A hyper-immunogenic and slow-growing fungal strain induces a murine granulomatous response to cryptococcal infection

Running title: A murine cryptococcal granuloma model

Calla L. Telzrow, a,b Shannon Esher Righi, c Natalia Castro-Lopez, d,e# Althea Campuzano, d Jacob T. Brooks, f John M. Carney, a,g Floyd L. Wormley Jr., d,e# J. Andrew Alspaugh a,b*

a Department of Medicine, Duke University School of Medicine, Durham, NC
b Department of Molecular Genetics and Microbiology, Duke University School of Medicine, Durham, NC
c Department of Microbiology and Immunology, Tulane University School of Medicine, New Orleans, LA
d Department of Biology, University of Texas at San Antonio, San Antonio, TX
e Department of Biology, Texas Christian University, Fort Worth, TX
f Department of Physics and Astronomy, University of North Carolina at Chapel Hill,
Chapel Hill, NC
g Department of Pathology, Duke University School of Medicine, Durham, NC
# Current address: Department of Biology, Texas Christian University, Fort Worth, TX
*Corresponding author:

J. Andrew Alspaugh

337 Sands Research Building

303 Research Drive, DUMC 102359

Durham, NC 27710, USA

Tel: (919) 684-0045

Fax: (919) 684-8902

andrew.alspaugh@duke.edu

Keywords: Cryptococcus neoformans, granuloma, GM-CSF, cell wall, Titan cell, cell cycle defects, hypoxia
ABSTRACT

Many successful pathogens cause latent infections, remaining dormant within the host for years but retaining the ability to reactivate to cause symptomatic disease. The human opportunistic pathogen Cryptococcus neoformans is a ubiquitous yeast that establishes latent pulmonary infections in immunocompetent individuals upon fungal inhalation from the environment. These latent infections are frequently characterized by granulomas, or foci of chronic inflammation, that contain dormant cryptococcal cells. Immunosuppression causes these granulomas to break down and release viable fungal cells that proliferate, disseminate, and eventually cause lethal cryptococcosis. This course of C. neoformans dormancy and reactivation is understudied due to limited models, as chronic pulmonary granulomas do not typically form in most mouse models of cryptococcal infection. Here, we report that a previously characterized Cryptococcus-specific gene which is required for host-induced cell wall remodeling, MAR1, inhibits murine granuloma formation. Specifically, the mar1Δ loss-of-function mutant strain induces mature pulmonary granulomas at sites of infection dormancy in mice. Our data suggest that the combination of reduced fungal burden and increased immunogenicity of the mar1Δ mutant strain stimulates a host immune response that contains viable fungi within granulomas. Furthermore, we find that the mar1Δ mutant strain has slow growth and hypoxia resistance phenotypes, which may enable fungal persistence within pulmonary granulomas. Together with the conventional primary murine infection model, latent murine infection models will advance our understanding of cryptococcal disease progression and define fungal features important for persistence in the human host.
Granulomas are complex foci of chronic inflammation that form in response to many stimuli, including microbial infections. A hallmark of indolent infections such as tuberculosis disease, granulomas are often characterized by epithelioid macrophages, multinucleated giant cells, and dormant and/or slowly proliferating microorganisms (1–4). The traditional understanding of the granuloma considered it to be a host-directed defense response that restricts microbial access to nutrients and oxygen, resulting in an immune microenvironment that limits microbial proliferation and prevents dissemination (3, 4). However, more recent work has demonstrated that granulomas are a dynamic component of the complex host-microbial “arms race”. In addition to serving as a host-directed protection mechanism, microorganisms can exploit the granuloma as a micro-niche for long-term survival in the host, where they remain shielded from immune detection until microbial reactivation (3–5). Although most work on granulomas has been conducted in the context of mycobacterial infections, many other infectious microorganisms induce granuloma formation in the human lung (6).

The fungal pathogen Cryptococcus neoformans is a significant cause of pneumonia and fatal meningoencephalitis in immunocompromised populations around the world, resulting in more than 180,000 deaths annually (7). Primary infection occurs upon inhalation of environmental C. neoformans cells and/or spores, often early in life (4, 8). Immunocompetent hosts typically control the primary infection, with fungi remaining dormant but viable within lung-associated granulomas (9). As a result, immunocompetent hosts do not manifest infection-related symptoms with disease during this stage of latency (10). However, this latent infection can reactivate when a
previously exposed individual becomes immunocompromised, especially in the setting of CD4+ T cell functional deficiency due to HIV infection, organ transplantation, and immunosenescence (2, 4, 7, 11). Breakdown of the cryptococcal granuloma structure results in microbial proliferation and systemic dissemination, including to the central nervous system.

The reactivation of fungal cells from granulomas is an understudied facet of cryptococcal disease, largely due to limited reactivation models. Although the mouse is the most well-characterized and commonly used animal model to study Cryptococcus-host interactions, many murine models do not form sustained granulomas in response to clinically relevant isolates of C. neoformans (12). As a result, most murine experiments focus on primary cryptococcal infection and subsequent systemic dissemination. To explore cryptococcal latency and reactivation, investigators have adopted models of cryptococcosis in rabbits (13) and rats (14, 15) or employed less virulent C. neoformans strains in mice (12, 16, 17). Recently, a novel latent model was reported in which pulmonary granulomas form in mice in response to infection with the gcs1Δ mutant cryptococcal strain lacking the glucosylceramide synthase (18–21). Mimicking the typical course of human disease, gcs1Δ cells induce well-formed granulomas in the lungs which contain dormant gcs1Δ cells that become reactivated from granulomas and disseminate upon immunosuppression (22).

We recently reported the identification and characterization of the C. neoformans MAR1 gene that is required for cell surface remodeling in response to the host environment (23). The mar1Δ loss-of-function mutant strain displays altered cell surface features when exposed to physiological conditions, including decreased cell wall
glucans and mannans, increased exposure of cell wall chitin, and impaired polysaccharide capsule attachment (23). The cell surface alterations of the \( \text{mar}1\Delta \) mutant strain result in enhanced macrophage activation \textit{in vitro} and hypovirulence in a murine inhalation model of cryptococcosis (23). We report here that this hyper-immunogenic \( \text{mar}1\Delta \) mutant strain induces pulmonary granulomas in mice, resulting in a chronic and indolent infection. Furthermore, we describe both fungal and host factors that contribute to this granuloma response. From the fungal perspective, the combination of reduced fungal burden and hyper-immunogenicity of the \( \text{mar}1\Delta \) mutant strain stimulates a host immune response that contains \( \text{mar}1\Delta \) mutant cells within well-circumscribed granulomas. From the host perspective, we find that host GM-CSF signaling, a known contributor to granuloma formation (17, 24–26), is required for the formation of these granulomas. Finally, \textit{in vitro} studies demonstrate that the \( \text{mar}1\Delta \) mutant strain has cell cycle defects that may contribute to a slow growth phenotype and hypoxia resistance, two features which likely enable cryptococcal persistence within pulmonary granulomas. Because \( \text{MAR}1 \) is a \textit{Cryptococcus}-specific gene, this model represents a unique addition to the limited tools available to study the reactivation model of cryptococcal disease.

\section*{MATERIALS \& METHODS}

\subsection*{Strains, media, and growth conditions}

All strains used in this study were generated in the \textit{C. neoformans} var. \textit{grubii} H99 (MAT\( \alpha \)) (13) background and are included in Table 1. Strains were maintained on yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% dextrose,
and 2% agar for solid medium). Unless otherwise indicated, strains were incubated at 30°C.

**Histology analyses**

The murine inhalation model of cryptococcosis was exclusively used in this study (27). For initial histological examination, C57BL/6 female mice were acquired from Charles River Laboratories. Mice were anesthetized with 2% isoflurane utilizing a rodent anesthesia device (Eagle Eye Anesthesia, Jacksonville, FL) and were infected via the intranasal route with $1 \times 10^4$ CFU of either the wild-type (WT) (H99) or the mar1Δ mutant (MAK1) strain. Mice were sacrificed at predetermined endpoints (3, 7, 14, and 40 DPI) by CO$_2$ inhalation followed by an approved secondary method of euthanasia. Lungs were perfused with and stored in 10% neutral buffered formalin. Lungs were subsequently paraffin-embedded, sectioned, mounted, and stained with hematoxylin and eosin by the Duke University School of Medicine Research Immunohistochemistry Shared Resource.

To determine the role of GM-CSF signaling in granuloma formation in this model, lungs from male and female Csf2rb$^{-/-}$ mice (The Jackson Laboratory # 005940) were prepared as described above, with a few alterations. Mice were sacrificed at the predetermined endpoints of 3, 7, and 14 DPI by CO$_2$ inhalation followed by an approved secondary method of euthanasia and lungs were perfused with PBS. The right lung was stored in 10% neutral buffered formalin for future histopathology preparation, while the left lung was used for fungal burden quantification analyses, as described below.

**Fungal burden quantification**
Mice were infected as described above. Mice were euthanized at predetermined endpoints by CO₂ inhalation followed by cervical dislocation, and lung tissues and/or brain tissues were excised. The left lobe of the lung and/or the brain was removed and homogenized in 1 mL of sterile PBS as previously described (28) followed by culture of 10-fold dilutions of each homogenate on YPD agar medium supplemented with chloramphenicol. Colony-forming units (CFU) were enumerated following incubation at 30°C for 48 hours. Statistical significance was determined using Student's t test (GraphPad Software, San Diego, CA).

**Pulmonary cytokine analyses**

C57BL/6 female mice acquired from Charles River Laboratories were infected and sacrificed as described above. Cytokine levels within the lung homogenates of infected mice were analyzed using the Bio-Plex protein array system (Luminex-based technology, Bio-Rad Laboratories, Hercules, CA). Briefly, lung tissues were excised and homogenized in 1 mL ice-cold sterile PBS. An aliquot (50 µl) was taken to quantify the pulmonary fungal burden, and an anti-protease buffer solution (1 mL) containing PBS, protease inhibitors, and 0.05% Triton X-100 was added to the homogenate. Samples were then clarified by centrifugation (3,500 rpm) for 10 minutes. Supernatants from pulmonary homogenates were assayed for the presence of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, KC (CXCL1), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), Eotaxin (CCL11), IFN-γ, tumor necrosis factor (TNF)-α, granulocyte macrophage-colony stimulating factor (GM-CSF), and granulocyte-colony stimulating factor (G-CSF) according to the
manufacturer’s instructions. Statistical significance between strains at each timepoint was determined using Student’s *t* test (GraphPad Software, San Diego, CA).

