

ABSTRACT

Title of Dissertation: **HOST-PATHOGEN INTERACTION
DURING *CRYPTOCOCCUS NEOFORMANS*
CNS INFECTION**

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Dissertation directed by: Associate Professor, Meiqing Shi, Department
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Cryptococcus neoformans (*C. neoformans*) is an opportunistic fungal pathogen widely distributed in the environment globally. *C. neoformans* infection initiates from the lung through inhaling the spores. While most healthy individuals can clear the fungus or contain the fungus in the granuloma, immunosuppressed patients and a small group of healthy populations fail in controlling the cryptococcal fungal pulmonary infection. In those cases, *C. neoformans* transmigrates from the lung to the central nervous system (CNS) and causes fatal meningoencephalitis, which accounts for 112,000 deaths each year worldwide. However, we have a very limited understanding of the transmigration of *C. neoformans* from the bloodstream to the brain *in vivo*, and the mechanism involved in the clearance of the organism in the brain remains poorly understood.

In this study, we first report a novel approach to quantitatively analyze the interactions between *C. neoformans* and brain endothelial cells in a mouse model using flow cytometry. Using this system, we show that *C. neoformans* was internalized by brain endothelial cells *in vivo* and that mice infected with acapsular or heat-killed *C. neoformans* yeast cells displayed a lower frequency of brain endothelial cells containing the yeast cell compared to mice infected with wild-type or viable yeast cells, respectively. We further demonstrate that brain endothelial cells were invaded by the serotype A strain (H99 strain) at a higher rate compared to the serotype D strain (52D strain).

Moreover, we found that clearance of *C. neoformans* in the brain correlates with accumulation and pro-inflammatory M1 polarization of Ly6C^{hi} mononuclear phagocytes and that these phagocytes play a critical role in the clearance of *C. neoformans* in the brain. Notably, the accumulation of Ly6C^{hi} mononuclear phagocytes coincides with enhanced secretions of TNF and IFN- γ in the brain. TNF receptor (TNFR) signaling, but not IFN- γ receptor (IFN- γ R) signaling, mediates the recruitment of Ly6C^{hi} mononuclear phagocytes to the brain in a cell-intrinsic manner. By contrast, IFN- γ induces M1 polarization of Ly6C^{hi} mononuclear phagocytes. Disruption of TNFR or IFN- γ R signaling enhances cryptococcal growth in the brain. Thus, Ly6C^{hi} mononuclear phagocytes act as effector cells for cryptococcal clearance in the brain, involving TNFR as well as IFN- γ R signaling.

Collectively, our study established that 1) internalization of *C. neoformans* by brain endothelial cells occurred *in vivo* and offered a powerful approach to quantitatively

analyze fungal migration into the brain; 2) Ly6C^{hi} mononuclear phagocytes accumulate in the brain during brain infection with *C. neoformans* and function as effector cells for clearance of *C. neoformans* in the brain involving TNFR signaling and IFN- γ signaling.

**HOST-PATHOGEN INTERACTION DURING *CRYPTOCOCCUS NEOFORMANS*
CNS INFECTION**

by

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Preface

Time flies, especially during a Ph.D. journey. My doctoral studies began in 2018, and over the past six and a half years, I have witnessed significant events including the falling of Cathédrale Notre-Dame de Paris, changes in national leadership, Brexit, and the first image of a black hole, and the unforgettable pandemic. Through these events, I've learned one thing for certain – change is inevitable. Reflecting on the past six and a half years, that's exactly how I would describe myself seeing how much I've evolved. While my college major was in biological sciences, my understanding of immunology was quite limited. However, over time, I transitioned into becoming a fungal immunologist.

Upon joining the lab in 2018, I was fortunate to have the guidance of my advisor, Dr. Meiqing Shi, and my talented colleagues, Ashley, Donglei, and Gongguan. At that point, my knowledge of fungi and immunology was minimal. It felt akin to learning a new language, starting from the basics like learning the alphabet as I was learning CD3, MHCII, *etc.* I'm grateful to my advisor, Dr. Shi, for entrusting me with a small project early on. Despite initial doubts about my abilities, I persevered, spending countless days and nights on experiments that now seem trivial compared to my current capabilities. Every small experiment, every late night in the lab, ultimately paid off. I gained valuable experience in *in vivo* experiments and flow cytometry, which laid the foundation for my subsequent studies.

During the pandemic, I faced numerous challenges and stresses. However, it was during this time that I found solace in the lab, where I was designated as an essential worker due to animal husbandry duties. This solitude provided me with the opportunity to focus on my research, particularly on cryptococcal brain infection. I amassed a wealth of data and deepened my understanding of *C. neoformans*. Though I struggled to piece together the puzzle alone, the unwavering support and guidance of Dr. Shi and my labmates illuminated the path forward. Through their assistance, I began to unravel the complexities of my research, asking crucial questions like: How does *C. neoformans* invade the brain *in vivo*? What's the potentially killing mechanism during cryptococcal brain infection?

My dissertation represents a portion of my Ph.D. work, focusing on the intricate interplay between host and pathogen in cryptococcal brain infection. The dissertation aims to provide novel insights into potential immunotherapeutic targets for cryptococcal brain infection. I hope this work contributes to our understanding and treatment of cryptococcosis.

Yanli Chen

May 2024

Dedication

This dissertation is dedicated to my family, friends, and people who generously love and support me for all these years. I would love to dedicate this work to all the people who suffered or are suffering from the disease and people who devote their time and efforts to conquer the disease. Last but not least, this work is dedicated to me, for surviving, pondering, growing, rising, and shining.

Acknowledgments

I am deeply grateful to my advisor, Dr. Meiqing Shi. I would like to extend my heartfelt gratitude to the members of my dissertation committee, Dr. Wenxia Song, Dr. Daniel Nelson, Dr. Utpal Pal, and Dr. Sean Riley.

I am also grateful to the University of Maryland, College Park for providing me with the resources and academic environment.

My sincere appreciation goes to my colleagues and peers who have offered their support and continuous hard work.

I also would like to express my gratitude to my family and all of my friends who have been giving me emotional support.

Disclaimer

All experiments were performed in accordance with the guidelines approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee. All experiments were performed in accordance with the biosafety level 2 guidelines.

Table of Contents

Preface.....	i
Dedication	iii
Acknowledgments	iv
Disclaimer	v
List of Figures.....	viii
List of Videos	ix
List of Abbreviations	x
Chapter 1: General introduction	1
<i>Cryptococcus neoformans</i> : history and evolution	1
<i>Cryptococcus neoformans</i> : capsule and serotypes.....	5
Cryptococcal meningoencephalitis: overview	7
Invasion of <i>C. neoformans</i> into the central nervous system	9
Trojan horse	11
Transcytosis	13
Paracellular crossing	14
Host immune responses to <i>C. neoformans</i> in the brain	15
Microglia.....	16
Inflammatory monocytes/macrophages	18
NK/NKT Cells	21
CD4/8 ⁺ T Cells.....	22
Proinflammatory cytokines	24
Fungal virulence factors as immune modulators	26
Polysaccharide capsule	27
Laccase activity and melanin formation	29
Phospholipase B1 activity.....	30
Urease activity	31
Chitin.....	31
CPL1	32
Concluding remarks.....	32
Chapter 2: Quantitative analysis reveals internalization of <i>Cryptococcus neoformans</i> by brain endothelial cells <i>in vivo</i>	34
Abstract.....	34
Introduction.....	35
Material and methods.....	37
Results.....	42
Internalization of <i>C. neoformans</i> by brain endothelial cells occurs <i>in vivo</i>	42
Using CFSE-labeled <i>C. neoformans</i> to quantitatively analyze the interaction between <i>C. neoformans</i> and brain endothelial cells <i>in vivo</i>	46
Mice infected with acapsular <i>C. neoformans</i> exhibit a lower frequency of endothelial cells containing the yeast cell.....	49
Mice infected with heat-killed <i>C. neoformans</i> display a lower frequency of endothelial cells containing the yeast cell.....	51
Brain endothelial cells are invaded by serotype A of <i>C. neoformans</i> at a higher rate than serotype D strain	52

Mice deficient in CD44 show a slightly lower frequency of brain endothelial cells containing <i>C. neoformans</i>	54
Discussion	56
Supporting information	60
Chapter 3: Recruited Ly6C^{hi} mononuclear phagocytes are effector cells for the clearance of <i>Cryptococcus neoformans</i> in the brain involving cell-intrinsic TNFR and IFN-γR signaling.....	62
Abstract	62
Introduction	63
Material and methods	66
Results	76
Clearance of <i>C. neoformans</i> in the brain correlates with accumulation and M1 polarization of Ly6C ^{hi} mononuclear phagocytes.	76
Recruited Ly6C ^{hi} mononuclear phagocytes are effector cells for clearance of <i>C. neoformans</i> in the brain	80
TNFR signaling mediates the accumulation of Ly6C ^{hi} mononuclear phagocytes in the brain	82
TNF directly signals on Ly6C ^{hi} mononuclear phagocytes accompanied by enhanced expressions of chemokines and adhesion molecules	86
ICAM-1 deficiency does not affect the fungal burden in the brain	90
IFN- γ R signaling is essential for M1 polarization of Ly6C ^{hi} mononuclear phagocytes.....	91
Disruption of TNFR or IFN- γ R signaling enhances the fungal growth in the brain	93
Discussion	95
Chapter 4: Conclusion and perspectives	103
Bibliography	108
Curriculum vitae.....	I

List of Figures

- Figure. 1.1 The structure of cryptococcal polysaccharide capsule GXM and GalXM
- Figure. 1.2 Invasion of *C. neoformans* to the central nervous system.
- Figure. 1.3 Host immune responses to *C. neoformans* in the brain.
- Figure. 1.4 Fungal virulence factors function as immune modulators.
- Figure. 2.1 Quantitative analysis of internalization of GFP-expressing *C. neoformans* by brain endothelialization *in vivo*.
- Figure. 2.2 Quantitative analysis of internalization of CFSE-labeled *C. neoformans* by brain endothelialization *in vivo*.
- Figure. 2.3 Mice infected with acapsular *C. neoformans* display a lower frequency of infected brain endothelial cells compared to wild-type *C. neoformans*.
- Figure. 2.4 Mice infected with heat-killed *C. neoformans* display a lower frequency of infected brain endothelial cells compared to viable *C. neoformans*.
- Figure. 2.5 The frequency of brain endothelial cells invaded by serotype A of *C. neoformans* is higher compared to serotype D.
- Figure. 2.6 CD44^{-/-} mice exhibit a slightly lower frequency of brain endothelial cells invaded by *C. neoformans* compared to wild-type mice.
- Figure. 3.1 Clearance of *C. neoformans* in the brain correlates with accumulation and M1 polarization of Ly6C^{hi} mononuclear phagocytes.
- Figure. 3.2 Recruited Ly6C^{hi} mononuclear phagocytes are effector cells for clearance of *C. neoformans* in the brain.
- Figure. 3.3 TNFR signaling mediates the accumulation of Ly6C^{hi} mononuclear phagocytes in the brain.
- Figure. 3.4 TNF directly signals on Ly6C^{hi} mononuclear phagocytes accompanied by enhanced expressions of chemokines and adhesion molecules.
- Figure. 3.5 ICAM-1 deficiency does not affect fungal burdens in the brain.
- Figure. 3.6 IFN- γ R signaling is essential for M1 polarization of Ly6C^{hi} mononuclear phagocytes.
- Figure. 3.7 Disruption of TNFR or IFN- γ R signaling enhances the fungal growth in the brain.
- Figure. S3.1 Gating strategy.
- Figure. S3.2 Disruption of IFN- γ R signaling does not affect the accumulation of Ly6C^{hi} mononuclear phagocytes in the brain.
- Figure. S3.3 Disruption of TNFR signaling enhances the size of fungal clusters and disruption of IFN- γ R signaling enhances the size of lesions.

List of Videos

Supplemental Video 1. 3D reconstructive movie of Figure 2.1 D

Supplemental Video 2. 3D reconstructive movie of Figure 2.2 B

List of Abbreviations

Ammonium-Chloride-Potassium	ACK
Activator Protein 1	AP-1
Antigen-Presenting Cells	APCs
American Type Culture Collection	ATCC
Brain-associated Macrophages	BAMs
Blood-cerebrospinal Fluid Barrier	BCSFB
Brain Infiltrating Leukocytes	BIL
Brain Microvascular Endothelial Cell	BMECs
Caspase Recruitment Domain-containing Protein 9	CARD9
Cryptococcus-associated Immune Reconstitution Inflammatory Syndrome	C-IRIS
C-C Chemokine Receptor Type 2	CCR2
Cluster of Differentiation	CD
Carboxyfluorescein Di-acetate Succinimidyl Ester	CFSE
Colony Forming Units	CFU
Central Nervous System	CNS
Cerebrospinal Fluid	CSF
C-X3-C Motif Chemokine Receptor 1	CX3CR1
C-X-C Chemokine Receptor 3	CXCR3
4',6-Diamidino-2-Phenylindole	DAPI
Dendritic Cells	DCs
Damage-Associated Molecular Patterns	DAMPs

Days Post-Infection	dpi
Extracellular Matrix	ECM
Enzyme-Linked Immunosorbent Assay	ELISA
Extracellular Signal-regulated Kinases 1 and 2	ERK1/2
Fluorescence-Activated Cell Sorting -	FACS
Fc Receptor	FcR
Flow Cytometry Staining Buffer	FSB
Galactoxylomannan	GalXM
Granulocyte Colony-Stimulating Factor	G-CSF
Green Fluorescent Protein	GFP
Granulocyte-Macrophage Colony-Stimulating Factor	GM-CSF
Granulysin	GNLY
Glucuronoxylomannan	GXM
Highly Active Antiretroviral Therapy	HAART
Human Brain Microvascular Endothelial Cells	HBMECs
Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome	HIV/AIDS
Heat Shock Protein 70	Hsp70
Intercellular Adhesion Molecule 1	ICAM-1
Interferon-gamma	IFN- γ
Interferon-gamma Receptor	IFN- γ R
Interleukin	IL
Institutional Animal Care and Use Committee	IACUC
Immunohistochemistry	IHC

Interleukin-1 Alpha	IL-1 α
Interleukin-1 Beta	IL-1 β
Interleukin-12	IL-12
Interleukin-17A	IL-17A
Interleukin-18	IL-18
Interferon Regulatory Factor 1	IRF1
Interferon Regulatory Factor 5	IRF5
Major Histocompatibility Complex	MHC
Major Histocompatibility Complex Class II	MHCII
Macrophage Inflammatory Protein 1 Alpha	MIP-1 α
Macrophage Inflammatory Protein 1 Beta -	MIP-1 β
Monoclonal Antibodies	mAbs
Median Fluorescent Intensity-	MFI
Metalloprotease 1	Mpr1
Natural Killer T cells	NKT cells
Natural Killer	NK
Natural Killer protein 30	NKp30
Nuclear Magnetic Resonance	NMR
Phosphate Buffered Saline	PBS
Pathogen-Associated Molecular Patterns	PAMPs
Polymerase Chain Reaction	PCR
Phosphoinositide 3-kinase	PI3K
Postinfectious Inflammatory Response Syndrome	PIIRS

Phospholipase B1	PLB1
Revolutions Per Minute	rpm
Ribonucleic Acid	RNA
Reverse Transcription Quantitative Polymerase Chain Reaction	RT-qPCR
Single-cell RNA Sequencing	scRNA-seq
Standard Error of the Mean	SEM
Signal Transducer and Activator of Transcription 3	STAT3
T cell receptor	TCR
T Helper 1	Th1
T Helper 17	Th17
T Helper 2	Th2
Toll-like Receptor	TLR
Tumor Necrosis Factor	TNF
Tumor Necrosis Factor Receptor	TNFR
Vascular Cell Adhesion Molecule 1	VCAM-1
Very Late Antigen 4	VLA4
Wild-type	WT

Chapter 1: General introduction

This chapter is based on a published paper [Chen Y, Shi ZW, Strickland AB, Shi M. *Cryptococcus neoformans* Infection in the Central Nervous System: The Battle between Host and Pathogen. *Journal of Fungi*. 2022; 8(10):1069. <https://doi.org/10.3390/jof8101069>]. Yanli Chen contributed to Conceptualization, Writing – original draft preparation, and Writing – review and editing.

Cryptococcus neoformans: history and evolution

Cryptococcus neoformans (*C. neoformans*) is an encapsulated pathogenic fungus, which infects humans and animals (1). Annually, it's estimated there are 152,000 (IQR 111,000 to 185,000) cases of cryptococcal meningitis, resulting in 112,000 cryptococcal-related deaths (IQR 79,000 to 134,000). Globally cryptococcal disease results in 19% (IQR 13% to 24%) of AIDS-related mortality.

C. neoformans resides in many environmental niches from the harsh Chernobyl to pigeon dropping to the mammalian hosts. *C. neoformans* was isolated over 100 years ago initially from fermenting peach juice and identified as a human pathogen in bone lesions. Fungi usually requires strict environmental and geographical niches. However, *C. neoformans* is widespread globally which may be due to the seafaring centuries ago when people brought carrier pigeons along with them. Now, *C. neoformans* in the pigeon dropping resides throughout the world. The increased prevalence of human cryptococcosis is accompanied by human advances. Increased organ transplants lead to an increasing number of cryptococcosis patients later in their life. However, the

epidemic of HIV infection is the main reason that drives the revivification of cryptococcosis. The fungal colonization in humans seems random since *C. neoformans* does not require a host environment to survive or proliferate. However, there are clues that cryptococcal natural habitat has prepared it to be a human pathogen.

C. neoformans' intracellular survival ability may be related to its infection in amoebae in nature (2). Amoeba is a unicellular natural predator with pseudopods and phagocytosis properties. Previous studies have been carried out using two main strains of amoebae (*Acanthamoeba Castellanii* and *Dictyostelium Discoindeum*). Studies with *A. castellanii* show that *C. neoformans* enlarges the capsule upon contact with the amoebae (3). The interaction between the yeast cells and *A. castellanii* could also lead to fungal melanin production (4). Fungal interaction with the amoebae usually leads to phagocytosis, and ingested fungus could proliferate inside the amoebae and eventually kill the organism (5). Amoebae share some similarities with macrophages which may explain the emergence of fungal intracellular pathogenic strategy during cryptococcal infection (4). Both of their phagocytosis is receptor-mediated and they use acidification and reactive oxygen species to degrade the organism inside the food vacuole and phagolysosome. A recent study shows that Amoeba predation of *C. neoformans* results in pleiotropic changes associated with virulence (6). The selective pressures from amoebae contribute to the maintenance or even regain of fungal virulence in animal hosts (4,5).

After infecting humans, *C. neoformans* could adapt to the environment quickly and colonize the organs. *C. neoformans* could not only cause pulmonary infection after patients inhale the spores. It could cause life-threatening meningoencephalitis. After escaping from the lung either by free fungus or through trojan horse, *C. neoformans* travels through the bloodstream. Microvasculature in the brain favors the mechanical arrest of the free fungus and promotes transcytosis and paracytosis. After invasion into the BBB, yeast cells could proliferate not only in the perivascular space but also in the brain parenchyma. Compared to the peripheral organs, there are fewer immune cells present inside the brain and less inflammation that could potentially contribute to the fungal clearance. The lack of complement in the brain could also favor the survival of *C. neoformans* from being phagocytosed.

Except for the fact that the brain is an organ with less immune crosstalk with the peripheral immune system, the brain has been shown to be an excellent environment for fungal growth for the abundant inositol. *C. neoformans* could utilize myo-inositol as a sole carbon source and the human central nervous system is compacted with inositol. Published studies have shown that a myo-inositol transporter gene family (ITR) with 10 or more members has been identified in *C. neoformans* (7). ITR genes are inositol transporters and important for myo-inositol stimulation of mating (7). Mutation on *itr1* or *itr1a* comprised fungal virulence. A follow-up study showed that inositol stimulates *C. neoformans* blood-brain barrier crossing. *C. neoformans* with a mutation on *itr1a* and *itr3c* reduced the crossing of brain endothelial cells by the organism *in vitro* and decreased fungal lesions in the brain (8). Utilizing inositol for mating is a

strategy of *C. neoformans* during its colonization in the trees. *C. neoformans* undergoes mating on plants and the inositol secreted from plants stimulates the fungal mating (9). Inositol is crucial for the regulation of neurological responses and psychological feedback (10). Astrocytes, which are closely related to the BBB, contain over 8 mM inositol (10), which could be released upon neuron inflammation and pathogen invasion (11).

The progeny of brain-resident fungi is not likely to contribute to the evolution of the species as a whole. However, there are microevolutions observed during laboratory and clinical infection settings. A study analyzed multiple H99 strains derived from the lab passage and the clinic samples. The results show that the virulence attenuation in the lab strain may be due to the loss of *sgf29*, a SAGA-associated factor. The loss of *sgf29* was also observed in the fungus isolated from two samples out of fourteen patients' samples (12). Another study analyzing fungal isolates from patients demonstrated an increase in mixed infections in humans. This mixed infection includes infection with different serotypes, genotypes, and different mating types. It was proposed that the ploidy changes could result from endoreplication and shuttling between haploid and diploid states which suggests *in vivo* evolution (13). A passage of *C. neoformans* in mouse infection is known to increase virulence. Researchers found that increased expression of a *FRE3*-encoded iron reductase is related to increased virulence (14). However, *C. neoformans* is not a contagious pathogen with absent or rare human-human transmission. The evolution we saw in the clinical isolates is not

due to the *in vivo* evolution but mostly due to the gene adaptation during the environment-mammal transition (15).

Cryptococcus neoformans: capsule and serotypes

Cryptococcus spp. was grouped into two varieties that included five serotypes: *C. neoformans* for serotypes A, D, and AD (a hybrid strain between A and D strains), and *C. neoformans var. gattii* for serotypes B and C decades ago. In 2000, *C. neoformans var. gattii* (*C. gattii*) was elected to be a separate species. All serotypes are known to cause cryptococcal meningitis. While the cryptococcosis caused by *C. gattii* is less than 20%, the majority of cryptococcosis is caused by *C. neoformans*. *C. neoformans* has five molecular types VNI, VNII, VNIII, VNIV, and VNB. Serotype A consists of VNI, VNII, and VNB which diverged from serotype D of VNIV and serotype AD (VNIII) is the hybrids of the two varieties. Serotype AD is a diploid strain and goes through asexual fertilization in an experimental setting but produces hyphae in the mating medium.

Studies regarding cryptococcal capsules started back in the 1950s, identifying mannose, xylose, glucuronic acid, and galactose which are the main components of cryptococcal capsules (16,17). The following studies showed that the dominant polysaccharide (88% of the total capsule) consists of glucuronoxylomannan (GXM; 10^6 Da), which lacks galactose (18–23). Nuclear magnetic resonance (NMR) shows that GXM consists of a linear alpha-1,3-linked mannose backbone bearing beta-xylose, beta-1,2-glucuronic acid, and 6-O-acetyl moieties. Xylose residues could be either 1,2-

or 1,4-linked. The xylose/mannose/glucuronic acid ratio of serotypes A, B, C, and D is 2:3:1, 3:3:1, 4:3:1, and 1:3:1. After analyzing over 100 isolates, six repeating mannosyl triads were identified, with each substituting with a single beta-1,2-glucuronic acid residue and different patterns of xylose (24). O-acetylated on mannose was further investigated and the results showed that O-acetylated on mannose are 12%, 6.5-9.5%, 3%, and 9% for serotype A, B, C, and D which varies in growth conditions (25,26). Using a mutant serotype D strain lacking O-acetylation, it was demonstrated that the O-acetylation only happens in two mannoses.

Another polysaccharide galactoxylomannan (GalXM) represents the other 12% of the capsule along with some mannoproteins. GalXM is 275,000 Da in size, less acidified, and includes galactose. Purified Methylation analysis shows that GalXM has a complex branched structure with xylose and galactofuranose terminal along with mannose, xylose, and galactose (27). A study of *cap67*, an acapsular serotype D strain, shows that GalXM consists of an alpha-1,6-linked polymer of galactose, being substituted at alternative residue with beta-1,3-linked galactose (as shown in panel B, the polymer of galactose could reside with either beta-1,3-linked galactose at two different locations). The latter galactose is extended by an alpha-1,4-linked dimer of mannose-alpha 1,3- mannose, and the side chain moieties could be substituted with xylose (28). Further study using the same strain shows that GalXM from *cap67* contains glucuronic acid which is linked to the side chain galactose in a position occupied by xylose (19). A recent study shows that GalXM from serotypes D and A is structurally and antigenically variable. Immune serum generated against GalXM from

surveillance and start to proliferate. Eventually, *C. neoformans* could escape from the lung to the bloodstream as extracellular fungus or as intracellular fungus residing in the phagolysosome of lung macrophages (32). After entering the systemic circulation with the bloodstream, *C. neoformans* could transmigrate from the bloodstream into the brain, across the blood-brain barrier (BBB), leading to the infection of the central nervous system (CNS) and meningoencephalitis (1,30,31).

Most cases of cryptococcal meningoencephalitis emerge in immunocompromised patients, including patients with HIV infection and organ transplants, although rare immunocompetent patients can also develop the illness (30,31). Cryptococcal meningoencephalitis is fatal without timely medical care, which involves long-term treatment with amphotericin B, flucytosine, and fluconazole (33). Even with successful therapy, survivors of this illness often develop neurologic deficits and have other adverse effects (34,35). It was estimated that this fungal disease affects nearly 152,000 people each year and leads to 112,000 deaths annually (36).

Cellular immune responses play an essential role in the clearance of cryptococcal infection (37). Inflammatory monocytes/macrophages, microglial cells, and antigen-specific T cells including CD4⁺ and CD8⁺ T cells are critically involved in host immune responses to *C. neoformans* infections (38,39). Th1 immune responses, characterized by IFN- γ , and Th17 immune responses, characterized by IL-17A, mediate protection against *C. neoformans* (37,40). IFN- γ activates macrophages and promotes classical activation of macrophages, correlating with fungal clearance (37,41).

