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TSC1 Suppresses Macrophage Necroptosis for the Control of Infection by Fungal Pathogen Candida albicans

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ABSTRACT

Candida albicans is the most common, opportunistic human fungal pathogen whose complex interplay with the host innate immune system remains incompletely understood. In this study, we revealed that infection macrophages with C. albicans triggers prominent cell death, which is largely attributed to the RIPK3/MLKL–mediated necroptosis. Our results further demonstrated that the TSC1-mTOR pathway plays a pivotal role in the control of macrophage necroptosis upon engaging the Dectin-1/2 and TLR-2/4 pathways through fungal components β-glucan/α-mannan or Sel1, respectively. Notably, the rapamycin-sensitive mTORC1 pathway, rather than the rapamycin-insensitive mTORC2 pathway, was responsible for elevated activation of RIPK1, RIPK3, and MLKL in TSC1-deficient macrophages. Following systemic infection with C. albicans, mice with macrophage/neutrophil–specific deletion of Tsc1 (Tsc1<sup>M/N</sup>−/−) showed heightened fungal burden in multiple organs, such as the kidney, liver, and spleen, severe morbidity, and mortality. Notably, Tsc1<sup>M/N</sup>−/− kidneys exhibited prominent cell death and concomitant loss of tissue-resident macrophages, which likely contributing to a dampened phagocytosis of fungal pathogens. Together, our data demonstrate a crucial role for the TSC1-mTOR pathway in the regulation of macrophage necroptosis and suggest that both Dectin- and TLRs-induced necroptosis may undermine the immune defense effector functions of these innate receptors during C. albicans infection. ImmunoHorizons, 2021, 5:90–101.

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; HKCA-Y, heat-killed C. albicans yeast; LDH, lactate dehydrogenase; MOI, multiplicity of infection; PAMP, pathogen-associated molecular pattern; PI, propidium iodide; PRR, pattern-recognition receptor; WT, wild-type; ZymD, zymosan depleted.

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INTRODUCTION

Fungal infection has been constantly increasing worldwide, causing invasive candidiasis and mortality (1). Nevertheless, our current understanding on the complex interplay between Candida albicans and the host immune system remains limited, and the antifungal therapy now available is often ineffective. Therefore, a better understanding of the host defense mechanisms against fungal infection is of great importance. The C-type lectin receptors are pattern-recognition receptors (PRRs) designated for the recognition of C. albicans. Among them, dectin-1 and dectin-2/3 mainly recognize the fungal cell wall components β-glucan and α-mannan, respectively (2). In addition, TLRs such as TLR2 and TLR4 can also be involved in sensing fungal infection via recognizing the secreted protein Sel1 or cell wall components phospholipomannan and O-linked mannosyl proteins (3, 4). The ability of C. albicans to upregulate expression of the small, secreted cysteine-rich protein Sel1 upon encountering limited nitrogen and abundant serum enables the induction of TLR2- and TLR4-dependent immune responses in macrophages (4). Engagement of these PRRs results in the activation of NF-kB and MAPKs, which leads to the induction of a myriad of proinflammatory cytokines and chemokines (5). Additional regulators, such as SHP-2, TRAF6, TAK1, as well as Cbl-b have been demonstrated to be critical components of C-type lectin receptor signaling for antifungal defense (5–9). Tissue-resident macrophages are the first responders to C. albicans infection (10), and the survival of macrophages has also been implicated in host defense against C. albicans. Notably, CX3CRI-deficient mice are susceptible to systemic C. albicans infection because of reduced macrophage survival (11). In the meanwhile, C. albicans is capable of triggering caspase-1-dependent pyroptosis to escape macrophage-conducted surveillance (12, 13). However, whether and how C. albicans can induce other types of cell death in macrophages remain to be elucidated.