**Pulmonary leukocyte isolation**

C57BL/6 female mice acquired from Charles River Laboratories were infected and sacrificed as described above. Lungs of infected mice were excised on 1, 3, 7, 14, and 21 DPI as previously described (28). Lungs were then digested enzymatically at 37°C for 30 minutes in 10 mL digestion buffer (RPMI 1640 and 1 mg/mL collagenase type IV [Sigma-Aldrich, St. Louis, MO]) with intermittent (every 10 minutes) stomacher homogenizations. The digested tissues were then successively filtered through sterile 70- and 40-μm nylon filters (BD Biosciences, San Diego, CA) to enrich for leukocytes, and the cells were then washed three times with sterile Hank’s Balanced Salt Solution (HBSS). Erythrocytes were lysed by incubation in NH₄Cl buffer (0.859% NH₄Cl, 0.1% KHCO₃, 0.0372% Na₂EDTA [pH 7.4]; Sigma-Aldrich) for 3 minutes on ice followed by a 2-fold excess of sterile PBS.

**Flow cytometry analyses**

Pulmonary leukocytes were isolated from infected mice as described above. Standard methodology was employed for the direct immunofluorescence of pulmonary leukocytes (28, 29). Briefly, in 96-well U-bottom plates, 100 μl containing 1 x 10⁶ cells in PBS were incubated with yellow Zombie viability dye (1:1000 dilution, Cat. No 423104, Biolegend, San Diego, CA) for 15 minutes at room temperature followed by washing in FACS buffer. Cells were then incubated with Fc block (1:500 dilution, Cat. # 553142, clone 2.4G2, BD Biosciences) diluted in FACS buffer for 5 minutes to block nonspecific binding of antibodies to cellular Fc receptors. Cells were then incubated with
fluorochrome-conjugated antibodies in various combinations to allow for multi-staining for 30 minutes at 4°C. Cells were washed three times with FACS buffer and fixed in 200 μl of 2% ultrapure formaldehyde (Polysciences, Warrington, PA) diluted in FACS buffer (fixation buffer). Fluorescence minus one (FMO) controls or cells incubated with either FACS buffer alone, or single fluorochrome-conjugated antibodies were used to determine positive staining and spillover/compensation calculations, and background fluorescence was determined with FlowJo v.10.8 Software (FlowJo, LLC, Ashland, OR). Raw data were collected with a Cell Analyzer LSRII (BD Biosciences) using BD FACSDiva v8.0 software at the University of North Texas Health Sciences Center (UNTHSC) Flow Core, and compensation and data analyses were performed using FlowJo v.10.8 Software. Cells were first gated for lymphocytes (SSC-A vs. FSC-A) and singlets (FSC-H vs. FSC-A). The singlets gate was further analyzed for the uptake of live/dead yellow stain to determine live vs. dead cells. From live cells, cells were gated on CD45+ cell expression. For data analyses, 100,000 events (cells) were evaluated from a predominantly leukocyte population identified by back gating from CD45+ stained cells. Statistical significance between strains at each timepoint was determined using Student’s t test (GraphPad Software, San Diego, CA).

**Macrophage activation analyses**

Intracellular staining of markers of macrophage activation was performed as described previously (29). Leukocytes isolated from infected mice as described above were incubated with cell stimulation cocktail (eBioscience Cat. # 00-4970-03) according to the manufacturer’s recommendation and incubated at 37°C in 5% CO₂ in cRPMI for two hours in a six-well plate. Golgi plug (1:100 dilution, Brefeldin A, Cat. # 51-2301KZ,
BD Biosciences) was added according to the manufacturer’s recommendations and incubated for an additional four hours (6 hours total). Cells were washed with PBS and stained with yellow Zombie viability dye in PBS at room temperature in the dark for 15 minutes. Cells were then washed with FACS buffer and incubated with Fc block (BD Biosciences) diluted in FACS buffer for 5 minutes. For nitric oxide (iNOS) and Arginase 1 (Arg1) production in macrophages, cells were stained for surface markers CD45, CD11b, CD64, F4/80, and CD24, and incubated at 4°C for 30 minutes. Cells were then washed and fixed with 2% ultra-pure formaldehyde (Polysciences, Warrington, PA) for 20 minutes. Subsequently, cells were washed with 0.1% saponin buffer and stained with antibodies for iNOS and Arg1 for 30 minutes at 4°C. Finally, cells were washed with saponin buffer and fixed with 2% ultra-pure formaldehyde. Samples were processed using a Cell Analyzer LSRII (BD Biosciences) using BD FACSDiva v8.0 software at the UNTHSC Flow Core, and 100,000 events were collected for analysis using FlowJo v.10.8 Software. Statistical significance between strains at each timepoint was determined using Student’s t test (GraphPad Software, San Diego, CA).

**Titan cell assay and quantification**

A previously described in vitro titanization assay was used here (30). In brief, the WT (H99), the mar1Δ mutant (MAK1), and the mar1Δ + MAR1 (MAK11) strains were incubated for 18 hours at 30°C, 150 rpm in 5 mL yeast nitrogen base (YNB) without amino acids prepared according to the manufacturer’s instructions plus 2% glucose. Cultures were washed six times with PBS. An optical density at 600 nm (OD\textsubscript{600}) of 0.001 for each strain was transferred to 5 mL 10% heat-inactivated fetal bovine serum (HI-FBS) in PBS and incubated at 37°C, 5% CO\textsubscript{2} for 96 hours. Cells were imaged by
differential interference contrast (DIC) microscopy using a Zeiss Axio Imager A1 microscope equipped with an Axio-Cam MRm digital camera. Cell diameter was measured using the ImageJ software (FIJI), and cells with a diameter > 10 μm were considered Titan cells. A minimum of 400 cells were analyzed across three biological replicates for each fungal strain. Statistical significance was determined using one-way analysis of variance (ANOVA) and the Tukey-Kramer test (GraphPad Software, San Diego, CA).

**SEM polysaccharide capsule visualization**

The WT (H99), the mar1Δ mutant (MAK1), the mar1Δ + MAR1 (MAK11), and the cap59Δ mutant (cap59) strains were incubated in YPD medium at 30°C and CO2-independent medium (Gibco) at 37°C until saturation. Samples were fixed with 2.5% glutaraldehyde for 1 hour at room temperature and were subsequently washed 3 times with PBS. Each sample was mounted onto 12 mm poly-L-lysine-coated coverslips (Neuvitro Corporation) and subsequently dehydrated by immersing the coverslips in ethanol (30% for 5 minutes, 50% for 5 minutes, 70% for 5 minutes, 95% for 10 minutes, 100% for 10 minutes, and 100% for 10 minutes). Samples were then critical point dried with a Tousimis 931 critical point dryer (Rockville, Maryland) and coated with gold-palladium using a Cressington 108 sputter-coater (Watford, United Kingdom). Coverslips containing the prepared samples were mounted and imaged on a Hitachi S-4700 scanning electron microscope (Tokyo, Japan).

**Cellular morphology defect quantification**

The WT (H99), the mar1Δ mutant (MAK1), and the mar1Δ + MAR1 (MAK11) strains were incubated for 18 hours in YPD medium at 30°C with shaking at 150 rpm. An
OD$_{600}$ of approximately 0.2 for each strain was transferred to fresh YPD medium and subsequently incubated at either 30°C or 37°C for 18 hours with shaking at 150 rpm. Cells were then pelleted, washed with PBS, and imaged by differential interference contrast (DIC) microscopy. DIC images were captured using a Zeiss Axio Imager A1 microscope equipped with an Axio-Cam MRm digital camera. A minimum of 500 cells were analyzed across three biological replicates for each strain using the ImageJ software (FIJI). Statistical significance was determined using two-way analysis of variance (ANOVA) and the Tukey-Kramer test (GraphPad Software, San Diego, CA).

**Growth curve analysis**

The WT (H99), the $mar1\Delta$ mutant (MAK1), and the $mar1\Delta + MAR1$ (MAK11) strains were incubated for 18 hours in YPD medium at 30°C with 150 rpm shaking. Cultures were normalized to an OD$_{600}$ of 0.01 in fresh YPD medium and added to wells of a 96-well plate. Growth was then measured at an absorbance of 595 nm every 10 minutes for 40 hours with shaking between readings and incubation at 37°C. Control wells containing YPD medium alone were also included to eliminate any background absorbance.

**Hypoxia resistance analyses**

The WT (H99), the $mar1\Delta$ mutant (MAK1), the $mar1\Delta + MAR1$ (MAK11), and the $sre1\Delta$ mutant (HEB6) strains were incubated in YPD medium at 30°C until mid-logarithmic growth phase. Strains were washed once in PBS, normalized to an OD$_{600}$ of 0.6 in PBS, and serially diluted onto YES (0.5% [w/v] yeast extract, 2% glucose, and 225 µg/mL uracil, adenine, leucine, histidine, and lysine) medium agar plates with or without cobalt chloride (0.7 mM) (31). Microaerophilic conditions were generated using...
a sealed chamber (BD GasPak™) and two activated GasPak™ EZ Campy Container System sachets (31). Plates were placed in the chamber (microaerophilic) or outside the chamber (ambient air), incubated at 30°C, and imaged daily for 96 hours.

**Mouse isolate recovery and phenotypic characterization**

C57BL/6 female mice acquired from Charles River Laboratories were infected as described above. At 61 DPI and 100 DPI, mice were sacrificed by CO₂ inhalation followed by an approved secondary method of euthanasia and fungi were subsequently isolated from the lungs as described above. Single fungal colonies were plated onto YPD agar medium and subsequently frozen in separate wells of 96-well plates at -80°C. Isolated fungi were stamped onto YPD agar medium incubated at 30°C, YPD agar medium incubated at 37°C, YPD agar medium supplemented with nourseothricin (NAT) (100 µg/mL) incubated at 30°C, and YPD agar medium buffered (150 mM HEPES) to pH 8.15 incubated at 30°C. All plates were imaged daily. Mouse isolates were determined to be mar1Δ mutant strain isolates based on growth on YPD + NAT medium and dry colony morphology on YPD pH 8.15 medium (23). The original WT (H99) and mar1Δ mutant (MAK1) strains were included on each plate as controls.

**Ethical use of animals**

All animal experiments in this manuscript were approved by the University of Texas at San Antonio Institutional Animal Care and Use Committee (IACUC) (protocol #MU021), the Texas Christian University and the University of North Texas Health Sciences Center (UNTHSC) IACUC (protocol #1920-9), and the Duke University IACUC (protocol #A102-20-05). Mice were handled according to IACUC guidelines.

