, and monitored daily for 7 days.

Histopathology of the kidneys

For histopathology analyses, kidneys were fixed in 4% paraformaldehyde for up to 48 h, and then embedded into paraffin and sectioned. 4 μm thick sections were stained with Hematoxylin & Eosin (H&E) staining and Periodic Acid-Schiff (PAS) staining.

Data analysis and statistical analysis

Statistical significance was calculated by the using Student's t test. Survival curves were plotted using the Kaplan-Meyer log rank test. A *P* value of 0.05 or less was considered significant. No animals were excluded from the analysis. Mice were allocated to experimental groups based upon their genotypes and randomized within their sex and age matched groups. No statistical method was used to predetermine sample size. It was assumed that normal variance occurs between experimental groups.

Results

Mice deficient for Akt-2 but not Akt-1 are protected from systemic *C. albicans* infection via a myeloid cell-dependent manner

Previously, we have demonstrated that Akt-1 and Akt-2 isoforms differentially regulate the proliferation of tTregs, and the susceptibility of mice to experimental autoimmune encephalomyelitis (EAE) (17). The role of these Akt isoforms in innate immunity, particularly in anti-fungal innate immunity, is completely undefined. To determine whether Akt-1 and Akt-2 isoforms also function differently in anti-fungal innate immunity, we chose

disseminated candidiasis as a model. To this end, WT mice, and mice lacking Akt-1 or Akt-2, were infected with a lethal dose of *C. albicans* (4×10^5 CFU) by tail vein injection to monitor survival rate, and with a sublethal dose (2.5×10^5 CFU) to measure serum cytokines and fungal burden. While all WT and $Akt1^{-/-}$ mice died within 3–5 days after infection, 40% of $Akt2^{-/-}$ mice survived to 7 days after infection (Fig. 1A). The improved survival rate observed in $Akt2^{-/-}$ mice correlated with lower kidney and spleen fungal burdens, less inflammatory cell infiltration, and fewer *C. albicans* hyphae in the kidneys, (Fig. 1B and C). We did not observe any significant alteration of survival and fungal burden in $Akt1^{-/-}$ mice relative to WT mice (Fig. 1A and B). Surprisingly, serum TNF-α and IL-6 levels were significantly lower in $Akt2^{-/-}$ mice than in WT and $Akt1^{-/-}$ mice upon *C. albicans* infection (Fig. 1D). There was no difference in IL-1β among the serum of WT, $Akt1^{-/-}$, and $Akt2^{-/-}$ mice (Fig. 1D), suggesting that Akt-2 is not involved in the non-canonical inflammasome activation mediated by Dectin-1 and/or other Dectin receptors. We therefore focused our studies on the Akt-2 isoform.

To determine whether innate immune cells lacking Akt-2 confer the protection to *C. albicans* infection, we crossed $Akt2^{-/-}$ mice to a $Rag1^{-/-}$ background to generate $Rag1^{-/-}Akt2^{-/-}$ mice which lack T and B cells. Indeed, $Rag1^{-/-}Akt2^{-/-}$ mice were also protected from a lethal dose of *C. albicans* (Fig. 1E). To further confirm whether Akt-2 deficiency in myeloid cells is responsible for the protection, we crossed *LysM Cre* mice to $Akt2^{f/f}$ mice to generate *LysM Cre*-$Akt2^{f/f}$ mice which delete Akt-2 in myeloid cell lineage (thereafter, $Akt2^{\Delta M}$). Consistent with the data shown in $Rag1^{-/-}Akt2^{-/-}$ mice, $Akt2^{\Delta M}$ mice were also protected from a lethal dose of *C. albicans* (Fig. 1F). Therefore, our data collectively indicate that Akt-2 expression in myeloid cells inhibits anti-fungal innate immune responses.

Akt-2 inhibits the fungal killing activities of neutrophils and macrophages

Recognition and uptake of fungal pathogens by professional phagocytes, such as neutrophils and macrophages, induces pro-inflammatory responses and the production of ROS, which are essential in controlling fungal infections by phagocytosis and killing mechanisms (6, 25, 26). ROS plays an important role in the initial step of fungal killing in the phagosomes (27). To define the cellular mechanism(s) by which Akt-2 deficiency protects mice from systemic *C. albicans* infection, we quantified the recruitment of monocytes, neutrophils, macrophages, and dendritic cells (DCs) to spleens and kidneys in response to infection with a sub-lethal dose of *C. albicans.* The populations of monocytes, macrophages and neutrophils were significantly increased in the spleens but significantly decreased in the kidneys in $Akt2^{-/-}$ mice relative to those in WT and $Akt1^{-/-}$ mice following *C. albicans* infection (Fig. 2A and B and Supplemental Fig. 1). The reduced neutrophils, monocytes, and macrophages in the $Akt2^{-/-}$ kidneys correlated with fewer inflammatory cells in the kidneys (Fig. 1C). These data suggest that *C. albicans* are most likely to be killed prior to their entry to the kidneys by phagocytes that lack Akt-2. To test this idea, we measured ROS expression in neutrophils and macrophages in the spleens and kidneys from mice lacking Akt-1 and Akt-2 upon *C. albicans* infection. We found that ROS expression was significantly increased in neutrophils and macrophages from spleens of mice that lack Akt-2 (Fig. 2C). ROS expression was also higher in neutrophils in the kidneys (Fig. 2D). To further define the cellular mechanism, we performed an in vitro fungal killing assay by co-culturing BMNs

and BMDMs with *C. albicans* hyphae and *C. albicans* yeast (cells of the *cap1* mutant). The *cap1* mutants are defective in the adenylate-cyclase-associated protein-1, which is required for cAMP generation and yeast-hyphal transition. Therefore, the *cap1* mutant is often used as *C. albicans* yeast in vitro (18). Neutrophils and macrophages from $Akt2^{-/-}$ mice displayed augmented fungicidal activity (Fig. 2E).

Akt-2 facilitates inflammatory responses against *C. albicans* infection

To gain more insight into the cellular basis of the observed protective effect, we assessed cytokine production by BMNs and BMDMs from WT, $Akt1^{-/-}$, and $Akt2^{-/-}$ mice. The BMNs and BMDMs were infected in vitro with *C. albicans* yeast mutant and hyphae, respectively, for 2, 4, and 6 h. The production of TNF-α and IL-6 in the culture supernatants were measured by ELISA. Although loss of Akt-1 did not impair the production of either TNF-α or IL-6, loss of Akt-2 in neutrophils and macrophages resulted in defective production of both TNF-α and IL-6 upon *C. albicans* yeast and hyphal infections (Fig. 3), suggesting that Akt-2, but not Akt-1, is required for inflammatory responses. These data are consistent with the serum cytokine data shown in Figure 1D. Taken together, although loss of Akt-2 leads to an impaired inflammatory response against *C. albicans* infection, it significantly enhances fungal killing activities.