Necroptosis is a form of regulated necrotic cell death dependent on the activation of RIPK3 and MLKL. Upon phosphorylated by RIPK1, RIPK3 then phosphorylates and activates MLKL, the executor of necroptosis, whereby triggering cell membrane disruption (14–18). Although TNF is the widely studied trigger of necroptosis, other stimuli such as poly(I:C), LPS, dsDNA, glucan, and IFNs are also known to elicit necroptosis in a variety of cell types, especially when caspase-8 is inhibited (19–21). Like cytokines and pathogen-associated molecular patterns (PAMPs), live viruses and bacteria, such as HSV, murine CMV, influenza virus, vaccinia virus, Staphylococcus aureus, and Listeria monocytogenes can also trigger necroptosis, which can contribute to host defense or immunopathology (22–29). Although numerous negative and positive regulators have been identified for necroptosis, the stringent control of necroptosis has just begun to unveil.

mTOR is an evolutionarily conserved serine/threonine kinase, which forms two multicomponent complexes, namely mTORC1 and mTORC2. mTOR integrates numerous extracellular and intracellular signals ensuring the precision control of immune responses, development, and tumorigenesis. Multiple growth factors and cytokines can activate mTOR by phosphorylating and inactivating the upstream repressor TSC1/TSC2 complex through kinases such as PI3K-Akt, ERK, and p90RSK (30, 31). TSC1 has been implicated in the induction of proinflammatory cytokines, macrophage polarization and the maintenance of tissue macrophages (32–37). Recent studies showed that the fungal cell wall component β-glucan can activate mTOR to induce metabolic-epigenetic reprogramming in monocytes and macrophages. Inhibition of mTOR activity by rapamycin or AMPK can severely suppress β-glucan induced trained immunity (38–40). Furthermore, TSC1/mTOR has been shown to regulate RIPK3 expression and gut epithelial necroptosis (41). In this study, we uncovered an important role for macrophage-derived TSC1 in the regulation of necroptosis during C. albicans infection. Moreover, our in vivo data also implicate that TSC1 can protect the host from C. albicans infection by suppressing necroptotic cell death of macrophages. Hence, these results provide important insight into the interplay between fungal pathogens and the host innate immune system.

MATERIALS AND METHODS

Mice

Tsc1-floxed (42), mTor-floxed (43), Riptor-floxed (44), Rictor-floxed (45) and LysM-cre mice (46) were purchased from The Jackson Laboratories. Ripk3−/− and Mlkl−/− mice on C57BL/6 background were described previously (47, 48). In all experiments, littermates carrying floxed alleles but without Cre recombinase were used as controls (wild-type [WT]). All the mice were bred and maintained in a pathogen-free animal facility at Institut Pasteur of Shanghai. All the procedures were conducted in compliance with a protocol approved by the Institutional Animal Care and Use Committee at Institut Pasteur of Shanghai.

Reagents

Zymosan depleted (ZymD, obtained by treating zymosan [an insoluble preparation of Saccharomyces cerevisiae cell wall] with hot alkali to remove its TLR-stimulating properties), ZymA (zymosan), LPS, and Pam3CSK4 were purchased from InvivoGen. Mannan, poly(I:C), and propidium iodide (PI) were obtained from Sigma-Aldrich. Rapamycin, z-VAD-FMK, z-DEVD-FMK, ferrostatin-1, GSK872, and necrostatin-1 were from Selleck Chemicals. Ac-YVAD-CMK was from Apexbio. Abs for TSC1 (4906), p-S6 (4858), S6 (2317), p-4E-BP1 (2855), 4E-BP1 (9644) and p-RIPK1 (31122) were from Cell Signaling Technology. Abs for p-RIPK3 (ab195117) and p-MLKL (ab196436) were purchased from Abcam. Ab against RIPK3 (2283) was from ProSci Antibody Services. Ab for RIPK1 (610458) was from BD Biosciences. Ab against MLKL (MABC604) was from MilliporeSigma. Ab against actin (SC-1616) was from Santa Cruz Biotechnology.

Generation of bone marrow–derived macrophages

Bone marrow cells were flushed out from the femurs and tibia of 6- to 10-wk-old C57BL/6 mice, and the RBCs were lysed using
ACK lysis buffer (0.15 M NaH2O4, 1 mM KHCO3, and 0.1 mM Na2EDTA [pH 7.3]). The bone marrow cells were then seeded at a concentration of 10^6 cells per milliliter with 1640 medium (Invitrogen) supplemented with 30% medium conditioned with L929 mouse fibroblasts (containing the cytokine M-CSF), as well as 10% FBS (HyClone). On day 4, nonadherent cells were removed and fresh medium was added. On day 7–8, adherent bone marrow–derived macrophages (BMDMs) were collected for use.