**Data availability**
All fungal strains and reagents are available upon request.

RESULTS

Pulmonary granulomas are formed and maintained in mice infected with the
mar1Δ mutant strain.

Based on our recent observations that the mar1Δ mutant strain displays a highly
immunogenic cell surface, we hypothesized that the mar1Δ mutant strain would have
unique interactions with the host in vivo. We previously observed that the mar1Δ mutant
strain is hypovirulent compared to the wild-type (WT) strain in a murine inhalation model
of cryptococcosis (23). Highly immunogenic fungal strains often induce a
hyperinflammatory response that is detrimental to the host, resulting in hypervirulence
(32–34). We therefore explored in greater detail the mechanisms by which the highly
immunogenic mar1Δ mutant strain simultaneously activates and is controlled by the
host immune response.

As an initial investigation into the interactions between the mar1Δ mutant strain
and the host, we assessed the gross appearance of infected lungs from our previously
reported mar1Δ mutant strain murine inhalation infection experiment. At the time of
sacrifice, generally between 24-40 days post-inoculation (DPI), we observed that the
lungs of mar1Δ-infected C57BL/6 mice displayed large, well-circumscribed inflammatory
foci surrounded by healthy-appearing lung tissue (Figure 1A). This contrasts starkly with
WT-infected lungs, which typically exhibit uncontrolled fungal proliferation accompanied
by a diffuse inflammatory response.
We examined histopathological features of infected murine lungs at specific timepoints throughout the course of infection to further characterize the unique pathology observed in mar1Δ-infected lungs. To do so, we replicated the experimental approach used in Figure 1A; we inoculated C57BL/6 mice by inhalation with the WT strain or the mar1Δ mutant strain and subsequently harvested lungs for analysis throughout infection. At 3 DPI, an early timepoint in infection at which all inoculated mice still appear healthy, the WT-infected and mar1Δ-infected lungs appear similar, with the only notable exception being the increased number of fungal cells observed in the WT-infected lungs (Figures 1B & S1). By 7 DPI, a timepoint in infection in which WT-infected mice begin to show signs and symptoms of fungal disease but the mar1Δ-infected mice still appear healthy, WT-infected lungs display numerous small foci of inflammation (mean diameter = 535 μm) that contain some, but not all, fungal cells (Figures 1B, 1C, & S1). As described previously, many of the WT fungal cells exhibit signs of titanization (35). These foci of inflammation also occasionally display hallmarks of early granuloma formation, such as the presence of epithelioid macrophages (1, 2, 4) (Figure 1B). This type of immature granulomatous inflammatory response has been reported previously in the C57BL/6 background infected with the C. neoformans serotype D strain, 52D (17). In contrast, the mar1Δ-infected lungs have few visible fungal cells and display a more uniform pattern of inflammation throughout the lungs at 7 DPI (Figures 1B). These observations demonstrate that distinct characteristics of the mar1Δ mutant strain pathology emerge early in infection.

At 14 DPI, a timepoint in infection in which the WT-infected mice begin to succumb to fungal infection and the mar1Δ-infected mice still appear healthy, WT cells,
many of which are titanized, proliferate throughout the lungs resulting in a scattered, unorganized inflammatory response with mixed cell infiltrates (Figure 1B). Additionally, most nascent granulomas have broken down, which may explain why this timepoint also corresponds to a period of accelerating clinical symptoms and imminent mortality in WT-infected mice (Figures 1B & 1C). In contrast, \( \text{mar1}\Delta \)-infected lungs begin to form granulomas by 14 DPI. Specifically, foci of inflammation appear (mean diameter = 866 \( \mu \)m), containing few fungal cells which are surrounded by regions of normal-appearing lung tissue without fungal or inflammatory cells (Figures 1B & 1C). Additionally, these inflammatory foci contain hallmarks of granulomas, such as epithelioid macrophages surrounded by lymphocytes (1, 2, 4) (Figure 1B). In contrast to those infected with the WT strain, the \( \text{mar1}\Delta \)-infected mice display few infection-related symptoms at this timepoint. Many \( \text{mar1}\Delta \)-infected mice survive to 40 DPI (23). At this late timepoint in infection, mature granulomas are frequently observed (mean diameter = 1355 \( \mu \)m), containing fungal cells, multinucleated giant cells, and palisading epithelioid macrophages (Figures 1B, 1C, & S1). Additionally, no fungal cells are observed in lung tissue outside of these granulomas. Collectively, these observations suggest differences in the immune response in the context of WT and \( \text{mar1}\Delta \) mutant strain infection. WT-infected mice show a consistently robust mixed inflammatory response and variable Titan cell response with vague granuloma formation during early stages of infection (7 DPI) (Figures 1B, 1C, & S1). This response is ineffective and is quickly overcome by fungal growth, resulting in fungal proliferation throughout the lungs (14 DPI) (Figures 1B & 1C). In contrast, the \( \text{mar1}\Delta \)-infected mice show an absent to minimal inflammatory
response, absent Titan cell formation, and minimal granulomatous inflammatory response during early stages of infection (7 DPI), with a more well-formed granulomatous response in mice that survive to later timepoints in infection (40 DPI) (Figures 1B, 1C, & S1). These mar1Δ-induced granulomas appear sufficient to contain fungal proliferation.

The mar1Δ mutant strain has a reduced fungal burden and hyper-immunogenicity in vivo.

To explore possible mechanisms by which mar1Δ mutant strain infections induce pulmonary granuloma formation, we assessed fungal burden and the pulmonary immune response at timepoints relevant to granuloma formation. To do so, we replicated the experimental approaches used in Figure 1; we inoculated C57BL/6 mice by inhalation and harvested lungs for analysis throughout infection. We previously reported a decrease in fungal burden in mar1Δ-infected lungs compared to WT-infected lungs as early as 1 and 4 DPI, despite identical doses being used for both strains (23).

In this work, at all tested timepoints (3, 7, 14, & 21 DPI), we find that mar1Δ-infected lungs have a significantly reduced fungal burden compared to WT-infected lungs. Specifically, the mar1Δ-infected lungs have a 10-fold reduction in fungal burden at 3 DPI, a 100-fold reduction in fungal burden at 7 DPI, and a >500-fold reduction in fungal burden at 14 and 21 DPI compared to WT-infected lungs (Figure 2). These observations support the reduced number of mar1Δ mutant cells observed at these same timepoints in our histopathology analyses (Figure 1B). As a result of the drastic reduction in pulmonary fungal burden throughout infection, we observed that the mar1Δ mutant
strain rarely disseminates to the brain (Figure 2). When the mar1Δ mutant strain does disseminate to the brain, the fungal burden is markedly lower than that of the WT strain (Figure 2). Together, these observations indicate that the mar1Δ mutant strain has reduced fungal burden in the murine lung and brain, reinforcing our previous reports that the mar1Δ mutant strain has reduced fitness in host-relevant conditions.

Based on the drastic differences in fungal burden observed between WT-infected and mar1Δ-infected lungs, we hypothesized that the immune microenvironment within the lungs would also differ significantly. We replicated the experimental approaches used in Figure 1; we inoculated C57BL/6 mice by inhalation and harvested lungs for analysis throughout infection. At early timepoints in infection, 1 and 3 DPI, we observed similar pulmonary cytokine profiles and leukocyte infiltrates within WT-infected lungs and mar1Δ-infected lungs (Figures 3A, 3B, S2, & S3). The only significant difference observed between the two infections was in the production of granulocyte macrophage-colony stimulating factor (GM-CSF), a cytokine required for maturation of myeloid cells. Specifically, mar1Δ-infected lungs display a significant increase in GM-CSF production compared to WT-infected lungs at 3 DPI, an early timepoint in infection at which the pulmonary immune response is being actively developed (Figures 3A & S2). Despite the drastic reduction in mar1Δ mutant fungal burden at these early timepoints, the mar1Δ mutant strain induces a cytokine and cellular response comparable to that of the WT strain, likely due to the increased immunogenicity of the mar1Δ mutant cells.

As infection progressed to 7, 14, and 21 DPI, we observed marked reductions in multiple cytokines (including IL-1β, IL-4, and GM-CSF) and leukocytes (including CD45+ cells, alveolar macrophages [AM], and CD4+ T cells) in mar1Δ-infected lungs.
compared to WT-infected lungs (Figures 3A, 3B, S2, & S3). These observations
demonstrate that by these timepoints in infection, the overall cytokine and cellular
response is reduced in mar1Δ-infected lungs compared to WT-infected lungs, likely due
to the sustained reduction in fungal burden present in the mar1Δ-infected lungs. This is
further supported by our histopathological observations made at the same timepoints
demonstrating more localized regions of inflammation in mar1Δ-infected lungs than in
WT-infected lungs (Figure 1B). We further explored macrophage polarization at these
same timepoints to determine whether the reduction in mar1Δ mutant strain fungal
burden, and the subsequent reduction in the pulmonary immune response, are due to
differences in macrophage activation (36). At each tested timepoint (7, 14, and 21 DPI),
we observed that the mar1Δ-infected lungs have a comparable number of or fewer
classically-activated (M1) and alternatively-activated (M2) alveolar and interstitial
macrophages compared to WT-infected lungs (Figure 3C). These observations
demonstrate that the mar1Δ mutant strain does not induce differential macrophage
polarization that results in a reduction in fungal burden and a more protective immune
response. Collectively, these data suggest that mar1Δ-induced pulmonary granuloma
formation appears to be a largely fungal-driven phenomenon. Despite reductions in
fungal burden, the mar1Δ mutant strain induces a WT strain-like immune response early
in infection that results in fungal containment within granulomas during mid-late stages
of infection. As infection matures and progresses, there is a marked decrease in many
cytokines and leukocytes infiltrating the mar1Δ-infected lung that corresponds with the
sustained reduction in fungal burden.
Host GM-CSF signaling is required for pulmonary granuloma formation.