In contrast, Th2 cytokines such as IL-4, IL-5, and IL-13 drive alternative activation of macrophages, associated with disease progression (37,42).

C. neoformans has developed sophisticated mechanisms to enable pathogenesis by escaping host defense mechanisms and modulating immune responses (43). The polysaccharide capsule and self-producing melanin are the major virulence factors of *C. neoformans* (44–46). In addition, *C. neoformans* secretes a number of virulence-associated enzymes (47). Those virulence factors are critically involved in *C. neoformans*' invasion into the CNS and suppression of host immune responses. The outcome of the interaction between the host and *C. neoformans* determines the progression of the disease.

Invasion of *C. neoformans* into the central nervous system

As a neurotropic pathogen, *C. neoformans* invade the brain and cause meningoencephalitis, which represents the main clinical manifestation of cryptococcosis. Two relevant entry sites have been described for neurotropic pathogens including viruses and bacteria: the BBB and the blood-cerebrospinal fluid barrier (BCSFB) (48). *C. neoformans* has demonstrated limited ability to migrate through the choroid plexus to invade the CNS, based on evidence that the choroid plexus remains normal and free of fungal cells in infected mice (49,50). Furthermore, there have been limited clinical cases of choroid plexitis reported in human patients (51–54), which supports the idea that crossing through BCSFB plays a minor role during cryptococcal dissemination into the CNS. Instead, cryptococcal clusters have been often observed

next to the cortical microvasculature of the brain and cerebellum (49,50), demonstrating that *C. neoformans* crosses the BBB for brain invasion.

The BBB is a selectively permeable barrier that separates the brain parenchyma from the vascular compartment (55). The major components of the BBB are endothelial cells, pericytes, and astrocytes (56). Endothelial cells line the lumen side of the brain vasculature and are connected by tight junctions. During inflammation, circulating leukocytes can transmigrate into the brain parenchyma paracellularly through the tight junction or transcellularly through endocytosis mediated by the endothelial cells (57). To enter the brain, *C. neoformans* must cross the BBB and three major pathways have been proposed for crossing the BBB including Trojan horse, transcytosis, and paracellular crossing (58–60) (Figure 1.2).

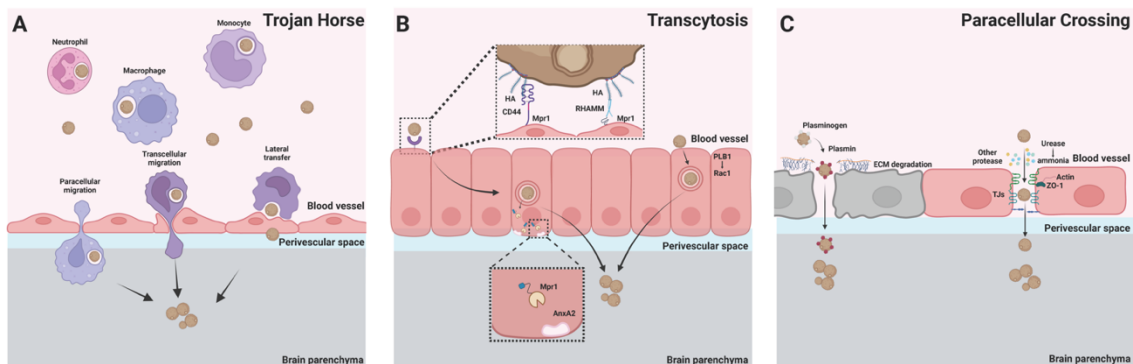


Figure. 1.2 Invasion of *C. neoformans* to central nervous system. *C. neoformans* transmigration to the brain across the BBB. Three pathways have been proposed for the BBB crossing of *C. neoformans*. **(A)** Trojan horse. Phagocytes can carry ingested *C. neoformans* across the BBB through paracellular and transcellular migration. Phagocytes containing *C. neoformans* can also directly transfer the fungal cell to brain endothelial cells through lateral transfer, leading to BBB crossing of the organism. **(B)** Transcytosis. Interactions of cryptococcal hyaluronic acid with endothelial CD44 or receptor of hyaluronan-mediated motility (RHAMM) lead to endocytosis of the yeast cell. Following endocytosis, cryptococcal Mpr1 interacts with Annexin A2 (AnxA2) to facilitate exocytosis of the organism from the endothelial cells. In addition, cryptococcal phospholipase B1 (PLB1) promotes transcytosis through

activation of host cell Rac1. (C) Paracellular crossing. Host plasminogen (white) binds to *C. neoformans* and is converted to the serine protease plasmin (red). Plasmin degrades the extracellular matrix (ECM), facilitating fungal crossing of the BBB. The tight junction of the BBB can be damaged by cryptococcal urease (due to the toxic effects of urease-induced ammonia) and other secreted cryptococcal proteases. *C. neoformans* can invade the brain across the damaged BBB. Figures were generated using BioRender.

Trojan horse

C. neoformans is a facultative intracellular pathogen that can survive and grow inside phagocytes (61). Expulsion of live *C. neoformans* (known as vomocytosis) by macrophages has been observed *in vitro* and *in vivo* following phagocytosis of the fungus (62–64). In addition, *C. neoformans* can move directly from infected to uninfected macrophages (65,66). These unique properties of *C. neoformans* support the hypothesis that phagocytes contribute to the spread and dissemination of *C. neoformans* from the infected lung to the CNS. Indeed, mononuclear phagocytes harboring *C. neoformans* have been detected in the perivascular space of the brain (67,68). Deletion of alveolar macrophages or CD11c⁺ cells (alveolar macrophage and dendritic cells (DCs)) in the lung significantly reduced the dissemination of *C. neoformans* to the lung lymph node and the brain (69,70), suggesting a role for these cells in extrapulmonary dissemination. In addition, the intravenous transfer of *C. neoformans*-infected macrophages to recipient mice enhanced brain colony-forming unit (CFU) (71,72), while depletion of monocytes decreased brain CFU (68,71), providing further evidence that phagocytes are capable of transporting fungal cells to the CNS.

Using a monolayer of human cerebral microvascular endothelial cells (hCMEC/D3) cultured *in vitro*, monocytes infected with *C. neoformans* were directly

seen to cross brain endothelial cells (73,74), demonstrating that these cells can facilitate neuroinvasion. Supporting this, intravital microscopy revealed that a substantial number of CX3CR1⁺Ly6C^{low} monocytes were recruited to the brain microvasculature 12 hours after *C. neoformans* infection and were seen to engulf *C. neoformans*, adhere to the luminal wall of the brain microvasculature, and transmigrate to the parenchyma (75). In contrast, CCR2⁺Ly6C^{hi} monocytes were observed to accumulate in the brain starting 14 days post intravenous *C. neoformans* infection, and instead drive brain inflammation in mice as well as in humans (76,77).

Further support of the Trojan horse mechanism of brain invasion by *C. neoformans*, comes from clinical studies that demonstrated that efficient internalization of *C. neoformans* by phagocytes positively correlated with CSF fungal burdens and the risk of death in HIV-associated cryptococcosis patients (78). Moreover, monocyte-derived macrophages from HIV/AIDS patients displayed a higher phagocytosis efficiency of *C. neoformans* along with more intracellular fungal growth compared to those of healthy individuals (79). With the use of miRNA transcriptomics, it has been recently shown that the cytoskeleton and myocilin, encoded by *MYOC*, were involved in cryptococcal brain dissemination by modulating the Trojan horse pathway in mice and macaques, and provides a possible explanation for higher incidences of cryptococcal meningoencephalitis in HIV/AIDS patients, who often have dysfunctional immune cell cytoskeletons (79).

Transcytosis

In a murine model of *C. neoformans* infection, fungal cells were observed inside endothelial cells of the brain, suggesting that *C. neoformans* invade the brain through transcytosis (67). *In vitro* studies using a monolayer of human brain microvascular endothelial cells (HBMECs) have shown that *C. neoformans* can adhere to endothelial cells and cross HBMEC monolayers via a transcellular pathway (50). Studies have shown that the cryptococcal *CPS1* gene encodes a hyaluronic acid synthase and that its product, hyaluronic acid, is critically involved in the adhesion of *C. neoformans* to brain endothelial cells (80). It was further demonstrated that the adhesion of *C. neoformans* to HBMEC monolayers and subsequent transcytosis was mediated by interactions between cryptococcal hyaluronic acids and CD44 expressed on HBMECs (81), and involved protein kinase C- α (82). Moreover, *C. neoformans* enhanced the activity of EphA2, a tyrosine kinase receptor, through CD44, facilitating fungal migration into the brain (83). In support of these *in vitro* observations, mice deficient in CD44 displayed lower brain fungal burdens following *C. neoformans* infection (84). Despite this, CD44 deficient mice still had *C. neoformans* present in their brains, and in fact, another hyaluronic acid receptor, RHAMM (receptor of hyaluronan-mediated motility), which is present in CD44 deficient mice, was also found to mediate the association of *C. neoformans* cells with mouse brain microvascular endothelial cells (BMECs) (84). Interestingly, the brain has high levels of sugar inositol, and inositol acquisition by *C. neoformans* leads to upregulation of the cryptococcal *CPS1* gene and

higher production of hyaluronic acids, thus promoting adherence and transcytosis of *C. neoformans*, which may explain *C. neoformans* predilection for the brain (85).

Apart from hyaluronic acids, cryptococcal phospholipase B1 (PLB1) has been shown to promote transcytosis of *C. neoformans* in cultured HBMECs through activation of host cell Rac family small GTPases, Rac1 and its association with STAT3 (86). In addition, a secreted metalloprotease (Mpr1) of *C. neoformans* was reported to be required for crossing hCMEC/D3 monolayers by interacting with cytoskeleton-endocytosis-associated protein Annexin A2, and consequently, mice infected with a strain of the fungus lacking the gene encoding Mpr1 survived longer due to reduced brain fungal burdens (87,88). Recently, an approach based on flow cytometry to quantitatively analyze fungal migration into the brain has been established, and using this approach, it has been confirmed that internalization of *C. neoformans* by brain endothelial cells occurs *in vivo* (89). Interestingly, quantitative analysis revealed that brain endothelial cells were invaded by *C. neoformans* at a higher rate compared to *C. neoformans*, which may reflect the higher virulence of *C. neoformans* during brain infection in mice (89).

Paracellular crossing

In addition to the Trojan horse and transcytosis pathways, it has been proposed that *C. neoformans* can migrate into the brain through paracellular crossing between brain endothelial cells and across damaged endothelial barriers (59,60). Early studies have shown that there was vessel damage and leakage at fungal arrest sites (49). In

addition, there is evidence that *C. neoformans* induces alterations of the tight junction and the cytoskeleton of endothelial cells and ultimately induces endothelial cell necrosis (90–92). In this context, the cryptococcal capsule and secreted enzymes may contribute to the damage of tight junction and endothelial cells (60). For example, cryptococcal urease is a major virulence factor and promotes fungal invasion into the CNS (58,93–95), likely by damaging tight junctions due to the toxic effects of urease-induced ammonia (95). *C. neoformans* has also been found to secrete a number of proteases, which have been shown to disrupt the BBB during brain infection (96,97). In addition, *C. neoformans* is able to bind and activate host plasminogen, leading to the conversion of plasminogen to the serine protease plasmin, which is also capable of degrading the BBB (98,99).

Host immune responses to *C. neoformans* in the brain

Following invasion into the brain, *C. neoformans* cells begin to grow. *C. neoformans* will first encounter microglia, the macrophages residing in the brain. Interactions between microglia and invading *C. neoformans* lead to neuroinflammation including the production of proinflammatory cytokines and chemokines, which promotes the recruitment and accumulation of innate immune cells as well as adaptive

immune cells. These immune cells, along with cytokines secreted by them, are critically involved in fighting the fungal cells (37,41) (Figure 1.3).

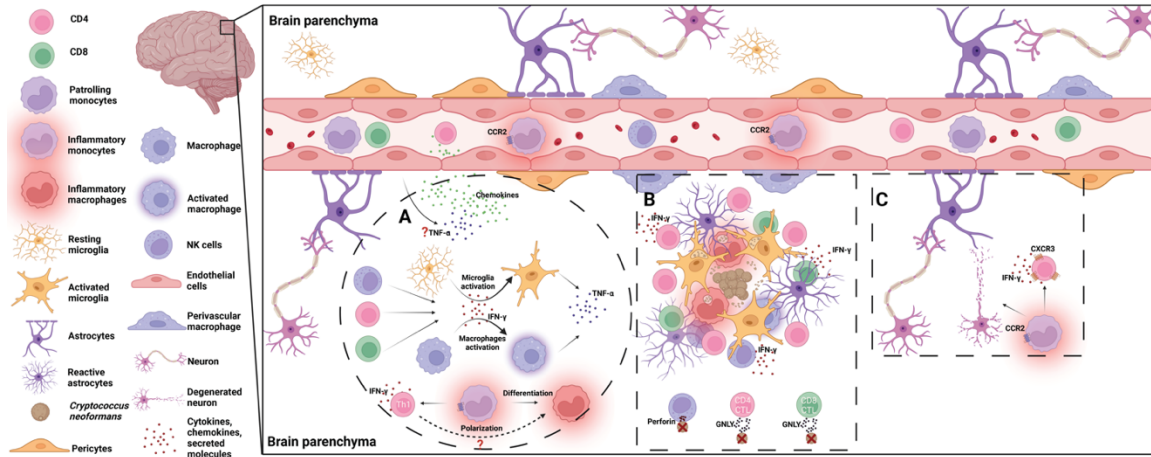


Figure. 1.3 Host immune responses to *C. neoformans* in the brain. (A) Following brain infection of *C. neoformans*, TNF and chemokines are produced, facilitating leukocyte migration to the brain parenchyma. IFN- γ produced by CD4⁺/CD8⁺ T cells and NK/NKT cells promotes the activation of microglia and macrophages. Activated microglia and macrophages secrete TNF, further driving brain inflammation. Recruited inflammatory monocytes differentiate into inflammatory macrophages and help shape Th1 immune responses, which are required for fungal clearance. (B) As the resident glial cells, microglia and astrocytes are the first cells to respond to invading yeast cells. Later, recruited leukocytes including CD4⁺/CD8⁺ T cells, NK/NKT cells, and inflammatory monocyte/macrophages accumulate in and around the fungal clusters. As effector cells, microglia and macrophages internalize the fungal cells. T cells and NK cells secrete IFN- γ to promote the fungicidal activity of these phagocytes. NK cells and T cells are also involved in the direct killing of the fungal cells through the release of perforin or granulysin (GNLY). (C) Inflammatory responses are required for fungal clearance in the brain but must be tightly controlled. Inflammatory monocytes and IFN- γ -secreting CXCR3⁺ CD4⁺ T cells facilitate neuron damage and cause immunopathology during cryptococcal meningoencephalitis. Figures were generated using BioRender.

Microglia

Tissue-resident macrophages are a diverse population of leukocytes that reside in mammalian tissues and play a prominent role in tissue homeostasis and host defense (100). Tissue-resident macrophages are usually embryonic in origin and self-renewing

under steady-state conditions (101,102). As brain-resident macrophages, microglia are found throughout the brain parenchyma and play a central role in immune responses in the brain. During the resting state, microglia extend their dendrites to survey the brain (103). Upon activation, microglia enlarge their cell bodies and adopt an “amoeboid” morphology with thicker ramifications and can engulf and clear dead neurons, pathogens, as well as pathogen-infected cells (104–109)

During brain infection with the fungal pathogen *Candida albicans*, CARD9⁺ microglia mediate protection by promoting neutrophil recruitment in an IL-1 β - and CXCL1-dependent manner (110). Studies on the interactions between microglia and *C. neoformans* revealed that microglia can internalize *C. neoformans* *in vitro* (111–115) as well as in the human brain (116). Internalization of nonopsonized *C. neoformans* by swine microglia was reported to involve the CD14 receptor (113). The presence of capsule binding antibodies was shown to enhance phagocytosis of *C. neoformans* by human microglial cells (114), and in the presence of anti-cryptococcal antibodies, *C. neoformans*-stimulated microglia secreted proinflammatory chemokines MIP-1 α and MIP-1 β (117). However, the cryptococcal capsule has been found to inhibit phagocytosis by murine microglial cells (115), as well as down-regulate the production of MIP-1 α and MIP-1 β (117).

Stimulation of microglia by TLR agonists also promotes phagocytosis and killing of *C. neoformans* and is associated with enhanced secretions of proinflammatory cytokines including TNF (112). In addition, the killing of *C.*

neoformans by microglia was positively correlated with nitric oxide secretion (118,119). Interestingly, the intracellular iron load of microglia, rather than the stimulation by IFN- γ , enhanced the anticryptococcal activity of microglia *in vitro* (120,121). As a facultative intracellular pathogen, *C. neoformans* can also survive and proliferate within microglial cells depending on the activation status of microglia (116,122).

Murine studies showed that MHC class II-positive perivascular microglial cells were involved in resistance to *C. neoformans* brain infection (123). In line with this report, immunotherapy with combined administration of anti-CD40 and IL-2 during *C. neoformans* infection reduced fungal burdens in the brain, which correlated with an increase of MHC class II expression on microglia in an IFN- γ -dependent manner (124,125). More recently, it has been shown that the number of microglial cells is significantly enhanced following upregulation of MHC class II and CD11c on the cells during brain infection of *C. neoformans* (126).

Inflammatory monocytes/macrophages

Monocytes consist of two major populations. These include CD14^{hi}CD16⁻ and CD14^{low}CD16^{hi} monocytes in humans, and the corresponding CCR2^{hi}CX3CR1^{low}Ly6C^{hi} inflammatory monocytes and CCR2^{low}CX3CR1^{hi}Ly6C^{low} monocytes in mice (127–129). During infection and inflammation, Ly6C^{hi} inflammatory monocytes migrate from the bone marrow to the periphery blood through CCR2 signaling and are recruited to the infected or inflamed tissues (127,130). The

recruited Ly6C^{hi} inflammatory monocytes can differentiate to monocyte-derived Ly6C^{hi}CD11b⁺ inflammatory macrophages or DCs (127). These recruited Ly6C^{hi} mononuclear phagocytes (i.e., Ly6C^{hi} monocytes and their derivatives) secrete high levels of proinflammatory cytokines and promote inflammation, as well as act as effector cells and play an essential role in the clearance of pathogens (127).

As signaling of CCR2 with its ligands, including CCL2 and CCL7, is required for emigration of Ly6C^{hi} monocytes from the bone marrow, CCR2^{-/-} mice have been widely used to study the functions of Ly6C^{hi} inflammatory monocytes and macrophages (127,130). Previous studies have shown that Ly6C^{hi} inflammatory monocytes and macrophages display a prominent role in the clearance of various fungal pathogens in the lung, including *Aspergillus fumigatus* (131,132), *Candida albicans* (133), *Histoplasma capsulatum* (134,135), and *Blastomyces dermatitidis* (134).

During pulmonary infection with *C. neoformans*, disruption of CCR2 signaling led to higher fungal burdens and coincided with the development of detrimental Th2 responses in the infected lung (136,137). CCR2 signaling promoted Ly6C^{hi} inflammatory monocyte migration to the infected lung, leading to differentiation and accumulation of Ly6C^{hi} inflammatory macrophages and DCs (138–140). These effector cells secrete nitric oxide and TNF and have been shown to kill *C. neoformans* in the lung (138). Moreover, recent studies showed that Ly6C^{hi} inflammatory monocytes and macrophages are critically involved in the protection generated by

candidate vaccine strains of *C. neoformans* (141–144). However, the fungicidal activity of Ly6C^{hi} inflammatory monocytes and macrophages must be tightly controlled to avoid tissue destruction (127). In this regard, more recent studies have shown that enhanced accumulation of Ly6C^{hi} inflammatory monocytes and macrophages mediated detrimental immune responses in the lung in a murine model of acute infection with *C. neoformans* (145,146).

CCL2 is expressed by microglia, astrocytes, and endothelial cells in the brain at physiological conditions (147). An internal crosstalk between inflammatory monocytes with microglia was observed during West Nile Virus-induced encephalitis (148), although whether it occurs during cryptococcal meningoencephalitis remains unknown. *In vitro* studies have shown that inflammatory monocytes transmigrate across brain microvascular endothelial cells through CD44-mediated extravasation (100). In addition, clinical studies have shown that inflammatory monocytes are recruited to the CNS of human patients during cryptococcal meningitis (76,77). Impaired recruitment of leukocytes to the brain in MIP-1 α knockout mice correlated with impaired clearance of *C. neoformans* in the brain (149), suggesting that accumulation of leukocytes including inflammatory monocytes in the brain is required for fungal clearance. However, the brain inflammatory responses mediated by inflammatory monocytes/macrophages and other leukocytes must be tightly controlled to avoid neuropathology during brain infection with *C. neoformans* (126,150).

NK/NKT Cells

Natural killer cells (NK cells) are innate immune cells and are well known to have the capability of killing virus-infected cells and tumor cells. There are limited NK cells in the brain parenchyma under steady-state conditions, but NK cells have been shown to be recruited in a CX3CR1-dependent manner to glioblastomas, including both common and aggressive brain tumors. Both resident and recruited NK cells are activated by cytokines IL-2, IL-15, and IL-19 to produce TNF and IFN- γ , as well as perforin and granzymes which are able to lyse target cells (151). Early studies have shown that NK cells can bind to *C. neoformans* and directly kill the fungus (152–156). It was later found that NK cells use perforin but not granulysin to kill *C. neoformans* (157), and require PI3K-dependent ERK1/2 signaling (158). It was further demonstrated that β -1,3-glucan, a component of the fungal cell wall, binds to NKp30 expressed on NK cells, mediating recognition and killing of *C. neoformans* (159,160). In addition to the direct killing of *C. neoformans*, NK cells can secrete IFN- γ upon stimulation with IL-12 and IL-18 to activate phagocytes such as macrophages, leading to the indirect killing of *C. neoformans* (161–163).

NKT cells are a unique subset of T lymphocytes and can be identified by their expression of both T cell receptors (TCR) along with NK cell lineage receptors. Invariant NKT (iNKT) cells reside in the brain parenchyma during homeostasis (164). While iNKT cells are CD1d-restricted T cells, both recruitment and activation of peripheral NKT cells to the CNS are CD1d-independent (165). iNKT cells accumulate

in the lung in an MCP-1-dependent manner during pulmonary cryptococcal infection and contribute to the development of protective Th1 immune responses (166). It was reported that α -Galactosylceramide activated NKT cells enhanced their secretions of IFN- γ in the absence of IL-18 signaling during systematic cryptococcal infection (167). Aged C57BL/6 mice with enhanced mature NKT cells displayed stronger fungal resistance in the lungs, indicating that NKT cells play a role in mediating protection (168). However, their role during cryptococcal CNS infection has not yet been elucidated.

CD4/8⁺ T Cells

Following antigen presentation, naïve T cells proliferate and differentiate into antigen-specific T cells, which migrate to infected tissues. Cryptococcal meningoencephalitis primarily affects immunocompromised hosts such as HIV/AIDS patients whose CD4⁺ T cell responses are impaired (1,169), demonstrating the essential role of T cells in host defense against *C. neoformans* infection.

During pulmonary infection with *C. neoformans*, both CD4⁺ and CD8⁺ T cells were substantially recruited to the lung, and depletion of either CD4⁺ or CD8⁺ T cells prevented pulmonary clearance and resulted in significant colonization of the brain in mice, demonstrating that both CD4⁺ and CD8⁺ T cells are required to clear *C. neoformans* infection (170–173). Depletion of either CD4⁺ or CD8⁺ T cells markedly reduced the influx of myeloid cells including monocytes and neutrophils to the infected lung (171). Interestingly, depletion of CD4⁺ T cells did not affect the influx of CD8⁺ T

cells to the infected lung (170–172) and CD8⁺ T cells functioned independently of CD4⁺ T cells to control fungal growth by limiting the survival of *C. neoformans* within macrophages through IFN- γ production (171,174).

During brain infection with *C. neoformans*, a substantial accumulation of CD4⁺ T cells, and to a lesser extent CD8⁺ T cells, was observed in the brain at weeks 3 and 4 post infection (126). Following cryptococcal infection, chemokines such as CCL2, CXCL1, CCL5, and CXCR3 ligands (CXCL9, 10, and 11) were secreted by astrocytes and T cells were recruited into the brain in a CXCR3-dependent manner (175). *In vivo* depletion of CD4⁺ T cells markedly reduced leukocyte accumulation in the brains of immunized mice and was associated with exacerbated CNS infection (176). Another study showed that CD4⁺ T cells were important for optimal infiltration of inflammatory cells into the brain and were required for optimal regional IFN- γ secretion and inducible nitric oxide synthase (iNOS) expression in *C. neoformans*-infected brains of immunized mice (177). However, recent studies have shown that depletion of CD4⁺ T cells prevented brain immunopathology and significantly enhanced the survival of mice despite enhanced fungal growth during brain infection with *C. neoformans* (126). It was further demonstrated that CXCR3⁺ CD4⁺ T cells mediated lethal brain pathology without contribution to fungal clearance in the brain during cryptococcal meningoencephalitis (175), emphasizing the importance of immune balance in the CNS.

In addition to regulating immune responses, T cells have been shown to bind to *C. neoformans* and directly kill the fungus. Human peripheral CD8⁺ T cells were found

to use granulysin to kill *C. neoformans* *in vitro* and this fungicidal activity was dependent on CD4⁺ T cells and IL-15 (178). Likewise, cytotoxic CD4⁺ T cells mediated the killing of *C. neoformans* using granulysin, and activation of this pathway was defective in HIV⁺ patients (179). Moreover, expression of granulysin by cytotoxic CD4⁺ T cells required PI3K- and STAT5-dependent expression of IL-2R β which is also defective in HIV-infected patients (180).