Akt-2 inhibits MPO activation and NETosis

Clearance of *C. albicans* by the hosts not only depends on ROS but also to a large extent on myeloperoxidase (MPO) activity (28). We therefore measured MPO activity using an MPO colorimetric activity assay kit. Our new data showed that MPO activity was significantly augmented in neutrophils lacking Akt-2 upon *C. albicans* yeast and hyphal infections (Fig. 4A).

Neutrophil extracellular traps (NETs) are networks of extracellular fibers, primarily composed of DNA from neutrophils, which bind pathogens. NET activation and release, or NETosis, is a dynamic process that can come in two forms, suicidal and vital NETosis (29). Neutrophils undergo a rapid NETosis in response to *C. albicans* mediated by the β2 integrin, complement receptor 3 (CR3) which is also essential for containment and clearance of a complex infection involving both yeast and hyphal forms (30). To assess the effect of Akt-2 in NETosis, we infected *LysM Cre* and $Akt2^{\Delta M}$ BMNs with *C. albicans* yeasts and hyphae, respectively. The NETosis was determined by a NETosis assay kit. We found that BMNs lacking Akt-2 displayed increased levels of NETosis upon infection with *C. albicans* yeasts and hyphae (Fig. 4B).

Loss of Akt-2 enhances the expression of CXCR2 in splenic neutrophils and macrophages

Immune cell recruitment to sites of infection or tissue injury plays a crucial role for the inflammatory response (31). Chemokine-chemokine receptor interaction in immune cells is essential for integrin activation and leukocyte recruitment. CXCR2 is a prominent chemokine receptor on neutrophils (32, 33), and to a lesser degree macrophage (34). The binding of CXCR2 to its ligands CXCL1 and CXCL2 regulates neutrophil mobilization from the bone marrow to the blood and promotes the migration of neutrophils into the inflammatory sites (35). To determine the potential cause of increased recruitment of

neutrophils and macrophages to the spleen, we measured the expression of CXCR2 in splenic neutrophils and macrophages. We found that the expression levels of CXCR2 in splenic neutrophils and macrophages of *C. albicans*-infected *Akt2*$^{-/-}$ mice were significantly higher than those in *C. albicans*-infected WT or *Akt1*$^{-/-}$ mice (Fig. 5A-D).

To determine whether CXCL1, the ligand for CXCR2, is also increased in spleens and kidneys, we infected WT and *Akt2*$^{-/-}$ mice with *C. albicans*. At day 2, mice were sacrificed, and the expression of CXCL1 in the homogenates of spleens and kidneys was detected by ELISA. We found that CXCL1 expression was significantly increased in the spleens lacking Akt-2, but there was no difference in its expression in the kidneys of WT and *Akt2*$^{-/-}$ mice post *C. albicans* infection (Fig. 5E). These data suggest that increased interaction between CXCR2/CXCL1 in neutrophils and macrophages in the absence of Akt-2 may facilitate their recruitment to the spleen for *C. albicans* clearance.

Akt-1 and Akt-2 differentially regulate the pathways leading to the activation of ROS and inflammatory cytokines.

Recognition of pathogen-associated molecular patterns (PAMPs) such as β-glucans, α-mannans, and chitin in fungal cell walls by pattern recognition receptors (PRRs), including CLRs, results in the release of inflammatory cytokines from innate immune cells, which is critical for anti-fungal immunity (5, 12). ROS generated by NADPH oxidase plays an important role in antimicrobial host defense (36–38). NADPH oxidase activation depends on the assembly of multi-subunit complexes consisting gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox}, p40^{phox}, and Rac-2 (37, 38). p40^{Phox} is complexed to p67^{Phox} in resting cells and is translocated to the membrane during activation of the NADPH oxidase (38). Translocation of p40^{Phox} appears to be dependent on p47^{Phox} and is mediated by p67^{Phox} (37). We performed an extensive search for potential phospho-Akt substrate motif (RXRXXS*/T*) within the NADPH oxidase complex. We identified a very conserved RLRPRT motif at position 148–154 among human, chimpanzee, bison, rat, rabbit, and mouse (Supplemental Fig. 2), suggesting that this site is potentially phosphorylated by Akt isoforms. Previously, it has been shown that phosphorylation of p40^{Phox} at T154 by PKC (39, 40) is an important physiological signal for activation of the neutrophil NADPH oxidase (40, 41). However, using a cell-free system it was reported that phosphorylation of p40^{Phox} at T154 by PKC inhibits NADPH oxidase activity (42). It is unknown whether Akt isoforms also phosphorylate p40^{Phox} at T154, and whether Akt-mediated phosphorylation of p40^{Phox} at T154 exerts a negative effect on NADPH oxidase activation. Foxo1 is a well-known substrate of Akt (43) and is needed for the expression of CXCR2 and neutrophil trafficking (44).

We therefore focused on the signaling molecules that affect inflammatory responses: MAPKs (ERK, p38, and JNK) and NF-κB, CXCR2 expression (Foxo1), and ROS activity (p40^{Phox}). To this end, BMNs of WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice were infected with *C. albicans* yeast and hyphae, respectively, for various time-points and lysed. The cell lysates were blotted with phospho-antibodies against ERK, p38, JNK, NF-κB p65, Foxo1, and p40^{Phox}. Interestingly, BMNs lacking Akt-2, but not Akt-1, displayed defective phosphorylation of ERK, p38, JNK, NF-κB, p65, Foxo1, and p40^{Phox} upon infection with *C.*