Granuloma formation is dependent on GM-CSF signaling in the context of both mycobacterial (24–26) and cryptococcal infections (17). GM-CSF is the only cytokine that showed significant differential production in our cytokine analyses. Specifically, we observed that the mar1Δ mutant strain induces more pulmonary GM-CSF production than the WT strain at 3 DPI (Figures 3A & S2). We therefore hypothesized that GM-CSF signaling would also be required for the formation of pulmonary granulomas in our model. To test this hypothesis, we assessed the progression of infections with the WT strain and the mar1Δ mutant strain in the Csf2rb−/− mouse background, which is defective in GM-CSF signaling due to loss of the functional GM-CSF receptor. We inoculated Csf2rb−/− mice using the inhalation route and harvested lungs for analysis throughout infection. Overall, a similar pattern of inflammation was observed between mice infected with the WT strain and mice infected with the mar1Δ mutant strain. We observed that putative pulmonary granulomas are absent in Csf2rb−/− mice infected with either strain at every tested timepoint (3, 7, and 14 DPI) (Figures 4A & S1). Instead, inflammation appears unorganized and diffuse throughout the entirety of the lungs infected with either fungal strain. Contrasting with the C57BL/6 infections, the Csf2rb−/− infections appear to be characterized by fewer macrophages, which is expected based on previous work that demonstrated that GM-CSF is required for macrophage recruitment to the lung during early cryptococcal infection (17) (Figures 4A & S1). Like the C57BL/6 infections, however, WT fungal cells are abundant throughout the lung, many with signs of titanization, while mar1Δ mutant fungal cells are infrequently observed (Figures 4A & S1). Pulmonary fungal burden assessed at 3 DPI confirms that mar1Δ-infected lungs
have a significantly lower fungal burden, with a 10-fold reduction compared to WT-infected lungs, similar to what was observed in the C57BL/6 infections (Figure 4B). These data demonstrate that GM-CSF signaling is required for granuloma formation in both WT strain and mar1Δ mutant strain infections. However, because loss of GM-CSF signaling does not rescue the reduction of mar1Δ mutant strain fungal burden during early stages of infection, these data also suggest that GM-CSF signaling does not exclusively drive the impaired fitness of mar1Δ mutant cells in the murine lung.

The mar1Δ mutant strain is attenuated in the employment of various virulence factors.

In our fungal burden assays, we observed a modest increase in mar1Δ mutant strain fungal burden as infection progressed from 3 to 21 DPI (Figure 2). Despite this, we find that mar1Δ-infected mice can remain healthy-looking and survive to at least 100 DPI. Furthermore, viable mar1Δ mutant cells that retain previously reported mar1Δ mutant phenotypes, including dry colony morphology on alkaline pH and nourseothricin (NAT) resistance (23), can be recovered from the lung at extended timepoints in infection (61 and 100 DPI) (Figure S4). These observations indicate that the mar1Δ mutant strain can persist within murine lung granulomas for extended periods of time without causing any symptoms or signs of disease. Based on this observation, we sought to understand the mechanism by which the mar1Δ mutant strain can survive and persist in the mouse lung.

In both human and murine infections, a subset of cryptococcal cells form enlarged Titan cells, an important virulence factor that enables cryptococcal persistence.
in the lungs (35, 37). Using an established in vitro titanization assay (30), we observed that the mar1Δ mutant strain is unable to form Titan cells (Figure 5A). This observation supports our histopathology experiments, in which Titan cells were absent in mar1Δ-infected lungs, collectively demonstrating that Titan cell formation does not explain the persistence of mar1Δ mutant cells within granulomas.

We previously reported that the mar1Δ mutant strain is impaired in the implementation of the polysaccharide capsule, assessed by India ink staining (23). We utilized high-resolution scanning electron microscopy (SEM) to more rigorously study the mar1Δ mutant strain capsule architecture. In permissive growth conditions (YPD medium, 30°C), the capsule of the mar1Δ mutant strain is nearly indistinguishable from that of the WT strain, which contrasts starkly with the acapsular cap59Δ mutant strain (Figure 5B). However, in capsule-inducing conditions (TC medium, 37°C), the mar1Δ mutant strain lacks the degree of capsule fiber elongation observed in the WT strain, explaining the reduction in India ink exclusion previously reported for the mar1Δ mutant strain (23) (Figure 5B). The inability of the mar1Δ mutant strain to employ these two important virulence factors, Titan cells and polysaccharide capsule, likely drive the hyper-immunogenicity observed in our murine infection studies.

The mar1Δ mutant strain displays cell cycle defects that result in a slow growth phenotype and hypoxia resistance.

Both Titan cell formation (30, 38) and polysaccharide capsule elaboration (39–41) are known to be mediated by the cell cycle, suggesting that the mar1Δ mutant strain may be unable to properly employ these virulence factors due to defects in cell cycle
progression. To explore cell cycle progression in the mar1Δ mutant strain background, we observed mar1Δ mutant cell morphology during logarithmic growth phase. When incubated at the permissive temperature of 30°C, the mar1Δ mutant strain displays an increased incidence of cytokinesis defects (such as elongated cells, cells with wide bud necks, and cells that fail to complete cytokinesis), compared to both the WT strain and the mar1Δ + MAR1 complemented strain (Figure 6A). The frequency of these cytokinesis defects is significantly enhanced at the physiological temperature of 37°C (Figure 6A). We next determined the impact of these defects on the growth kinetics of the mar1Δ mutant strain. We observed that the mar1Δ mutant strain displays a reduction in growth during logarithmic phase at 37°C, compared to both the WT strain and the mar1Δ + MAR1 complemented strain (Figure 6B). These data demonstrate that the mar1Δ mutant strain has a slow growth phenotype at the physiological temperature of 37°C that is likely driven in part by cytokinesis defects.

Cell cycle regulation is also known to be related to fungal adaptation to hypoxia (42–44). Because C. neoformans is an obligate aerobe, WT fungal cells undergo G2-arrest in response to hypoxia (45, 46). We assessed the ability of the mar1Δ mutant strain to grow in an environment with reduced oxygen availability by observing growth in the presence of CoCl₂ and in a microaerophilic chamber. In both cases, we observed that the mar1Δ mutant strain displays enhanced growth compared to the WT strain and the mar1Δ + MAR1 complemented strain (Figure 6C). In these assays, the CoCl₂- and hypoxia-sensitive sre1Δ mutant strain was used as a control (31) (Figure 6C). Collectively, these observations suggest that the cell cycle defects of the mar1Δ mutant
strain may contribute to its ability to survive, slowly proliferate, and persist in the murine granuloma environment.

**DISCUSSION**

Here, we report and characterize the host response to a chronic and indolent *C. neoformans* lung infection, one distinguished by sustained granulomas. Using the inhalation route of infection in C57BL/6 mice, we observe granuloma formation in infections due to both the WT and *mar1Δ* mutant strains. However, the appearance, development, and maintenance of these granulomas differ significantly. In WT infections, small, immature granulomas form early in infection. As infection progresses, these nascent granulomas begin to degenerate, leading to fungal proliferation throughout the lungs, fungal dissemination to the brain, and eventually murine death. This type of early, immature granuloma formation has been observed previously in murine infections with other *C. neoformans* WT strains (16, 17). In contrast, in *mar1Δ* mutant strain infections we observe mature pulmonary granulomas that develop over several weeks in the absence of overt clinical symptoms. These granulomas differ from the WT-induced granulomas because they appear later in infection, are typically larger, and are more contained. The containment of these granulomas may be expected because *mar1Δ*-induced granulomas are associated with a significantly lower fungal burden compared to WT strain infections, suggesting that the granulomas effectively inhibit fungal proliferation throughout the lungs. Despite this drastic reduction in fungal burden, the *mar1Δ* mutant strain induces a comparable pulmonary cytokine and leukocyte response to that of the WT strain during early stages of infection. Previous
work reported by our group characterized the mar1Δ mutant strain as more immunogenic than the WT strain, due to its poorly organized cell wall and impaired polysaccharide capsule attachment (23). We posit that the combination of reduced fungal burden and increased immunogenicity drives mar1Δ-induced granuloma formation: the increased immunogenicity results in an immune response that contains the reduced number of mar1Δ mutant cells within granulomas during early stages in infection.

We further observe that mar1Δ-induced granulomas are maintained throughout infection, from 14 DPI to as late as 100 DPI. We find that the immune microenvironment associated with these granulomas has significantly reduced cytokine and leukocyte responses. Previous work has implicated classically-activated macrophage polarization in enhanced antifungal activity of macrophages (47–49). We find that mar1Δ-infected lungs have a comparable number of or fewer (depending on the timepoint) classically-activated (M1) and alternatively-activated (M2) macrophages compared to WT-infected lungs, suggesting that differential polarization of macrophages does not contribute to the reduced fungal burden and associated immune response in mar1Δ-infected lungs. Collectively, these observations demonstrate that mar1Δ-induced granulomas are largely a fungal-driven phenomenon, with the sustained reduction in mar1Δ mutant strain fungal burden resulting in a dampened immune response compared with WT-infected lungs. Using these approaches, we have defined a detailed timeline of granuloma formation, in both WT and mar1Δ mutant strain infections, and characterized multiple fungal factors that contribute to granuloma formation (Figure S5).
In addition to the fungal drivers of \( mar1\Delta \)-induced granuloma formation described above, we have also confirmed the role of GM-CSF as a host driver of cryptococcal granuloma formation. From our pulmonary cytokine analyses, we observed that GM-CSF is the only differentially produced cytokine in \( mar1\Delta \)-infected lungs compared to WT-infected lungs. Specifically, GM-CSF is elevated in \( mar1\Delta \)-infected lungs at 3 DPI, an early timepoint in infection at which the pulmonary immune response is being actively developed. This increased GM-CSF production may be a result of increased Dectin-1 activation by the \( mar1\Delta \) mutant strain. We previously reported that the \( mar1\Delta \) mutant strain is partially recognized by the pathogen recognition receptor Dectin-1, likely due its increased exposed surface \( \beta \)-glucan and chitin (23). Dectin-1 has been shown to be required for normal GM-CSF production in murine macrophages (50). Additionally, GM-CSF production is known to result in an increase in Dectin-1 expression by murine macrophages (50, 51). We also report that granuloma formation is dependent on GM-CSF signaling, as granulomas are absent in Csf2rb\(^{-}\) mouse background infections with either the WT or \( mar1\Delta \) mutant strains. These results are expected because GM-CSF plays a significant role in both \( C. gattii \) and \( C. neoformans \) infections, as individuals with GM-CSF autoantibodies are unusually susceptible to cryptococcal infection (52–54). Furthermore, previous work in both mycobacterial (24–26) and cryptococcal infections (17) has demonstrated that GM-CSF signaling is required for granuloma formation, likely due to its requirement for macrophage recruitment to the lung during early stages of infection. Our model enables further exploration of the requirement of GM-CSF for granuloma maintenance. For example, future experiments can introduce GM-CSF antibodies into \( mar1\Delta \)-infected mice to
determine whether GM-CSF is required for mar1Δ-induced granuloma maintenance and control of infection. Furthermore, WT strain infections can be supplemented with exogenous GM-CSF to determine whether increased GM-CSF can help maintain WT-induced granulomas.