Proinflammatory cytokines

Cytokines play critical roles in regulating immune responses to *C. neoformans* (37). Th1 immune responses, characterized by IFN- γ secretion and classical activation of macrophages, are required to control *C. neoformans* infection (141,181–183). In contrast, Th2 responses, characterized by IL-4, IL-5, and IL-13 production with alternative macrophage activation worsen the disease (184–187). As a signature cytokine of Th1 responses, IFN- γ plays a central role in combating *C. neoformans* infection. Early studies showed that IFN- γ activation of macrophages was critical for anticytotoxic activity (188–190). During *C. neoformans* pulmonary infection, IFN- γ R^{-/-} mice displayed significantly higher fungal burdens in the lung and were markedly more susceptible to *C. neoformans* infection than wild-type mice (181,191). Interestingly, infection of mice with a murine gamma interferon-producing *C. neoformans* strain completely protected mice from infection with wild-type *C. neoformans* (192).

During brain infection with *C. neoformans*, a substantial amount of IFN- γ was detected in the brains of mice (126,193). *In vivo* neutralization of IFN- γ exacerbated cryptococcal CNS infection (176,194). During intracranial infection of *C. neoformans*, IFN- γ was shown to mediate protection by activating microglial cells in the brains of mice (125). Human astrocytes activated by IFN- γ and IL-1 β *in vitro* inhibited cryptococcal growth by a nitric oxide-mediated mechanism (195). In clinical settings, higher secretions of IFN- γ by CD4⁺ T cells were associated with improved survival of patients (196) and the addition of IFN- γ to standard treatments significantly enhanced the rate of clearance of *C. neoformans* infection from the CSF during cryptococcal meningoencephalitis (197,198).

Th17 responses, characterized by IL-17A, also contribute to anti-cryptococcal immunity (37). The development of Th1/Th17 responses and classical activation of macrophages resulted in significant containment of *C. neoformans* cells in the lungs of mice (199). Using IL-17A^{-/-} mice, it was demonstrated that IL-17A mediated protection through the promotion of leukocyte recruitment, activation, and IFN- γ secretions (200). It was recently reported that type I IFN induction via poly-ICLC protected mice against cryptococcosis and that the protective effect was diminished by neutralization of IL-17A, demonstrating the protective function of IL-17A (201).

TNF is another cytokine that mediates protection during cryptococcosis (37). Neutralization of TNF reduced leukocyte influx to the lung and resulted in higher fungal burdens during pulmonary infection of *C. neoformans* (202); TNF was required

for induction of IL-12 and IFN- γ , which mediated protection during pulmonary cryptococcal infection (203). Moreover, TNF mediated protection in the lung infected with *C. neoformans* by inducing DC1 polarization and the initial Th1/Th17 responses at the early stages of infection (204). TNF expression by an engineered *C. neoformans* strain led to protective immune responses during pulmonary cryptococcosis (205). Enhanced secretion of TNF was detected in the brain during *C. neoformans* infection (193). During brain infection with *C. neoformans*, neutralization of TNF led to a remarked increase of brain fungal burdens, indicative of anti-fungal activity of TNF in cryptococcal meningoencephalitis (194). However, the mechanism of the protective role of TNF during cryptococcal CNS infection has not been elucidated.

Fungal virulence factors as immune modulators

To fight against host immune defense, *C. neoformans* develops immune evasion strategies. The major virulence factors of *C. neoformans* include its polysaccharide capsule, melanin production, and the secretion of extracellular enzymes (47). These virulence factors not only contribute to cryptococcal pathogenesis but also are involved in the modulation of host immune responses (206) (Figure 1.4).

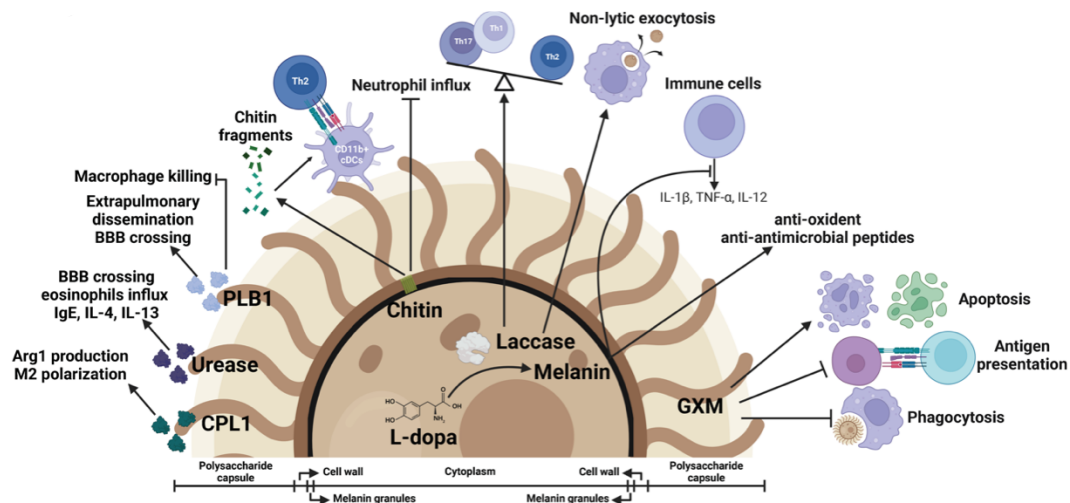


Figure. 1.4 Fungal virulence factors function as immune modulators. (A) Following brain infection of *C. neoformans*, TNF and chemokines are produced, facilitating leukocyte migration to the brain parenchyma. IFN- γ produced by CD4⁺/CD8⁺ T cells and NK/NKT cells promotes the activation of microglia and macrophages. Activated microglia and macrophages secrete TNF, further driving brain inflammation. Recruited inflammatory monocytes differentiate into inflammatory macrophages and help shape Th1 immune responses, which are required for fungal clearance. (B) As the resident glial cells, microglia and astrocytes are the first cells to respond to invading yeast cells. Later, recruited leukocytes including CD4⁺/CD8⁺ T cells, NK/NKT cells, and inflammatory monocyte/macrophages accumulate in and around the fungal clusters. As effector cells, microglia and macrophages internalize the fungal cells. T cells and NK cells secrete IFN- γ to promote the fungicidal activity of these phagocytes. NK cells and T cells are also involved in the direct killing of the fungal cells through the release of perforin or granulysin (GNLY). (C) Inflammatory responses are required for fungal clearance in the brain but must be tightly controlled. Inflammatory monocytes and IFN- γ -secreting CXCR3⁺ CD4⁺ T cells facilitate neuron damage and cause immunopathology during cryptococcal meningoencephalitis. Figures were generated using BioRender.

Polysaccharide capsule

One of the major virulence factors for *C. neoformans* is the polysaccharide capsule (47). The capsule is located outside of the fungal cell wall and protects the fungus from immune recognition and destruction. Studies have shown that the capsule

is mainly composed of two types of polysaccharides and that the dominant polysaccharide (>90% of the total capsule polysaccharides) is glucuronoxylomannan (GXM); another polysaccharide, known as galactoxylomannan (GalXM), represents the other 5-8% of capsule along with nonpolysaccharide components such as mannoprotein (44,206,207). The capsular polysaccharide is synthesized intracellularly and is believed to be translocated to the extracellular space within vesicles (207).

Cryptococcal capsule modulates host immune responses in multiple ways (44,206). The capsule prevents phagocytosis of the fungus by macrophages (61,208). GXM is known to inhibit the antigen-presenting capacity of human monocytes and monocyte-derived macrophages through the induction of IL-10 secretion and suppression of MHC-II expression, resulting in impaired T cell proliferation (209–211). GXM also induces apoptosis of macrophages and T cells through induction of Fas ligand expression (212,213) or induction of iNOS expression and NO production in a caspase-independent pathway (214). GXM also inhibits DC activation and maturation (215,216). Of note, the regulated release of capsular GXM of *C. neoformans* suppresses leukocyte migration to the brain, promoting fungal growth in the brain (217). In clinical settings, capsule shedding (exo-cellular GXM) can be detected in the serum and CSF of patients during cryptococcal meningitis (218,219). There is a positive correlation between serum GXM titers and mortality during HIV-associated cryptococcal meningitis (220). Moreover, the increased size of the capsule correlated with impaired fungal clearance and reduced CSF leukocytes and proinflammatory cytokines

including IL-6 and IFN- γ , confirming the immunosuppressive properties of the capsule in HIV-associated cryptococcal meningitis (218).

Laccase activity and melanin formation

In addition to capsule, laccase and melanin are known major virulence factors of *C. neoformans* (47,221–225). Laccase is required for the biosynthesis of melanin (226), which accumulates in the *C. neoformans* cell wall (47). Published work suggested that laccase facilitated extrapulmonary dissemination of *C. neoformans* (224). This is likely, at least in part, because laccase activity protected *C. neoformans* from killing by alveolar macrophages (222). Moreover, laccase activity enhanced pulmonary eosinophilia and shifted immune polarization from protective Th1/Th17 and M1 responses to deleterious Th2 and M2 responses, promoting fungal growth during pulmonary infection with *C. neoformans* (225). Recently, it has been shown that laccase activity dampened Th17 responses, neutrophil accumulation, and function during the early stages of infection with *C. gattii* (227). Interestingly, laccase is also critically involved in the regulation of nonlytic exocytosis of *C. neoformans* from macrophages (228). In HIV-associated cryptococcosis patients, cryptococcal laccase activity correlated with fungal survival and poor fungal clearance in CSFs (78), arguably through regulation of the interaction between *C. neoformans* and phagocytes. Thus, cryptococcal laccase modulates host defenses through multiple mechanisms involving innate and adaptive immunity.

Located in the cell wall, cryptococcal melanin is a powerful antioxidant and helps protect fungal cells against oxygen- and nitrogen-derived oxidants generated by host effector cells (46,229–231). Early studies have shown that melanization of *C. neoformans* correlated with higher levels of IL-4 and MCP-1 and enhanced leukocyte recruitment and interfered with phagocytosis, indicative of immunomodulation (232). Due to the presence of melanin, *C. neoformans* is less susceptible to cationic antimicrobial peptides released by phagocytes (233). Solubilized melanin has been recently shown to inhibit macrophage functions (234). In addition to modulating innate immunity, melanin inhibited antigen recognition and down-regulated T cell immunity and inflammation during *C. neoformans* pulmonary infection (235). During brain infection with *C. neoformans*, melanization suppressed the production of IL-12, IL-1 β TNF, IFN- γ , and iNOS, promoting mortality (236).

Phospholipase B1 activity

Phospholipases are extracellular enzymes that degrade cell membrane phospholipids (47). Amongst them, phospholipase B1 (PLB1) has been well characterized and is known as one of the major virulence factors of *C. neoformans* (47,72,237–242). Depletion of PLB1 significantly reduced fungal burdens during pulmonary infection with *C. neoformans* (240). In addition, PLB1 is essential for extrapulmonary dissemination of *C. neoformans* (241,242) and transmigration of the fungus across the BBB (72,86). Importantly PLB1 is required for the release of arachidonic acid from phospholipids and the production of cryptococcal eicosanoid, which down-regulates macrophage functions *in vitro* and during pulmonary infection

(242). In line with this finding, recent work suggested that PLB1 promoted the proliferation of *C. neoformans* within macrophages and inhibited the killing of the fungus within the phagosome (243).

Urease activity

Urease is an extracellular enzyme and is considered a major virulence factor of *C. neoformans* (93). *C. neoformans* secretes urease that catalyzes the hydrolysis of urea to ammonia and carbamate (47). Published work suggests that urease activity promotes cryptococcal brain invasion (58,94,95). In addition, infection with urease-producing *C. neoformans* correlated with enhanced eosinophil influx, higher levels of IgE, IL-4, and IL-13, and alternatively, activation of macrophages, suggesting that urease activity promotes Th2 immune responses (244).

Chitin

Chitin is an essential component of the cell wall of *C. neoformans*, and the fungus has eight putative chitin synthases (245). Mammalian hosts have chitotriosidase, an enzyme that can degrade chitin into chitin fragments (246,247). Early studies showed that degraded chitin fragments could trigger human macrophage activation (248). In a murine model of infection with *C. neoformans*, host chitotriosidase degraded fungal chitin into small fragments that led to Th2 differentiation by CD11b⁺ conventional DCs, demonstrating that chitin recognition via chitotriosidase promoted detrimental Th2 immune responses (145). Of note, chitotriosidase activity correlated

with cryptococcal infection in humans (145). In contrast to the negative impact of chitin on immune responses, it has been recently reported that mice infected with *C. neoformans* depleted of chitin synthase 3 died significantly earlier due to excessive neutrophil influx, demonstrating a beneficial role of chitin in preventing lethal immune responses to *C. neoformans* (249).

CPL1

CPL1 is a secreted cryptococcal protein encoded by CNAG_02797/*CPL1* (250). A recent study showed that CPL1 induced arginase-1 production in macrophages and enhanced macrophage sensitivity to IL-4 signaling through activation of TLR4 signaling and promotion of phosphorylation of STAT3. As a result, CPL1 drove alternative activation of macrophages and promoted harmful type 2 immunity during cryptococcosis (251).

Concluding remarks

Although cryptococcal infection starts in the lung, cryptococcosis commonly presents as meningoencephalitis (252). Cryptococcal meningoencephalitis is among the most devastating complications of HIV/AIDS patients and is a leading cause of mortality of HIV/AIDS patients (253,254). One of the critical steps to cause the illness is fungal dissemination and invasion into the brain across the BBB (60). Fungal invasion leads to host defenses including innate and adaptive immunity involving

resident microglial cells and recruited myeloid cells and T cells (41). In response to host defense, *C. neoformans* uses multiple virulence factors to suppress host immune responses (206,255). In the past decades, much progress has been achieved in understanding the interaction between host and *C. neoformans*. However, many questions remain unanswered. For example, which pathway is dominant during brain invasion by *C. neoformans*? How do microglial cells interact with recruited leukocytes in the brain during *C. neoformans* infection? What is the strategy that the fungus uses to survive and replicate in the brain? By addressing these questions, we will have a better understanding of the complex interaction between the host and *C. neoformans*.

Chapter 2: Quantitative analysis reveals internalization of *Cryptococcus neoformans* by brain endothelial cells *in vivo*

This chapter is based on a published paper [Chen Y, Li C, Sun D, Strickland AB, Liu G, Shi M. Quantitative analysis reveals internalisation of *Cryptococcus neoformans* by brain endothelial cells *in vivo*. *Cell Microbiol.* 2021;23(6):e13330. doi:10.1111/cmi.13330]. Yanli Chen contributed to Conceptualization, Designing and conducting all experiments, Collecting and analyzing all the data, Writing – original draft preparation, and Writing – review and editing.

Abstract

Migration of *Cryptococcus neoformans* (*C. neoformans*) from the blood to the brain parenchyma is crucial to cause fatal meningoencephalitis. Although mechanisms involved in the brain migration of *C. neoformans* have been widely studied *in vitro*, less is known about how the fungus crosses the blood-brain barrier (BBB) *in vivo*. This is in part because of the lack of an approach to quantitatively analyze the dynamics of fungal transmigration into the brain across the BBB *in vivo*. In this study, we report a novel approach to quantitatively analyze the interactions between *C. neoformans* and brain endothelial cells in a mouse model using flow cytometry. Using this system, we show that *C. neoformans* was internalized by brain endothelial cells *in vivo* and that mice infected with acapsular or heat-killed *C. neoformans* yeast cells displayed a lower frequency of brain endothelial cells containing the yeast cell compared to mice infected with wild-type or viable yeast cells, respectively. We further demonstrate that brain endothelial cells were invaded by the serotype A strain (H99 strain) at a higher rate

compared to serotype D strain (52D strain). Our experiments established that internalization of *C. neoformans* by brain endothelial cells occurred *in vivo* and offered a powerful approach to quantitatively analyze fungal migration into the brain.

Introduction

Cryptococcosis is caused by encapsulated fungal pathogen *Cryptococcus neoformans* (*C. neoformans*) (256). The fungal cells exist in the environment and when they are inhaled into the lung, they will initially induce lung infection. In immunocompetent individuals, the fungal cells are usually cleared by the immune cells or establish a latent infection in the lung. However, in immunocompromised individuals including AIDS patients, the organisms can disseminate from the lung to the brain, causing meningoencephalitis (256,257). Cryptococcal meningoencephalitis is often fatal without treatment, and even with treatment two-thirds of patients die within a few months of diagnosis (258,259). Worldwide, fatalities due to cryptococcal meningoencephalitis were recently estimated at more than 181,100 cases each year (260). It is believed that crossing of the blood-brain barrier (BBB) by *C. neoformans* is a critical step in causing meningoencephalitis (261). As such, the mechanism(s) of BBB crossing by *C. neoformans* is fundamental for understanding cryptococcal pathogenesis (262).

The BBB is a structural and functional barrier. It separates the brain parenchyma from the blood, maintaining the neural microenvironment by regulating

the passage of molecules and cells into the brain (263). Although *C. neoformans* has been shown to hijack phagocytes such as monocytes and macrophages to transmigrate into the brain across the BBB within phagocytes (264–268), free yeast cells have also been proposed to directly transmigrate to the brain parenchyma through transcytosis mediated by brain endothelial cells (265,269). Evidence of transcytosis is mainly derived from *in vitro* studies using a monolayer of human brain microvascular endothelial cells (HBMECs) cultured *in vitro*. In this *in vitro* system, HBMECs were grown in a collagen-coated glass coverslip or a transwell chamber (269–271). During the experiment, *C. neoformans* yeast cells were allowed to settle onto the top of HBMECs, the adhesion to and invasion of HBMECs by *C. neoformans* were observed by microscopy and the transmigration of the yeast cells via transcytosis was determined by counting colony forming units (CFU) in the bottom chamber. Recently, this system has been also used to study Trojan horse mechanisms by adding monocytes harboring *C. neoformans* onto the top of HBMECs (267,272). These *in vitro* models have made great contribution to our understanding of fungal transmigration to the brain, leading to the discovery of several important molecules involved in brain invasion of *C. neoformans*. However, it remains incompletely understood as to how *C. neoformans* transmigrates into the brain across the BBB *in vivo*, mainly because of technical challenges characterizing dynamic events of fungal BBB crossing *in vivo*.

The BBB contains not only endothelial cells, but also the basal lamina, pericytes and astrocytes (263). The previously used *in vitro* BBB model is composed of only a monolayer of endothelial cells; it therefore does not reflect the complexity of the BBB

in vivo. In addition, the *in vitro* BBB models cannot recapitulate some *in vivo* features in the brain microvasculature such as shear stress and interactions of cells with extracellular matrix molecules (273). Therefore, it is necessary to develop a novel approach to study fungal migration into the brain *in vivo*. In this study, we established a quantitative analysis of fungal migration into the brain in a mouse model of infection with GFP-expressing or CFSE (carboxyfluorescein di-acetate succinimidyl ester) labeled *C. neoformans*. The frequency of brain endothelial cells containing *C. neoformans* was analyzed by flow cytometry. The internalization of the yeast cell by brain endothelial cells was further confirmed by confocal microscopy after fluorescence-activated cell sorting of the endothelial cells. We analyzed the effect of fungal capsule and viability on the frequency of brain endothelial cells invaded by the yeast cell. Furthermore, we compared the brain invasion between serotype A and serotype D of *C. neoformans*. Finally, we evaluated the effect of host CD44 expressed by brain endothelial cells on cryptococcal invasion to brain endothelial cells. We conclude that *C. neoformans* was internalized by brain endothelial cells *in vivo* and that flow cytometry is a powerful tool to study brain invasion by *C. neoformans in vivo* by quantitatively analyzing the interaction between the yeast cells and brain endothelial cells.

Material and methods

Mice

Eight- to twelve-week-old wild-type C57BL/6 mice were purchased from the National Cancer Institute (NCI). CD44^{-/-} mice in the C57BL/6 background (Catalog No. 005085) (274) were purchased from The Jackson Laboratory and bred in-house in the animal facilities of the University of Maryland, College Park. Mice were maintained in pathogen-free individually ventilated cages. All experiments were performed in accordance with the guidelines approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee.

Fungal strains and culture

Encapsulated *C. neoformans* H99 strain (serotype A), its isogenic derivative with deletion in the *cap59* gene (acapsular strain, a gift of Dr. Kyung J Kwon-Chung, NIH), the temperature-sensitive strains *ras1* and *cdc24* mutant in H99 background (a gift of Dr. J. Andrew Alspaugh, Duke University Medical Center), *C. neoformans* 52D strain (serotype D), and *C. neoformans* XL280 (serotype D, a gift of Dr. Xiaorong Lin, University of Georgia) were used in this study. GFP-expressing H99 strain (a gift of Dr. Robin May, University of Birmingham) was also used in this study. The yeast cells were grown in Sabouraud dextrose broth (BD) to exponential phase at 30°C with gentle rotation in an orbital shaker (200 r.p.m). The yeast cells were harvested by centrifugation at 500 × g for 5 min and washed twice in sterile 1× PBS. The concentration of the yeast cells was determined using a hemocytometer. All experiments were performed in accordance with the biosafety level 2 guidelines.

Staining of *C. neoformans*

C. neoformans yeast cells were labeled with carboxyfluorescein di-acetate succinimidyl ester (CFSE). 100 μ L of 5 mM CFSE stock solution was used to stain 10 mL of cell suspension with 1×10^7 cells, for a working concentration of 50 μ M. The cell suspension was incubated at 37°C for 15 min. The yeast cells were then collected by centrifugation at $500 \times g$ for 5 min and washed twice with sterile $1 \times$ PBS. In some experiments, *C. neoformans* yeast cells were labeled with Unvitex 2B *in vitro* as described previously (275) or were heat-killed at 55°C for 20 min before use.

Infection

Mice were intravenously infected with 5×10^6 , 10×10^6 or 20×10^6 *C. neoformans* yeast cells in 200 μ L sterile $1 \times$ PBS.

Quantification of fungal burdens in the brain

For fungal burden determination, mice were intravenously infected with 20×10^6 *C. neoformans* H99 or *C. neoformans* 52D and euthanized 3 days after infection. To determine fungal burdens, mice were transcardially perfused from the left ventricle with 20 mL of $1 \times$ PBS, and brain tissues were collected into 15 mL tubes each containing 2 mL sterile $1 \times$ PBS. The tissues were homogenized with a tissue homogenizer (Omni International, USA). After serial dilution and plating on Sabouraud dextrose agar plate (BD), colony forming units (CFU) were counted after fungal growth for 36 h at 30°C in the incubator.

Isolation of mouse endothelial cells from the brain

Mouse brain endothelial cells were isolated as described previously with adjustments (276). Mice were euthanized and perfusion was performed with 20 mL 1 × PBS. Mouse brains were collected, and brain stems were removed prior to homogenization. The brain was placed in 2 mL of ice cold RPMI 1640 Medium (Corning™) supplemented with L-Glutamine. Brains were minced into small pieces with surgical scissors, digested with Collagenase II and Collagenase IV (Worthington) with 1 mg/mL as working concentration in the incubator at 37°C for 60 min, and then forced gently through a 70 µM cell strainer using a sterile syringe plunger and washed with 15 mL of RPMI-1640 medium. The cell suspension was centrifuged at 500 × g for 5 min at 4°C. The supernatant was discarded, and the pellet was re-suspended in 2 mL of 25% Percoll and the cell suspension was layered onto 2 mL of 100% Percoll (GE, USA) and then centrifuged at 1500 × g with the brake off for 15 min at room temperature. The myelin layer was carefully removed with a serological pipette. The cell suspension at the interface was harvested and washed with 12 mL 1 × PBS by centrifugation at 500 × g for 5 min at 4°C to remove the Percoll residue. The pellet was resuspended in 400 µL of erythrocyte lysing (ACK) buffer, and incubated at room temperature for 2 min, then diluted with 10 mL 1 × PBS, followed by centrifugation at 500 × g for 5 min at 4°C. Cells were resuspended with 1 mL of flow cytometry staining buffer and counted with a hemocytometer. Cells were diluted with flow cytometry staining buffer to concentration of 5 × 10⁶ cells/mL prior to staining.

Flow cytometry analysis of brain endothelial cells

The cell suspension prepared as described above was incubated with purified anti-mouse CD16/CD32 ([Fc γ III/II Receptor], clone: 2.4G2, Biolegend) for 15 min at 4°C to block nonspecific binding of antibodies to FcRs and washed with staining buffer. After centrifugation at 500 $\times$ g for 5 min at 4°C, the cells were suspended with staining buffer and stained with anti-mouse CD31 (clone: 390, Biolegend) and anti-mouse CD45 (clone: 30-F11, Biolegend) mAbs for cell surface markers for 30 min at 4°C and washed with staining buffer by centrifugation. The pellet was suspended with staining buffer and kept at 4°C until analysis by BD FACSCanto™ II. Results were analyzed using FlowJo software.

Fluorescent-activated sorting of CD31⁺GFP⁺ or CD31⁺CFSE⁺ cells

The preparation for samples were described above. The samples were sorted with BD FACSAira™ II cell sorter. The CD31⁺GFP⁺CD45⁻ or CD31⁺CFSE⁺ CD45⁻ cells were collected in a well of 24-well plate containing 1 mL FBS. The samples collected were kept on ice.

Confocal microscopy

Samples sorted using the BD FACSAira™ II were transferred to a 1.5 mL Eppendorf tube and centrifuged at 500 $\times$ g for 5 min at 4°C. The supernatant was discarded, and the pellet was resuspended with 10 μ L staining buffer and pipetted onto the glass bottom of a 35 mm microwell dish (MatTek Corporation) and a cover glass (Fisherbrand) was placed on the samples. The samples were examined under a Zeiss LSM 800 system.