albicans yeast and hyphae, respectively (Fig. 6A and B). In support of this finding, phospho-p40^{Phox} was also observed in BMNs lacking Akt-2 upon curdlan stimulation (Fig. 6C). Our data suggest that defective activation of MAPKs (ERK, p38, and JNK) in neutrophils lacking Akt-2 may be responsible for the impaired inflammatory response, whereas impaired phosphorylation of Foxo1 allows Foxo1 to remain in the nucleus to upregulate CXCR2 which is essential for the recruitment of neutrophils, and to a lesser content, macrophages, to the spleen. To confirm whether Foxo1 expression is increased in BMNs lacking Akt-2, we infected WT and *Akt2*$^{-/-}$ mice with *C. albicans* yeasts and hyphae, and analyzed cytosolic and nuclear fractions for Foxo1 expression by immunoblotting. Our data showed that BMNs lacking Akt-2 displayed increased nuclear expression of Foxo1 (Fig. 6D). Consistent with these data, CXCR2 mRNA expression was also augmented in BMNs deficient for Akt-2 (Supplemental Fig. 3). In addition, failure to induce phosphorylation of p40^{Phox} at T154 may lead to enhanced NADPH oxidase activity and augmented ROS production. Therefore, Akt-2, but not Akt-1, is the major isoform in neutrophils and (possibly macrophages) that facilitates the phosphorylation of MAPKs, NF-κB, Foxo1, and p40^{Phox}, thus differentially regulating inflammatory responses, CXCR2 expression, and ROS activity, the latter two being crucial for anti-fungal innate responses against systemic *C. albicans* infection.

Targeting Akt-2 as a potential therapeutic approach for disseminated candidasis

The above data strongly indicate that inactivation of Akt-2 may represent a potential therapeutic approach for disseminated candidiasis. To address this, we treated WT mice with specific inhibitors for Akt-1 and Akt-2 at the time of *C. albicans* infection, and monitored the survival rates. Indeed, treating WT mice with the Akt-2 inhibitor, but not Akt-1 inhibitor, significantly protected WT mice from the lethal systemic *C. albicans* infection (Fig. 7).

Discussion

Akt has been shown to play a key role in multiple cellular processes including but not limited to cell proliferation, cell trafficking, cell death, transcription, and glucose metabolism (14). There are three isoforms of Akt, Akt-1, Akt-2, and Akt-3 that are conserved in mammalian genomes and each has cell- and tissue-specific functions (14). Previously, we have demonstrated that Akt-1 and Akt-2 have differential functions in the development of iTregs and tTreg proliferation (16, 17). Akt-1 and Akt-2 have been shown to differentially regulate M1/M2 macrophage polarization (45, 46) and loss of Akt-2 protects mice from acute lung injury via the M2 macrophage phenotype (46). However, Akt-2 is essential for host defense against *S. typhimurium* infection (47). In this report, we first show that Akt-2, but not Akt-1, inhibits essential inflammatory responses against *C. albicans* infection. We demonstrate that Akt-2, but not Akt-1, attenuates neutrophil and macrophage recruitment to spleens, and limits the production of ROS in neutrophils and macrophages, and hinders fungal killing.

It is well documented that both PI3K/Akt and Ras-ERK regulate cell proliferation and survival. Cross-talk between the two pathways has also been reported (48, 49). However, it is unclear whether Akt isoforms differentially regulates NF-κB and MAPK signaling. In

addition, it has been shown that in T cells Akt can phosphorylate CARD11, a homolog of CARD9, thus facilitating the assembly of CARD11/Bcl-10/MALT1 complex and subsequent activation of NF-κB (50). Indeed, Akt-1 has been shown to be essential for the activation of NF-κB in primary chicken embryonic fibroblasts overexpressing a constitutive active form of Akt-1 or dominant negative form of Akt-1 (51). In this study, we found that the activation of ERK, JNK, and p38 MAPK, as well as NF-κB, is dependent on Akt-2, but not Akt-1, in neutrophils upon infection with *C. albicans* yeasts and hyphae (Fig. 6), which correlates with the defective production of TNF-α and IL-6 by these cells in culture (Fig. 3). Consistent with this data, lower levels of serum TNF-α and IL-6 were observed in mice lacking Akt-2, but not Akt-1, infected with *C. albicans* (Fig. 1D). Therefore, our data collectively indicate that Akt-2 is essential for anti-fungal inflammatory responses downstream of Dectin CLRs.

Although impaired inflammatory responses are observed in $Akt2^{-/-}$ mice, these mice are protected from lethal systemic *C. albicans* infection (Fig. 1). We found that loss of Akt-2 facilitates the recruitment of neutrophils, and to a lesser extent macrophages, to the spleen (Fig. 2), which is the most important lymphoid organ responsible for immune surveillance against bloodstream infections. We found that CXCR2, a major chemokine receptor that facilitates neutrophil trafficking into inflammatory sites via binding to its ligands CXCL1 and CXCL2 (35), is significantly increased in neutrophils, and to a lesser extent macrophages, in the spleen (Fig. 5A-D). Indeed, CXCL1 expression is increased significantly in spleens but not kidneys (Fig. 5E). Foxo1, a well-known substrate of Akt (43), is required for the expression CXCR2 and neutrophil trafficking (44). Foxo1 phosphorylation at Thr^{24} and Ser^{319} by Akt is critical for its nuclear exclusion, and prevent activation of its target genes, including CXCR2, in the nucleus (43, 52–54). We also confirmed that the failure to induce Foxo1 phosphorylation in $Akt2^{-/-}$ neutrophils upon infection with *C. albicans* yeasts or hyphae results in elevated CXCR2 transcription (Supplemental Fig. 3). Up-regulation of CXCR2 provides a reasonable explanation for the enhanced recruitments of neutrophils and macrophages to the spleen and strengthened fungal clearance within the spleen. Our results strongly support the notion that Akt-2 is a bona fide Akt isoform that phosphorylates Foxo1.

Loss of Akt-2, but not Akt-1, potentiates ROS activation in kidney (Fig. 2C). ROS activation is regulated by NADPH oxidase which consists of a complex between $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac-2. We identified a very conserved RLRPRT motif at position 148–154 in $p40^{phox}$ among humans and a range of other mammalian species (Supplemental Fig. 2). Defective phosphorylation of $p40^{phox}$ in the absence of Akt-2 in neutrophils upon infection with *C. albicans* yeasts or hyphae may impair NADPH oxidase activation. However, the role of $p40^{phox}$ in the control of ROS expression is still controversial since neutrophils lacking $p40^{phox}$ display defective expression of ROS (36, 40), although a cell-free system study suggested that $p40^{phox}$ negatively regulates ROS expression (42). Further studies are needed to confirm whether $p40^{phox}$ potentiates or inhibits ROS expression/activation using a *C. albicans* infection model.