Despite the reduced fungal burden of the mar1Δ mutant strain compared to the WT strain, the mar1Δ mutant strain persists in the murine lung long-term, up to 100 DPI. Titan cell formation is a well-characterized persistence mechanism that is specific to Cryptococcus species (35, 37). Results from an established in vitro titanization assay (30) in combination with our histopathological observations demonstrate that the mar1Δ mutant strain is unable to form Titan cells, and as a result, Titan cells do not explain the persistence of the mar1Δ mutant strain in the murine lung. We also observed that the mar1Δ mutant strain is attenuated in the implementation of another important virulence factor, the polysaccharide capsule. Although the mar1Δ mutant strain has a similar basal level of capsule to the WT strain, it is unable to extend its capsule to the level of the WT strain in response to capsule-inducing signals.

The expression of many virulence factors is known to be mediated by the cell cycle (41). Furthermore, recent work has proposed that C. neoformans undergoes a unique cell cycle in vivo, the “stress cell cycle”, that regulates the employment of various virulence factors (55). Titan cells are polyploid cryptococcal cells that form in both human and mouse lungs during infection (35, 37). This polyploidization and concomitant cell body enlargement is negatively regulated by the transcription factor Usv101, which acts downstream of the cell cycle regulator Swi6 (30, 38). Furthermore, recent work has found that the cyclin, Cln1, contributes to Titan cell formation by regulating DNA
replication and cell division after G$_2$-arrest in vivo (55). Similarly, capsule elongation is also regulated by the cell cycle, with the majority of capsule elongation occurring in G$_1$ phase of the cell cycle (40). The dysregulation of these cell cycle-mediated virulence factors suggests that the mar1$\Delta$ mutant strain harbors cell cycle defects.

We indeed observed that the mar1$\Delta$ mutant strain displays a marked increase in cytokinesis defects compared to the WT strain, at both 30°C and 37°C, leading to a decreased growth rate. In various cell types, including stem cells (56), tumor cells (57), bacteria (58), and fungi (43), a reduction in growth rate is required for survival in the presence of hypoxia. It is possible that its inherent decreased growth rate predisposes the mar1$\Delta$ mutant strain to growth in a hypoxic environment. The mammalian environment is known to limit oxygen availability to invading microorganisms, as a stressor used to contain microbial proliferation (42). This important resource is likely even further restricted within the pulmonary granuloma, which is known to have suboptimal oxygen levels in the context of mycobacterial infection (59). Recent work by the Alanio laboratory has demonstrated that cryptococcal dormancy can be induced by a combination of nutrient and oxygen deprivation (44, 60). Furthermore, the Dromer laboratory has found that dormant cryptococcal cells are characterized by reduced metabolic activity and delayed growth (43). With these observations in mind, it is possible that the slow growth and hypoxia resistance phenotypes of the mar1$\Delta$ mutant strain enable its survival and persistence within granulomas in the model described here. Further work will be required to determine whether these phenotypes are necessary and/or sufficient for fungal survival and persistence within granulomas.
The Del Poeta laboratory has developed the most well-characterized murine pulmonary granuloma model of cryptococcal disease to date using the $gcs1$$\Delta$ mutant strain. From the fungal perspective, the $gcs1$$\Delta$ mutant strain lacks the membrane sphingolipid glucosylceramide, making it an obligate intracellular pathogen and, as a result, completely avirulent in a murine inhalation model, the route of infection that most closely replicates the course of human infection (18, 19). It is noteworthy that both the $gcs1$$\Delta$ mutant strain and the $mar1$$\Delta$ mutant strain are constructed in the same WT strain background, and as a result, these two mutant strains are comparable and can potentially be used together to explore the complex characteristics of granuloma formation. For example, both strains display cell cycle defects in the presence of physiological stress: the $gcs1$$\Delta$ mutant strain arrests at alkaline pH (18) and the $mar1$$\Delta$ mutant strain displays cytokinesis defects at 37°C. These similarities suggest that a slow growth phenotype in the host environment may favor fungal containment with granulomas. Virulence potential is a notable difference between the strains. The $gcs1$$\Delta$ mutant strain is unable to initiate infection and disease via the inhalation route of infection (18), categorizing $GCS1$ as a disease initiation factor (61). In contrast, the $mar1$$\Delta$ mutant strain can establish infection and cause fatal disease in nearly half of the infected mice (23), making $MAR1$ a disease progression factor (61). This may be related to the fact that $GCS1$ orthologs are found in many pathogenic fungi (18), while $MAR1$ appears to be a Cryptococcus-specific gene (23). These contrasting features suggest that granuloma formation is a highly complex process that relies on the interplay between many fungal and host factors.
From the host perspective, \( gcs1\Delta \)-induced granuloma formation requires host sphingosine kinase 1-sphingosine 1-phosphate (SK1-S1P) signaling (20, 21). Most recently, the Del Poeta laboratory has applied this model to explore cryptococcal reactivation. Mimicking human disease, \( gcs1\Delta \) mutant cells become reactivated from granulomas and disseminate upon immunosuppression with the multiple sclerosis therapeutic FTY720, which suppresses SK1-S1P signaling (22). This model has enabled the first murine reactivation studies of cryptococcal infection. Future work with \( mar1\Delta \)-induced granulomas can similarly explore reactivation in the context of immunosuppression, to better understand the typical course of cryptococcal disease in humans. One of the populations most vulnerable to cryptococcal reactivation includes untreated HIV/AIDS patients (7). In our leukocyte infiltrate analyses, we observed that \( mar1\Delta \)-infected lungs have an enhanced CD4+ T cell response compared to WT-infected lungs at 21 DPI. This observation is particularly striking because \( mar1\Delta \)-infected lungs have a decreased or equivalent response compared to WT-infected lungs for all other leukocytes tested at this same timepoint. CD4+ T cells are present in pulmonary granulomas of immunocompetent individuals (62). Furthermore, CD4+ T cells border the periphery of pulmonary granulomas in HIV+ individuals receiving antiretroviral therapy, but they are lost in individuals with advanced HIV/AIDS, suggesting that CD4+ lymphocytes may be involved in granuloma maintenance (2, 62). By inducing CD4+ T cell depletion, and as a result mimicking the HIV/AIDS disease state, we can probe the role of CD4+ T cells in the maintenance of granulomas in this model. Following immunosuppression, we can observe \( mar1\Delta \)-infected mice to track granuloma breakdown and fungal proliferation with the same approaches used here.
Considering both the fungal and host drivers of granuloma formation outlined here, this model harbors features that make it unique from other existing cryptococcal granuloma models.

ACKNOWLEDGEMENTS

We thank the Duke University School of Medicine for the use of the Research Immunohistochemistry Laboratory Shared Resource, which prepared all histopathology samples. We thank Dr. Joseph Heitman and Anna Floyd-Averette for providing the Csf2rb-/- mice. Flow cytometry was performed in the Flow Cytometry and Laser Capture Microdissection Core Facility at The University of North Texas Health Science Center (UNTHSC) (which is supported by National Institutes of Health award ISI0R018999-01A1) and the Cell Analysis Core at The University of Texas at San Antonio. Scanning electron microscopy was performed at the Chapel Hill Analytical and Nanofabrication Laboratory, CHANL, a member of the North Carolina Research Triangle Nanotechnology Network, RTNN, which is supported by the National Science Foundation, Grant ECCS-1542015, as part of the National Nanotechnology Coordinated Infrastructure, NNCI. This work was supported by R01 AI074677 from the National Institutes of Health to JAA and FLW.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

REFERENCES
1. Adams DO. 1976. The granulomatous inflammatory response. A review. *Am J Pathol* 84:164–191.

2. Shibuya K, Hirata A, Omuta J, Sugamata M, Katori S, Saito N, Murata N, Morita A, Takahashi K, Hasegawa C, Mitsuda A, Hatori T, Nonaka H. 2005. Granuloma and cryptococcosis. *J Infect Chemother* 11:115–122.

3. Ramakrishnan L. 2012. Revisiting the role of the granuloma in tuberculosis. *Nat Rev Immunol* 12:352–366.

4. Ristow LC, Davis JM. 2021. The granuloma in cryptococcal disease. *PLoS Pathog* 17:e1009342.

5. Oehlers SH, Cronan MR, Scott NR, Thomas MI, Okuda KS, Walton EM, Beerman RW, Crosier PS, Tobin DM. 2015. Interception of host angiogenic signalling limits mycobacterial growth. *Nature* 517:612–615.

6. Zumla A, James DG. 1996. Granulomatous infections: etiology and classification. *Clin Infect Dis* 23:146–158.

7. Rajasingham R, Smith RM, Park BJ, Jarvis JN, Govender NP, Chiller TM, Denning DW, Loyse A, Boulware DR. 2017. Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. *Lancet Infect Dis* 17:873–881.

8. Goldman DL, Khine H, Abadi J, Lindenberg DJ, Priofski LA, Niang R, Casadevall A. 2001. Serologic evidence for *Cryptococcus neoformans* infection in early childhood. *Pediatrics* 107:E66.

9. McDonnell JM, Hutchins GM. 1985. Pulmonary cryptococcosis. *Hum Pathol* 16:121–128.
10. Warr W, Bates JH, Stone A. 1968. The spectrum of pulmonary cryptococciosis. *Ann Intern Med* 69:1109–1116.

11. Grebenciucova E, Reder AT, Bernard JT. 2016. Immunologic mechanisms of fingolimod and the role of immunosenescence in the risk of cryptococcal infection: a case report and review of literature. *Mult Scler Relat Disord* 9:158–162.

12. Normile TG, Bryan AM, Poeta MD. 2020. Animal models of *Cryptococcus neoformans* in identifying immune parameters associated with primary infection and reactivation of latent infection. *Front Immunol* 11:581750.

13. Perfect JR, Lang SD, Durack DT. 1980. Chronic cryptococcal meningitis: a new experimental model in rabbits. *Am J Pathol* 101:177–194.

14. Goldman D, Cho Y, Zhao M, Casadevall A, Lee SC. 1996. Expression of inducible nitric oxide synthase in rat pulmonary *Cryptococcus neoformans* granulomas. *Am J Pathol* 148:1275–1282.

15. Kobayashi M, Ito M, Sano K, Koyama M. 2000. Granulomatous and cytokine responses to pulmonary *Cryptococcus neoformans* in two strains of rats. *Mycopathologia* 151:121–130.