Statistics

GraphPad Prism 8.0 software (GraphPad) was used for all statistical analyses. Data are presented as the mean $\pm$ SEM. Significance of differences was determined by two-tailed Student's *t* test or Log-rank (Mantel-Cox) test. A *p* value of < 0.05 is considered statistically significant.

Results

Internalization of *C. neoformans* by brain endothelial cells occurs *in vivo*

C. neoformans was observed to initially adhere to cultured endothelial cells and then become internalized by endothelial cells (269). To study the interaction of *C. neoformans* yeast cells with brain endothelial cells and their migration to the brain *in vivo*, we developed a quantitative analysis method in a mouse model of infection with *C. neoformans* by using flow cytometry. We infected mice with GFP-expressing *C. neoformans* H99 via the tail vein and then isolated brain endothelial cells by using a Percoll gradient. We stained the cells with anti-CD45 and anti-CD31 mAbs. After selecting single cells, the CD31⁺CD45⁻ population was identified as endothelial cells (Figure. 2.1A and B). We further gated on GFP⁺CD31⁺ cells among the endothelial cell population to identify the endothelial population harboring *C. neoformans* (Figure. 2.1C). To further confirm internalization of *C. neoformans* by brain endothelial cells,

GFP⁺CD31⁺ endothelial cells were sorted using fluorescence-activated cell sorting technique and subjected to analysis by confocal microscopy. The results showed that GFP⁺ cells detected were inside CD31⁺ cells, suggesting co-localization of *C. neoformans* and endothelial cells and confirming internalization of *C. neoformans* by brain endothelial cells (Figure. 2.1D, Video S1). To determine the kinetics of interactions between *C. neoformans* and brain endothelial cells, we next infected mice with GFP-expressing *C. neoformans* and euthanized the mice at various time points. Consistent with *in vitro* studies (277), there was a tendency for the frequency of brain endothelial cells harboring *C. neoformans* to increase with time (Figure. 2.1E and F). Moreover, we infected mice with different dosages of fungal cells and found that the frequency of internalization correlated with the number of fungal cells injected (Figure. 2.1G and H). To determine the portion of yeast cells expressing GFP *in vivo*, we labeled GFP-expressing yeast cells with Uvitex 2B (275,278) and then infected mice with the double labeled yeast cells. The results confirmed that *C. neoformans* continuously expressed GFP (Figure. 2.1I). Collectively, we established a novel system to quantitatively analyze the interactions of *C. neoformans* with brain endothelial cells *in vivo* in a mouse model of infection by using flow cytometry and demonstrated that internalization of *C. neoformans* occurred *in vivo* by using this system.

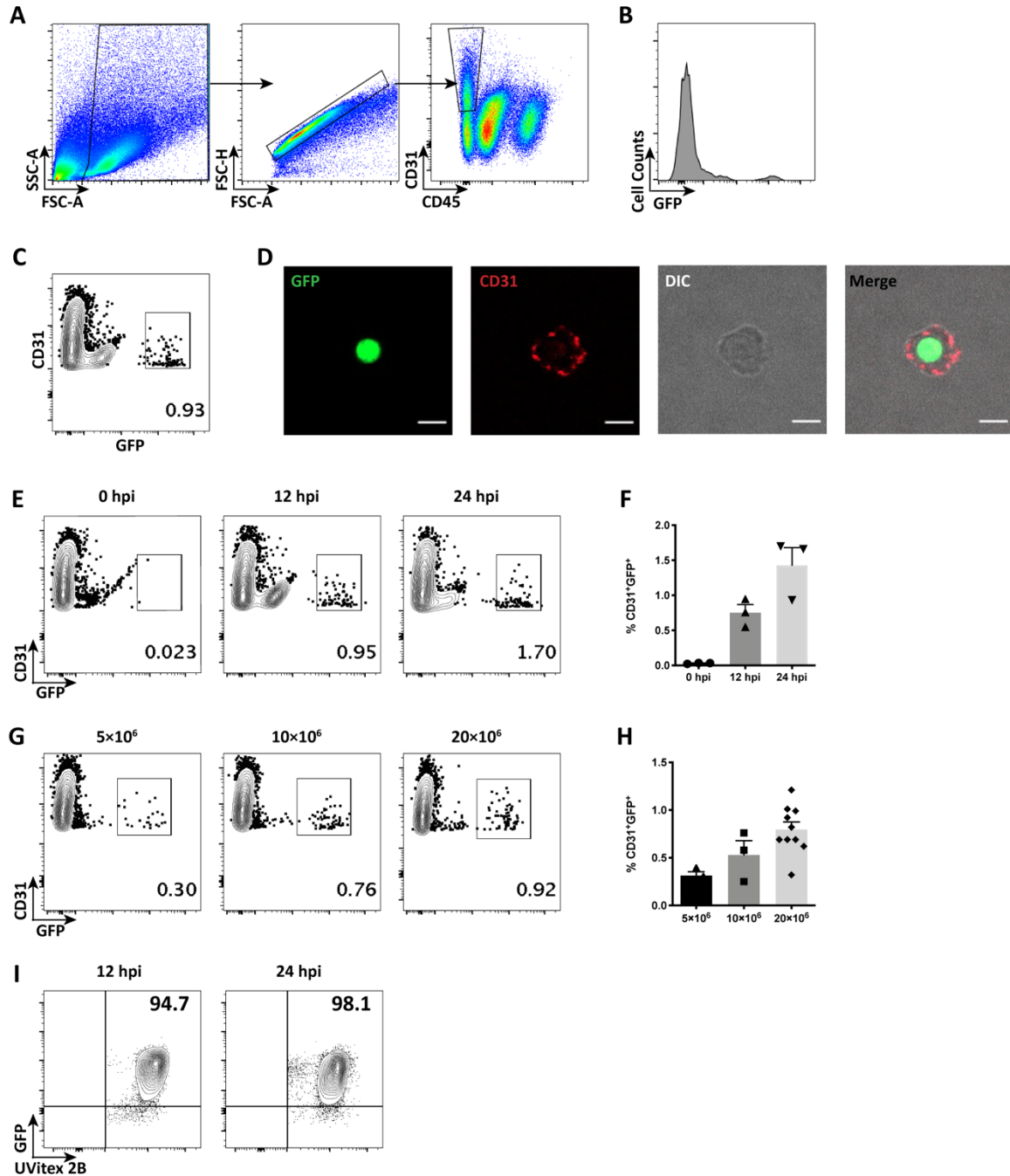


Figure. 2.1 Quantitative analysis of internalization of GFP-expressing *C. neoformans* by brain endothelialization *in vivo*. (A and B) Gating strategy of CD31⁺GFP⁺ cells. Endothelial cells were isolated from the brain of mice 24 h after i.v. infection with 20×10⁶ GFP-expressing *C. neoformans*. Endothelial cells were defined as CD31⁺CD45⁻. CD45⁺ leukocytes and CD45 intermediate microglia were gated out after selecting CD45⁻ population (A). A representative flow cytometry histogram showing the expression of GFP by CD31⁺CD45⁻ cells (B). (C and D) Mice were infected with 20×10⁶ GFP-expressing *C. neoformans* H99. Brain endothelial cells were purified 24 h after infection. The frequency of

CD31⁺GFP⁺ cells (gated on CD31⁺ CD45⁻ cells) was determined by flow cytometry **(C)**. CD31⁺GFP⁺ cells were sorted by fluorescence-activated cell sorting (FACS). The sorted cells were subjected to analysis by confocal microscopy to determine the colocalization of brain endothelial cells (CD31⁺) and the yeast cells (GFP⁺) **(D)**. **(E and F)** Mice were infected with 20×10⁶ GFP-expressing *C. neoformans* H99. The frequency of CD31⁺GFP⁺ cells (within CD31⁺CD45⁻ cells) was determined 0, 12, and 24 h post infection by flow cytometry. Representative dot plots I and quantification **(F)**. **(G and H)** Mice were infected with 5×10⁶, 10×10⁶, or 20×10⁶ GFP-expressing *C. neoformans* H99. The frequency of CD31⁺GFP⁺ cells (within CD31⁺CD45⁻ cells) was determined 24 h post infection by flow cytometry. Representative dot plots **(G)** and quantification **(H)**. **(I)** GFP-expressing H99 yeast cells were labeled with Uvitex 2B to determine the GFP fluorescent efficiency *in vivo* by flow cytometry. Mice were infected with 20×10⁶ Uvitex 2B labeled GFP-expressing H99. Representative dot plots showing the frequency of GFP⁺Uvitex 2B⁺ cells in the brain 12 h and 24 h post infection. Data shown are the mean ± SEM. Dots represent individual mice. Data are a representative of two independent experiments. Scale bars: 5 μm.

Using CFSE-labeled *C. neoformans* to quantitatively analyze the interaction between *C. neoformans* and brain endothelial cells *in vivo*

The above results demonstrated that *C. neoformans* yeast cells expressing GFP are useful for analyzing the interaction of *C. neoformans* with brain endothelial cells by flow cytometry, particularly, when we study how host factors affect cryptococcal transmigration into the brain. However, if *C. neoformans* mutant strains are used to analyze effects of fungal factors on BBB crossing, the fungal cells must be labeled *in vitro* with fluorescent dyes. After comparison of various dyes including fluorescein isothiocyanate, tetramethylrhodamine, and CFSE, we decided to label the yeast cells with CFSE. CFSE is not toxic to cells and has been widely used to label T cells for *in vivo* proliferation assay, since the fluorescence of cells can be detected even after cell proliferation.

Based on *in vitro* and *in vivo* studies, *C. neoformans* started to cross the BBB within hours (269,279), and did not proliferate until 24 h after intravenous infection (279,280). We confirmed that *C. neoformans* did not lose CFSE-labeling within 24 hours (Fig. 2C). Thus, we intravenously infected mice with CFSE-labeled *C. neoformans* H99 yeast cells and isolated brain endothelial cells 24 h after infection. Following staining with anti-CD45 and anti-CD31 mAbs, CFSE⁺CD31⁺ cells were detected in the population of brain endothelial cells (Figure. 2.2A). We sorted those double positive cells by flow cytometry for the examination of co-localization of *C. neoformans* and brain endothelial cells by confocal microscopy. The results showed that, like GFP-expressing *C. neoformans*, CFSE-labeled yeast cells were located inside

brain endothelial cells, suggesting internalization of CFSE-labeled *C. neoformans* by brain endothelial cells in the infected brain (Figure. 2.2B, Video S2). Furthermore, we confirmed that CFSE can be used to label other wild-type (XL280) and mutant (temperature sensitive *ras1* and *cdc24*) strains of *C. neoformans* for *in vivo* quantitative assays (Figure. 2.2D).

Together, those results confirmed the internalization of *C. neoformans* by brain endothelial cells *in vivo* and demonstrated that CFSE-labeled yeast cells can be used for quantitative analysis of fungal brain migration by flow cytometry.

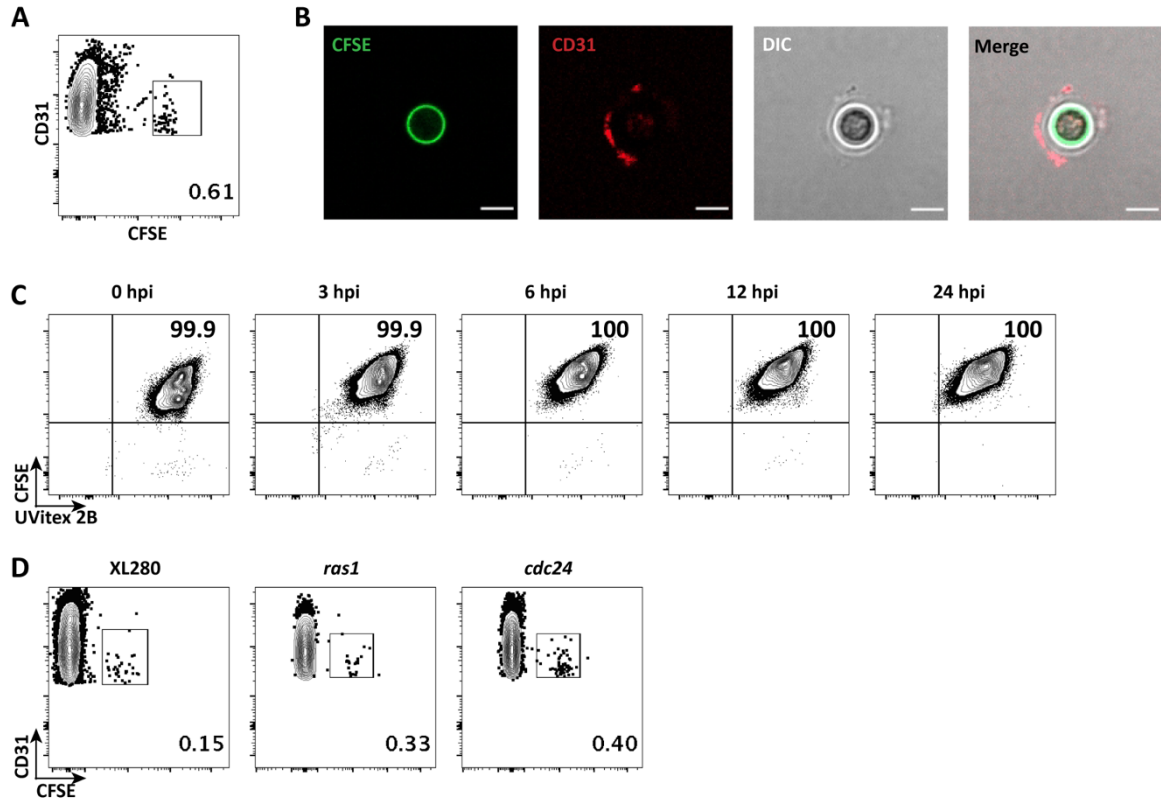


Figure. 2.2 Quantitative analysis of internalization of CFSE-labeled *C. neoformans* by brain endothelialization *in vivo*. (A and B) Mice were infected with 20×10^6 CFSE-labeled *C. neoformans* H99. Brain endothelial cells were purified 24 h after infection. The frequency of CD31⁺CFSE⁺ cells (gated on CD31⁺ CD45⁻ cells) was determined by flow cytometry (A). CD31⁺CFSE⁺ cells were sorted by fluorescence-activated cell sorting (FACS). The sorted cells were subjected to analysis by confocal microscopy to determine the colocalization of brain endothelial cells (CD31⁺) and the yeast cells (CFSE⁺) (B). Scale bars: 5 μ m. CFSE-labeled H99 cells were stained with Uvitex 2B to determine the CFSE fluorescent efficiency. Yeast cells labeled with CFSE and Uvitex 2B were cultured *in vitro* and collected at 0 h, 3 h, 6 h, 12 h, 24 h post culture for flow cytometry (C). Mice were infected with CFSE-labeled *C. neoformans* XY280 and mutant strains *ras1* and *cdc24*. The frequency of CD31⁺CFSE⁺ cells (gated on CD31⁺CD45⁻ cells) in the brain was determined 24 h post infection by flow cytometry (D).

Mice infected with acapsular *C. neoformans* exhibit a lower frequency of endothelial cells containing the yeast cell

Having established the quantitative analysis of fungal transmigration, we next examined how fungal factors affect the interaction of *C. neoformans* with brain endothelial cells using this novel approach. As a major virulence factor, cryptococcal capsule is involved in pathogenesis of the fungus through multiple mechanisms (281). However, the role of capsule in fungal transmigration into the brain across the BBB *in vivo* is largely unknown.

Mice were infected with CFSE-labeled *C. neoformans* H99 and its isogenic acapsular mutant *cap59*. Internalization of the yeast cells by brain endothelial cells was quantitatively analyzed by flow cytometry at 3 h, 6 h, 12 h and 24 h post infection. Overall, we detected a significant reduction in the frequency and absolute number of brain endothelial cells containing *C. neoformans* after infection with acapsular mutant *cap59* as compared to infection with wild-type H99 (Figure. 2.3).

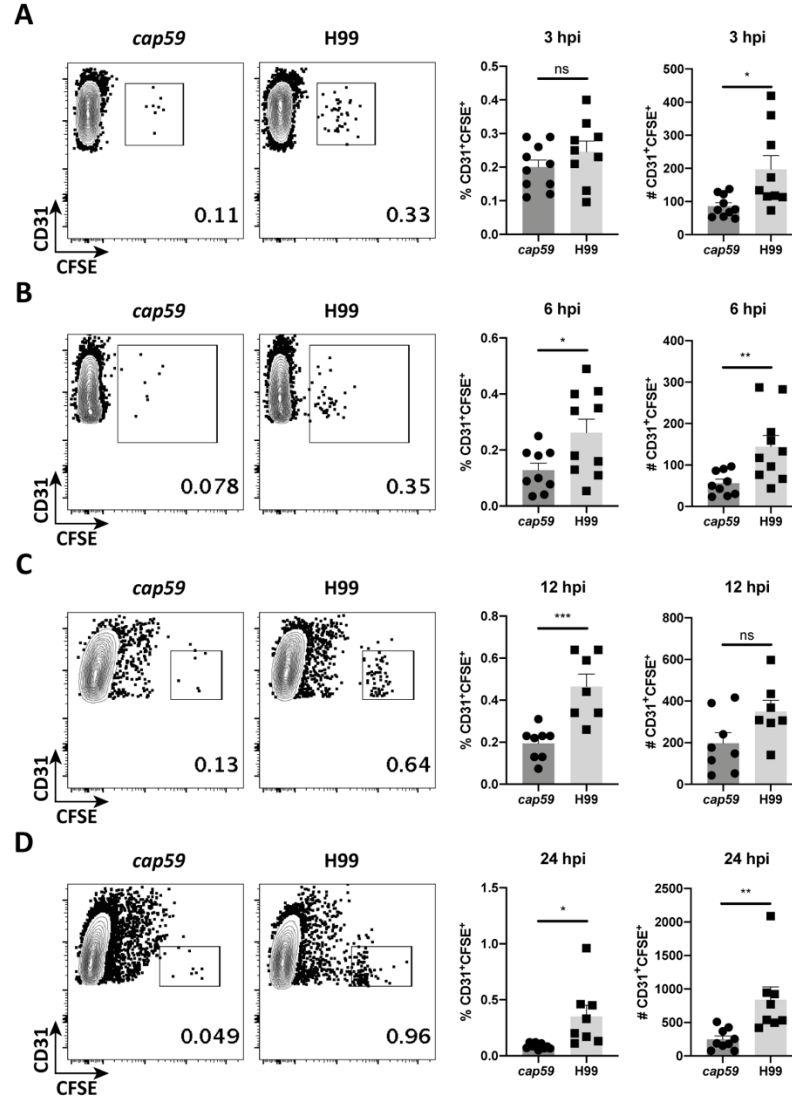


Figure. 2.3 Mice infected with acapsular *C. neoformans* display a lower frequency of infected brain endothelial cells compared to wild-type *C. neoformans*. Mice were infected with 20×10^6 CFSE-labeled acapsular strain *cap59* or wild-type *C. neoformans* H99. Brain endothelial cells were purified 3 h (A), 6 h (B), 12 h (C) or 24 h (D) after infection. The frequency of CD31⁺CFSE⁺ cells (gated on CD31⁺CD45⁻ cells) was determined by flow cytometry. Left panels: representative dot plots; right panels: frequency (within CD31⁺CD45⁻ cells) and absolute number. Data from two independent experiments were pooled and are presented as mean $\pm$ SEM. Dots represent individual mice. Ns, not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Mice infected with heat-killed *C. neoformans* display a lower frequency of endothelial cells containing the yeast cell

It has been shown that viability did not affect the arrest of *C. neoformans* in the brain vasculature (280). To examine the effect of viability of *C. neoformans* on the internalization of the yeast cell by brain endothelial cells, mice were intravenously injected with CFSE-labeled viable *C. neoformans* H99 yeast cells or heat-killed yeast cells. Internalization of the yeast cells was quantitatively analyzed 24 h post infection by flow cytometry. The results showed that the frequency and absolute number of brain endothelial cells containing *C. neoformans* were significantly lower in mice injected with heat-killed *C. neoformans* as compared to mice injected with viable yeast cells (Figure. 2.4).

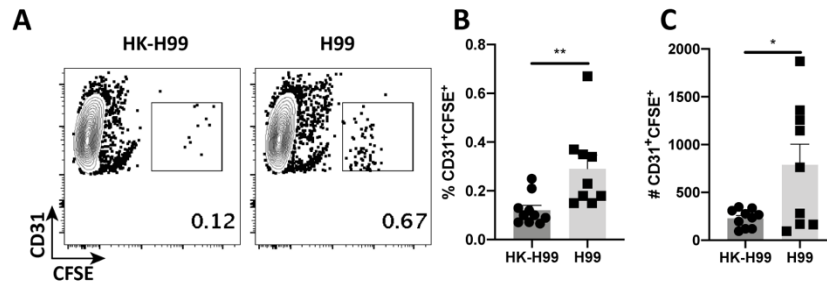


Figure. 2.4 Mice infected with heat-killed *C. neoformans* display a lower frequency of infected brain endothelial cells compared to viable *C. neoformans*. Mice were infected with 20×10^6 CFSE-labeled heat-killed (HK-99) or viable *C. neoformans* H99. Brain endothelial cells were purified 24 h after infection. The frequency of CD31⁺CFSE⁺ cells (gated on CD31⁺CD45⁻ cells) was determined by flow cytometry. (A) representative dot plots, (B) frequency (within CD31⁺CD45⁻ cells), and (C) absolute number. Data from two independent experiments were pooled and are presented as mean $\pm$ SEM. Dots represent individual mice. * $p < 0.05$, ** $p < 0.01$.

Brain endothelial cells are invaded by serotype A of *C. neoformans* at a higher rate than serotype D strain

C. neoformans has two serotype strains: serotype A and serotype D, which have different capsule structures. It has been reported that serotype A H99 strain is more virulent than serotype D 52D strain in a mouse model of pulmonary infection (282). Although these two strains induce different polarization of CD4⁺ T cells which may account for the different outcomes of infections (282), it remains to be determined whether they have different capacities of crossing the BBB. To address this question, we infected mice with CFSE-labeled *C. neoformans* H99 strain or *C. neoformans* 52D strain and compared the fungal invasion into brain endothelial cells. The results showed that the frequency and absolute number of endothelial cells harboring *C. neoformans* were significantly higher following infection with H99 strain as compared to infection with 52D strain (Figure. 2.5A-C), suggesting that the serotype of *C. neoformans* affected the internalization of the yeast cell by brain endothelial cells. Accordingly, mice infected with H99 strain died earlier (Figure. 2.5D), associated with a higher fungal burden in the brain (Figure. 2.5E), as compared to mice infected with 52D strain. Collectively, these data suggested that H99 strain entered brain endothelial cells at a higher rate than 52D strain.

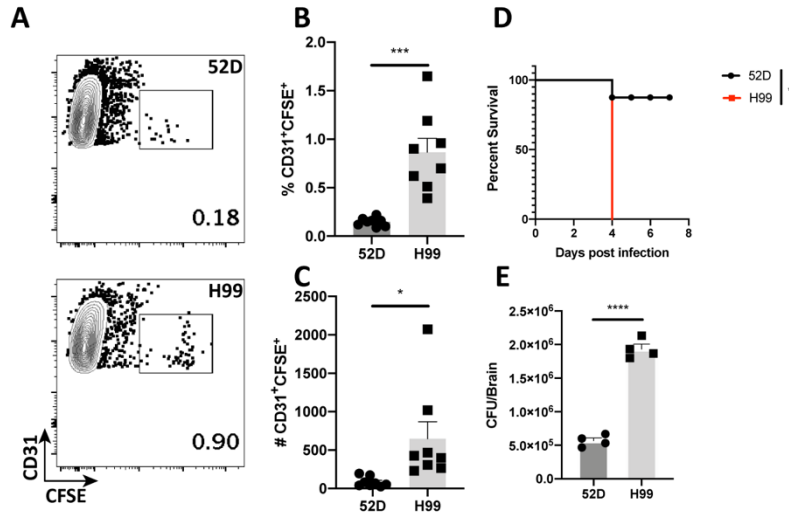


Figure. 2.5 The frequency of brain endothelial cells invaded by serotype A of *C. neoformans* is higher compared to serotype D. (A-C) Mice were infected with 20×10^6 CFSE-labeled *C. neoformans* H99 (serotype A) or 52D (serotype D). Brain endothelial cells were purified 24 h after infection. The frequency of CD31⁺CFSE⁺ cells (gated on CD31⁺CD45⁻ cells) was determined by flow cytometry. **(A)** representative dot plots, **(B)** frequency (within CD31⁺CD45⁻ cells), and **(C)** absolute number. **(D and E)** Mice were infected with 20×10^6 *C. neoformans* H99 or *C. neoformans* 52D. The survival was determined (n=5 mice/group) **(D)**. The fungal burdens in the brain were determined on day 3 after infection (n=4 mice/group) **(E)**. Data from two independent experiments were pooled for **(B)** and **(C)**, and data are presented as mean $\pm$ SEM. Dots represent individual mice. * p<0.05, ** p<0.01, ***p<0.001, **** p<0.0001.