The current limitation for anti-fungal treatment is the resistance to anti-fungal agents, and this contributes to high morbidity and mortality associated with invasive candidiasis (5). Our data showed that mice deficient for Akt-2 are protected from disseminated candidiasis,

suggesting that inhibition of Akt-2 could be used as a potential therapeutic approach for disseminated candidiasis. Indeed, inhibition of Akt-2, but not Akt-1, in mice receiving systemic *C. albicans* infection protects these mice from lethality. The advantage for the inhibition of Akt-2 as a therapeutic approach is the lack of hyper-systemic inflammation which may contributes to higher mortality. Therefore, our data strongly indicate that targeting Akt-2 may represent a potential therapeutic approach for disseminated candidiasis.

In summary, in this study we showed that Akt-2 and Akt-1 function differently during systemic *C. albicans* infection, and that Akt-2, but not Akt-1, deficiency in myeloid cells, in particular neutrophils, confers protection to invasive *C. albicans* infection. Furthermore we showed that Akt-2, but not Akt-1, is the major Akt isoform that phosphorylates Foxo1 and $p40^{Phox}$ (at T154), that possibly negatively regulate CXCR2 expression and ROS activity. In addition, Akt-2 also inhibits MPO activity and NETosis during *C. albicans* infection. Therefore, targeting Akt-2 may be a potential therapeutic approach for disseminated candidiasis without causing a hyper-inflammatory response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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KEY POINTS

- Akt-2 but not Akt-1 inhibits anti-fungal innate immune responses.
- Akt-2 suppresses fungal killing in neutrophils and macrophages.
- Targeting Akt-2 may represent a therapeutic approach for disseminated candidiasis.

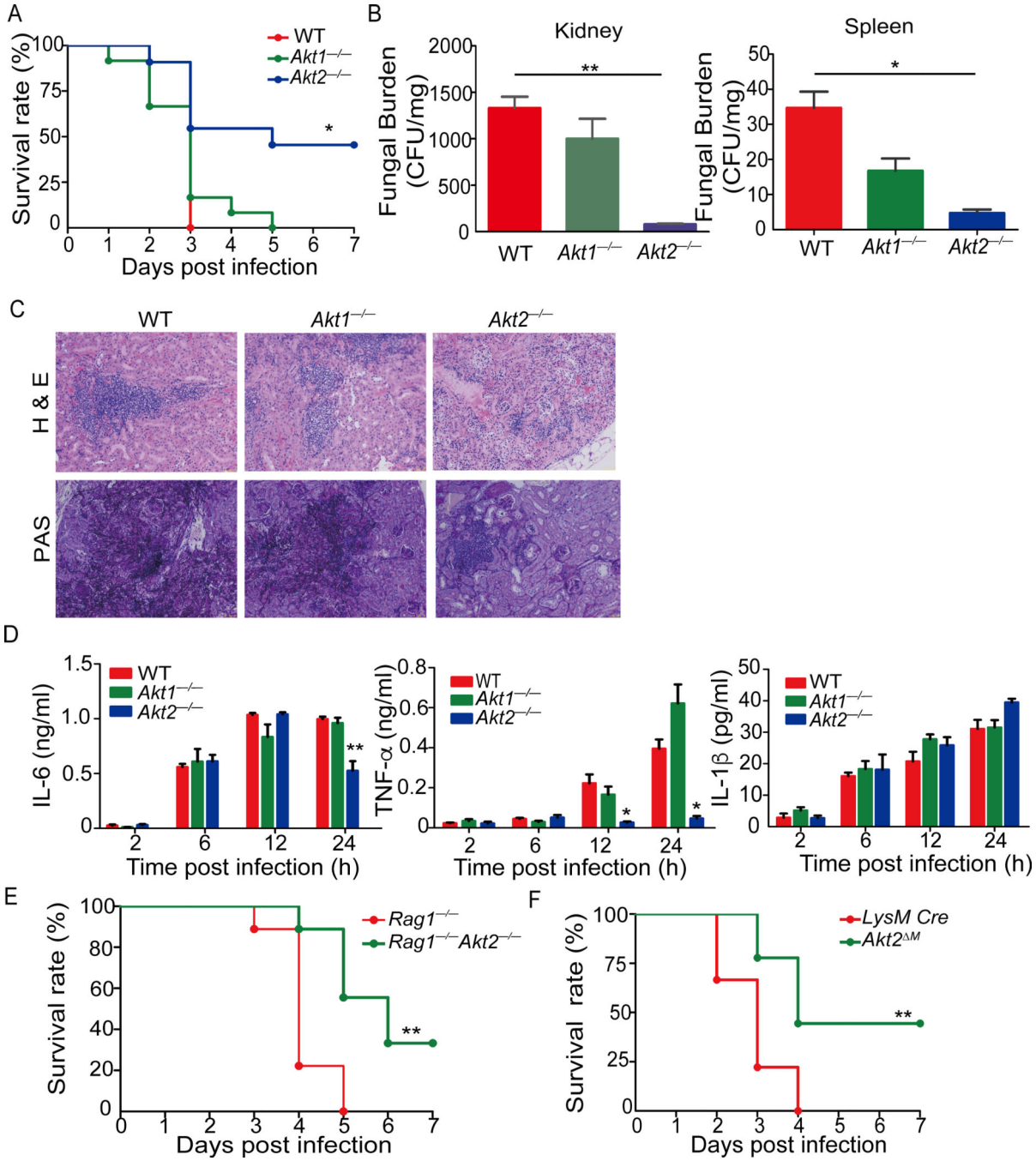

Figure 1. Akt-2 deficiency in myeloid cells protects mice from lethal systemic *C. albicans* infection

(A) Kaplan–Meier survival curve of WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice (n = 11–12 per group) after infection with 4 × 10 ^{5} CFU of *C. albicans* (SC5314). Data are representative of three independent experiments. WT mice vs *Akt2*$^{-/-}$mice. *p < 0.05, by log-rank test. (B) Fungal burden in paired kidneys (left) and spleens (right) of WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice (n = 10 per group) at day 2 after infection with 2.5 × 10^{5} CFU of *C. albicans*. Data are representative of three independent experiments. *p < 0.05; **p < 0.01; by unpaired two-tailed Student's t-test. (C) Kidney histopathology analysis by H&E staining (top) and PAS staining to

visualize fungal burden (hyphae) (bottom) (n = 5 mice per group). Images are representative of two independent experiments (biological replicates). Scale bars, 50 μm. (D) Serum IL-6, TNF-α, and IL-1β production in WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice infected with 2.5× 10^5 CFU of *C. albicans* at 2, 6, 12, and 24 h after infection (n = 10 mice per group; each with three repeated wells). Data are representative of three independent experiments (biological replicates). Error bars are mean ± s.d., *p < 0.05, ** p < 0.01, by Student t test. (E) Survival rate of *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Akt2*$^{-/-}$ mice infected with 4 × 10^5 CFU of *C. albicans* (n = 9 mice per group). Data are representative of three independent experiments (biological replicates). **p < 0.01; by log-rank test. (F) Survival analysis of *Akt2*$^{f/f}$ and *Akt2*$^{\Delta M}$ mice infected with *C. albicans* (4 × 10^5 CFU) by i.v. injection (n = 9 mice per group). Data are representative of three independent experiments (biological replicates). **p < 0.01, by log-rank test.