16. Feldmesser M, Casadevall A, Kress Y, Spira G, Orlofsky A. 1997. Eosinophil-*Cryptococcus neoformans* interactions *in vivo* and *in vitro*. *Infect Immun* 65:1899–1907.

17. Chen GH, Olszewski MA, Mcdonald RA, Wells JC, Paine R, Huffnagle GB, Toews GB. 2007. Role of granulocyte macrophage colony-stimulating factor in host defense against pulmonary *Cryptococcus neoformans* infection during murine allergic bronchopulmonary mycosis. *Am J Pathol* 170:1028–1040.
18. Rittershaus PC, Kechichian TB, Allegood JC, Merrill AH, Hennig M, Luberto C, Poeta MD. 2006. Glucosylceramide synthase is an essential regulator of pathogenicity of Cryptococcus neoformans. J Clin Invest 116:1651–1659.

19. Kechichian TB, Shea J, Poeta MD. 2007. Depletion of alveolar macrophages decreases the dissemination of a glucosylceramide-deficient mutant of Cryptococcus neoformans in immunodeficient mice. Infect Immun 75:4792–4798.

20. McQuiston T, Luberto C, Poeta MD. 2010. Role of host sphingosine kinase 1 in the lung response against cryptococcosis. Infect Immun 78:2342–2352.

21. Farnoud AM, Bryan AM, Kechichian T, Luberto C, Poeta MD. 2015. The granuloma response controlling cryptococcosis in mice depends on the sphingosine kinase 1–sphingosine 1-phosphate pathway. Infect Immun 83:2705–2713.

22. Bryan AM, You JK, McQuiston T, Lazzarini C, Qiu Z, Sheridan B, Nuesslein-Hildesheim B, Poeta MD. 2020. FTY720 reactivates cryptococcal granulomas in mice through S1P receptor 3 on macrophages. J Clin Invest 130:4546–4560.

23. Esher SK, Ost KS, Kohlbrenner MA, Pianalto KM, Telzrow CL, Campuzano A, Nichols CB, Munro C, Wormley FL, Alspaugh JA. 2018. Defects in intracellular trafficking of fungal cell wall synthases lead to aberrant host immune recognition. PLoS Pathog 14:e1007126.

24. Gonzalez-Juarrero M, Hattle JM, Izzo A, Junqueira-Kipnis AP, Shim TS, Trapnell BC, Cooper AM, Orme IM. 2005. Disruption of granulocyte macrophage-colony stimulating factor production in the lungs severely affects the ability of mice to control Mycobacterium tuberculosis infection. J Leukoc Biol 77:914–922.
25. Szeliga J, Daniel DS, Yang CH, Sever-Chroneos Z, Jagannath C, Chroneos ZC. 2008. Granulocyte-macrophage colony stimulating factor-mediated innate responses in tuberculosis. *Tuberculosis* 88:7–20.

26. Benmerzoug S, Marinho FV, Rose S, Mackowiak C, Gosset D, Sedda D, Poisson E, Uyttenhove C, Van Snick J, Jacobs M, Garcia I, Ryffel B, Quesniaux VFJ. 2018. GM-CSF targeted immunomodulation affects host response to *M. tuberculosis* infection. *Sci Rep* 8:8652.

27. Cox GM, Mukherjee J, Cole GT, Casadevall A, Perfect JR. 2000. Urease as a virulence factor in experimental cryptococciosis. *Infect Immun* 68:443–448.

28. Leopold Wager C, Hole CR, Wozniak KL, Olszewski MA, Mueller M, Wormley FL. 2015. STAT1 signaling within macrophages is required for antifungal activity against *Cryptococcus neoformans*. *Infect Immun* 83:4513–4527.

29. Hole CR, Leopold Wager CM, Castro-Lopez N, Campuzano A, Cai H, Wozniak KL, Wang Y, Wormley FL. 2019. Induction of memory-like dendritic cell responses in vivo. *Nat Commun* 10:2955.

30. Dambuza IM, Drake T, Chapuis A, Zhou X, Correia J, Taylor-Smith L, LeGrave N, Rasmussen T, Fisher MC, Bicanic T, Harrison TS, Jaspars M, May RC, Brown GD, Yuecel R, MacCallum DM, Ballou ER. 2018. The *Cryptococcus neoformans* Titan cell is an inducible and regulated morphotype underlying pathogenesis. *PLoS Pathog* 14:e1006978.

31. Brown HE, Telzrow CL, Saelens JW, Fernandes L, Alspaugh JA. 2020. Sterol-response pathways mediate alkaline survival in diverse fungi. *mBio* 11:e00719-20.
32. Al-Bader N, Vanier G, Liu H, Gravelat FN, Urb M, Hoareau CMQ, Campoli P, Chabot J, Filler SG, Sheppard DC. 2010. Role of trehalose biosynthesis in *Aspergillus fumigatus* development, stress response, and virulence. *Infect Immun* 78:3007–3018.

33. Ost KS, O’Meara TR, Huda N, Esher SK, Alspaugh JA. 2015. The *Cryptococcus neoformans* alkaline response pathway: identification of a novel Rim pathway activator. *PLoS Genet* 11:e1005159.

34. Zacharias CA, Sheppard DC. 2019. The role of *Aspergillus fumigatus* polysaccharides in host–pathogen interactions. *Curr Opin Microbiol* 52:20–26.

35. Zaragoza O, García-Rodas R, Nosanchuk JD, Cuenca-Estrella M, Rodríguez-Tudela JL, Casadevall A. 2010. Fungal cell gigantism during mammalian infection. *PLoS Pathog* 6:e1000945.

36. McQuiston TJ, Williamson PR. 2012. Paradoxical roles of alveolar macrophages in the host response to *Cryptococcus neoformans*. *J Infect Chemother* 18:1–9.

37. Cruickshank JG, Cavill R, Jelbert M. 1973. *Cryptococcus neoformans* of unusual morphology. *Appl Microbiol* 25:309–312.

38. Hommel B, Mukaremera L, Cordero RJB, Coelho C, Desjardins CA, Sturny-Leclère A, Janbon G, Perfect JR, Fraser JA, Casadevall A, Cuomo CA, Dromer F, Nielsen K, Alanio A. 2018. Titan cells formation in *Cryptococcus neoformans* is finely tuned by environmental conditions and modulated by positive and negative genetic regulators. *PLoS Pathog* 14:e1006982.

39. Zaragoza O, Telzak A, Bryan RA, Dadachova E, Casadevall A. 2006. The polysaccharide capsule of the pathogenic fungus *Cryptococcus neoformans*
enlarges by distal growth and is rearranged during budding. *Mol Microbiol* 59:67–83.

40. García-Rodas R, Cordero RJB, Trevijano-Contador N, Janbon G, Moyrand F, Casadevall A, Zaragoza O. 2014. Capsule growth in *Cryptococcus neoformans* Is coordinated with cell cycle progression. *mBio* 5:e00945-14.

41. Kelliher CM, Leman AR, Sierra CS, Haase SB. 2016. Investigating conservation of the cell-cycle-regulated transcriptional program in the fungal pathogen, *Cryptococcus neoformans*. *PLoS Genet* 12:e1006453.

42. Grahl N, Shepardson KM, Chung D, Cramer RA. 2012. Hypoxia and fungal pathogenesis: to air or not to air? *Eukaryot Cell* 11:560–570.

43. Alanio A, Vernel-Pauillac F, Sturny-Leclère A, Dromer F. 2015. *Cryptococcus neoformans* host adaptation: toward biological evidence of dormancy. *mBio* 6:e02580-14.

44. Hommel B, Sturny-Leclère A, Volant S, Veluppillai N, Duchateau M, Yu CH, Hourdel V, Varet H, Matondo M, Perfect JR, Casadevall A, Dromer F, Alanio A. 2019. *Cryptococcus neoformans* resists to drastic conditions by switching to viable but non-culturable cell phenotype. *PLoS Pathog* 15:e1007945.

45. Ohkusu M, Raclavsky V, Takeo K. 2001. Deficit in oxygen causes G₂ budding and unbudded G₂ arrest in *Cryptococcus neoformans*. *FEMS Microbiol Lett* 204:29–32.

46. Ohkusu M, Raclavsky V, Takeo K. 2004. Induced synchrony in *Cryptococcus neoformans* after release from G₂-arrest. *Antonie Van Leeuwenhoek* 85:37–44.

47. Arora S, Hernandez Y, Erb-Downward JR, McDonald RA, Toews GB, Huffnagle
GB. 2005. Role of IFN-gamma in regulating T<sub>2</sub> immunity and the development of alternatively activated macrophages during allergic bronchopulmonary mycosis. *J Immunol* 174:6346–6356.

48. Hardison SE, Ravi S, Wozniak KL, Young ML, Olszewski MA, Wormley FL. 2010. Pulmonary infection with an interferon-gamma-producing *Cryptococcus neoformans* strain results in classical macrophage activation and protection. *Am J Pathol* 176:774–785.

49. Leopold Wager CM, Wormley FL. 2014. Classical versus alternative macrophage activation: the Ying and the Yang in host defense against pulmonary fungal infections. *Mucosal Immunol* 7:1023–1035.

50. Walachowski S, Tabouret G, Fabre M, Foucras G. 2017. Molecular analysis of a short-term model of β-glucans-trained immunity highlights the accessory contribution of GM-CSF in priming mouse macrophages response. *Front Immunol* 8:1089.

51. Williment JA, Lin HH, Reid DM, Taylor PR, Williams DL, Wong SYC, Gordon S, Brown GD. 2003. Dectin-1 expression and function are enhanced on alternatively activated and GM-CSF-treated macrophages and are negatively regulated by IL-10, dexamethasone, and lipopolysaccharide. *J Immunol* 171:4569–4573.

52. Rosen LB, Freeman AF, Yang LM, Jutivorakool K, Olivier KN, Angkasekwinai N, Suputtamongkol Y, Bennett JE, Pyrgos V, Williamson PR, Ding L, Holland SM, Browne SK. 2013. Anti-GM-CSF autoantibodies in patients with cryptococcal meningitis. *J Immunol* 190:3959–3966.

53. Saijo T, Chen J, Chen SCA, Rosen LB, Yi J, Sorrell TC, Bennett JE, Holland SM,
Browne SK, Kwon-Chung KJ. 2014. Anti-granulocyte-macrophage colony-stimulating factor autoantibodies are a risk factor for central nervous system infection by Cryptococcus gattii in otherwise immunocompetent patients. mBio 5:e00912-14.