**Preparation of C. albicans**

The culture and heat inactivation of C. albicans were previously described (5). Briefly, single colonies of C. albicans strain SC5314 from yeast-peptone-dextrose agar plates were inoculated into yeast-peptone-dextrose medium, followed by culture overnight at 30°C (yeast form). For heat inactivation, yeast cells were washed and the kidneys, livers and spleens were collected and homogenized, then the tissue homogenates were serially diluted and measured 3 d postinfection. The infected mice were sacrificed, then the tissue homogenates were serially diluted and plated on yeast-extract-peptone-dextrose-agar. Fungal CFUs were counted after 24 h.

For in vitro infection, macrophages were infected with WT C. albicans strain SC5314 (multiplicity of infection [MOI]: 1) for 4 h; after that, fresh medium with PI (5 µg/ml) was added for another 10 min, and then the images were collected under a fluorescence microscope.

**Western blotting**

To make whole-cell lysates, cells were lysed in lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% NP-40, and 1 mM EDTA [pH 8.0]) supplemented with protease inhibitor Complete mini (Roche Diagnostics) and 1 mM PMSF, 1 mM Na3VO4, and 1 mM NaF for 30 min on ice, and cell debris was cleared by centrifugation at 13,000 rpm for 15 min. The cell lysates were mixed with SDS-loading buffer and boiled for 5 min, then the samples were separated by 10% SDS-PAGE and were transferred to PVDF membrane and then probed with the appropriate Abs.

To obtain detergent-insoluble fraction of the cell lysates, cells were first lysed with NP-40 lysis buffer. After centrifugation, the pellet was washed with ice-cold PBS and then further extracted with freshly made NP-40 lysis buffer containing 6 M urea on ice for 30 min. After centrifugation at 13,000 rpm for 15 min, the supernatant of detergent-insoluble fraction was collected.

**Cell cytotoxicity and viability assay**

The cell cytotoxicity and cell viability of BMDMs after necroptosis induction were measured by a CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega) and a CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega), respectively.

**Flow cytometry**

After the mice were euthanized, kidneys were isolated, minced, and then digested with 1 mg/ml of type IV collagenase (Invitrogen) and 0.15 mg/ml DNasel (Sigma-Aldrich) at 37°C for 45 min and subsequently passed through a 70-µm cell strainer. After RBCs lysis with ACK lysis buffer, leukocytes from the kidneys were isolated by Percoll gradient centrifugation (GE Healthcare).

Fluorochrome-labeled Abs for CD11b (M1/70), F4/80 (BM8), and Ly-6C (H1k.4) were from eBioscience. Abs for CD45.2 (104), MHC class II (M5/114.15.2), and Ly-6G (1A8) were from BD Biosciences. All Abs were tested for their specificities with respective isotype controls. All the samples were processed with an LSR-Fortessa Flow Cytometer (BD Biosciences), and the data were analyzed by FlowJo software (Tree Star).

**Histological analysis**

Fresh kidney tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections were cut in 5 µm and stained with H&E for histological analysis.

**TUNEL assay**

To assess cell death in kidney, fresh kidney tissues were embedded in OCT at −20°C. Tissue sections were cut in 5-µm sections and TUNEL assay was conducted with In-Situ Cell Death Kit (Roche Diagnostics) according to the manufacturer’s instructions. Sections were then stained with CD11b (M1/70; BD Biosciences) or Gr-1 (RB6-8C5; BD biosciences) or CD68 (KPI; Abcam) Abs and anti-Cy3 Abs. Stained sections were then analyzed under a fluorescence microscope.

**Statistical analysis**

The survival curve was analyzed by log-rank (Mantel–Cox) test. The unpaired, two-tailed Student t test was used to compare two groups. One-way ANOVA was used for multiple groups. A p value <0.05 is considered significant.

**RESULTS**

C. albicans induce necroptosis in macrophages

As engulfing of C. albicans can lead to the death of macrophages, we sought to investigate the nature of cell death upon fungal infection. At first, live C. albicans was applied to murine macrophages, and prominent macrophage cell death was observed in both RAW 264.7 cells and BMDMs (Fig. 1A, IB). Concomitant treatment with either caspase-1 or caspase-3 inhibitor alleviated C. albicans–elicited macrophage death, characterized by fewer PI-positive RAW cells or reduced lactate dehydrogenase (LDH) release from BMDMs (Fig. 1A, 1B). Additionally, blockade of ferroptosis with ferrostatin-1 (Fer-1), or necroptosis with RIPK1 or RIPK3 inhibitor (necrostatin-1 [Nec-1] or GSK872) also reduced macrophage cell death (Fig. 1A, IB). Of note, with much worsened cell death (>80%) 12 h postinfection, neither caspase-1 or caspase-3 inhibitor, nor blockade of ferroptosis or necroptosis was able to prevent the massive cell death triggered by C. albicans (Fig. 1B). Hence, these results indicate that a variety of
FIGURE 1. Macrophage death triggered by *C. albicans*.