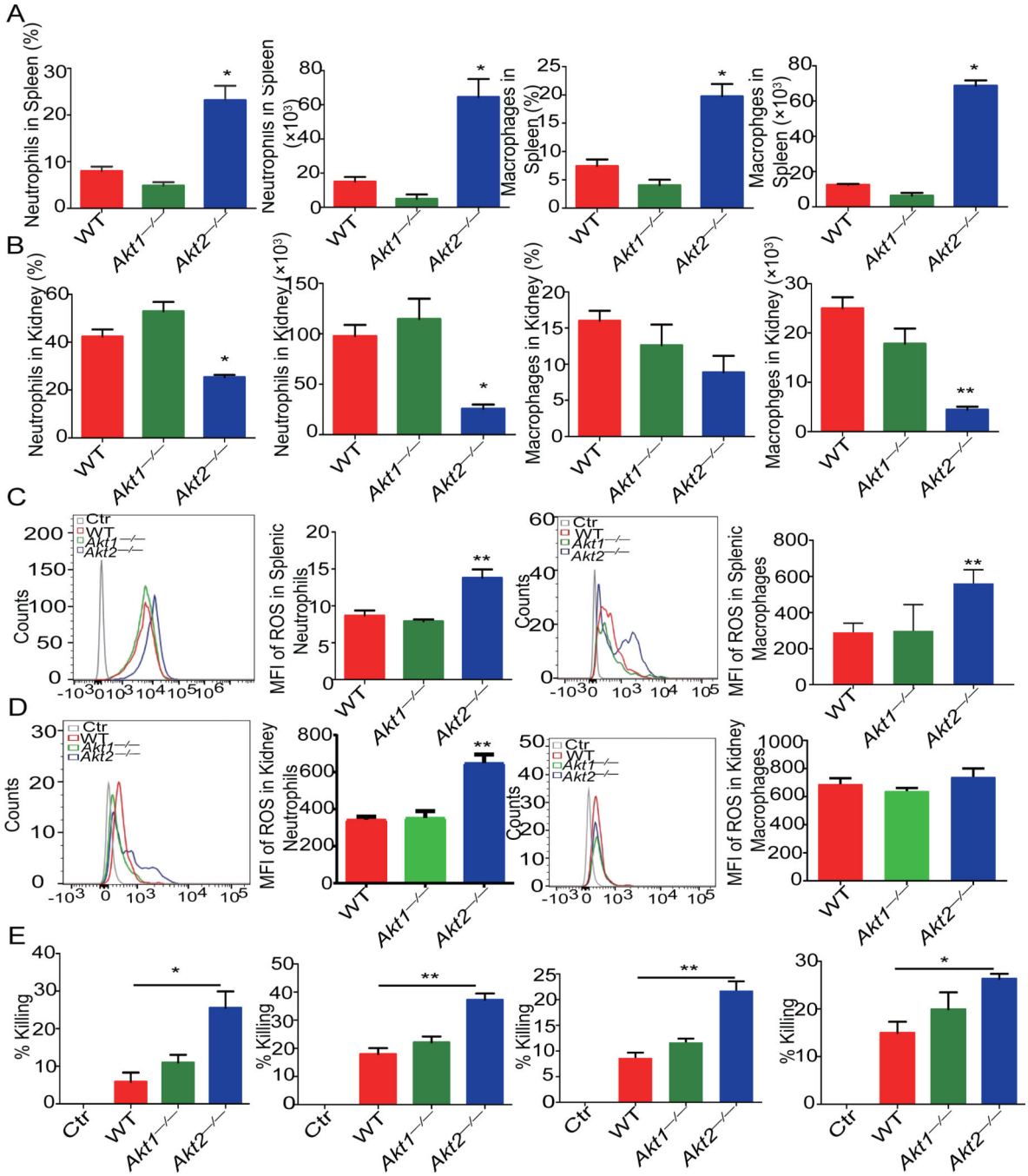

Figure 2. Akt-2 inhibits neutrophil and macrophage recruitment to spleens and their expression of ROS and fungal killing

(A) Percentages and absolute numbers of neutrophils (left) and macrophages (right) in the spleens from WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice (n = 5 mice per group) at 48 h after infection with *C. albicans* (2.5×10^5 CFU). Data are shown as means ± s.d., *p < 0.05, by Student's t test. (B) Percentages and absolute numbers of neutrophils (left) and macrophages (right) in the kidneys from WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice (n = 5 mice per group) at 48 h after infection with *C. albicans* (2.5×10^5 CFU). Data are shown as means ± s.d., *p < 0.05, ** p < 0.01, by Student's t test. (C and D) ROS staining in BMNs (C) and BMDMs (D) infected

with 2.5×10^5 CFU at day 2 after infection. Data are shown as means ± s.d. ** $P < 0.01$, by Student's t test. (E) Fungal killing capacity of WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ BMNs (left panel) and BMDMs (right panel) as assessed by 24 h co-culture with *C. albicans* yeast or hyphae (MOI = 1:100). Data are shown as means ±s.d., *$p < 0.05$, **$p < 0.01$, by Student test.

