The Role Of Host Variability In Determining Macrophage Responses To Cryptococcosis

By

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“Your pain is the breaking of the shell that encloses your understanding.”

- Khalil Gibran.

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Preface

Cryptococcosis remains the leading cause of fungal meningitis worldwide, caused by the intracellular pathogens *Cryptococcus neoformans* and *Cryptococcus gattii*. Although the majority of cryptococcosis cases occur in HIV patients, there is growing incidence of this disease in otherwise healthy individuals. To date, however, few studies address how this fungal pathogen is able to evade host innate immune responses in individuals with intact antifungal defenses. Interestingly, most healthy individuals are thought to harbor infections from early childhood onwards that are either resolved or become latent. Macrophages are a key host cell for cryptococcal infection; however, *C. neoformans* has evolved mechanisms to evade killing by macrophages, including vomocytosis (non-lytic expulsion from phagosomes). We sought to quantify the extent of individual variation in this early phagocyte response within a small cohort of healthy volunteers. Here we discuss how underlying host innate immune responses vary between human hosts in response to cryptococcal disease caused by *C. neoformans*. We also assess current understanding of how immune responses in different hosts may be predictive of protection from, or susceptibility to cryptococcal meningitis (CM). We find that rates of both intracellular fungal proliferation and non-lytic expulsion (vomocytosis) are remarkably variable between individuals, irrespective of gender, and that *in vitro* host inflammatory cytokine profiles are not determinants of this variation. We also provide data associating TLR4 with the vomocytosis of *C. neoformans* from mouse bone marrow derived macrophages (bMDMs). We thereafter questioned whether genetic variation in known innate immune genes (Toll-like receptor 2 (TLR2);

TLR4; the C-type Lectin, Dectin-1; the mitogen-activated protein (MAP) kinase, ERK5; and the autophagy protein, Galectin-8 (GAL8) may underlie some of the person-to-person variation we had observed. Our analysis detected a TLR4 polymorphism (rs4986791) in one individual among 9 healthy adults that was of no observed immune consequence during *in vitro* challenge of host macrophages with *C. neoformans*; suggesting that larger GWAS studies be carried out to verify the association of these polymorphisms with susceptibility to cryptococcosis in immunocompetent hosts. Lastly, given the ability of *C. neoformans* to manipulate phagosome maturation, we screened 13 off-patent drugs from the Prestwick Chemical Library®, for activity against intracellular *C. neoformans*. We present data that proposes Fendiline Hydrochloride as a potential anticryptococcal drug capable of eliminating intracellular fungi, but not extracellular cryptococci, and recommend calcium channel blockers as potential targets for future anticryptococcal treatments.

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List of Abbreviations

AA ..... arachidonic acid
AIDS ..... Acquired Immunodeficiency Syndrome
AmB ..... Amphotericin B
AMI ..... Antibody-mediated Immunity
AML ..... Acute Myeloid Leukemia
ANXA2 ..... Annexin A2
ARV ..... Anti-Retroviral therapy
BBB ..... Blood-brain barrier
BCG ..... Bacille Calmette-Guerin Vaccine
BCVs ..... bacteria-containing vesicles
BECs ..... bladder epithelial cells
bMDMs ..... Bone marrow-derived macrophages
BSA ..... Bovine Serum Albumin
CAT ..... Clot Activator Tubes
CD14 ..... Cluster of Differentiation
CD18 ..... Cluster of Differentiation 18
CFU ..... Colony Forming Units
CLR ..... C-type lectin receptor
CM ..... Cryptococcal Meningitis
CMV ..... cytomegalovirus
CNS ..... Central Nervous System
CrAg ..... Cryptococcal Antigen Test
CSF ..... Cerebral Spinal fluid
DMEM ..... Dulbecco's modified Eagle's medium
DMSO ..... dimethyl sulphoxide
DRF ..... Damage-response framework
ELISA ..... Enzyme-linked immunosorbent assay
ERK5 ..... Extracellular-signal-regulated kinase 5
FBS ..... Fetal Bovine Serum
FCGR ..... Fc gamma receptor
FCGR3A ..... Fc Gamma Receptor 3A
FcγR ..... Fc gamma receptor
FDA ..... US Food and Drug Administration
GAL8 ..... Galectin-8
GXMGal ..... Galactoxylomannan
GFP ..... Green fluorescence protein
GlcCer ..... Glucosylceramides
GM-CSF ..... granulocyte-macrophage colony-stimulating factor
GWAS ..... genome-wide association study
GXM ..... Glucuronoxylomannan
H99 ..... C. neoformans var. grubii serotype A strain
HIV ..... Human Immunodefeciency Virus
HMDMs ..... Human Monocyte derived macrophages
IFN-γ ..... Interferon Gamma
IL-12RB1 ..... Interleukin 12 Receptor Subunit Beta 1