(A and B) RAW 264.7 cells and BMDMs were either untreated or pretreated with Ac-YVAD-CMK (YVAD, 50 μM), z-DEVD-FMK (DEVD, 30 μM), ferrostatin-1 (Fer-1, 5 μM), GSK872 (5 μM), or necrostatin-1 (Nec-1, 30 μM) for 1 h, then infected with live *C. albicans* (MOI: 1) for various times, then cell death was measured by PI-staining or LDH release (*n* = 3). (C and D) WT, *Mlkl*<sup>−/−</sup>, and *Ripk3*<sup>−/−</sup> BMDMs were uninfected (UI) or infected with live *C. albicans* (MOI: 1) for 4 h. PI (5 μg/ml) was added 10 min prior to harvest. Images were collected under a fluorescence microscope and percentage of PI+ macrophages (PI+ macrophage numbers per field/total macrophage numbers per field) were calculated (UI, *n* = 3; *C. albicans*, *n* = 15). White arrows indicate PI-positive BMDMs. Scale bar, 50 μm. (E) WT, *Mlkl*<sup>−/−</sup> and *Ripk3*<sup>−/−</sup> BMDMs were uninfected (UI) or infected with live *C. albicans* (MOI: 1) for 4 and 8 h with or without z-VAD (40 μM), then cell cytotoxicity was measured by LDH release (*n* = 3). The data are representative of three independent experiments and shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, by one-way ANOVA.

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FIGURE 2. Tsc1\(^{-/-}\) BMDMs are highly susceptible to C. albicans–induced necroptosis. 

(A) Immunoblots of protein lysates from WT and Tsc1\(^{MN/-}\) BMDMs infected with live C. albicans (MOI: 1) for various times with indicated Abs. (B) WT and Tsc1\(^{MN/-}\) BMDMs were uninfected (UI) or infected with live C. albicans (MOI: 1) for 8 h with or without z-VAD (40 \(\mu\)M). Cell death was measured by LDH release (n = 3). (C–E) WT and Tsc1\(^{MN/-}\) BMDMs (C and D) or WT, Tsc1\(^{MN/-}\), and Tsc1\(^{MN/-}\) Mlk1\(^{+/}\) BMDMs (E and F) were uninfected (UI) or infected with live C. albicans (MOI: 1) for 4 h. PI (5 \(\mu\)g/ml) was added 10 min prior to harvest. Images were collected under a fluorescence microscope.
cell death pathways are simultaneously involved in \textit{C. albicans}–elicited macrophage death.

Next, we took a genetic approach to further interogate the role of necroptosis in fungus-elicited macrophage death. Although WT BMDMs infected with live \textit{C. albicans} exhibited massive cell death (Fig. 1C, 1D), BMDMs deficient in either Ripk3 (\textit{Ripk3} \textsuperscript{−/−}) or \textit{Mlkl} (\textit{Mlkl} \textsuperscript{−/−}) showed a notable decrease in cell death (Fig. 1C, 1D). Although numerous studies have shown that the pan-caspase inhibitor z-VAD is dispensable for \textit{C. albicans}–induced necroptosis (Fig. 1E). Together, these results demonstrated that \textit{C. albicans} can trigger RIPK3-MLKL–dependent necroptotic cell death in macrophages.