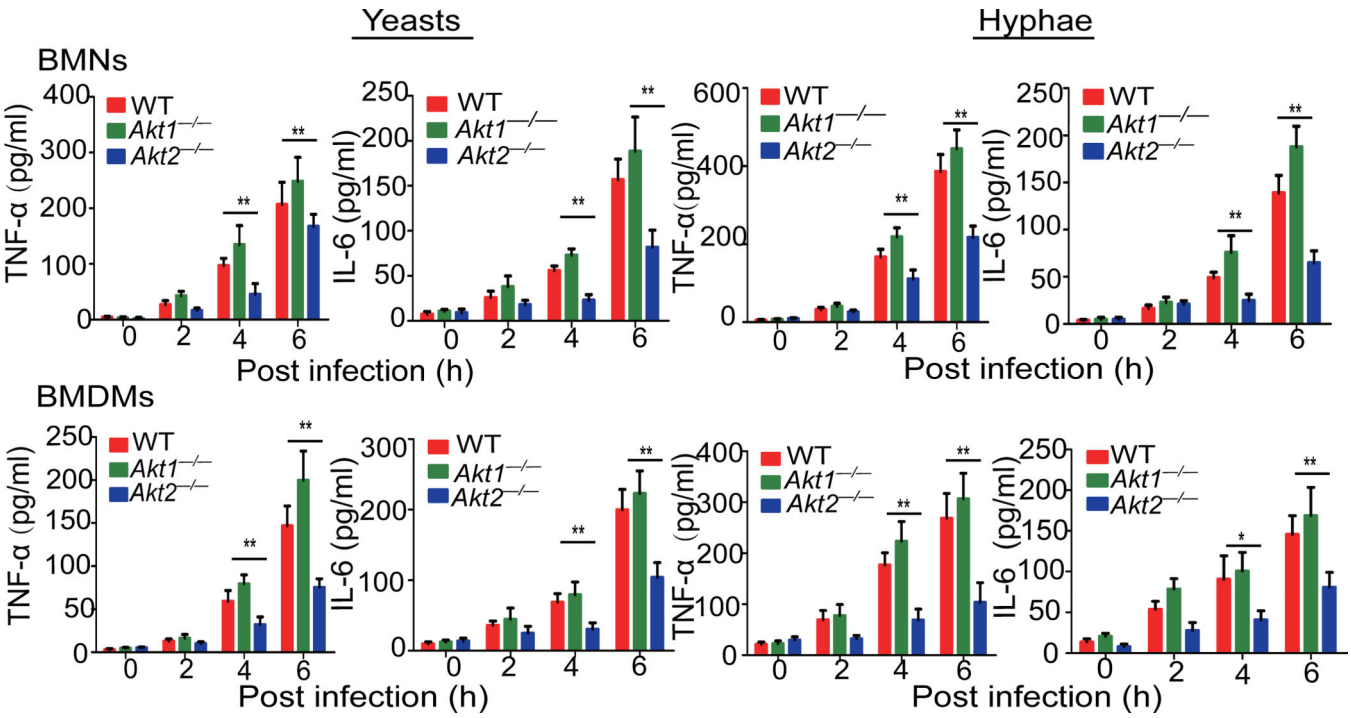

Figure 3. Akt-2 facilitates inflammatory responses against *C. albicans* infection

ELISA of TNF-α and IL-6 production in the supernatants collected from BMNs or BMDMs of WT *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice infected with *C. albicans* yeast and hyphae forms (MOI = 1:1) for 0, 2, 4, and 6 h. Data are representative of three experiments. Data are shown as means ±s.d., *$p < 0.05$, **$p < 0.01$, by Student test.

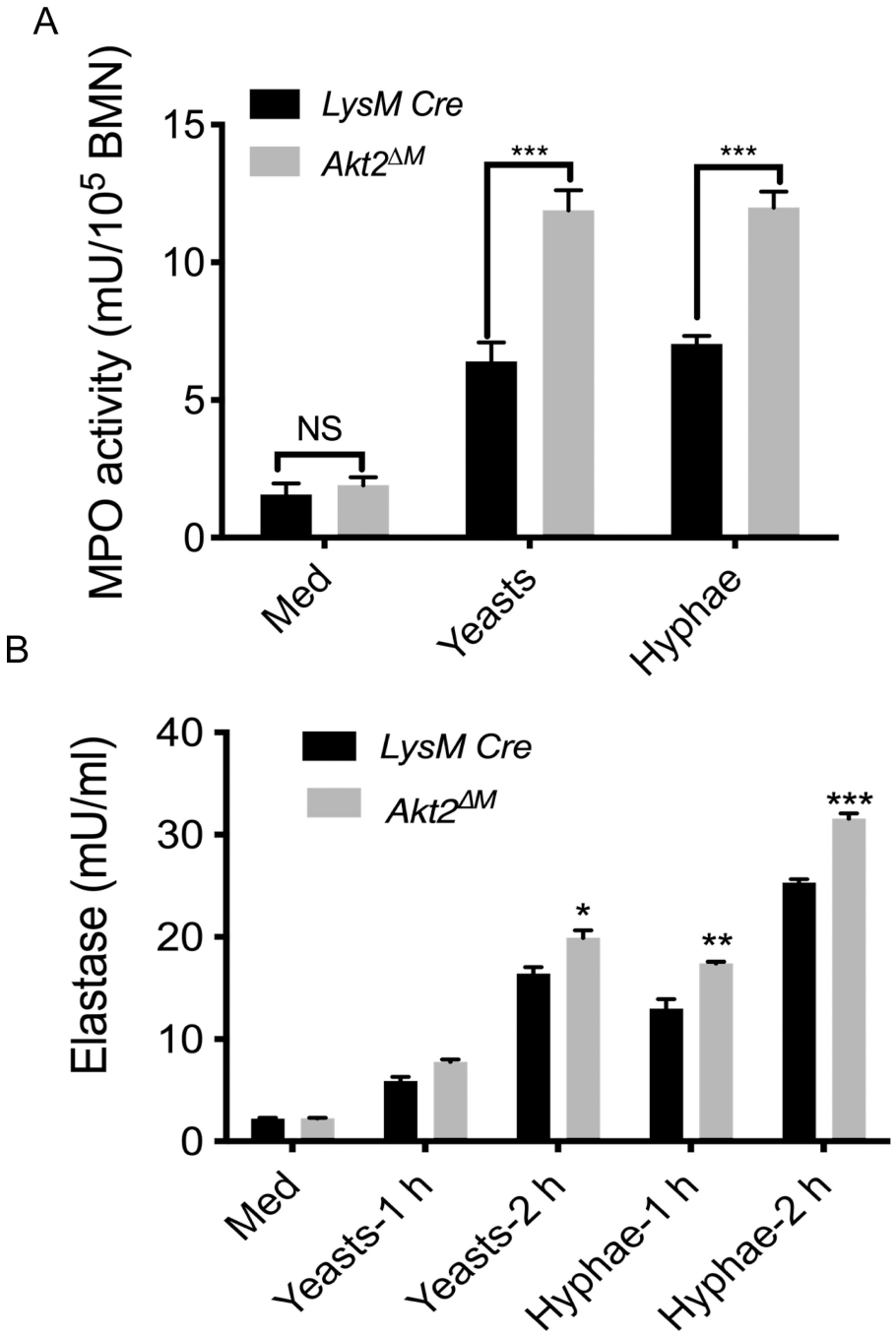

Figure 4. Loss of Akt-2 facilitates MPO activation and NETosis in BMNs upon *C. albicans* yeast and hyphal infections