IL-1α ..... Interleukin- 1 alpha
IL-1β ..... Interleukin - 1 beta
IL-6 ..... Interleukin - 6
iNOS ..... Inducible Nitric Oxide
IPR ..... Intracellular proliferation rate
IRIS ..... Immune Reconstitution Inflammatory Syndrome
LDH ..... Lactate dehydrogenase
L-DOPA ..... levodopa or L-3,4-dihydroxyphenylalanine
LFA ..... Lateral Flow Assay
LOAD ..... late-onset Alzheimer's Disease
LP ..... Lumbar Puncture
LPS ..... Lipopolysaccaride
M1 ..... Classically Activated macrophages
M2 ..... Alternatively Activated macrophages
MAb ..... Monoclonal antibody
MALDI-TOF ..... matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MAP ..... mitogen-activated protein
MAPKs ..... mitogen-activated protein kinases
MBL ..... Mannose-binding lectin
MDMs ..... Monocyte-derived macrophages
miRNAs ..... MicroRNAs
MLST ..... Multilocus Sequence Typing
MOI ..... multiplicity of infection
MyD88 ..... Myeloid differentiation primary response 88
NF-κB ..... Nuclear Factor-Kappa B
NO ..... Nitric Oxide
PAMPs ..... pathogen-associated molecular patterns
PBMCs ..... Primary peripheral blood monocytes
PCR ..... Polymerase chain reaction
PHS ..... Pooled human Serum
PLB ..... Phospholipase B
PMA ..... phorbol myristate acetate
PRRs ..... Pattern Recognition Receptors
RBC ..... Red Blood Cell
RNS ..... Reactive Nitrogen Species
ROS ..... Reactive Oxygen Species
SCID ..... Severe Combined Immunodeficiency
SNPs ..... small nucleotide polymorphisms
SOT ..... Solid Organ Transplant
STAT1 ..... Signal transducer and activator of transcription 1
TB ..... Trypan blue
Th1 ..... Type 1 immune response
Th17 ..... Type 17 immune reactions
Th2 ..... Type 2 immune response
THP-1 ..... Human macrophage-like cell line
TLR2 ..... Toll-like receptor 2
TLR4 ..... Toll-like receptor 4
TLRs ..... Toll-like Receptors

TNFα.....Tumor Necrosis Factor alpha
TRAF.....TNF receptor associated factorprotein family
UPEC.....UroPathogenic Escherichia coli
YPD .....Yeast Extract–Peptone–Dextrose

Chapter 1 – Introduction

Parts of this chapter have been adapted from the review below:

GARELNABI, M. and MAY, R.C. 2018. Variability In Innate Host Immune Responses To Cryptococcosis. Manuscript in press, Memórias do Instituto Oswaldo Cruz.

Abstract

Cryptococcosis is a fungal disease caused by members of the genus *Cryptococcus*, with a high frequency of cases being due to the opportunistic pathogens *Cryptococcus neoformans* and *Cryptococcus gattii*. Although the majority of cryptococcosis cases occur in HIV patients, there is growing evidence to support the pathogenicity of Cryptococcus spp. towards otherwise healthy individuals. To date, however, few studies address how this fungal pathogen is able to evade host innate immune responses in individuals with more robust antifungal defenses. Here we discuss how underlying host innate immune responses vary between human hosts in response to cryptococcal disease caused by *C. neoformans*. We also assess current understanding of how immune responses in different hosts may be predictive of protection from, or susceptibility to cryptococcal meningitis (CM).