\textbf{TSC1 suppresses \textit{C. albicans}–induced macrophage necroptosis}

Given our recent finding that TSC1/mTOR regulates intestinal epithelial necroptosis and inflammation (41), we went on to examine whether TSC1/mTOR has a role in \textit{C. albicans}–induced necroptosis in macrophages. To this end, we bred \textit{Tsc1}−/− mice onto a transgenic strain expressing \textit{LysM-cre} to generate \textit{Tsc1} \textsuperscript{−/−}/\textit{LysM-cre} whose macrophage/neutrophil compartment is deficient in \textit{Tsc1} (hereafter named \textit{Tsc1} \textsuperscript{M/N−/−}). Immunoblotting revealed drastic reduction in TSC1 expression in \textit{Tsc1} \textsuperscript{−/−}/\textit{LysM-cre} BMDMs (Fig. 2A), indicating an efficient ablation of \textit{Tsc1} alleles in the macrophage compartment. Consistent with a negative role of TSC1 in mTOR activation, \textit{Tsc1} \textsuperscript{−/−} BMDMs also showed increased phosphorylation on S6 and 4E-BP1, two well-established substrates for mTOR (Fig. 2A).

Next, we differentiated WT and \textit{Tsc1} \textsuperscript{M/N−/−} BMDMs in vitro and infected them with live \textit{C. albicans}. Western blot analysis revealed that \textit{C. albicans} infection augmented mTOR activity, as demonstrated by increased phosphorylation of S6 and 4E-BP1 (Fig. 2A). Remarkably, \textit{Tsc1}−/− BMDMs had much higher percentage of dead cells than WT BMDMs (Fig. 2B–D), characterized by pronounced PI-positive staining or LDH release. To test whether the increased cell death in \textit{Tsc1}−/− BMDMs were attributable to necroptosis, we crossed \textit{Tsc1} \textsuperscript{M/N−/−} mice with \textit{Mlkl}−/− mice to obtain \textit{Tsc1} \textsuperscript{M/N−/−}/\textit{Mlkl}−/− mice. Compared with \textit{Tsc1}−/− BMDMs, \textit{C. albicans}–triggered cell death was lessened in \textit{Tsc1}−/−/\textit{Mlkl}−/− BMDMs, supporting a crucial role for necroptosis in aggravating \textit{Tsc1}−/− BMDMs death (Fig. 2E, 2F). Hence, these data suggest a crucial role for TSC1 in the regulation of \textit{C. albicans}–elicited necroptosis.

\textbf{TSC1-mTOR suppresses dectin-1 and TLR4-induced necroptosis in macrophages}

\textit{C. albicans}–derived PAMPs can be recognized by PRRs such as TLR2/4 and Dectin-1/2. Therefore, we wondered which PRRs TSC1 might impinge on to curb macrophage death. First, we stimulated BMDMs with heat-killed \textit{C. albicans} yeast (HKCA-Y) along with various fungal PAMPs, such as dectin-1 ligand ZymD, dectin-2 ligand α-mannan, and zymosan A (ZymA, a ligand for both dectin-1 and TLR2). In the presence of the pan-caspase inhibitor z-VAD, all the above ligands induced cell death in WT BMDMs, as measured by LDH release (Fig. 3A). In contrast, \textit{Mlkl}−/− BMDMs were markedly resistant, exhibiting negligible cell death to all the stimuli (Fig. 3A). Furthermore, ATP release assay also support the notion that all these stimuli elicited MLKL-dependent necroptotic cell death in BMDMs (Supplemental Fig. 1A). Consistently, LPS (TLR4 ligand), poly(I:C) (ligand for TLR3), and Pam3 (TLR2 ligand) were also capable of triggering necroptosis in BMDMs (Fig. 3B, Supplemental Fig. 1B).

We further stimulated WT and \textit{Tsc1}−/− BMDMs with HKCA-Y, ZymD, mannan, and ZymA, as well as LPS, poly(I:C), and Pam3 in the presence of z-VAD, and found that these stimuli elicited more pronounced necroptosis in \textit{Tsc1}−/− BMDMs, which could be blocked by Nec-1 (Fig. 3C). Recently, we found \textit{C. albicans}–secreted, small cysteine–rich protein Sell can elicit TLR2– and TLR4–dependent immune responses (4). Similar to Pam3 and LPS, Sell also elicited potent necroptosis in \textit{Tsc1}−/− BMDMs (Fig. 3C). The massive cell death exhibited in \textit{Tsc1}−/− BMDMs (Fig. 3C) was alleviated by ablating \textit{Mlkl} (Fig. 3D), further supporting a role for Sell in triggering necroptosis. Collectively, these data revealed a crucial role for TSC1 in suppressing dectin-1/2– and TLR2/4–induced necroptosis in macrophage.