54. Viola GM, Malek AE, Rosen LB, DiNardo AR, Nishiguchi T, Okhuysen PC, Holland SM, Kontoyiannis DP. 2021. Disseminated cryptococcosis and anti-granulocyte-macrophage colony-stimulating factor autoantibodies: an underappreciated association. Mycoses 64:576–582.

55. Altamirano S, Li Z, Fu MS, Ding M, Fulton SR, Yoder JM, Tran V, Nielsen K. 2021. The cyclin Cln1 controls polyploid Titan cell formation following a stress-induced G2 arrest in Cryptococcus. mBio 12:e0250921.

56. Latil M, Rocheteau P, Châtre L, Sanulli S, Mémet S, Ricchetti M, Tajbakhsh S, Chrétien F. 2012. Skeletal muscle stem cells adopt a dormant cell state post mortem and retain regenerative capacity. Nat Commun 3:903.

57. Kinoshita M, Johnson DL, Shatney CH, Lee YL, Mochizuki H. 2001. Cancer cells surviving hypoxia obtain hypoxia resistance and maintain anti-apoptotic potential under reoxygenation. Int J Cancer 91:322–326.

58. Bagchi G, Das TK, Tyagi JS. Molecular analysis of the dormancy response in Mycobacterium smegmatis: expression analysis of genes encoding the DevR-DevS two-component system, Rv3134c and chaperone alpha-crystallin homologues. FEMS Microbiol Lett 211:231–237.

59. Qualls JE, Murray PJ. 2016. Immunometabolism within the tuberculosis granuloma: amino acids, hypoxia, and cellular respiration. Semin Immunopathol
60. Alanio A. 2020. Dormancy in Cryptococcus neoformans: 60 years of accumulating evidence. J Clin Invest 130:3353–3360.

61. Cramer RA, Kowalski CH. 2021. Is it time to kill the survival curve? A case for disease progression factors in microbial pathogenesis and host defense research. mBio 12:e03483-20.

62. Shibuya K, Coulson WF, Naoe S. 2002. Histopathology of deep-seated fungal infections and detailed examination of granulomatous response against cryptococci in patients with acquired immunodeficiency syndrome. Nihon Ishinkin Gakkai Zasshi 43:143–151.

63. O’Meara TR, Holmer SM, Selvig K, Dietrich F, Alspaugh JA. 2013. Cryptococcus neoformans Rim101 is associated with cell wall remodeling and evasion of the host immune responses. mBio 4:e00522-12.

FIGURE LEGENDS

Figure 1. Pulmonary granuloma formation in murine cryptococcal infections. A. Lung dissections of female C57BL/6 mice infected with the mar1Δ mutant strain were performed to display macroscopic lung pathology, specifically granulomas (white arrowheads). Cartoon adapted from BioRender.com (2021). B. The lungs of female C57BL/6 mice inoculated with 1 x 10⁴ cells of the WT strain or the mar1Δ mutant strain sacrificed at predetermined endpoints (3, 7, 14, and 40 DPI) were harvested for histopathological analyses. Hematoxylin and eosin staining were utilized to visualize microscopic lung pathology (fungal cells [yellow arrowheads], multinucleated giant cells
[yellow circle], epithelioid macrophages (yellow arrows), inset [yellow boxes]). 5x scale bar (left), 250 μm. 10x scale bar (right), 50 μm. C. Granuloma diameter (μm) was measured using FIJI. σ, standard deviation (μm). Gray box, no experimental subjects can be assessed at this timepoint.

Figure 2. Fungal burden throughout infection. Pulmonary fungal burden of female C57BL/6 mice \((n = 15)\) inoculated with \(1 \times 10^4\) cells of the WT strain or the \(mar1\Delta\) mutant strain was measured by quantitative cultures throughout infection: 3, 7, 14, and 21 DPI. Brain fungal burden of female C57BL/6 mice \((n = 15)\) inoculated with \(1 \times 10^4\) cells of the WT strain or the \(mar1\Delta\) mutant strain was measured by quantitative cultures at 21 DPI. Error bars represent standard error of the mean (SEM). Statistical significance was determined using Student's \(t\) test \((**, P < 0.001; ****, P < 0.0001; \text{ns, not significant})\).

Figure 3. Pulmonary cytokine profile and leukocyte infiltrate associated with granuloma formation. A. Pulmonary cytokine responses of female C57BL/6 mice inoculated with \(1 \times 10^4\) cells of the WT strain or the \(mar1\Delta\) mutant strain were measured using the Bio-Plex protein array system throughout infection: 1 \((n = 15)\), 3 \((n = 15)\), 7 \((n = 10)\), 14 \((n = 10)\), and 21 \((n = 10)\) DPI. Error bars represent SEM. Statistical significance between strains at each timepoint was determined using Student's \(t\) test \((*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; \text{no designation between strains, not significant})\). Only a subset of data is shown; refer to Figure S2 for full analysis. B. Pulmonary leukocyte infiltrates of female C57BL/6 mice inoculated with \(1 \times 10^4\) cells of
the WT strain or the *mar1*Δ mutant strain were measured by flow cytometry throughout infection: 1, 3, 7, and 21 DPI. Data shown are the mean ± of absolute cell numbers from three independent experiments (*n* = 3) performed using five mice per group per timepoint per experiment. Error bars represent SEM. Statistical significance between strains at each timepoint was determined using Student's *t* test (*, *P* < 0.05; no designation between strains, not significant). Only a subset of data is shown; refer to Figure S3 for full analysis. C. Pulmonary macrophage activation of female C57BL/6 mice (*n* = 3) inoculated with 1 x 10⁴ cells of the WT strain or the *mar1*Δ mutant strain were measured by flow cytometry throughout infection: 7, 14, and 21 DPI. Inducible nitrogen oxide synthase (iNOS) was used as a marker for M1 macrophages and Arginase 1 (Arg1) was used as a marker for M2 macrophages. The percentage of total iNOS+ cells and Arg1+ cells is shown. Error bars represent the SEM. Log transformation was used to normally distribute the data for statistical analysis. Statistical significance between strains at each timepoint was determined using Student's *t* test (*, *P* < 0.05; no designation between strains, not significant). AM = alveolar macrophage (CD45+, CD11b-). IM = interstitial macrophage (CD45+, CD11b+). Cartoons adapted from BioRender.com (2021).

**Figure 4. Contributions of GM-CSF signaling to pulmonary granuloma formation.**

A. The lungs of female (*n* = 2) (shown) and male (*n* = 2) (not shown) Csf2rb<sup>−/−</sup> mice inoculated with 1 x 10⁴ cells of the WT strain or the *mar1*Δ mutant strain sacrificed at predetermined endpoints (3, 7, and 14 DPI) were harvested for histopathological analyses. Hematoxylin and eosin staining were utilized to visualize microscopic lung
pathology (fungal cells [yellow arrowheads], inset [yellow boxes]). 5x scale bar (left),
250 μm. 10x scale bar (right), 50 μm. B. Pulmonary fungal burden of female (n = 2) and
male (n = 2) Csf2rb−/− mice inoculated with 1 x 10^4 cells of the WT strain or the mar1Δ
mutant strain sacrificed at 3 DPI was measured by quantitative cultures. Error bars
represent the SEM. Statistical significance was determined using Student’s t test (*, P <
0.05).

Figure 5. Cell cycle-mediated virulence factor phenotypes of the mar1Δ mutant
strain. A. Titan cell formation was induced in the WT strain, the mar1Δ mutant strain,
and the mar1Δ + MAR1 complemented strain. Cells were pre-grown in YNB medium at
30°C and an OD_{600} of 0.001 was transferred to 10% HI-FBS in PBS incubated at 5%
CO₂, 37°C for 96 hours. Cells were imaged by DIC microscopy (Zeiss Axio Imager A1).
Cell diameter was measured using FIJI, and cells with a diameter > 10 μm were
considered Titan cells (red arrowheads). The number of Titan cells per 10,000 cells was
calculated for each strain. A minimum of 400 cells were analyzed across three biological
replicates (n = 3). Error bars represent the SEM. Statistical significance was determined
using a one-way ANOVA (*, P < 0.05; ns, not significant). 63x scale bar, 10 μm. B. The
WT strain, the mar1Δ mutant strain, the mar1Δ + MAR1 complemented strain, and the
cap59Δ mutant strain were incubated in YPD medium at 30°C and CO₂-independent
medium (TC) at 37°C until saturation. Samples were subsequently fixed, mounted,
dehydrated, and sputter-coated. Samples were imaged with a Hitachi S-4700 scanning
electron microscope to visualize capsule organization and elaboration.
Figure 6. Slow growth phenotypes of the mar1Δ mutant strain. A. Morphological
defects were analyzed in the WT strain, the mar1Δ mutant strain, and the mar1Δ +
MAR1 complemented strain through incubation in YPD medium at either 30°C or 37°C.
Cells were imaged by DIC microscopy (Zeiss Axio Imager A1) and were subsequently
visually inspected for morphological defects, such as elongated cells (red squares),
wide bud necks (red arrowhead), and cytokinesis failure (red circle). The percentage of
total cells displaying morphological defects was quantified for each strain at each
temperature. A minimum of 500 cells were analyzed across three biological replicates
(n = 3). Error bars represent the SEM. Log transformation was used to normally
distribute the data for statistical analysis (two-way ANOVA; *, P < 0.05; **, P < 0.01; ns,
not significant). 63x scale bar, 10 μm. B. Growth of the WT strain, the mar1Δ mutant
strain, and the mar1Δ + MAR1 complemented strain was assessed in YPD medium at
37°C. Growth was tracked for 40 hours and was measured by absorbance at OD_{600}.
Figure summarizes data across three biological replicates (n = 3). Error bars represent
the SEM. C. Hypoxia resistance was assessed by growth on YES medium in the
presence of CoCl₂ (0.7 mM) and in a microaerophilic chamber. Serial dilutions of the WT
strain, the mar1Δ mutant strain, the mar1Δ + MAR1 complemented strain, and the sre1Δ
mutant strain were spotted onto agar plates and incubated at 30°C. Results were
compared to the same strains grown in ambient air conditions.