(A) BMNs (1×10^5) from *LysM Cre* and *Akt2*$^{\Delta M}$ mice were infected with *C. albicans* yeasts (MOI:1:1) and hyphae (MOI:1:1) for 1 h, and lysed. The MPO activity in BMN lysates of *LysM Cre* and *Akt2*$^{\Delta M}$ mice were measured by an MPO colorimetric activity assay kit. (B) BMNs (2×10^6) from *LysM Cre* and *Akt2*$^{\Delta M}$ mice were infected with *C. albicans* yeasts (MOI:1:1) and hyphae (MOI:1:1) for 1 h and 2 h. The NETosis in BMNs *LysM Cre* and *Akt2*$^{\Delta M}$ mice was detected using a NETosis assay kit.

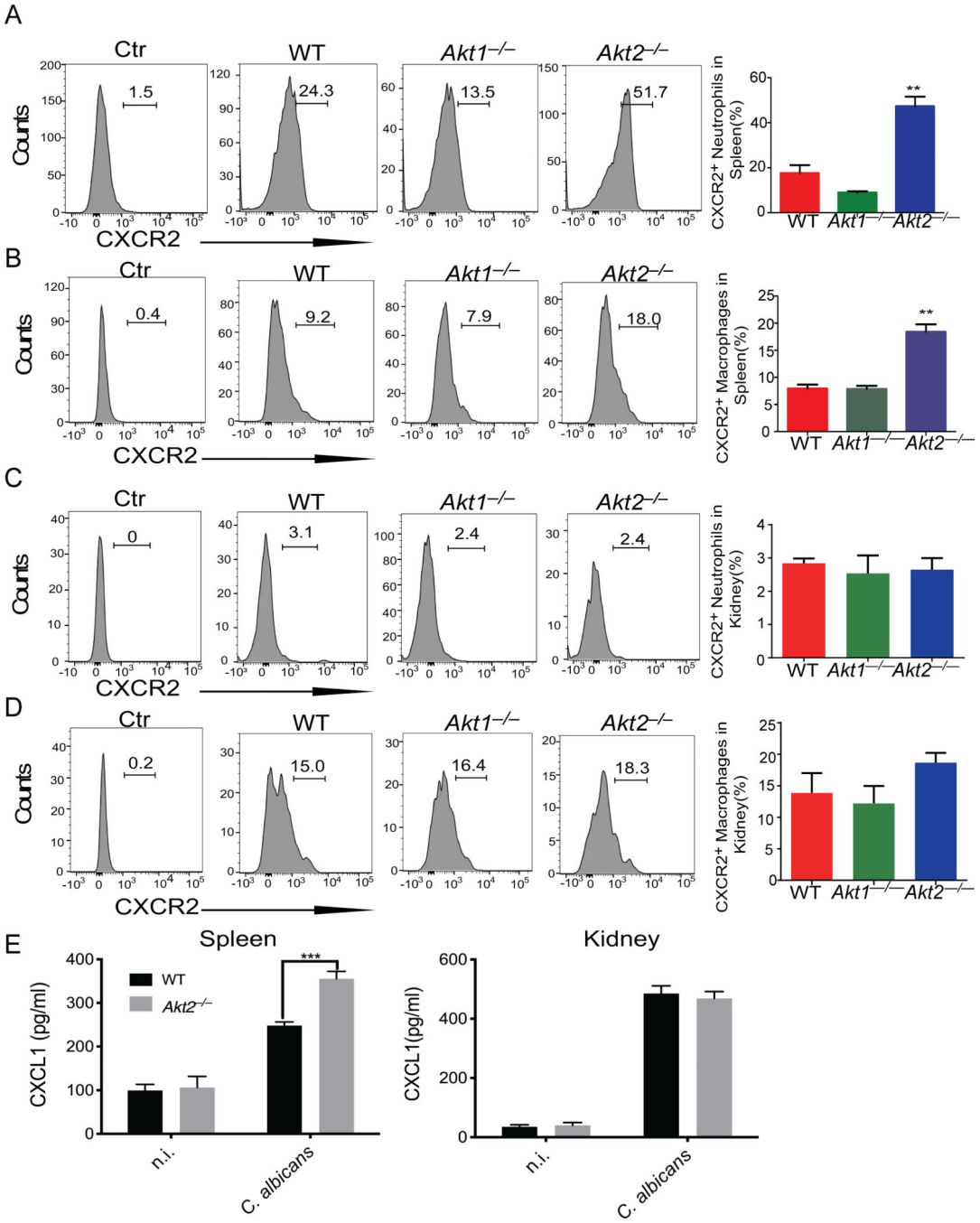

Figure 5. Enhanced expression of CXCR2 on neutrophils and macrophages lacking Akt-2 in spleens

(A-D) Expression of CXCR2 on neutrophils and macrophages were determined in spleens and kidneys from WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice using flow cytometry at day 2 after infection with 2.5 × 10 ^{5} CFU of *C. albicans*. (n = 5 mice per group; each with three repeated wells). (E) CXCL1 expression in spleens and kidneys of WT and *Akt2*$^{-/-}$ mice (n = 5 mice per group) were determined by ELISA. Data are shown as means ± s.d. and analyzed using the student's t test. **p < 0.01; *** p < 0.001. n.i., no infection.