Next, we interrogated whether hyperactive mTORC1 might have contributed to heightened necroptosis in \textit{Tsc1}−/− BMDMs. To this end, we pretreated WT and \textit{Tsc1}−/− BMDMs with mTORC1 inhibitor rapamycin, then followed with ZymD or LPS stimulation. We found that rapamycin inhibited ZymD– or LPS–induced necroptosis, especially in \textit{Tsc1}−/− BMDMs (Fig. 4A), indicative of the involvement of mTORC1 in this process. Next, we generated \textit{Tsc1}/\textit{mTor} \textsuperscript{M/N−/−} mice to further investigate the role of mTOR in this process. Following stimulation with either ZymD, mannan, LPS, or poly(I:C) in the presence of z-VAD, the aberrant necroptosis in \textit{Tsc1}−/− BMDMs was largely rescued by concomitant ablation of \textit{mTor} (Fig. 4B). mTOR can form two complexes mTORC1 and mTORC2, whose activities rest on adaptors Raptor and Rictor, respectively. Interestingly, ZymD–, LPS–, or poly(I:C)–elicited necrotic cell death decreased in \textit{Riptor}−/− BMDMs (Fig. 4C). Together, these data demonstrated that mTORC1 has a crucial role in regulating macrophage necroptosis.

\textbf{TSC1 regulates the activation of necrototic signaling}

The initiation of necroptosis relies on the activation of kinases RIPK1 and RIPK3, which subsequently phosphorylate and activate MLKL. We then compared the necroptosis signaling cascade...
between WT and Tsc1<sup>−/−</sup> BMDMs. Postinfection with C. albicans, the phosphorylation of RIPK3 and MLKL were increased in Tsc1<sup>−/−</sup> BMDMs (Fig. 5A). When cells were treated with C. albicans plus z-VAD, the phosphorylation of RIPK1, RIPK3, and MLKL were further strengthened in Tsc1<sup>−/−</sup> BMDMs (Fig. 5B). Consistently, Tsc1<sup>−/−</sup> BMDMs also showed elevated phosphorylation of RIPK1, RIPK3, and MLKL upon stimulation with ZymD, Sell, or LPS plus z-VAD (Fig. 5C, 5D, Supplemental Fig. 2A). Activated RIPK1 has been found in a detergent-insoluble fraction during necroptosis as detergent-insoluble ubiquitylated RIPK1 (iuRIPK1) (49). To this end, we stimulated WT and Tsc1<sup>−/−</sup> BMDMs with LPS plus z-VAD for various times and extracted the NP-40–insoluble pellets with 6 M urea–containing buffer. In the NP-40–insoluble fraction, phosphorylated RIPK1, RIPK3, and MLKL proteins increased over time, and were more abundantly present in Tsc1<sup>−/−</sup> BMDMs than WT BMDMs (Supplemental Fig. 2B). Additionally, higher molecular weights of RIPK1 and RIPK3 proteins were more prominent in Tsc1<sup>−/−</sup> BMDMs, suggestive of increased poly-ubiquitination on RIPK1 and RIPK3 (Supplemental Fig. 2B).

**TSC1-deficiency in macrophages compromises the host defense to C. albicans**

To determine whether TSCI-mediated necroptosis regulates antifungal immune responses in vivo, we infected mice i.v. with...
C. albicans. Compared with WT mice, Tsc1<sup>M/N--</sup> mice suffered aggravated morbidity and mortality (Fig. 6A, 6B). Conversely, ablating Mlkl protected mice from C. albicans–induced mortality (Fig. 6C). Furthermore, Tsc1<sup>M/N--</sup> mice also had higher fungal burden in multiple tissues, including the kidney, liver, and spleen (Fig. 6D). Next, we assessed the immune responses in WT and Tsc1<sup>M/N--</sup> mice upon infection with C. albicans. One day postinfection, Tsc1<sup>M/N--</sup> sera demonstrated more prominent IL-6, but similar IL-12p40 and CXCL1 presence to WT sera (Supplemental Fig. 3A). In addition, H&E staining revealed more immune cells infiltrating into the kidneys of Tsc1<sup>M/N--</sup> mice (Supplemental Fig. 3B). Further analyses by flow cytometry (Supplemental Fig. 3C) indicated that both WT and Tsc1<sup>M/N--</sup> mice suffered loss of their resident macrophages (CD11b<sup>+</sup>F4/80<sup>lo</sup>) after fungal infection, with Tsc1<sup>M/N--</sup> mice lost more resident macrophages than WT mice (Fig. 6E, 6F). The numbers of monocyte-derived macrophages (CD11b<sup>+</sup>F4/80<sup>hi</sup>) after fungal infection, with Tsc1<sup>M/N--</sup> mice had markedly higher neutrophils than WT counterparts postinfection (Fig. 6E, 6F).