Mice deficient in CD44 show a slightly lower frequency of brain endothelial cells containing *C. neoformans*

We next sought to examine host factors affecting internalization of *C. neoformans* by brain endothelial cells *in vivo*. CPS1 has been identified as the hyaluronic acid synthase in *C. neoformans*; and hyaluronic acid is a component of the cryptococcal capsule (283,284). *In vitro* studies have shown that *C. neoformans* adhered to cultured HBMECs through the interaction of hyaluronic acids expressed by the yeast cells with CD44 expressed on HBMECs (284,285). In addition, cryptococcal hyaluronic acid production can be enhanced by stimulation with inositol that is known to promote brain invasion of *C. neoformans* (286). To compare the frequency of internalization of *C. neoformans* by brain endothelial cells between CD44^{-/-} mice and wild-type mice, we performed three independent experiments by infecting mice with GFP-expressing *C. neoformans* H99. We repeatedly observed a tendency of decrease in the frequency of brain endothelial cells harboring *C. neoformans* in infected CD44^{-/-} mice compared to wild-type mice. We pooled the data together and observed a slightly lower frequency of brain endothelial cells harboring *C. neoformans* in CD44^{-/-} mice (Figure. 2.6A and B), although the absolute number of infected brain endothelial cells between CD44^{-/-} and wild-type mice did not reach statistical difference (Figure. 2.6C).

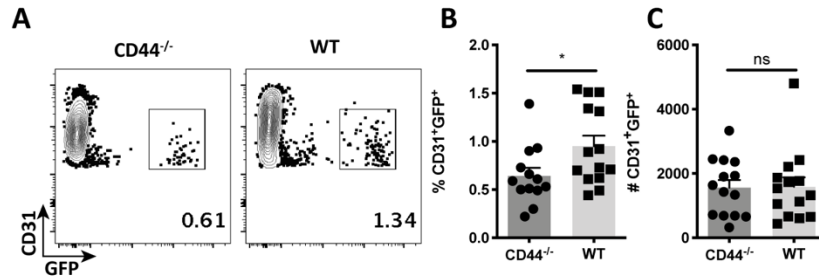


Figure. 2.6 CD44^{-/-} mice exhibit a slightly lower frequency of brain endothelial cells invaded by *C. neoformans* compared to wild-type mice. CD44^{-/-} and wild-type (WT) mice were infected with 20×10^6 GFP-expressing *C. neoformans* H99. Brain endothelial cells were purified 24 h after infection. The frequency of CD31⁺GFP⁺ cells (gated on CD31⁺CD45⁻ cells) was determined by flow cytometry. **(A)** representative dot plots, **(B)** frequency (within CD31⁺CD45⁻ cells), and **(C)** absolute number. Data from three independent experiments were pooled and are presented as mean $\pm$ SEM. Dots represent individual mice. Ns, not significant; * $p < 0.05$.

Discussion

Although *C. neoformans* infection starts in the lung by inhalation of the yeast cell, the most devastating event is that the fungus eventually disseminates to the brain via crossing the BBB in conditions of immune deficiency, resulting in fatal meningoencephalitis (256,262). To study fungal transmigration into the brain, an *in vitro* system has been widely used in the past. The *in vitro* BBB model provided direct evidence that *C. neoformans* can penetrate the BBB through transcytosis (269) and the Trojan horse mechanism (267,272). It was further demonstrated that the interaction of CD44 expressed by endothelial cells with cryptococcal hyaluronic acid mediates the adhesion of *C. neoformans* to brain endothelial cells (284,285). In line with this finding, inositol enhanced the production of hyaluronic acid by the yeast cells and promoted their traversal of the BBB (286). Following binding, internalization of the yeast cell occurred in an actin-dependent manner (287–290). In addition to hyaluronic acid, cryptococcal phospholipase B1 (Plb1) and metalloprotease 1 (Mpr1) have been reported to be involved in yeast transmigration across the BBB (270,291).

Compared to *in vitro* findings, less is known about the BBB crossing of *C. neoformans in vivo*. This is in part because of the lack of an *in vivo* system to quantify the kinetics of fungal transmigration. One major contribution of the current study is the development of an approach by using flow cytometry and labeling *C. neoformans* to quantitatively analyze the events of cryptococcal transmigration in the infected brain *in vivo*. In the past, *in vitro* BBB models have advanced our knowledge of fungal migration to the brain. However, the *in vitro* BBB models cannot recapitulate the *in*

vivo BBB features due to the absence of shear stress and the absence of the basal lamina, pericytes and astrocytes. Therefore, it is unknown to what extent the findings of *in vitro* studies reflect what occurs *in vivo* regarding fungal brain invasion. Several studies have been performed to observe fungal brain invasion *in vivo* using histology (265,279,292). However, fungal brain migration is a transient and dynamic process, and histological studies have a limitation in addressing this dynamic process (293). In this regard, intravital microscopy has been recently used to study fungal brain migration (268,280,294). However, intravital microscopy can image only a very small area and a very limited number of fungal cells, which may not be representative for most of the fungal cells. In some experiments, counting brain CFU has been used to determine fungal transmigration (270,286,291,295). However, CFU analysis cannot distinguish yeast cells inside brain vessels and those in the brain parenchyma. In contrast, our study offers a powerful approach to study fungal transmigration *in vivo* by quantitatively analyzing the frequency of brain endothelial cells containing *C. neoformans*, with the potential to be widely used for studying brain migration of other fungal as well as bacterial species.

Although uptake of *C. neoformans* by endothelial cells has been widely studied *in vitro*, this is the first study to quantitatively identify the internalization of fungal cells by brain endothelial cells *in vivo*. Interestingly, mice infected with acapsular or heat-killed *C. neoformans* displayed a lower frequency of endothelial cells containing the yeast cell compared to mice infected with wild-type or viable *C. neoformans*, respectively. Our *in vivo* observations contrasted with previous findings that loss of

capsule and viability did not affect binding and uptake of *C. neoformans* by HBMECs *in vitro* (277). The discrepancy may reflect the difference in the microenvironments between brain microvasculature *in vivo* and cell culture *in vitro*. It is worth noting that the frequency of brain endothelial cells invaded by H99 strain was significantly higher compared to 52D strain, associated with higher brain CFU in infected mice. These observations may explain, at least in part, why H99 strain is more virulent than 52D strain. Of note, the results suggest that the serotype and capsule structure of *C. neoformans* influence the invasion of the yeast cell to endothelial cells, identifying capsule as a virulence factor for fungal transmigration into the brain. As a major virulence factor, the capsule of *C. neoformans* plays a central role in causing disease by multiple mechanisms (281). It is conceivable that cryptococcal capsule is also involved in brain invasion of this organism.

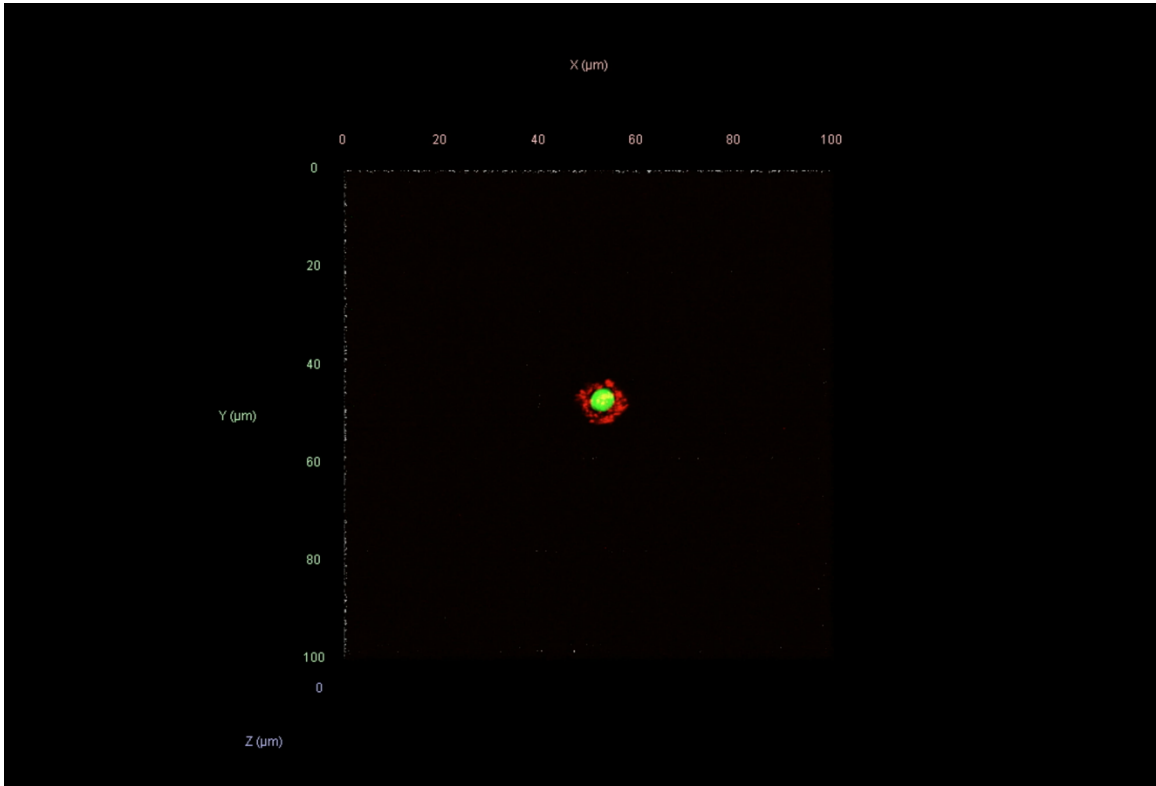
It has been previously shown that ligation of CD44 with hyaluronic acid facilitates cryptococcal transmigration across the monolayer of HBMECs (284,285,290). In our *in vivo* experimental setting, we observed a slight reduction in the frequency of brain endothelial cells invaded by *C. neoformans* in the absence of CD44 signaling. It should be pointed out that there is a difference in microenvironments around arrested *C. neoformans* between *in vitro* and *in vivo*. In the *In vitro* system, *C. neoformans* yeast cells are required to adhere to HBMECs prior to transmigration, and the adhesion requires the interaction of hyaluronic acid with CD44 (284,285). It is likely that the subsequent internalization of the yeast involves multiple cryptococcal components including Plb1, Mpr1, and hyaluronic acid (270,291). In contrast, *C.*

neoformans was arrested in small brain capillaries *in vivo* (269,279); and the arrest of the yeast cells appears to be a mechanical process due to the narrow size of capillaries without CD44 involvement, i.e. adhesion is not a prerequisite for the yeast-endothelium contact (280). Further investigation may provide a better evaluation of the contribution of CD44 signaling to brain invasion of *C. neoformans in vivo*.

In summary, we have established an approach to quantitatively analyze the interaction between *C. neoformans* and brain endothelial cells *in vivo* during infection by using flow cytometry. This novel approach revealed internalization of *C. neoformans* by brain endothelial cells in the infected brain, a mechanism of fungal brain invasion which was extensively studied *in vitro* but poorly addressed *in vivo* due to the lack of a quantitative analysis method. We further demonstrated that the capsule, viability, and serotype of *C. neoformans* affect the frequency of brain endothelial cells invaded by the organism. Thus, our studies offer a novel approach to study fungal brain invasion *in vivo* and elucidate an *in vivo* mechanism of fungal transmigration.

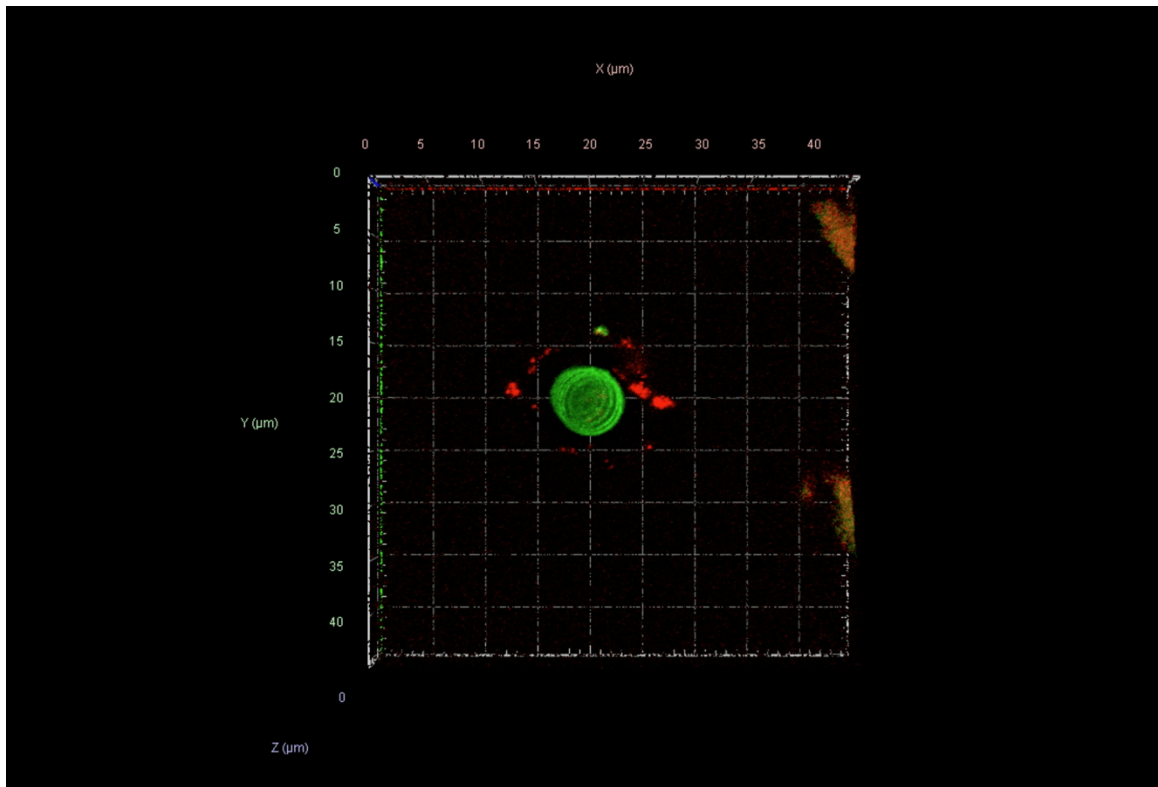
Supporting information

Supplemental Video 1



Supplemental Video 1 3D reconstructive movie of Figure 2.1 D showing colocalization of the brain endothelial cell (red) and GFP-expressing *C. neoformans* (green)

Supplemental Video 2



Supplemental Video 2 A 3D reconstructive movie of Figure 2.2 B showing colocalization of the brain endothelial cell (red) and CFSE-labelled *C. neoformans* (green)

Chapter 3: Recruited Ly6C^{hi} mononuclear phagocytes are effector cells for the clearance of *Cryptococcus neoformans* in the brain involving cell-intrinsic TNFR and IFN- γ R signaling

Abstract

Cryptococcus neoformans (*C. neoformans*) causes fatal meningoencephalitis and the mechanism involved in the clearance of the organism in the brain remains poorly understood. Here we report that clearance of *C. neoformans* in the brain correlates with the accumulation and M1 polarization of Ly6C^{hi} mononuclear phagocytes and that these phagocytes play a critical role in the clearance of *C. neoformans* in the brain. Notably, the accumulation of Ly6C^{hi} mononuclear phagocytes coincides with enhanced secretions of TNF and IFN- γ in the brain. TNF receptor (TNFR) signaling, but not IFN- γ receptor (IFN- γ R) signaling, mediates the recruitment of Ly6C^{hi} mononuclear phagocytes in a cell-intrinsic manner. By contrast, IFN- γ induces M1 polarization of Ly6C^{hi} mononuclear phagocytes. Disruption of TNFR or IFN- γ R signaling enhances cryptococcal growth in the brain. Thus, Ly6C^{hi} mononuclear phagocytes act as effector cells for cryptococcal clearance in the brain, involving cell-intrinsic TNFR as well as IFN- γ R signaling.

Introduction

Cryptococcus neoformans (*C. neoformans*) is an emerging fungal pathogen that affects human beings, especially the immunocompromised population (31). WHO recently ranked it the top fungal pathogen (296). Besides the immunocompromised population, including HIV/AIDS patients and solid organ transplant recipients, there are elevated reports of cryptococcosis in healthy hosts (297,298). Owing to its prevalent distribution in the environment, it imposes a relatively great threat to human society.

Treatment for cryptococcosis is very limited in the most affected areas (299). Even with sufficient resources, the treatment process is also painful and time-consuming. As advised by the CDC, initial cryptococcosis treatment is amphotericin B in combination with flucytosine. After initial treatment, patients usually need to take fluconazole for an extended time to clear cryptococcal infection. Finding new therapeutically regulating targets is urgently needed.

There is evidence that many immune cells are involved in fungal clearance (Chapter 1). During cryptococcal pulmonary infection, alveolar macrophages, interstitial macrophages, and DCs are known as the effector cells, whose fungicidal activities are regulated by CD4⁺ T cell immune responses (42). However, immune cells possessing fungicidal activities during cryptococcal central nervous system infection are greatly understudied (300).

In the brain, microglia are the major immune cells and are found throughout the brain parenchyma. Microglia play a central role in defense against infections. Following brain invasion, *C. neoformans* will first encounter the resident microglia. Previous *in vitro* studies revealed that microglia could function as killing cells with the help of TNF, nitric oxide secretions, and IFN- γ (112,118,119,301). However, further studies revealed that microglia could also serve as a growing reservoir for *C. neoformans in vitro* (116,122,302). Recent studies also suggested that microglia facilitate *C. neoformans* transmigration from the bloodstream to brain parenchyma in zebrafish and murine infection models (303,304). These *in vivo* results suggest that microglia may play a detrimental role during brain infection with *C. neoformans*.

Compared to the ongoing research regarding the roles of resident microglia during *C. neoformans* infection, there is relatively limited attention given to the recruited immune cells, including recruited mononuclear phagocytes. A recent study has shown that CCR2 signaling drives inflammatory monocytes to migrate to the brain in a mouse model that closely mimics post-infectious inflammatory response syndrome (PIIRS) and enhances brain inflammation and damage, despite enhanced fungal burdens in the brain, suggesting that inflammatory monocytes may contribute to immunopathology (150).

As a facultative intracellular pathogen, *C. neoformans* could survive both intracellularly and extracellularly. With an anti-acid polysaccharide capsule, *C. neoformans* could inhibit the acidification of macrophages when residing

intracellularly (37). However, with adjuvant LPS, proinflammation-polarized macrophages were shown to function as fungicidal activity (37), suggesting that proinflammatory macrophages are important for fungal clearance.

Recruited CD11b⁺ Ly6C^{hi} mononuclear phagocytes are differentiated from inflammatory monocytes. The egression of inflammatory monocytes from the bone marrow is mediated by CCR2 signaling (305,306). Recent studies have shown that CCR2^{-/-} mice exhibited a significantly lower number of inflammatory monocytes in the brain during *C. neoformans* brain infection (150), likely due to impaired emigration of monocytes from the bone marrow to the blood. Published literature showed that TNF facilitated the recruitment of inflammatory monocyte-derived DCs to the lung (204). However, the molecular mechanisms involved in the recruitment of CD11b⁺ Ly6C^{hi} mononuclear phagocytes to the brain parenchyma are largely unknown.

Successful fungicidal activity also relies on the proinflammatory polarization of CD11b⁺ Ly6C^{hi} mononuclear phagocytes. IFN- γ is an inflammatory cytokine that has been known to promote the proinflammatory polarization of macrophages (307). Mainly produced by CD4⁺ T cells, IFN- γ has been proven to be protective against cryptococcal pulmonary infection (308). IFN- γ producing *C. neoformans* has been shown as protective from sequential cryptococcal infection, indicating that IFN- γ mediates protection during *C. neoformans* lung infection (309). However, its role in the polarization of recruited brain macrophages is largely understudied.

The goal of the study is to identify the effector cells that kill *C. neoformans* in the brain and the mechanisms involved in the clearance of *C. neoformans* in the infected brain. Using a mouse model of chronic infection with *C. neoformans*, we found that clearance of *C. neoformans* in the brain correlated with accumulation of CD11b⁺ Ly6C^{hi} mononuclear phagocytes in the brain. Our results suggest that these CD11b⁺ Ly6C^{hi} mononuclear phagocytes function as effector cells for the clearance of *C. neoformans* in the brain, involving cell-intrinsic TNFR signaling and IFN- γ R signaling. Our studies provided novel insights into mechanisms involved in fungal clearance in the brain, suggesting a therapeutic strategy for cryptococcal meningoencephalitis aimed at targeting CD11b⁺ Ly6C^{hi} mononuclear phagocytes.

Material and methods

Mice

Wild-type C57BL/6 mice were purchased from the National Cancer Institute (NCI). TNFR^{-/-} (Catalog No. 003243), IFN- γ R^{-/-} (Catalog No. 003288), CCR2^{-/-} (Catalog No. 004999), and ICAM-1^{-/-} (Catalog No. 002867) mice in the C57BL/6 background were purchased from The Jackson Laboratory and bred in house in the animal facilities of University of Maryland, College Park. All mice were maintained in pathogen-free individually ventilated cages. All experiments were performed in accordance with the guidelines approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee.

Fungal strains and culture

Encapsulated *C. neoformans* 52D strain (serotype D) was used in this study. The yeast cells were kept in a -80°C freezer for long-term storage. The yeast cells were cultured in Sabouraud dextrose broth (BD, Catalog No. DF0382-17-9) at 30°C with gentle rotation in an orbital shaker (200 r.p.m) for 17-18 hrs. The yeast cells were harvested by centrifugation at $500 \times g$ for 5 min and washed twice in sterile $1 \times$ PBS. The concentration of the yeast cells was determined using a hemocytometer. All experiments were performed in accordance with the biosafety level 2 guidelines.

Infection and quantification of fungal burdens in the brain

Mice were intravenously infected through the tail vein with 1×10^6 *C. neoformans* 52D yeast cells diluted in 200 μ L sterile $1 \times$ PBS. To determine the fungal burden in the brains of infected mice, mice were anesthetized by i.p. injection of a Ketamine/Xylazine mixture (10 mg/kg xylazine and 200 mg/kg ketamine hydrochloride) in PBS. After confirmation of anesthesia, mice were perfused with 20 mL sterile $1 \times$ PBS through the left ventricle with an open femoral artery. Brains were then collected in 2 mL sterile $1 \times$ PBS. The tissues were homogenized. After serial dilutions, the tissue homogenate was plated on Sabouraud dextrose agar plates (BD, Catalog No. DF0109-17-1) and kept in the 30°C incubators for 36 – 48 hrs. Fungal colony forming units (CFU) in each brain tissue were determined.

Isolation of mouse leukocytes

Mouse brain leukocytes were isolated as described previously with adjustments (310). Mice were anesthetized and whole blood samples were collected through cardiac puncture. After collecting blood samples, mice were then perfused with ice-cold sterile 1 x PBS. For brain leukocytes, mouse brain tissues were collected. Brain stems were removed prior to homogenization. To get rid of the meninge macrophages, mouse brains were gently rolled over the sterile gauge. The brain was placed in 2 mL of ice-cold RPMI 1640 Medium (Corning™) supplemented with L-Glutamine. Brains were minced into small pieces with surgical scissors, and digested with Collagenase IV (Worthington) with 1 mg/mL as working concentration in the incubator at 37°C for 60 min, shaking at 200 r.p.m. The brain tissues were then forced gently through a 70 µM cell strainer (VWR, Catalog No. 76327-100) using a sterile syringe plunger and washed with 15 mL of RPMI-1640 medium. The cell suspension was centrifuged at $500 \times g$ for 5 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 2 mL of 30% Percoll (GE, USA) and the cell suspension was layered onto 1 mL of 70% Percoll and then centrifuged at $1500 \times g$ with the brake off for 15 min at room temperature. The myelin layer was carefully removed with a serological pipette. The cell suspension at the interface was harvested and washed with 15 mL 1 x PBS by centrifugation at $500 \times g$ for 5 min at 4°C to remove the Percoll residue. The pellet was resuspended in 400 µL of erythrocyte lysing (ACK) buffer (Gibco™), and incubated at room temperature for 2 min, then diluted with 10 mL 1 x PBS, followed by centrifugation at $500 \times g$ for 5 min at 4°C. Cells were resuspended with 1 mL of flow cytometry staining buffer and counted with a hemocytometer. Cells were diluted with flow cytometry staining buffer to a concentration of 5×10^6 cells/mL prior to staining.

For spleen leukocytes, mouse spleens were collected in 20 mL sterile RPMI medium and gently forced through a 70 μ M cell strainer on ice with ice-cold RPMI medium. Spleen cell suspension was centrifuged at $500 \times g$ for 5 min at 4°C. Supernatants were carefully discarded with serological pipettes. The cell pellet was resuspended in 1 mL ACK lysis buffer and incubated for 5 min at room temperature. After red blood cells lysis, 15 mL sterile $1 \times$ PBS was used to wash the cells with centrifugation at $500 \times g$ for 5 min at 4°C. After discarding the supernatant, the cell pellet was resuspended with 1 mL flow cytometry staining buffer and counted with a hemocytometer. Cells were diluted with flow cytometry staining buffer to a concentration of 5×10^6 cells/mL prior to staining.