Table 1. Fungal strains used in this study.

| Strain | Genotype            | Source |
|--------|---------------------|--------|
| H99    | MATα                | (13)   |
| MAK1   | MATα mar1Δ::NAT     | (23)   |
**Figure S1. Additional histopathology granuloma images.** A. Medium power image from a WT-infected C57BL/6 mouse at 7 DPI, demonstrating a moderate peribronchiolar neutrophilic and mononuclear inflammatory reaction with vague, early, and poorly formed granulomata formation (10X). B. Low power image from a mar1Δ-infected C57BL/6 mouse at 3 DPI, demonstrating an absence of a significant inflammatory reaction (4X). C, D. Low power image (C) from a mar1Δ-infected C57BL/6 mouse at 40 DPI showing a relatively well-circumscribed nodule containing well developed organizing lymphohistiocytic inflammation and medium power view (D) highlighting compact histiocytic aggregates and peripheral mononuclear cells, characteristic of granuloma formation (C; 4X, D; 10X). E. Medium power image from a WT-infected Csf2rb−/− mouse at 7 DPI showing a marked peribronchiolar neutrophilic and mononuclear inflammatory reaction without granuloma formation (10X). F. Low power image from a WT-infected Csf2rb−/− mouse at 14 DPI demonstrating an absence of a significant inflammatory reaction (4X). All images are of hematoxylin- and eosin-stained tissue sections.

**Figure S2. Complete pulmonary cytokine profile throughout infection.** Pulmonary cytokine responses of female C57BL/6 mice inoculated with 1 x 10⁴ cells of the WT strain or the mar1Δ mutant strain were measured using the Bio-Plex protein array system throughout infection: 1 (n = 15), 3 (n = 15), 7 (n = 10), 14 (n = 10), and 21 (n =
1036) DPI. Error bars represent SEM. Statistical significance between strains at each
timepoint was determined using Student's t test (*, P < 0.05; **, P < 0.01; ***, P < 0.001;
****, P < 0.0001; no designation between strains, not significant).

Figure S3. Complete pulmonary leukocyte infiltrate response throughout
infection. Pulmonary immune cell infiltrates of female C57BL/6 mice inoculated with 1 x
10^4 cells of the WT strain or the mar1Δ mutant strain were measured by flow cytometry
throughout infection: 1, 3, 7, 14, and 21 DPI. Data shown are the mean ± of absolute
cell numbers from three independent experiments (n = 3) performed using five mice per
group per timepoint per experiment. Error bars represent the SEM. Statistical
significance between strains at each timepoint was determined using Student's t test (*,
P < 0.05; **, P < 0.01; no designation between strains, not significant).

Figure S4. Recovery of mar1Δ mutant cells from murine lungs at extended
timepoints in infection. Lungs from female C57BL/6 mice infected with the mar1Δ
mutant strain were harvested at 61 and 100 DPI. Single fungal colonies were isolated
on YPD agar plates and subsequently incubated in various conditions that allowed for
identification of mar1Δ mutant isolates: YPD medium at 30°C, YPD medium at 37°C,
YPD medium + nourseothricin (NAT), and YPD medium pH 8.15. The original WT strain
(A1) and mar1Δ mutant strain (A2) are included as controls in each condition.

Figure S5. Granuloma formation and maintenance timeline. Chronological summary
of important observations about granuloma formation and maintenance in the WT strain
(top) and mar1Δ mutant strain (bottom) backgrounds. Cartoons adapted from BioRender.com (2021).
Figure 1. Pulmonary granuloma formation in murine cryptococcal infections. A. Lung dissections of female C57BL/6 mice infected with the mar1Δ mutant strain were performed to display macroscopic lung pathology, specifically granulomas (white arrowheads). Cartoon adapted from BioRender.com (2021). B. The lungs of female C57BL/6 mice inoculated with 1 x 10^4 cells of the WT strain or the mar1Δ mutant strain sacrificed at predetermined endpoints (3, 7, 14, and 40 DPI) were harvested for histopathological analyses. Hematoxylin and eosin staining were utilized to visualize microscopic lung pathology (fungal cells [yellow arrowheads], multinucleated giant cells [yellow circle], epithelioid macrophages [yellow arrows], inset [yellow boxes]). 5x scale bar (left), 250 μm. 10x scale bar (right), 50 μm. C. Granuloma diameter (μm) was measured using FIJI. σ, standard deviation (μm). Gray box, no experimental subjects can be assessed at this timepoint.
Figure 2. Fungal burden throughout infection. Pulmonary fungal burden of female C57BL/6 mice (n = 15) inoculated with 1 x 10^4 cells of the WT strain or the mar1Δ mutant strain was measured by quantitative cultures throughout infection: 3, 7, 14, and 21 DPI. Brain fungal burden of female C57BL/6 mice (n = 15) inoculated with 1 x 10^4 cells of the WT strain or the mar1Δ mutant strain was measured by quantitative cultures at 21 DPI. Error bars represent standard error of the mean (SEM). Statistical significance was determined using Student's t test (***, P < 0.001; ****, P < 0.0001; ns, not significant).
Figure 3. Pulmonary cytokine profile and leukocyte infiltrate associated with granuloma formation. A. Pulmonary cytokine responses of female C57BL/6 mice inoculated with $1 \times 10^4$ cells of the WT strain or the mar1Δ mutant strain were measured using the Bio-Plex protein array system throughout infection: 1 (n = 15), 3 (n = 15), 7 (n = 10), 14 (n = 10), and 21 (n = 10) DPI. Error bars represent SEM. Statistical significance between strains at each timepoint was determined using Student’s t test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; no designation between strains, not significant). Only a subset of data is shown; refer to Figure S2 for full analysis. B. Pulmonary leukocyte infiltrates of female C57BL/6 mice inoculated with $1 \times 10^6$ cells of the WT strain or the mar1Δ mutant strain were measured by flow cytometry throughout infection: 1, 3, 7, and 21 DPI. Data shown are the mean ± of absolute cell numbers from three independent experiments (n = 3) performed using five mice per group per timepoint per experiment. Error bars represent SEM. Statistical significance between strains at each timepoint was determined using Student’s t test (*, $P < 0.05$; no designation between strains, not significant). Only a subset of data is shown; refer to Figure S3 for full analysis. C. Pulmonary macrophage activation of female C57BL/6 mice (n = 3) inoculated with $1 \times 10^6$ cells of the WT strain or the mar1Δ mutant strain were measured by flow cytometry throughout infection: 7, 14, and 21 DPI. Inducible nitrogen oxide synthase (iNOS) was used as a marker for M1 macrophages and Arginase 1 (Arg1) was used as a marker for M2 macrophages. The percentage of total iNOS+ cells and Arg1+ cells is shown. Error bars represent the SEM. Log transformation was used to normally distribute the data for statistical analysis. Statistical significance between strains at each timepoint was determined using Student’s t test (*, $P < 0.05$; no designation between strains, not significant). AM = alveolar macrophage (CD45+, CD11b+). IM = interstitial macrophage (CD45+, CD11b+). Cartoons adapted from BioRender.com (2021).
Figure 4. Contributions of GM-CSF signaling to pulmonary granuloma formation.

A. The lungs of female (n = 2) (shown) and male (n = 2) (not shown) Csf2rb<sup>−/−</sup> mice inoculated with 1 x 10^4 cells of the WT strain or the mar1<sup>Δ</sup> mutant strain sacrificed at predetermined endpoints (3, 7, and 14 DPI) were harvested for histopathological analyses. Hematoxylin and eosin staining were utilized to visualize microscopic lung pathology (fungal cells [yellow arrowheads], inset [yellow boxes]). 5x scale bar (left), 250 μm. 10x scale bar (right), 50 μm.

B. Pulmonary fungal burden of female (n = 2) and male (n = 2) Csf2rb<sup>−/−</sup> mice inoculated with 1 x 10^4 cells of the WT strain or the mar1<sup>Δ</sup> mutant strain sacrificed at 3 DPI was measured by quantitative cultures. Error bars represent the SEM. Statistical significance was determined using Student’s t test (*, P < 0.05).
Figure 5. Cell cycle-mediated virulence factor phenotypes of the mar1Δ mutant strain.
A. Titan cell formation was induced in the WT strain, the mar1Δ mutant strain, and the mar1Δ + MAR1 complemented strain. Cells were pre-grown in YNB medium at 30°C and an OD$_{600}$ of 0.001 was transferred to 10% HI-FBS in PBS incubated at 5% CO$_2$, 37°C for 96 hours. Cells were imaged by DIC microscopy (Zeiss Axio Imager A1). Cell diameter was measured using FIJI, and cells with a diameter > 10 μm were considered Titan cells (red arrowheads). The number of Titan cells per 10,000 cells was calculated for each strain. A minimum of 400 cells were analyzed across three biological replicates (n = 3). Error bars represent the SEM. Statistical significance was determined using a one-way ANOVA (*, P < 0.05; ns, not significant). 63x scale bar, 10 μm. B. The WT strain, the mar1Δ mutant strain, the mar1Δ + MAR1 complemented strain, and the cap59Δ mutant strain were incubated in YPD medium at 30°C and CO$_2$-independent medium (TC) at 37°C until saturation. Samples were subsequently fixed, mounted, dehydrated, and sputter-coated. Samples were imaged with a Hitachi S-4700 scanning electron microscope to visualize capsule organization and elaboration.
Figure 6. Slow growth phenotypes of the mar1Δ mutant strain. A. Morphological defects were analyzed in the WT strain, the mar1Δ mutant strain, and the mar1Δ + MAR1 complemented strain through incubation in YPD medium at either 30°C or 37°C. Cells were imaged by DIC microscopy (Zeiss Axio Imager A1) and were subsequently visually inspected for morphological defects, such as elongated cells (red squares), wide bud necks (red arrowhead), and cytokinesis failure (red circle). The percentage of total cells displaying morphological defects was quantified for each strain at each temperature. A minimum of 500 cells were analyzed across three biological replicates (n = 3). Error bars represent the SEM. Log transformation was used to normally distribute the data for statistical analysis (two-way ANOVA; *, P < 0.05; **, P < 0.01; ns, not significant). 63x scale bar, 10 μm. B. Growth of the WT strain, the mar1Δ mutant strain, and the mar1Δ + MAR1 complemented strain was assessed in YPD medium at 37°C. Growth was tracked for 40 hours and was measured by absorbance at OD₆₀₀. Figure summarizes data across three biological replicates (n = 3). Error bars represent the SEM. C. Hypoxia resistance was assessed by growth on YES medium in the presence of CoCl₂ (0.7 mM) and in a microaerophilic chamber. Serial dilutions of the WT strain, the mar1Δ mutant strain, the mar1Δ + MAR1 complemented strain, and the sre1Δ mutant strain were spotted onto agar plates and incubated at 30°C. Results were compared to the same strains grown in ambient air conditions.