Next, we examined necroptosis in C. albicans–infected kidneys by TUNEL staining. Histology analysis revealed widespread presence of necrotic cells in Tsc1<sup>M/N--</sup> kidneys, but only scarce TUNEL-positive cells in WT kidneys upon C. albicans infection (Supplemental Fig. 3D). To substantiate this observation, we conducted double staining using anti-CD11b, -Gr1, or -CD68 Abs in conjunction with TUNEL assay, assessing the necroptotic death of neutrophils and macrophages, respectively. After C. albicans infection, the Tsc1<sup>M/N--</sup> kidneys exhibited more CD11b-/TUNEL-positive, Gr1-/TUNEL-positive, and CD68-/TUNEL–double-positive cells than WT kidneys, indicative of elevated necroptotic cell death among both macrophages and neutrophils (Fig. 7A–C). Also, Tsc1<sup>M/N--</sup>/Mlkl<sup>−/−</sup> mice were markedly protected from C. albicans infection as compared with Tsc1<sup>M/N--</sup> mice (Fig. 7D), further demonstrating a critical role for TSC1-regulated necroptosis in anti-C. albicans defense.

**DISCUSSION**

Being the most common human fungal pathogen, C. albicans has been extensively studied for their pathogenic mechanism and corresponding host countermeasures (50). In this study, we report that TSC1 functions as a critical regulator of macrophage function by controlling their necroptosis upon C. albicans infection. We further demonstrated that TSC1-deficiency in macrophages could disrupt the immune defense to C. albicans and cause severe pathologic condition. Collectively, our study provides important insights into disease and therapeutic strategies for fungal infections.
insight into the complex interplay between the host innate immune system and fungal pathogen.

TSC1 has been implicated in the apoptotic cell death of immune cells including T cells, dendritic cells, and peritoneal macrophages (36, 51–54). Our recent work further establishes a role for TSC1/mTOR in epithelial necroptosis and intestinal inflammation (41). Of note, the fungal cell wall component β-glucan can activate mTOR, whereby promoting trained immunity to subsequent encounter with C. albicans (38–40). Thus, it is not surprising that TSC1 is capable of regulating C. albicans–elicited macrophage necroptosis. Whereas Tsc1-deficient epithelial cells demonstrate elevated expression of RIPK3 and MLKL (41), in this study, we found that the levels of RIPK1 was also upregulated in Tsc1−/− BMDMs along with RIPK3 and MLKL. These results suggest that TSC1 may similarly regulate RIPK3 and MLKL through Trim11-mediated protein

FIGURE 6. Tsc1−/− mice are more vulnerable to C. albicans infection. (A and B) Six- to eight-week-old WT and Tsc1−/− littermates (n = 11) were infected with C. albicans (4 × 10^4 fungal cells per mouse) by i.v. injection; weight loss (A) and survival (B) were documented daily. (C) Six- to eight-week-old WT and Mlkl−/− littermates (n = 11–12) were infected with C. albicans (4 × 10^4 fungal cells per mouse) by i.v. injection, survival was documented daily. (D) Quantification of C. albicans in the kidney, liver, and spleen of WT and Tsc1−/− mice (n = 6) 3 d postinfection with C. albicans as in (A), analyzed by serial dilution of homogenized tissues and presented as CFU per gram of tissue. (E and F) The percentages (E) and numbers (F) of resident macrophages (CD11b+F4/80hi), monocyte-derived macrophages (CD11b+F4/80lo), and neutrophils of the kidney in WT and Tsc1−/− mice either uninfected (UI) or 3 d postinfection with C. albicans were analyzed by flow cytometry (n = 6–7), cells were pregated as CD45+. The data are representative of three independent experiments and shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, analyzed by unpaired Student t test (A, D, and F) or by log-rank test (B and C).
**(A–C)** Immunofluorescent staining on kidney sections from WT and Tsc1<sup>M/N</sup>–/– mice (n = 2–4) either uninfected (UI) or 3 d postinfection with C. albicans (4 × 10<sup>4</sup> fungal cells per mouse). Representative images were captured, and double-positive cells were counted (UI, n = 4; C. albicans, n = 8). White arrows indicate double-positive cells. Scale bar, 50 μm. **(D)** Six- to eight-week-old WT, Tsc1<sup>M/N</sup>–/–, and Tsc1<sup>M/N</sup>–/–/Mikl<sup>–/–</sup> mice (n = 12) were infected with C. albicans (5 × 10<sup>4</sup> fungal cells per mouse) by i.v. injection, and survival was monitored daily. The data are representative of three independent experiments and shown as mean ± SEM. *p < 0.05, **p < 0.01, analyzed by unpaired Student t test (A–C) or by log-rank test (D).