Flow cytometry analysis of lymphocytes and leukocytes

The cell suspension prepared as described above was incubated with purified anti-mouse CD16/CD32 ([Fc γ III/II Receptor], clone: 2.4G2, Biolegend) for 15 min at 4°C to block nonspecific binding of antibodies to FcRs and washed with staining buffer. After centrifugation at $500 \times g$ for 5 min at 4°C, the cells were suspended with staining buffer and stained with anti-mouse CD3 (Clone: 17A2, Biolegend), CD4 (Clone: GK1.5, Biolegend), CD8 (Clone: 53-6.7, Biolegend), CD11a (Clone: M17/4, Biolegend), CD11b (Clone: M1/70, Biolegend), CD11c (Clone: N418, Biolegend), CD44 (Clone: IM7, Biolegend), CD45 (Clone: I3/2.3, Biolegend), CD45.1 (Clone: A20, Biolegend), CD45.2 (Clone: 104, Biolegend), CD62L (Clone: MEL-14, Biolegend), NK1.1 (Clone: PK136, Biolegend), Ly6C (Clone: HK1.4, Biolegend), Ly6G (Clone: 1A8, Biolegend), MHC II (Clone: M5/114.15.2, Biolegend), F4/80 (Clone: BM8,

Biolegend), ICAM-1 (Clone: YN1/1.7.4, Biolegend), VCAM-1 (Clone: 429 (MVCAM.A), Biolegend) and VLA4 (Clone: R1-2, Biolegend) mAbs for cell surface markers for 30 min at 4°C and washed with flow cytometry staining buffer by centrifugation at $500 \times g$ for 5 min at 4°C. The pellet was suspended with staining buffer and kept at 4°C until analysis by BD FACSCelesta™. Results were analyzed using FlowJo software. For further intracellular markers staining, cell pellets were resuspended with 200 μ L 1 $\times$ fixation solution from eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (Invitrogen™). Cell suspensions were incubated at room temperature for 30 min in the dark. Cell pellets were washed with 1 mL 1 $\times$ permeabilization buffer with centrifugation at $500 \times g$ for 5 min at 4°C. Cell pellets were resuspended in 100 μ L 1 $\times$ permeabilization buffer and incubated with mAbs specific for iNOS (eBioscience™, 53-5920-82), Arginase 1 (Arg1) (Clone: W21047I, Biolegend) and Ki-67 (Clone: 11F6, Biolegend) for 30 min at 4°C in the dark. For flow cytometry staining of intracellular *C. neoformans*, brain leukocytes were incubated with mouse-anti-cryptococcus antibody 18B7 (a gift from Dr. Arturo Casadevall, Baltimore) conjugated with Alexa Fluor™ 647 fluorophore (Antibody Labeling Kits, Invitrogen™) to stain the polysaccharide capsule. Cell suspensions were washed with 1 mL 1 $\times$ permeabilization buffer with centrifugation at $500 \times g$ for 5 min at 4°C. Cell pellets were then resuspended with 1 $\times$ permeabilization buffer and kept at 4°C until analysis by BD FACSCelesta™ (BD Biosciences). For Annexin V and 7-AAD staining, leukocytes were first stained with cell surface makers and then the samples were washed with 1 mL Annexin V binding buffer (Invitrogen™) and centrifuged at $500 \times g$ for 5 min at 4°C, followed by resuspending in Annexin V binding

buffer and staining with Annexin V (Biolegend) for 10-15 min at room temperature in the dark. Cell samples were washed again with 1 mL Annexin V binding buffer and centrifuged at $500 \times g$ for 5 min at 4°C. Cells were resuspended in 200 μ L of 1X binding buffer and kept on ice in the dark. 7-AAD (Biolegend) was added in the samples 5-15 mins before FACSCelesta™ acquisition. Flow cytometry data were analyzed using FlowJo v.7.6 (TreeStar).

Immunohistochemistry

Mice were infected as described above. Mice were anesthetized by i.p. injection of a Ketamine/Xylazine mixture (10 mg/kg xylazine and 200 mg/kg ketamine hydrochloride) in PBS. After the confirmation of the anesthesia, mice were perfused with 10 mL sterile $1 \times$ PBS and then with 10 mL 4% paraformaldehyde through cardiac perfusion at the left ventricle with a cut opened right femoral artery. After perfusion, mouse brains were collected and further fixed in 20 mL 4% paraformaldehyde overnight at 4°C. Following the fixation, dehydration was performed at 15% and 25% sucrose diluted in sterile $1 \times$ PBS subsequently overnight at 4°C. Mouse brain tissues were further embedded in the Tissue-Tek O.C.T. solutions and snap-frozen with dry ice or liquid nitrogen. Tissue blocks were then cut into 15 μ m brain tissue sections using Microm HM550 Cryostat Microtome. Brain sections were further fixed in ice-cold acetone at 4°C for 10 min. Tissues were then washed for 5 min twice with a gentle shake in washing buffer with 0.05% Tween 20 (Sigma-Aldrich) diluted in sterile $1 \times$ PBS. Tissues were blocked with 2% goat serum diluted in sterile $1 \times$ PBS at room temperature for 30 min in the dark in a wet chamber. Tissue sections were then stained

with mouse-anti-cryptococcus E1 mAbs (a gift from Dr. F. Dromer, Paris, France), rabbit-anti-mouse Iba1 mAb (Clone: EPR16588, Abcam), or rat-anti-mouse CD11b mAbs (Clone: M1/70, Biolegend) diluted in blocking buffer (2% goat serum) at 4°C overnight in the dark in the wet chamber. After two washes with washing buffer, tissue sections were subsequently stained with corresponding goat-anti-mouse or goat-anti-rat secondary antibodies at room temperature in the dark for 2 hrs. After two final washes, tissue sections were then further incubated with 300 nM DAPI (Invitrogen™) at room temperature in the dark for 5 min. Slides were washed with washing buffer with a gentle shake and mounted with an anti-fade fluorescence mounting medium (Abcam). Finally, slides were sealed with coverslips and examined with Zeiss LSM 800 system.

H&E Staining

C57BL/6 wild-type and IFN- γ R^{-/-} mice were intravenously infected as described above. Brain samples were collected at 9-, 18-, and 27- days post infection and stored in 4% paraformaldehyde. Samples were later sent to American Histo Labs Inc (Rockville, Maryland) for H&E staining. Brain slides were examined under AxioLab A1 Microscope and analyzed in Fiji software.

Enzyme-linked immunosorbent assay (ELISA)

Mice were infected and euthanized as described above. Mouse brain tissues were collected in 2 mL sterile RPMI medium and homogenized through a 70 μ M cell strainer using a sterile syringe plunger on ice. Tissue homogenates were collected in a

15 mL falcon tube for centrifugation at $500 \times g$ for 5 min at 4°C. Supernatants were collected in 1.5 mL Eppendorf tubes and stocked at -20°C. ELISA was performed to measure the cytokine levels of TNF, IFN- γ , IL-12p40, IL-4, and IL-5 using commercial ELISA kits from the R&D System according to manufacturing instructions and protocols.

Adoptive transfer

Ly5.1 mice (B6.SJL-*Ptprc^aPepc^b*/BoyCrl) were purchased from Charles River and bred in-house in the animal facilities of the University of Maryland, College Park under IACUC instruction. Ly5.1 (CD45.1) and wild-type C57BL/6 (CD45.2) mice were cross-bred in-house to generate the Ly5.1 $\times$ B6 (CD45.1 $\times$ CD45.2) mice. Ly5.1 (CD45.1), TNFR^{-/-} (CD45.2), and Ly5.1 $\times$ B6 (CD45.1 $\times$ CD45.2) mice were infected as described above. CD11b⁺ Ly6C^{hi} monocytes were isolated from the bone marrow of Ly5.1 and TNFR^{-/-} mice at 14 and 19 dpi using the Monocyte Isolation Kit (Miltenyi Biotec), according to manufacture instructions. After isolation, CD11b⁺ Ly6C^{hi} monocytes from Ly5.1 (CD45.1) and TNFR^{-/-} (CD45.2) were mixed at a 1:1 ratio and a total of 4×10^6 cells were transferred to infected Ly5.1 $\times$ B6 (CD45.1 $\times$ CD45.2) mice; brain samples were then collected at 21 dpi for flow cytometry analysis.

RT-qPCR

50 μ L of brain homogenates were collected and reserved following homogenization during leukocyte isolation. Total RNA was extracted from brain homogenates using TriZol (InvitrogenTM) according to manufacture instructions.

SuperScript IV First-Strand Synthesis System (Invitrogen™) was used for cDNA synthesis. Quantitative real-time PCR (qPCR) was performed with appropriate primers on a Bio-Rad CFX96 real-time system using SYBR Green PCR master mix (Applied Biosystems). The gene expressions for each sample were normalized against the housekeeping gene GAPDH and the fold change was calculated using the $\Delta\Delta C_t$ method. Primers used for RT-qPCR were as follows:

iNOS: 5'- CAGCTGGGCTGTACAAACCTTC -3' (forward), 5'- CATTGGAAGTGAAGCGTTTCG -3' (reverse);

CD86: 5'- ACGATGGACCCCAGATGCACCA -3' (forward), 5'- GCGTCTCCACGGAAACAGCA -3' (reverse);

Fizz1: 5'- CCAATCCAGCTAACTATCCCTCC -3' (forward), 5'- ACCCAGTAGCAGTCATCCCA -3' (reverse);

CD206: 5'- TCAGCTATTGGACGCGAGGCA -3' (forward), 5'- TCCGGGTTGCAAGTTGCCGT -3' (reverse);

Arg1: 5'- CTCCAAGCCAAAGTCCTTAGAG -3' (forward), 5'- AGGAGCTGTCATTAGGGACATC -3' (reverse);

CCL2: 5'- CCCACTCACCTGCTGCTACTCA -3' (forward), 5'- GCTTCTTTGGGACACCTGCTG -3' (reverse);

CCL7: 5'- CCAATGCATCCACATGCTGC -3' (forward), 5'- GCTTCCCAGGGACACCGAC -3' (reverse);

KC: 5'- CCGAAGTCATAGCCACACTCAA -3' (forward), 5'- GCAGTCTGTCTTCTTTCTCCGTTAC -3' (reverse);

MIP2: 5'- AGACAGAAGTCATAGCCACTCTCAAG -3' (forward), 5'- CCTCCTTTCCAGGTCAGTTAGC -3'(reverse);

ICAM-1: 5'- GTGATGCTCAGGTATCCATCCA -3' (forward), 5'- CACAGTTCTCAAAGCACAGCG -3' (reverse);

VCAM-1: 5'- AGTTGGGGATTCTGGTTGTTCT -3' (forward), 5'- CCCCTCATTCCTTACCACCC -3' (reverse);

GAPDH: 5'- CGACTTCAACAGCAACTCCCACTCTTCC -3' (forward), 5'- TGGGTGGTCCAGGGTTTCTTACTCCTT -3'(reverse).

Statistics

GraphPad Prism 9.0 software (GraphPad) was used for all statistical analyses. Data are presented as the mean $\pm$ SEM. For single comparisons, an unpaired two-sided Student's *t* test was performed; for multiple comparisons, two-way ANOVA was used. In all cases, a *p* value of <0.05 is considered significant.

Results

Clearance of *C. neoformans* in the brain correlates with accumulation and M1 polarization of Ly6C^{hi} mononuclear phagocytes.

To investigate the immune cells combating the fungal infection in the brain, we adopted an established chronic *C. neoformans* infection model by intravenously infecting C57BL/6 mice with 1×10^6 *C. neoformans* 52D through tail vein injection (126). We collected brain samples at various time points after infection and determined the fungal burdens in the brain by counting the colony forming units (CFU). We observed that the fungal burden in the brain increased steadily and reached a plateau after 14 days post-infection (dpi), followed by resolution after 21 dpi (Figure 3.1A). The decrease in the fungal burden suggested that a fungal clearance mechanism(s) exists in the brains of infected mice. Next, we performed flow cytometry analysis on leukocytes in the brain. The results showed that the enhanced fungal burdens correlated with an increased influx of CD45⁺ brain infiltrating leukocytes (BIL) and CD11b⁺ Ly6C^{hi} mononuclear phagocytes (Figure 3.1 B). We also observed the presence of Iba1⁺ and CD11b⁺ cells surrounding the fungal cluster, cryptococcoma, in the brain during cryptococcal infection using immunohistochemistry staining (IHC) (Figure 3.1 C). These results suggest that *C. neoformans* infection in the brain results in the accumulation of Ly6C^{hi} mononuclear phagocytes in the brain, especially in the fungal clusters. Published studies have shown that proper proinflammatory polarization is required for mononuclear phagocytes to clear pathogens (311). M1 polarization is crucial for antifungal activity, while M2 polarization is detrimental in general (311).

To investigate the polarization of brain mononuclear phagocytes, we infected C57BL/6 mice and collected brain samples at 18 and 27 dpi. We performed iNOS and Arg1 intracellular staining to study the polarization of mononuclear phagocytes. The results show that Ly6C^{hi} mononuclear phagocytes expressed significantly higher iNOS at 18 dpi compared to 27 dpi (Figure 3.1 D), suggesting a correlation between the production of iNOS by Ly6C^{hi} mononuclear phagocytes and the fungal burden. There was a tendency of higher numbers of iNOS-producing Ly6C^{hi} mononuclear phagocytes at 18 dpi compared to 27 dpi. In addition, the percentage of iNOS-producing Ly6C^{hi} mononuclear phagocytes out of the total Ly6C^{hi} mononuclear phagocytes and the portion of iNOS-producing Ly6C^{hi} mononuclear phagocytes out of the total iNOS⁺ cells were significantly higher at 18 dpi compared to 27 dpi. In contrast, the number and percentage of Arg1-producing Ly6C^{hi} mononuclear phagocytes were significantly lower at 18 dpi compared to 27 dpi. These results suggest that fungal clearance in the brain correlated with the production of iNOS but not Arg1 (Figure 3.1 D). Taken together, our results suggest that clearance of *C. neoformans* in the brain correlated with accumulation and M1 polarization of Ly6C^{hi} mononuclear phagocytes.

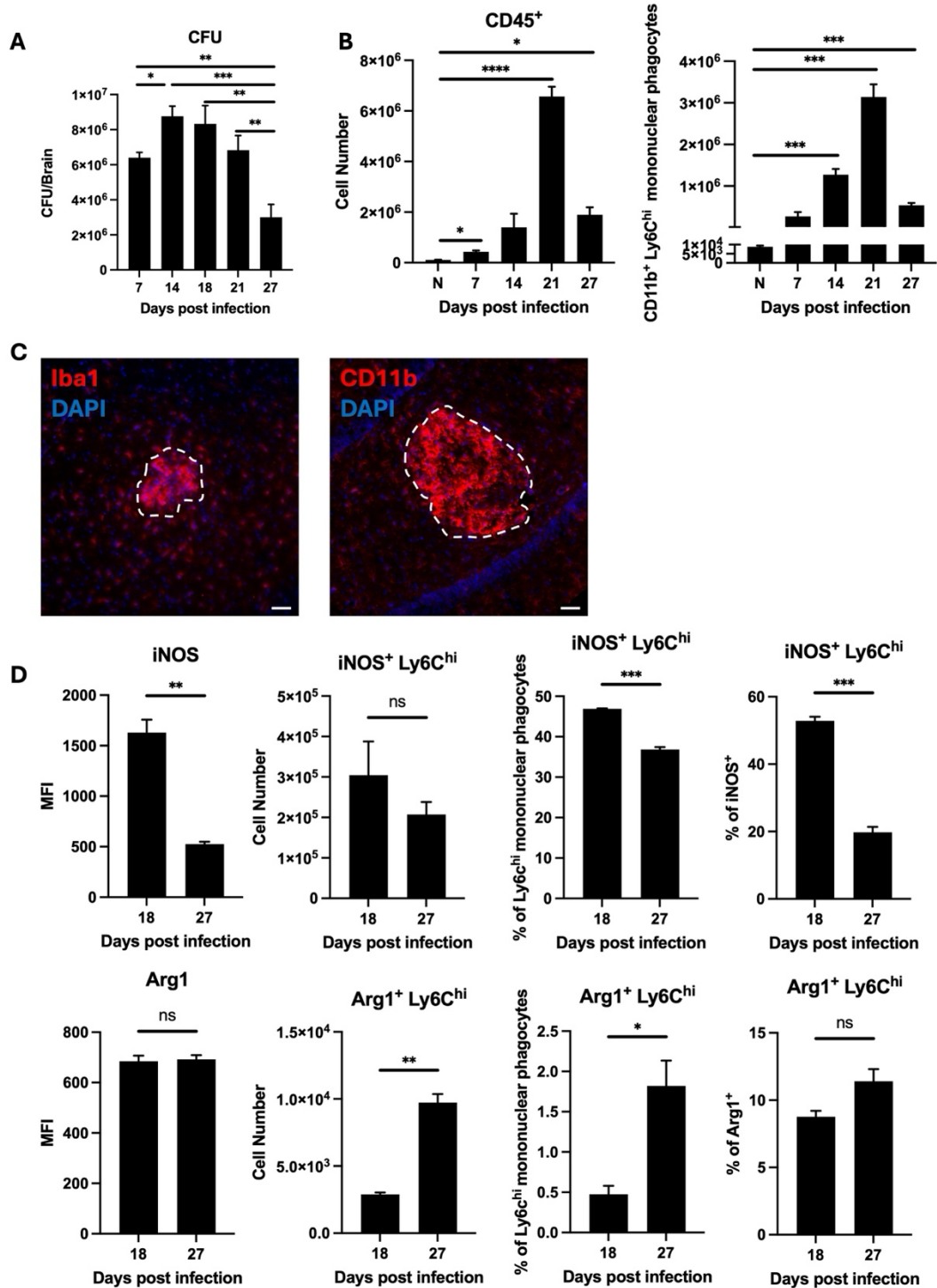


Figure. 3.1 Clearance of *C. neoformans* in the brain correlates with accumulation and M1 polarization of Ly6C^{hi} mononuclear phagocytes. C57BL/6 wild-type mice were infected with 1×10^6 *C. neoformans* 52D via tail vein intravenous injection. (A). The fungal burdens in the brains of infected C57BL/6 wild-type mice (n = 4-9) were determined at 7-, 14-, 18-, 21- and 27- days post infection. Data are representative of two independent experiments (7-, 14- and 18- days post infection) or pooled from

two independent experiments (21- and 27- days post infection). **(B)**. Naïve (N) and infected C57BL/6 wild-type mice (n = 3-13) were sacrificed at indicated time points. CD45⁺ brain infiltrating leukocytes (BIL) and CD11b⁺ Ly6C^{hi} mononuclear phagocytes were isolated and analyzed by flow cytometry at 7-, 14-, 21- and 27- days post infection. The absolute numbers of CD45⁺ cells and CD11b⁺ Ly6C^{hi} mononuclear phagocytes were determined. **(C)**. Representative immunohistochemistry images showing the distribution of Iba1⁺ and CD11b⁺ cells (red) in cryptococcal lesions in the brains of infected C57BL/6 wild-type mice at 3- weeks post infection. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Scale bars: 50 μ m. **(D)**. Quantification of iNOS and Arg1 expression on brain CD11b⁺ Ly6C^{hi} mononuclear phagocytes in infected C57BL/6 wild-type mice (n = 3) at 18- and 21- days post infection. The absolute numbers and frequencies of iNOS⁺ and Arg1⁺ Ly6C^{hi} mononuclear phagocytes were calculated. Data are presented as mean $\pm$ SEM and representative of two independent experiments. Ns, not significant, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Recruited Ly6C^{hi} mononuclear phagocytes are effector cells for clearance of *C. neoformans* in the brain

Although a few subsets of leukocytes have the capacity of killing *C. neoformans* (300), the effector cells accounting for *C. neoformans* clearance in the brain have not been identified. The above results showing the correlation between accumulation of Ly6C^{hi} mononuclear phagocytes and fungal clearance led us to formulate a hypothesis that Ly6C^{hi} mononuclear phagocytes are the major effector cells of killing *C. neoformans*. To test our hypothesis, by using flow cytometry and anti-cryptococcal capsule antibody (18B7), we found that CD11b⁺Ly6C^{hi}18B7⁺ cells were present in the brain of mice after infection, suggesting the direct interaction between CD11b⁺Ly6C^{hi} mononuclear phagocytes and *C. neoformans* (Figure 3.2 A). To investigate if recruited CD11b⁺Ly6C^{hi} mononuclear phagocytes are directly involved in the fungicidal activity, we infected wild-type C57BL/6 mice and CCR2^{-/-} mice which lack monocyte egression from bone marrow, and collected brain samples at 3 weeks post infection (wpi). Flow cytometry results showed that there was a significant reduction of Ly6C^{hi} mononuclear phagocytes in the brain in infected CCR2^{-/-} mice compared to infected wild-type mice (Figure 3.2 B). Importantly, infected CCR2^{-/-} mice exhibited significantly lower fungal burdens in the brain compared to infected wild-type mice (Figure 3.2 B). Collectively, these results suggest that Ly6C^{hi} mononuclear phagocytes are the effector cells for the clearance of *C. neoformans* in the brain.

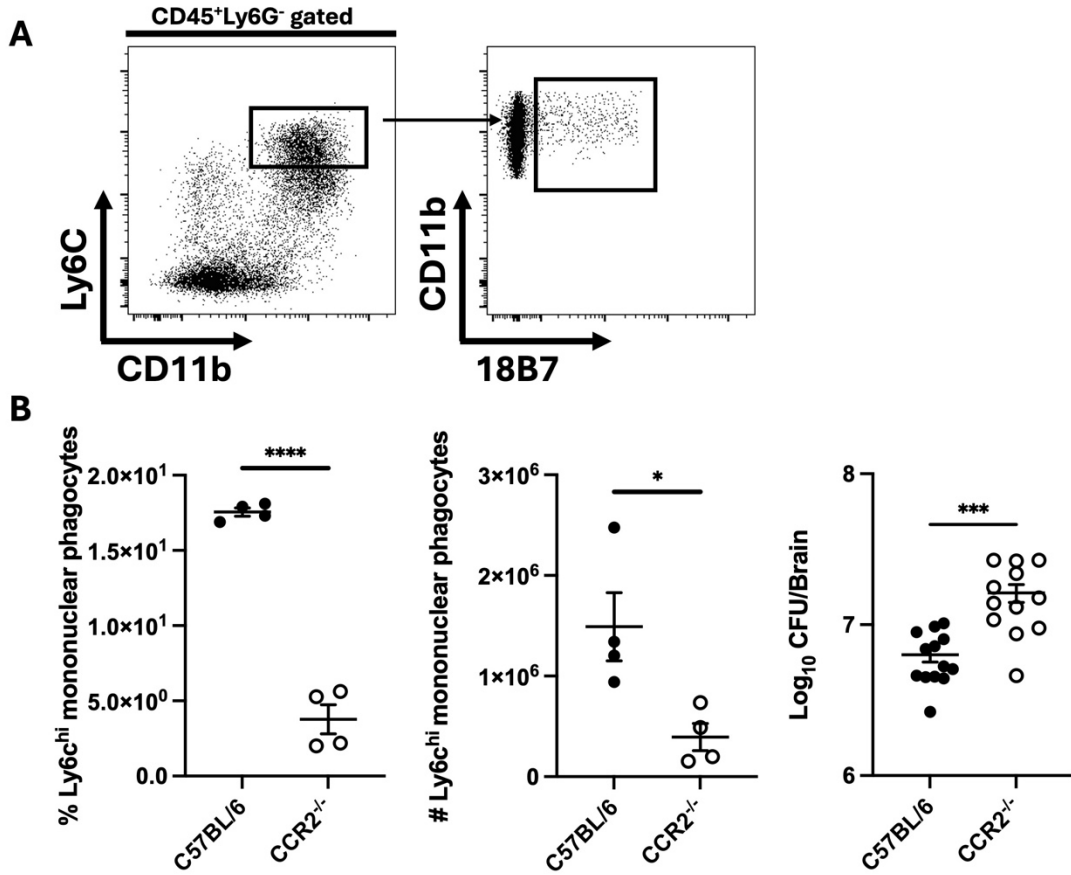


Figure. 3.2 Recruited Ly6C^{hi} mononuclear phagocytes are effector cells for clearance of *C. neoformans* in the brain. (A). C57BL/6 wild-type mice (n = 3) were infected with 1×10^6 *C. neoformans* 52D via tail vein intravenous injection. Brain samples were collected at 21 dpi. Representative FACS plots show that 18B7⁺ fungal cells were associated with Ly6C^{hi} mononuclear phagocytes (left panel, gated on CD45⁺Ly6G⁻ cells). (B). C57BL/6 wild-type and CCR2^{-/-} mice (n = 4) were infected with 1×10^6 *C. neoformans* 52D via tail vein injection. The infected mice were sacrificed at 21 dpi. Brain leukocytes were isolated and analyzed by flow cytometry. The frequency and absolute number of Ly6C^{hi} mononuclear phagocytes were calculated. The brain fungal burdens in the infected C57BL/6 wild-type and CCR2^{-/-} mice (n = 12-13) were determined at 21 dpi. Data are presented as mean $\pm$ SEM and are representative of two independent experiments (% Ly6C^{hi} mononuclear phagocytes and # Ly6C^{hi} mononuclear phagocytes) or pooled from two independent experiments (CFU). Dots represent individual mice. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

TNFR signaling mediates the accumulation of Ly6C^{hi} mononuclear phagocytes in the brain

The recruitment of Ly6C^{hi} mononuclear phagocytes to the brain plays a crucial role in facilitating their execution of fungicidal activity during cryptococcal brain infection. TNF is known as the cytokine mediating the recruitment of Ly6C^{hi} mononuclear phagocytes to the brain in various disease models (312,313). However, the role of TNF during cryptococcal brain infection, especially how it helps recruit the Ly6C^{hi} mononuclear phagocytes is unknown. We observed an increase in TNF production in the brains of infected C57BL/6 wild-type mice following infection (Figure 3.3 A). We next examined the cellular sources of TNF using flow cytometry following intracellular staining of TNF. The results showed that microglia were the major producer of TNF in mouse brains at 2 dpi. At 3 wpi, microglia, natural killer T cells (NKT cells), inflammatory macrophages, and natural killer cells (NK cells) contributed greatly to the TNF production (Figure 3.3 B). Our findings indicate that TNF is induced by cryptococcal brain infection and that both resident and recruited immune contribute to TNF production.