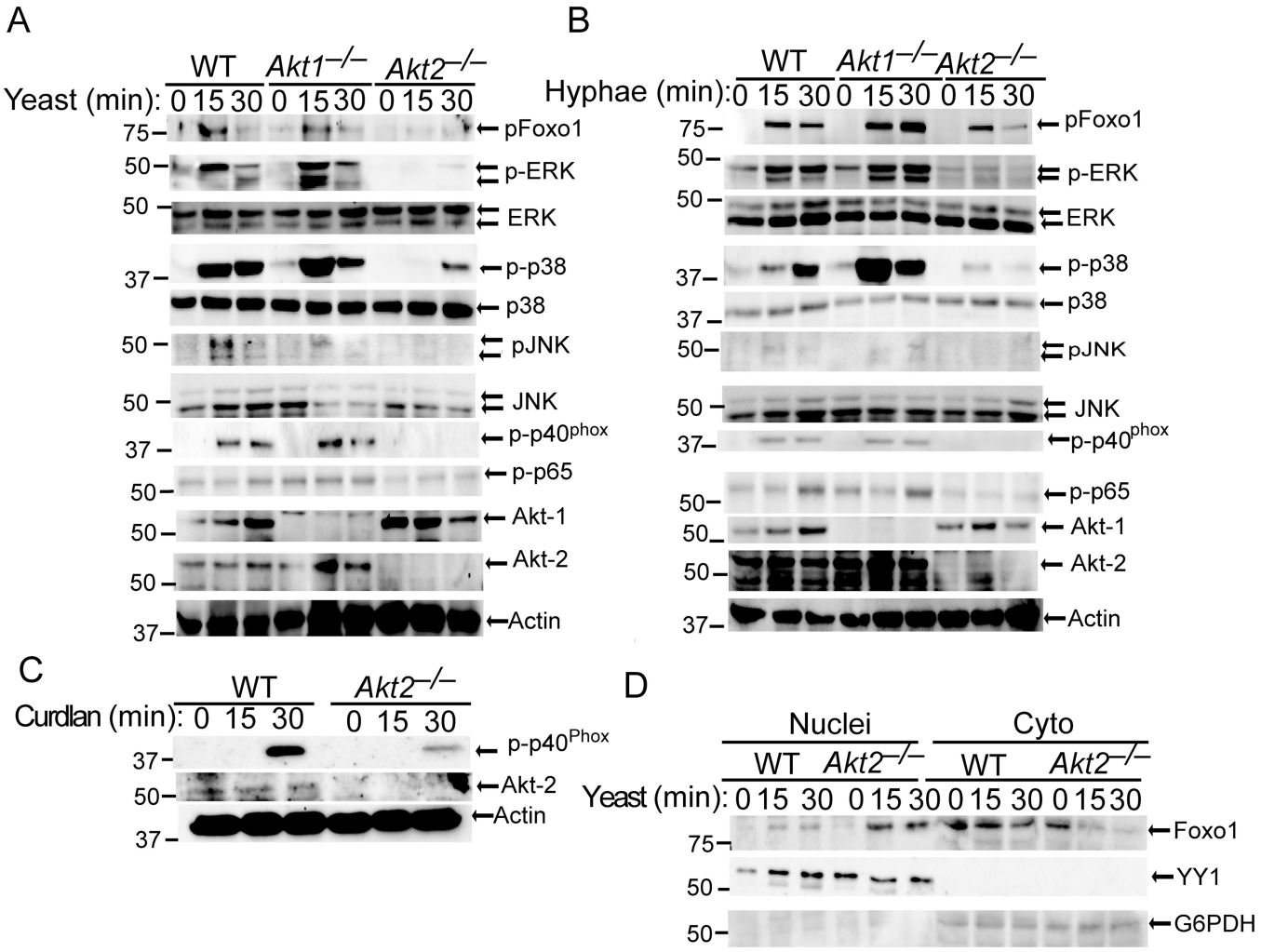

Figure 6. Loss of Akt-2 but not Akt-1 impairs MAPK and Foxo activation in neutrophils upon *C. albicans* yeast and hyphal infections

(A) BMNs from WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice infected with *C.albicans* yeast and hyphae, respectively, and lysed in 1% Triton X-100 buffer. The cell lysates were Immunoblotted with phospho-antibodies against Foxo1, ERK, JNK, p38, NF-κB p65, and p40^{phox}, and reprobed with anti-Akt-1, anti-Akt-2, and anti-actin. (B) BMNs of WT and *Akt2*$^{-/-}$ mice were stimulated with curdlan for 15 and 30 min, and lysed. The cell lysates were blotted with anti-phospho-p40^{phox}, and reprobed with anti-Akt-2 and anti-actin, respectively. (C) Cytosolic and nuclear fractions of BMNs of WT and *Akt2*$^{-/-}$ mice infected with *C.albicans* yeast were blotted with anti-Foxo1. The purity of cytosolic vs. nuclear fraction was determined by immunoblotting with anti-G6PDH (for detection of cytosolic protein) and anti-YY1 (for detection of nuclear protein). Images are representative of three independent experiments (biological replicates).

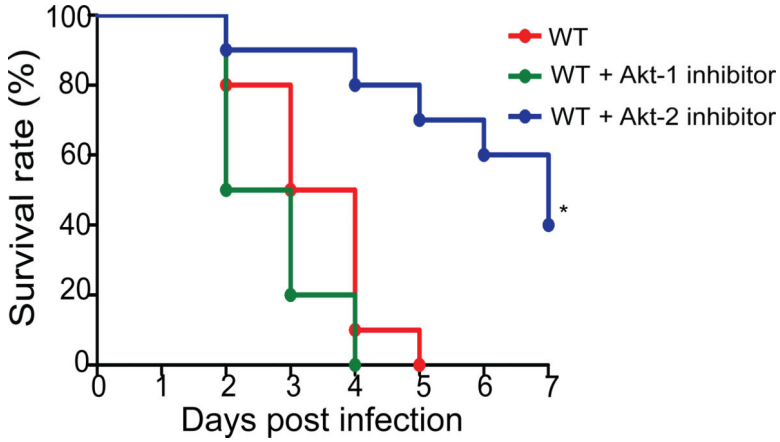

Figure 7. Akt-2 inhibitor protects WT mice from systemic *C. albicans* infection

Survival of C57BL/6 mice treated with A-674563, a selective Akt-1 inhibitor, or 124029 Akt Inhibitor XII, a selective Akt-2 inhibitor (15 mg per kg body weight per mouse) by i.p. injection 24 h after infection with *C. albicans* (4×10^5 CFU) (n =10 mice per group). Data are representative of three independent experiments (biological replicates). **p < 0.01 by log-rank test.