Tsc1<sup>M/N</sup>–/–/Mikl<sup>–/–</sup> mice were less susceptible than Tsc1<sup>M/N</sup>–/– mice implicates a pathogenic role for MLKL-dependent necroptosis in C. albicans infection. Therefore, our data support the notion that TSC1-controlled necroptosis is crucial for macrophage survival and host defense against C. albicans. Conceivably, the interplay between fungal pathogens and the host innate immune system is complex, and further investigations are needed to resolve this discrepancy.

Tissue-resident macrophages are key effector cells in antifungal defense, as they can either directly kill fungal pathogens by phagocytosis or indirectly control infection by producing a myriad of proinflammatory cytokines and chemokines (55). In this study, we found that the expression of proinflammatory cytokines and chemokines was comparable in WT and Tsc1<sup>M/N</sup>–/– kidneys, hence excluding a possible role of TSC1 in inflammation per se. In contrast, we found fewer resident macrophages present in the Tsc1<sup>M/N</sup>–/– kidney before and after C. albicans infection. For example, CD68<sup>+</sup>/TUNEL-double staining revealed massive macrophage necroptosis in the Tsc1<sup>M/N</sup>–/– kidney especially after C. albicans infection. Considering that Tsc1<sup>M/N</sup>–/–/Mikl<sup>–/–</sup> mice were more resistant to C. albicans than Tsc1<sup>M/N</sup>–/– mice, we propose that TSCI-regulated macrophage necroptosis might have compromised the phagocytosis and killing of C. albicans, resulting in heightened fungal burdens. Neutrophils also have a major role in host defense against C. albicans. In our study, we noticed that there were more neutrophils infiltrating into the kidney of Tsc1<sup>M/N</sup>–/– mice after C. albicans infection. However, Gr-1/TUNEL-double staining indicates that there was also significantly enhanced neutrophil death in Tsc1<sup>M/N</sup>–/– mice. Neutrophils can release neutrophil extracellular traps to combat hyphae, a much-enlarged fungal filament (2). Given that neutrophil extracellular trap release can also be regulated by necroptosis (56, 57), the role of neutrophils in Tsc1<sup>M/N</sup>–/– mice after C. albicans infection needs to be further elucidated.

RIPK1 activation is tightly controlled by phosphorylation and ubiquitination (58–63). Activated RIPK1 can be detected in the detergent-insoluble complex as detergent-insoluble ubiquitylated form (iuRIPK1) (49). In our study, we found that Tsc1<sup>–/–</sup> BMDMs had marked elevation in the expression and phosphorylation of RIPK1, RIPK3, and MLKL after stimulation with various necroptosis-inducing regimens. We also noticed that Tsc1<sup>–/–</sup> BMDMs had more abundant polyubiquitinated RIPK1 and RIPK3 proteins during the induction of necroptosis. It is possible that both enhanced phosphorylation and ubiquitination might have contributed to augmented necroptosis in Tsc1<sup>–/–</sup> BMDMs. However, the temporal order and causative relationship between phosphorylation and ubiquitination on RIPK1 or RIPK3 during necroptosis remain unclear at present, and certainly warrant further investigation in the future.

In summary, we determined TSC1 as a regulator controlling the necroptotic cell death of macrophages during C. albicans infection. We also provided compelling evidence advocating curbing necroptosis by TSC1 benefits the host containing fungal pathogen C. albicans, hence offering key insight into the understanding of antifungal immune defense.
DISCLOSURES
The authors have no financial conflicts of interest.

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REFERENCES
ImmunoHorizons

TSC1 SUPPRESSES FUNGI-INDUCED MACROPHAGE NECROPTOSIS