To investigate the potential role of TNF in the recruitment of Ly6C^{hi} mononuclear phagocytes during cryptococcal brain infection, C57BL/6 wild-type and TNFR^{-/-} mice were infected intravenously and brain samples were collected at 2 and 3 wpi for flow cytometry analysis. The results showed that the number of CD45⁺ BIL and Ly6C^{hi} mononuclear phagocytes was significantly reduced in the brain of infected TNFR^{-/-} mice compared to infected wild-type mice, while the number of CD45⁺ cells

and Ly6C^{hi} mononuclear phagocytes remained similar in the spleens (Figure 3.3 C-D). Immunohistochemistry studies confirmed that there was an accumulation of Iba1⁺ cells in the fungal cluster of infected wild-type mice but not TNFR^{-/-} mice (Figure 3.3 E). To exclude the possibility that the decreased number of CD45⁺ BIL and Ly6C^{hi} mononuclear phagocytes in TNFR^{-/-} mice is the result of a lower cell base level in the spleen and the blood due to cell apoptosis in the absence of TNFR (314), we performed apoptotic analysis. The results show that there were no differences in the apoptosis of Ly6C^{hi} mononuclear phagocytes in both spleen and blood between infected wild-type and TNFR^{-/-} mice (Figure 3.3 F). We also found that the proliferation of the Ly6C^{hi} mononuclear phagocytes in the brain was not affected by TNFR deficiency (Figure 3.3 G). Collectively, our results suggest that TNFR signaling mediates the recruitment of Ly6C^{hi} mononuclear phagocytes to the brain during *C. neoformans* infection.

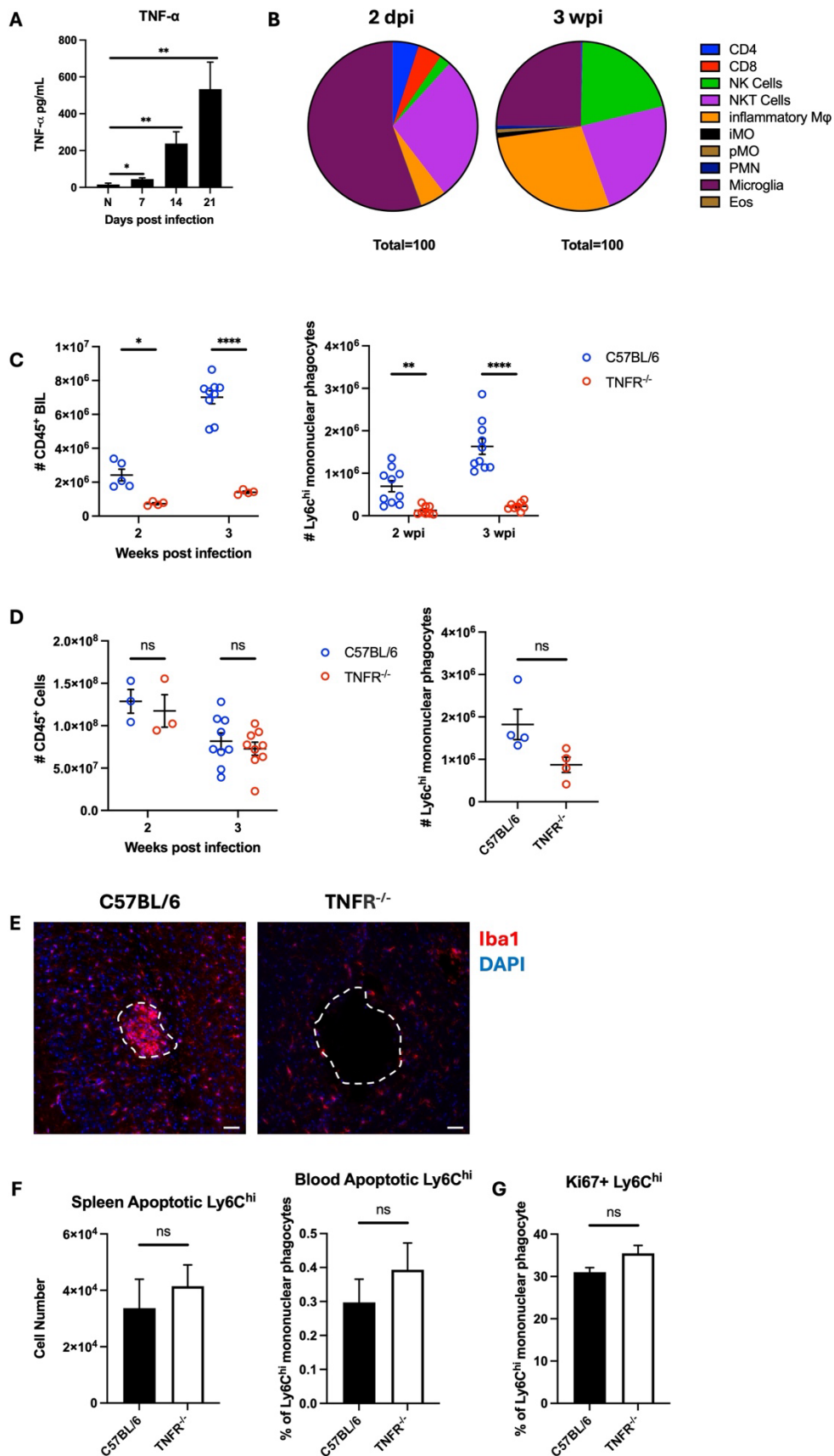


Figure. 3.3 TNFR signaling mediates the accumulation of Ly6C^{hi} mononuclear phagocytes in the brain. C57BL/6 wild-type mice were infected with 1×10^6 *C. neoformans* 52D via tail vein injection. **(A).** The concentrations of TNF in the brains of naïve (N) and infected C57BL/6 wild-type mice (n = 8-10) were measured by ELISA at 7-, 14-, and 21- days post infection. **(B).** Quantification of different subsets of the immune cells expressing TNF in infected C57BL/6 wild-type mice (n = 3) at 2 days and 3 weeks post infection. **(C-G).** C57BL/6 wild-type and TNFR^{-/-} mice were infected with 1×10^6 *C. neoformans* 52D via tail vein injection. **(C).** CD45⁺ brain infiltrating leukocytes (BIL) and brain CD11b⁺ Ly6C^{hi} mononuclear phagocytes from infected C57BL/6 wild-type and TNFR^{-/-} mice (n = 4-10) were analyzed by flow cytometry at 2- and 3- weeks post infection. **(D).** Spleen CD45⁺ cells from infected C57BL/6 wild-type and TNFR^{-/-} mice (n = 3-9) were analyzed by flow cytometry at 2- and 3- weeks post infection. Spleen Ly6C^{hi} mononuclear phagocytes from infected C57BL/6 wild-type and TNFR^{-/-} mice (n = 4) were analyzed by flow cytometry at 3- weeks post infection. **(E).** Representative immunohistochemistry images showing the distribution of Iba1⁺ cell (red) in the cryptococcal lesions in the brains of infected C57BL/6 wild-type and TNFR^{-/-} mice at 3- weeks post infection. Nuclei were stained with DAPI (blue). Scale bars: 50 μ m. **(F).** Apoptotic assay was performed on spleen and blood Ly6C^{hi} mononuclear phagocytes from infected C57BL/6 wild-type and TNFR^{-/-} mice (n = 3-4) at 3- weeks post infection using flow cytometry. **(G).** Proliferation assay was performed on Ly6C^{hi} mononuclear phagocytes from the brain of infected C57BL/6 wild-type and TNFR^{-/-} mice (n = 3-4) at 3- weeks post infection using flow cytometry. Data are expressed as mean $\pm$ SEM and representative of two independent experiments. Dots represent individual mice. Ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

TNF directly signals on Ly6C^{hi} mononuclear phagocytes accompanied by enhanced expressions of chemokines and adhesion molecules

To study the mechanism involved in TNF-mediating Ly6C^{hi} mononuclear phagocytes recruitment to the brain, we first examined the expression of chemokines and adhesion molecules in the brain of wild-type and TNFR^{-/-} mice. We collected the brain samples at 21 dpi and analyzed the gene expression of various chemokines and adhesion molecules using RT-qPCR. We found that expressions of chemokines CCL2, CCL7, KC, and MIP2 were significantly reduced in TNFR^{-/-} mice compared to C57BL/6 wild-type mice (Figure 3.4 A). Besides inducing the expressions of chemokines, we found that TNF also enhanced the mRNA expression of adhesion molecules ICAM-1 and VCAM-1 (Figure 3.4 B). We next examined if the expressions of their corresponding integrins CD11a and VLA4 on Ly6C^{hi} mononuclear phagocytes are also affected. We found that the expression of CD11a was greatly reduced on Ly6C^{hi} mononuclear phagocytes as shown in flow cytometry analysis while VLA4 expression was spared (Figure 3.4 C).

To study if TNF could directly signal on Ly6C^{hi} mononuclear phagocytes to mediate their recruitment to the brain, we collected Ly6C^{hi} mononuclear phagocytes from the bone marrows of infected wild-type Ly5.1 mice (CD45.1) and TNFR^{-/-} mice (CD45.2) at 14 and 19 dpi, respectively (Figure 3.4 D). We mixed the cells at a 1:1 ratio and confirmed it with flow cytometry (Figure 3.4 E). Then we adoptively transferred the mixture to infected wild-type Ly5.1 × C57BL/6 mice (CD45.1×CD45.2) at 14 and 19 dpi, respectively, and collected brain samples and analyzed the recruitment

of Ly6C^{hi} mononuclear phagocytes at 21 dpi. We found that there were significantly more donor CD45.1⁺ Ly6C^{hi} mononuclear phagocytes (TNFR^{+/+}) recruited to the brain compared to donor CD45.2⁺ Ly6C^{hi} mononuclear phagocytes (TNFR^{-/-}), suggesting that TNFR directly mediates the recruitment of Ly6C^{hi} mononuclear phagocytes in a cell-intrinsic manner during cryptococcal brain infection (Figure 3.4 E).

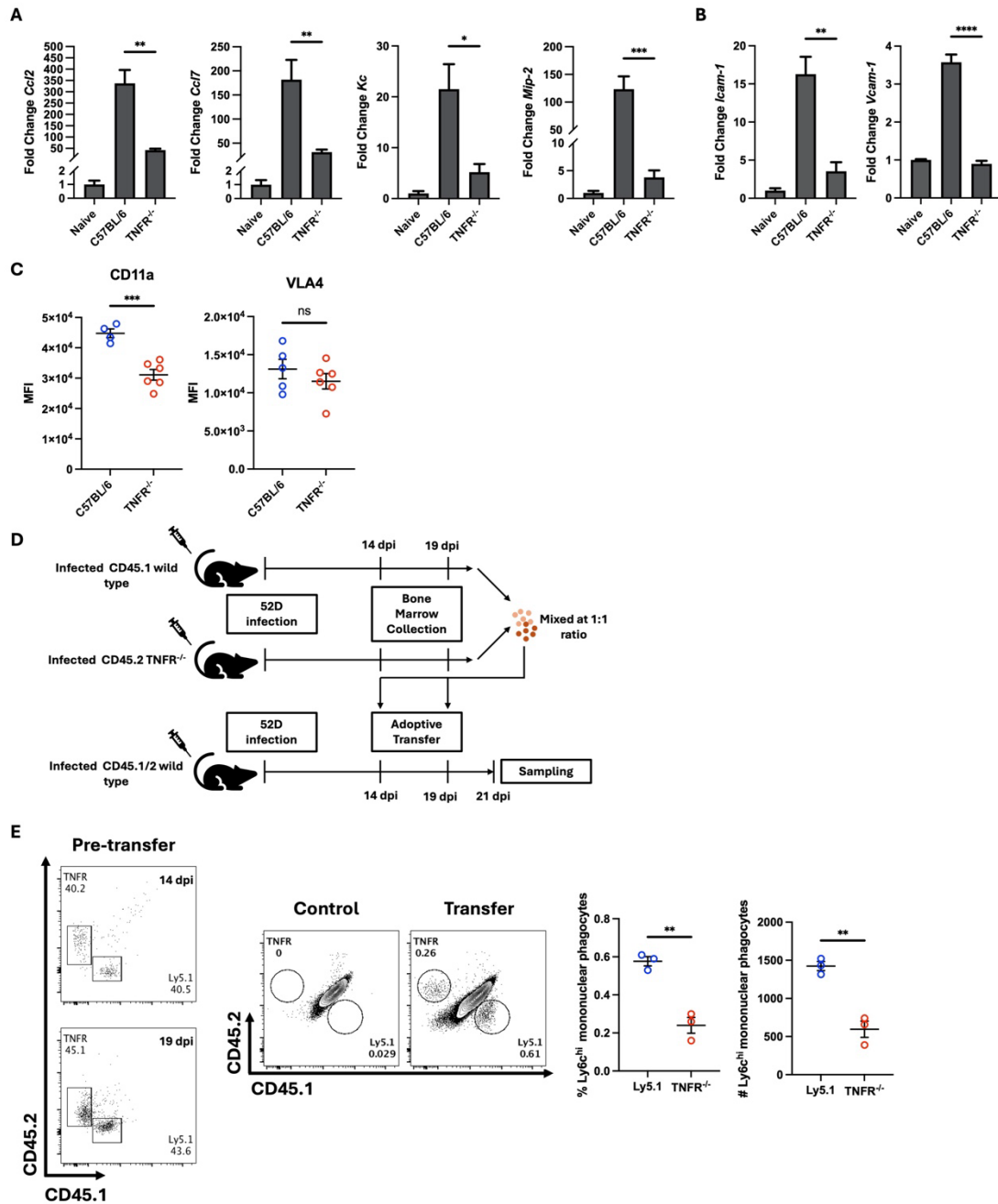


Figure 3.4 TNF directly signals on Ly6C^{hi} mononuclear phagocytes accompanied by enhanced expressions of chemokines and adhesion molecules. C57BL/6 wild-type and TNFR^{-/-} mice were infected intravenously with 1×10^6 *C. neoformans* 52D for 21 days. **(A & B)**. Gene expression of chemokine CCL2, CCL7, KC, and Mip-2 **(A)**, and adhesion molecules ICAM-1 and VCAM-1 **(B)** in the brains of naive C57BL/6 ($n = 3$), infected C57BL/6 wild-type and TNFR^{-/-} ($n = 5$) mice. **(C)**. Quantification of CD11a and VLA4 expression on brain CD11b⁺ Ly6C^{hi} mononuclear phagocytes in infected C57BL/6 wild-type mice ($n = 4-6$) at 21 days post infection. **(D)**. Schematic of the adoptive

transfer experiments. Infected CD45.1/2 wild-type mice received a mixture of isolated monocytes from the bone marrow of infected CD45.1 Ly5.1 WT and CD45.2 TNFR^{-/-} mice (mixed at 1:1 ratio) on day 14 and 19 post-infection (dpi) of *C. neoformans* 52D, respectively. Brain samples were collected for analysis at 21 dpi. **(E)**. Isolated bone marrow monocytes from infected Ly5.1 WT (CD45.1) or TNFR^{-/-} (CD45.2) mice were mixed at a 1:1 ratio and confirmed by flow cytometry prior to adoptive transfer (left panel). The frequency and the total number of donor Ly6C^{hi} mononuclear phagocytes from Ly5.1 WT and TNFR^{-/-} mice in the brain of recipient mice were analyzed by flow cytometry at 21 dpi (right two panels) (n=3). Representative flow cytometric dot plots are shown for both non-transferred control mice and transferred mice (gated on Ly6C^{hi} mononuclear phagocytes) (middle panel). Data are expressed as mean ± SEM and representative of two independent experiments. Dots represent individual mice. Ns, not significant, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

ICAM-1 deficiency does not affect the fungal burden in the brain

The above results showed that both ICAM-1 and its integrin CD11a were affected by the disruption of TNFR signaling and thus were potentially associated with the defects in Ly6C^{hi} mononuclear phagocytes recruitment. To study if ICAM-1 signaling could directly affect fungal clearance during *C. neoformans* infection, we infected C57BL/6 wild-type and ICAM-1^{-/-} mice with *C. neoformans* 52D through tail vein injection and measured brain fungal burden at 2 and 3 wpi. However, our results indicated that ICAM-1 deficiency could not sufficiently influence the fungal burdens in the brain, in terms, providing additional support for the notion that the primary role of TNF is to directly act on Ly6C^{hi} mononuclear phagocytes during recruitment (Figure 3.5).

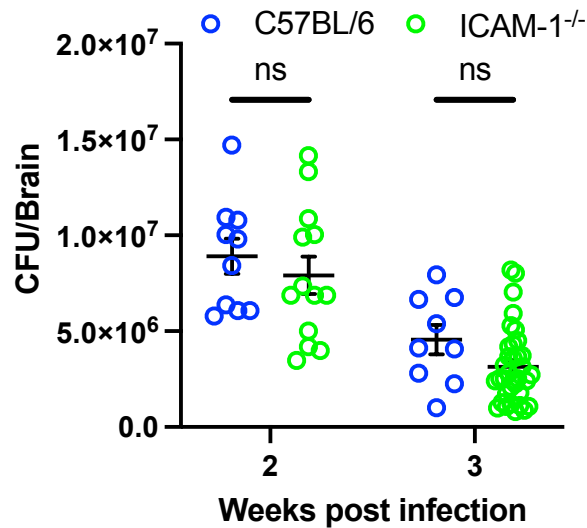


Figure. 3.5 ICAM-1 deficiency does not affect fungal burdens in the brain. C57BL/6 wild-type and ICAM-1^{-/-} mice (n = 9-35) were infected with 1×10^6 *C. neoformans* 52D via tail vein injection. C57BL/6 wild-type and ICAM-1^{-/-} mice were sacrificed at 2- and 3- weeks post infection. The brain fungal burdens were determined. Data are expressed as mean $\pm$ SEM and were pooled from 3 independent experiments. Dots represent individual mice. Ns, not significant.

IFN- γ R signaling is essential for M1 polarization of Ly6C^{hi} mononuclear phagocytes

Polarization of Ly6C^{hi} mononuclear phagocytes is essential for fungicidal activity (315–317). IFN- γ is known as the main cytokine that mediates the polarization of macrophages (307). Nevertheless, the role of IFN- γ in the polarization of Ly6C^{hi} mononuclear phagocytes during cryptococcal brain infection remains understudied. We found that the production of IFN- γ increased after 52D infection in the brain as determined by Enzyme-linked immunosorbent assay (ELISA) and that the induction of IFN- γ was dispensable for the recruitment of Ly6C^{hi} mononuclear phagocytes (Figure 3.6 A & Figure S3.2). Deficiency of IFN- γ R signaling led to lower expression of M1 markers iNOS and CD86; and higher expression of M2 markers CD206, FIZZ1, and Arg1 in the brain of infected mice (Figure 3.6 B). Compared to infected wild-type mice, infected IFN- γ R^{-/-} mice exhibited lower frequencies and absolute numbers of iNOS-producing Ly6C^{hi} mononuclear phagocytes but higher frequencies and absolute number of Arg1-producing Ly6C^{hi} mononuclear phagocytes in the brain (Figure 3.6 C). The median fluorescent intensity (MFI) of iNOS on Ly6C^{hi} mononuclear phagocytes was also decreased in the brain of infected IFN- γ R^{-/-} mice compared to infected C57BL/6 wild-type mice (Figure 3.6 C). Collectively, these results suggest that IFN- γ R signaling is essential for M1 polarization of Ly6C^{hi} mononuclear phagocytes during brain infection with *C. neoformans*.

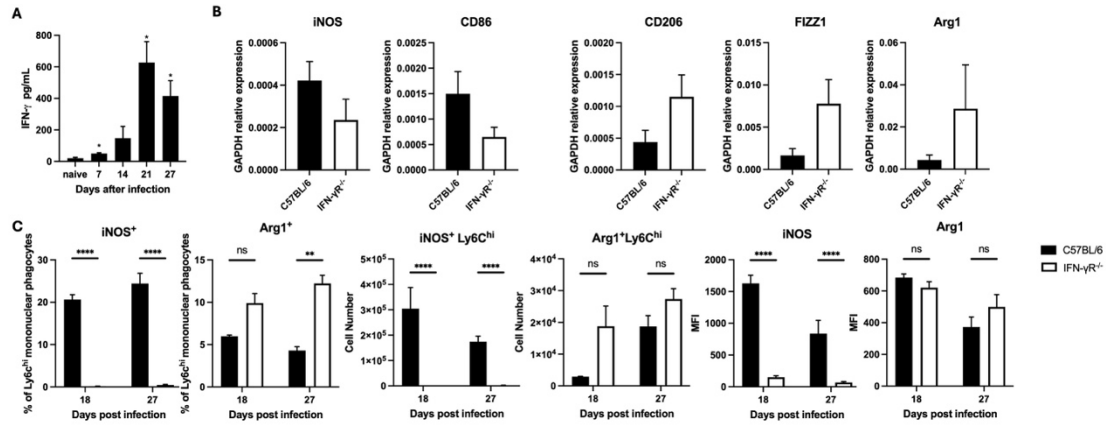


Figure 3.6 IFN-γR signaling is essential for M1 polarization of Ly6C^{hi} mononuclear phagocytes.

(A). C57BL/6 wild-type mice were infected with 1×10^6 *C. neoformans* 52D via tail vein injection. The concentrations of IFN-γ in the brains of naïve and infected C57BL/6 wild-type mice (n = 3-4) were measured by ELISA at 7-, 14-, 21- and 27-days post infection. (B-C). C57BL/6 wild-type and IFN-γR^{-/-} mice were infected with 1×10^6 *C. neoformans* 52D via tail vein injection. (B). Brain homogenates from infected C57BL/6 wild-type and IFN-γR^{-/-} mice (n = 4-5) were collected and gene expressions of iNOS, CD86, CD206, FIZZ1, and Arg1 were measured. (C). Brain leukocytes were isolated from infected C57BL/6 wild-type and IFN-γR^{-/-} mice (n = 3-15) and Ly6C^{hi} mononuclear phagocytes were analyzed by flow cytometry at 18- and 27- days post infection. Data are expressed as mean ± SEM and representative of two independent experiments or pooled from two independent experiments. Ns, not significant, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Disruption of TNFR or IFN- γ R signaling enhances the fungal growth in the brain

The above results demonstrate that TNFR and IFN- γ R signaling are essential for the recruitment and M1 polarization of Ly6C^{hi} mononuclear phagocytes, respectively. We next examined if TNFR and IFN- γ R signaling are essential for fungal clearance. C57BL/6 wild-type and TNFR^{-/-} mice were intravenously infected with *C. neoformans* 52D. The fungal burdens in the brains of infected mice were determined. We found that disruption of TNFR signaling led to higher brain CFU (Figure 3.7 A). Enhanced sizes of fungal clusters were also observed in infected TNFR^{-/-} mice compared to infected wild-type mice (Figure S3.3 A-B). We also infected C57BL/6 wild-type and IFN- γ R^{-/-} mice with *C. neoformans* 52D and examined brain CFU. The results show that infected IFN- γ R^{-/-} mice displayed significantly higher fungal burdens in the brain (Figure 3.7 B). Accordingly, larger fungal lesions were observed in the brains of infected IFN- γ R^{-/-} mice compared to infected wild-type mice (Figure S3.3 C-D). Taken together, these results suggest that TNFR and IFN- γ R signaling are essential for fungal clearance in the brain of mice infected with *C. neoformans*.

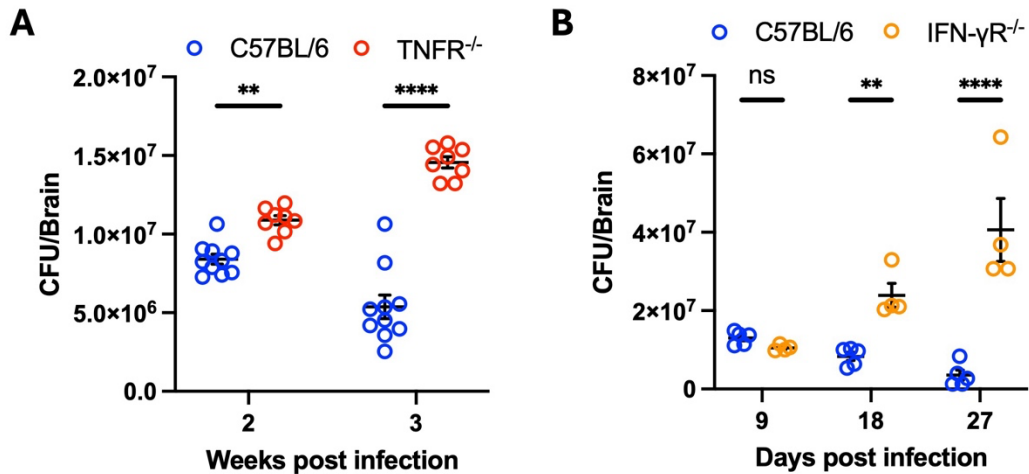


Figure. 3.7 Disruption of TNFR or IFN- γ R signaling enhances the fungal growth in the brain. (A). C57BL/6 wild-type and TNFR^{-/-} mice (n = 8-10) were infected with 1×10^6 *C. neoformans* 52D via tail vein injection. The fungal burdens in the brains of infected C57BL/6 wild-type and TNFR^{-/-} mice were determined at 2-, and 3- weeks post infection. Data are expressed as mean $\pm$ SEM and pooled from two independent experiments. Dots represent individual mice. ** $p < 0.01$, **** $p < 0.0001$. **(B).** C57BL/6 wild-type and IFN- γ R^{-/-} mice (n = 4-5) were infected with 1×10^6 *C. neoformans* 52D via tail vein injection. The fungal burdens in the brains of infected C57BL/6 wild-type and IFN- γ R^{-/-} mice were determined at 9-, 18-, and 27- days post infection. Data are expressed as mean $\pm$ SEM and representative of two independent experiments. Dots represent individual mice. Ns, not significant, ** $p < 0.01$, **** $p < 0.0001$.

Discussion

Identifying the effector cells involved in the clearance of cryptococcal brain infection is crucial for developing potential therapeutic strategies. While previous research has primarily relied on *in vitro* models, the complexity of fungal clearance in the brain necessitates more sophisticated *in vivo* approaches (112,117,122,209,210,318–320). Our study employed a chronic *C. neoformans* infection model using the less virulent strain 52D, providing a resolving systemic infection murine model with controlled fungal burden in the brain. By adopting this model, we found that clearance of *C. neoformans* in the brain is accompanied by accumulation of Ly6C^{hi} mononuclear phagocytes in the brain. Using flow cytometry, we demonstrated that there were interactions between Ly6C^{hi} mononuclear phagocytes and *C. neoformans*. Importantly, defects in inflammatory monocytes egressions from the bone marrow resulted in enhanced fungal burden in the brain, indicating that Ly6C^{hi} mononuclear phagocytes are essential for fungal control. We further demonstrated that TNFR signaling was essential for the recruitment of Ly6C^{hi} mononuclear phagocytes to the infected brain and that IFN- γ R signaling was required for M1 polarization of Ly6C^{hi} mononuclear phagocytes. Finally, we showed that disruption of TNFR signaling or IFN- γ R signaling significantly enhanced the fungal burdens in the brains of infected mice.

Little is known about the mechanism mediating the recruitment of Ly6C^{hi} mononuclear phagocytes from the bloodstream to brain parenchyma during cryptococcal CNS infection. TNF is a well-known cytokine with pleiotropic roles in

regulating the immune responses (321). During pulmonary infection with *C. neoformans*, TNF plays a protective role by reducing the fungal burden in the infected lung (202,205,322). Published studies show that TNF could regulate inflammatory monocyte-derived DCs during cryptococcal pulmonary infection by promoting the antigen presentation to the CD4⁺ T cells and mediating the trafficking of DCs to the lymph nodes (204). Less is known about the role of TNF during cryptococcal CNS infection. Our results showed that TNF signaling was critically involved in mediating the recruitment of Ly6C^{hi} mononuclear phagocytes to the brain in a cell-intrinsic manner. Accordingly, infected TNFR^{-/-} mice displayed significantly higher fungal burdens in the brain. Obviously, impaired fungal clearance observed in the brain of TNFR^{-/-} mice is attributed to reduced accumulation of Ly6C^{hi} mononuclear phagocytes. However, it still remains unknown as to how TNFR signaling enhances the recruitment of Ly6C^{hi} mononuclear phagocytes. In this regard, we found that TNF enhanced the expression of chemokines including CCL2, CCL7, KC, Mip-2, and adhesion molecules including VACM-1 and ICAM-1/CD11a. It is conceivable that these molecules are involved in the migration of Ly6C^{hi} mononuclear phagocytes to the brain either directly or indirectly. Interestingly, we found that ICAM-1 deficiency did not affect the fungal burdens in the brain, suggesting that multiple molecules are involved in the recruitment of Ly6C^{hi} mononuclear phagocytes and that there is a functional redundancy among multiple molecules. Nevertheless, TNFR^{-/-} monocytes displayed a cell-intrinsic defect in migrating to the brain, demonstrating that TNF signals directly on mononuclear phagocytes for their brain migration.

Besides the recruitment of Ly6C^{hi} mononuclear phagocytes, proinflammatory polarization of Ly6C^{hi} mononuclear phagocytes is crucial for fungal clearance as well. With the anti-phagocytic polysaccharide capsule, *C. neoformans* can escape phagocytosis and direct killing from phagocytes (323–325). Fungal-secreting proteins CPL1, urease, PLB1, *etc.* could all inhibit the proinflammatory immune responses (242,244,250,251). With the help of virulence factors, *C. neoformans* could successfully escape macrophages' fungicidal activities. Considering cryptococcal immunoregulating factors, hosts need sufficient pro-inflammatory immune response to clear the fungal cells. Published studies have shown that IFN- γ can induce M1 polarization of pulmonary macrophages, resulting in fungal clearance in the lung during pulmonary infection with *C. neoformans* (183). In this study, we found that IFN- γ R signaling is required for M1 polarization of Ly6C^{hi} mononuclear phagocytes in the brain. We also found that IFN- γ R^{-/-} mice exhibited significantly higher fungal burdens in the brain. The enhanced fungal burdens in the brain of IFN- γ R^{-/-} mice are likely because of the reduced M1 polarization of Ly6C^{hi} mononuclear phagocytes.

In summary, we have identified Ly6C^{hi} mononuclear phagocytes as being essential for the clearance of *C. neoformans* infection in the brain. Both TNF and IFN- γ are required for Ly6C^{hi} mononuclear phagocyte-mediated killing of *C. neoformans* in the brain. However, they play a different role in the clearance of *C. neoformans* infection in the brain, namely, TNF mediates the recruitment of Ly6C^{hi} mononuclear phagocytes, while IFN- γ promotes M1 polarization Ly6C^{hi} mononuclear phagocytes.

Both cytokines act synergistically to facilitate the clearance of *C. neoformans* brain infection mediated by Ly6C^{hi} mononuclear phagocytes.

Supporting information

Supplemental Figure 1

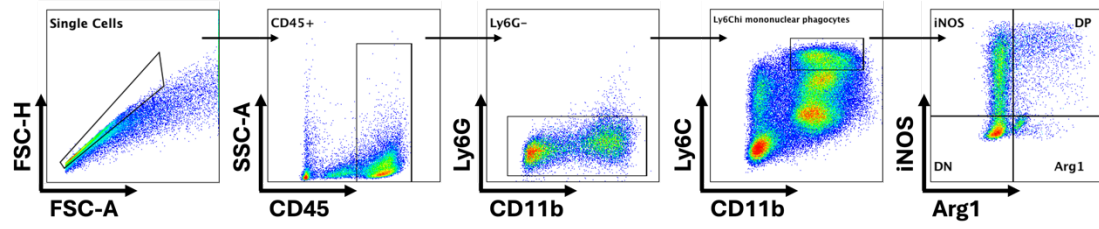


Figure S3.1. Gating strategy.

Supplemental Figure 2

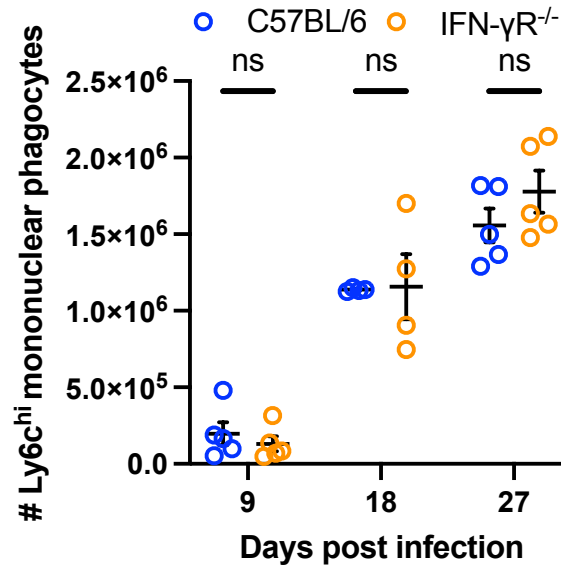


Figure. S3.2 Disruption of IFN-γR signaling does not affect the accumulation of Ly6C⁺ mononuclear phagocytes in the brain. C57BL/6 wild-type and IFN-γR^{-/-} mice (n = 4-5) were infected with 1 × 10⁶ *C. neoformans* 52D via tail vein injection. Brain CD11b⁺ Ly6C^{hi} mononuclear phagocytes were analyzed by flow cytometry at 9-, 18-, and 27- days post infection. Data are expressed as mean ± SEM and representative of three independent experiments. Dots represent individual Mice. Ns, not significant.

Supplemental Figure 3

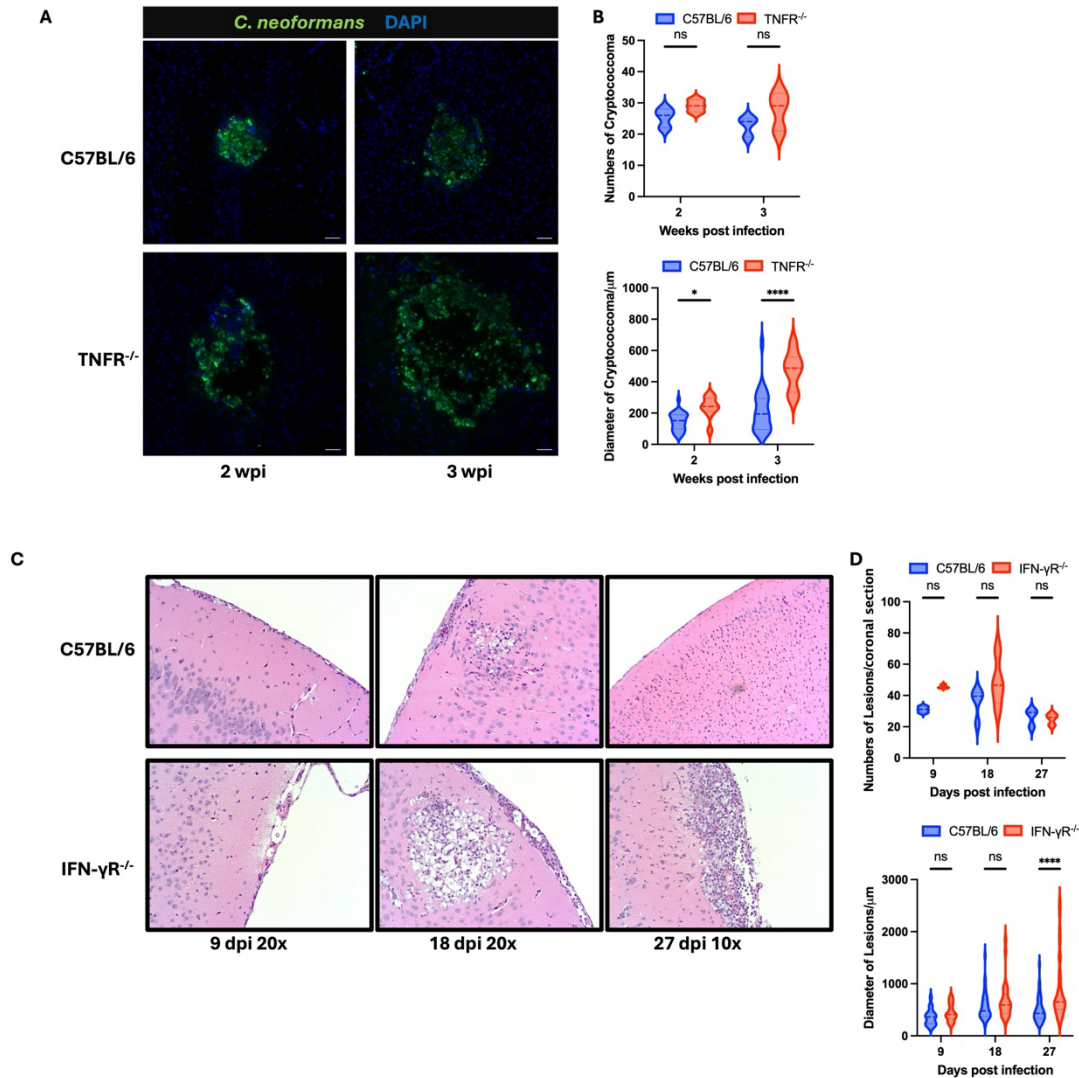


Figure. S3.3. Disruption of TNFR signaling enhances the size of fungal clusters and disruption of INF- γ R signaling enhances the size of lesions. (A). C57BL/6 wild-type and TNFR^{-/-} mice (n = 3) were infected with 1×10^6 *C. neoformans* 52D via tail vein injection. Brain samples from infected C57BL/6 wild-type and TNFR^{-/-} mice were collected at 2- and 3- weeks post infection. Immunohistochemistry was performed to stain *C. neoformans* cells (green). Nuclei were stained with DAPI (blue). Numbers of cryptococcoma in comparable coronal sections and the diameter of each cryptococcoma were measured using Zen software. **(B).** Violin plots of the quantifications of the numbers of cryptococcoma in comparable coronal sections and diameters of each cryptococcoma. Ns, not significant, $*p < 0.05$, $****p < 0.0001$. **(C).** C57BL/6 wild-type and IFN- γ R^{-/-} mice (n = 3-4) were infected with 1×10^6 *C. neoformans* 52D via tail vein injection. Brain samples from infected C57BL/6 wild-type and IFN- γ R^{-/-}

mice were collected at 9-, 18-, and 27- days post infection. H&E staining was performed to detect cryptococcal lesions. The number of lesions in comparable coronal sections and the diameter of each lesion were measured using Fiji software. **(D)**. Violin plots of the quantification of the numbers of lesions in comparable coronal sections and diameters of each lesion. Ns, not significant, $***p < 0.0001$. Data are representative of two independent experiments.

Chapter 4: Conclusion and perspectives

This study aims to determine how *C. neoformans* migrate to the brain and how host immune cells respond to the invading organism. *C. neoformans* is a significant contributor to mortality in fungal diseases (1). Cryptococcosis poses a threat to both immunocompromised and immunocompetent individuals, with increasing cases observed in non-HIV-infected populations, signaling a potential spread of the disease (326). Treatment for cryptococcosis typically spans six months or longer, highlighting the urgent need for more effective therapeutic approaches. Understanding the mechanisms involved in brain invasion by *C. neoformans* and host immune responses to the organism may help us develop novel strategies to treat this illness.

To investigate the transmigration of *C. neoformans* across the blood-brain barrier (BBB), researchers have extensively relied on multiple *in vitro* infection models to simulate natural cryptococcal infection conditions (73,74,84,88,90). The *in vitro* models are an important step to study the interactions between *C. neoformans* and brain endothelial cells and have significantly advanced our understanding of brain invasion by *C. neoformans*. However, these simplified *in vitro* models failed to capture the intricacies of the BBB structure. With this in mind, we have developed an *in vivo* mouse model to quantitatively analyze brain invasion of *C. neoformans* following intravenous infection of the fungal cells using flow cytometry. While humans become infected by inhaling the fungal cells, *C. neoformans* migrate to the blood and gain access to the brain directly from the blood (60,300). Intravenous inoculation eliminates the variable transmigration from the lung and mimics the process of brain invasion once the

organisms enter the blood. Thus, our studies established a powerful and physiologically relevant murine model to study brain invasion of *C. neoformans*, with the potential to study brain invasion by other microbes. Using this *in vivo* model, we demonstrate that internalization of *C. neoformans* by brain endothelial cells occurs *in vivo*, supporting that *C. neoformans* invades the brain via a transcytosis pathway. Notably, we found that the efficiency of *C. neoformans* endocytosis by brain endothelial cells is influenced by factors such as capsule presence, viability, and serotypes. These results provide novel insights into mechanisms underlying brain invasion of *C. neoformans* and improve our understanding of cryptococcal pathogenesis (Chapter 2).

It is worthy noting that *C. neoformans* may use multiple strategies to cross the BBB (60,73,74,88,90,300). In addition to transcytosis, *C. neoformans* may transmigrate to the brain through the Trojan horse mechanism or paracellular crossing (73,74,90). It remains unknown as to which is the dominant pathway that *C. neoformans* employs to cross the BBB. In addition, little is known about the molecular mechanism(s) involved in brain invasion of *C. neoformans*, especially *in vivo*. In the past, lab strains of *C. neoformans* have been used to study BBB crossing of *C. neoformans*. Do clinical strains use the same strategies to migrate to the brain as the lab strains do? There are many questions left unanswered in the field. It is challenging to study *C. neoformans* transmigration to the brain across the BBB *in vivo* because it is a dynamic and transit process. Nevertheless, with the development of new technologies and approaches, we will be in a better position to address the complex mechanisms of brain invasion by *C. neoformans*.

Following brain invasion, *C. neoformans* will encounter different subsets of immune cells including resident microglia and recruited leukocytes. It has been a long-standing question in the field as to which immune cells kill *C. neoformans* in the brain and what is the underlying mechanism(s). Inbred C57BL/6 mice but not outbred mice have been widely used to study immunology because of the availability of a large pool of transgenic and knockout mice in this background of mice. Taking advantage of knockout mice in C57BL/6 background, we next explored the host immune responses to the invading *C. neoformans* cells in the brain, with the focus on identifying effector cells for fungal clearance and the underlying mechanism(s). Our results suggest there is a correlation between fungal clearance and accumulation of Ly6C^{hi} mononuclear phagocytes in the brain following brain infection with *C. neoformans*. Notably, reducing the accumulation of Ly6C^{hi} mononuclear phagocytes by disrupting CCR2 signaling significantly enhanced fungal burdens in the infected brain, suggesting that these phagocytes function as effector cells for the clearance of *C. neoformans*. We also found that cytokines TNF and IFN- γ are produced in the infected brain and that TNFR and IFN- γ R are critically involved in the clearance of *C. neoformans* in the brain. Further studies suggest that TNFR signaling mediates the recruitment of Ly6C^{hi} mononuclear phagocytes in the brain in a cell-intrinsic manner and that IFN- γ R signaling promotes M1-polarization of these phagocytes. Thus, we identified Ly6C^{hi} mononuclear phagocytes as being essential for the clearance of *C. neoformans* in the brain involving TNFR signaling and IFN- γ signaling. These results advanced our

understanding of mechanisms involved in the clearance of *C. neoformans* in the brain (Chapter 3).

Regarding immune responses to invading *C. neoformans* cells in the brain, many questions still remain unanswered. For example, microglia, the major resident immune cells, have been recently shown to not provide protection during acute cryptococcal meningoencephalitis (302). Why do Ly6C^{hi} mononuclear phagocytes but not microglia mediate protection, as both are phagocytes? How do microglia interact with recruited Ly6C^{hi} mononuclear phagocytes during brain infection with *C. neoformans*? HIV/AIDS patients are more susceptible to *C. neoformans* infection due to the depletion of CD4⁺ T cells. It is believed that CD4⁺ T cells are critically involved in *C. neoformans* clearance in the brain. What is the crosstalk between CD4⁺ T cells and Ly6C^{hi} mononuclear phagocytes during clearance of cryptococcal infection in the brain? We believe that we will have a better understanding of the sophisticated mechanisms involved in the clearance of *C. neoformans* in the brain by addressing these important questions.

In summary, we have developed a powerful *in vivo* model to quantitatively analyze brain invasion of *C. neoformans* and demonstrated the internalization of the organism by brain endothelial cells *in vivo*. We also studied the immune responses to *C. neoformans* after the organism invades the brain and identified Ly6C^{hi} mononuclear phagocytes as effector cells for clearance of the fungal cells in the brain involving

TNFR signaling and IFN- γ R signaling. These studies improve our understanding of the complex interactions between host and *C. neoformans* in the brain.

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Curriculum vitae

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Current Position

Graduate Assistant
University of Maryland

January 2018-Present
College Park, MD

Education

01/2018-07/2024	Ph. D., <i>Veterinary Microbiology and Immunology</i> College of Agriculture College of Agriculture and Natural Resources, University of Maryland, College Park (Maryland, USA)
09/2013-07/2017	B.S., <i>Biological Sciences</i> College of Life Sciences, Sichuan University (Sichuan, China)

Research Experience

Graduate Research Assistant, 01/2018-Present
University of Maryland, College Park

- Investigating host innate immune responses and host-pathogen interaction during brain infection with fungal pathogen *Cryptococcus neoformans*.
 - Investigating the roles of brain macrophages during cryptococcal central nervous system (CNS) infection.
 - Investigating the regulation of cytokines TNF and IFN- γ during cryptococcal CNS infection.
 - Developing multiple *in vivo* systemic mouse models to investigate the host-pathogen interaction.
 - Using multiparametric flow cytometry assays to phenotype different subsets of primary brain macrophages.
 - Conducting ELISA, RT-qPCR, intracellular cytokine flow cytometry to study the expression and regulation of key immune markers and cytokines.
 - Performing intravital microscopy, immunohistochemistry and spatial transcriptomics to study the spatial and temporal dynamics of cellular interactions and molecular expressions within tissues.
 - Supervising new lab members and interns in designing and executing experiments and projects.
 - Participating and in charge of daily lab management, responsible for all inspections, lab orders, organizing and keeping track of supplies, documenting lab files as well as standard protocols, etc.

Research Assistant, 04/2014-05/2015&10/2016-05/2017

Laboratory of Stem Cell and Tissue Engineering, State Key Laboratory of Biotherapy, Sichuan University

- Isolating and characterizing the phenotype of human urine derived stem cells and

evaluated the application of the stem cells for wound healing as qualified tissue engineering seed cells using flow cytometry.

Research Assistant, 09/2015-01/2016

Research Center of Growth Mechanism and Aging, Sichuan University

- Investigating cancer-related genes p53 and p63 and learned to conduct basic experiments, including cell transformation, *in vitro* cell digestion, western blotting, etc.

Honors and Awards

- Outstanding Graduate Assistant Award, University of Maryland, 2023 – 2024
- Ann G. Wylie Dissertation Fellowship, University of Maryland, 2023 – 2024
- The Basil and Anne Hatzios Scholarship Fund for Veterinary Medical Research, 2022
- AAI Trainee Abstract Award, The American Association of Immunologists, 2022
- Dean's Fellowship, University of Maryland, 2018, 2019, 2020, 2022
- Jacob K. Goldhaber Travel Award, University of Maryland, 2022

Mentoring

- Yiquan Wu, MS 02/2021 – 09/2022 PhD Student, University of Maryland, College Park
- Zoe Shi 07/2022 – 09/2022 Undergraduate Intern, University of Maryland, College Park
- Cindy Wang 07/2022 – 08/2022 High School Intern, Atholton High School
- Krystal Ogoke 07/2022 0 05/2023 High School Intern, Science and Technology Internship Program, Eleanor Roosevelt High School
- Hope Pruitt 09/2023 – present MS Student, University of Maryland, College Park

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Peer-reviewed publications:

1. Xiao K, Liu H, Dong S, Fan X, **Chen Y**, Xu H. Interfacial effect of *Stropharia rugoso-annulata* in liquid medium: interaction of exudates and nickel-quintozone. *RSC Adv.* 2016 Sep 9;6(89):86068–81.
2. Sun D, Sun P, Li H, Zhang M, Liu G, Strickland AB, **Chen Y**, et al. Fungal dissemination is limited by liver macrophage filtration of the blood. *Nature Communications* 2019 10:1. 2019 Oct 8;10(1):1–14.
3. Liu G, Fu Y, Yosri M, **Chen Y**, Sun P, Xu J, et al. CRiG plays an essential role in intravascular clearance of bloodborne parasites by interacting with complement. *Proc Natl Acad Sci USA.* 2019 Nov 26;116(48):24214–20.
4. Sun D, Zhang M, Sun P, Liu G, Strickland AB, **Chen Y**, et al. VCAM1/VLA4 interaction mediates Ly6Clow monocyte recruitment to the brain in a TNFR signaling dependent manner during fungal infection. *PLoS Pathog.* 2020;16(2): e1008361.
5. Liu G, Abas O, Fu Y, **Chen Y**, Strickland AB, Sun D, et al. IL-27 negatively regulates Tip-DC development during infection. *mBio.* 2021 Jan 1;12(1):1–15.
6. **Chen Y**, Li C, Sun D, Strickland AB, Liu G, Shi M. Quantitative analysis reveals internalisation of *Cryptococcus neoformans* by brain endothelial cells *in vivo*. *Cell Microbiol.* 2021 Jun 1; 23(6): e13330.
7. Strickland AB, Sun D, Sun P, **Chen Y**, Liu G, Shi M. IL-27 Signaling Promotes Th1 Responses and Is Required to Inhibit Fungal Growth in the Lung during Repeated Exposure to *Aspergillus fumigatus*. *Immunohorizons.* 2022 Jan 1;6(1):78–89.
8. **Chen Y**, Shi ZW, Strickland AB, Shi M. *Cryptococcus neoformans* Infection in the Central Nervous System: The Battle between Host and Pathogen. *J Fungi (Basel).* 2022 Oct 1; 8(10).

9. Shi ZW*, **Chen Y***, Ogoke KM, Strickland AB, Shi M. Cryptococcal Immune Reconstitution Inflammatory Syndrome: From Clinical Studies to Animal Experiments. *Microorganisms*. 2022 Dec 1;10(12). * indicates joint first authors with equal contributions.
10. Strickland AB, **Chen Y**, Sun D, Shi M. Alternatively activated lung alveolar and interstitial macrophages promote fungal growth. *iScience*. 2023 Apr 23;26(5):106717.

Poster Presentation:

Chen Y, Sun D, Strickland AB, Liu G and Shi M, IFN- γ signaling promotes proinflammatory microglia polarization during brain infection with *Cryptococcus neoformans*, 30th Annual Research Symposium of Virginia-Maryland College of Veterinary Medicine, November 9, 2019.

Chen Y, Li C, Sun D, Strickland AB, Liu G and Shi M, Quantitative analysis reveals internalization of *Cryptococcus neoformans* by brain endothelial cells *in vivo*, 3rd AGNR annual Cornerstone event, November 9–13, 2020

Chen Y, Li C, Sun D, Strickland AB, Liu G and Shi M, Quantitative analysis reveals internalization of *Cryptococcus neoformans* by brain endothelial cells *in vivo*, 31st Annual Research Symposium of Virginia-Maryland College of Veterinary Medicine, March 25-26, 2021

Chen Y, Strickland AB and Shi M, TNF- α signaling is required for fungal clearance during brain infection with *Cryptococcus neoformans* via promoting the recruitment of CD4⁺ T cells and inflammatory monocytes, 105th AAI annual meeting, May 6-10, 2022

Oral Presentation:

Chen Y, Strickland AB and Shi M, TNF- α signaling is required for fungal clearance during brain infection with *Cryptococcus neoformans* via promoting the recruitment of CD4⁺ T cells and inflammatory monocytes, 105th AAI annual meeting, May 6-10, 2022

Chen Y and Shi M, The functions of mononuclear phagocytes during *Cryptococcus neoformans* CNS infection, INFECTIOUS DISEASES: FIGHTING THE GLOBAL CHALLENGES, Cell Press, April 26-28, 2023