Literature Review

THE IMMUNE SYSTEM

The immune system is a combination of immune cells and molecules that defend the body from physical, chemical and biological invasion by pathogens such as viruses, bacteria and fungi. It spans a number of tissue groups and organ systems, and consists of highly orchestrated cells and substances that are able to detect differences between invasive pathogens and the body's own cells and microbiome (self) [1]. Immune cells are generated in organs such as the spleen, bone marrow and lymph nodes, and monitor how the body responds to an invasive pathogen in order to mount the most effective defense against it. This is known as the immune response. Immune responses are classified as either innate or adaptive (explained below); the two arms of immunity that interact with each other to effectively bring about pathogen clearance. However, in some cases, the immune system may fail to self-recognize and mount an attack against the body's own cells, leading to the development of autoimmune diseases.

The innate immune system

Upon infection, the first line of defense is the innate immunity. This arm of the immune system is inherited, rapid, and considered to be 'non-specific'; it includes anatomical and chemical deterrents such as the epithelial and mucous membranes, and internal defenses such as myeloid cells (macrophages, dendritic cells, masT-cells and natural killer (NK) cells). Innate immunity also consists of

molecular components such as the complement system, cytokines and acute-phase proteins which may act as inflammatory mediators [1]. Pertaining to this thesis, the characteristics of macrophages, the complement system and cytokines will be described below.

Macrophages

A major component of innate immune cells are macrophages. These are phagocytic cells which develop from blood-borne monocytes with the ability to distinguish between host and pathogen cells via highly specialized cell surface receptors [1]. They are able to engulf pathogens within an intracellular compartment known as a phagosome, that matures into a phagolysozome capable of destroying a phagocytosed pathogen following acidification of the phagolysosomal lumen [2, 3].

Aside from their phagocytic abilities, macrophages comprise a dynamic population of cells capable of other functions such as tissue repair and remodeling, that are determined by signals in the macrophage's local microenvironment [4]. These stimuli influence macrophages to differentiate and express cell surface receptors that enable them to respond to cytokines released by other macrophages and surrounding cells, and pattern recognition receptors (PRRs) to detect pathogen-associated molecular patterns (PAMPs) and trigger phagocytosis. Macrophages derived from tissue-surrounding monocytes are able to differentiate into multiple phenotypes to fulfill different roles in host defense, tissue repair and immune modulation [5]. In general, macrophages are

considered as being either classically (M1) or alternatively activated (M2), although other macrophage activation states have been suggested [6, 7]. The M1 macrophage activation state is considered microbicidal and arises following exposure to GM-CSF and inflammatory stimuli such as IFN-γ and LPS *in vitro*; whereas M2 macrophages are activated in the presence of M-CSF and IL-4 and demonstrate functions in tissue restoration.

The macrophage arsenal to destroy engulfed pathogens includes the release of proinflammatory cytokines and chemokines that extend cell mediated immunity by increasing monocyte and neutrophil recruitment to the site of infection, and antigen presentation to T-cells (described below). Response to cytokine signaling results in the production of reactive nitrogen species such as nitric oxide (NO) [8], reactive oxygen species (ROS) and hydrogen peroxide into the phagolysozome, along with other enzymes to eliminate the pathogen from the host. In macrophages, NO is made by inducible nitric oxide synthase (iNOS) enzyme. NO plays a role in intracellular and extracellular signaling, free radical damage, and is crucial in the destruction of intracellular pathogens [8, 9].

The complement system

The complement system is a soluble component of the innate immune system. It consists of a number of plasma proteins that interact with each other to mark pathogens for destruction by innate immune cells in a process known as opsonization. Macrophages have receptors for both antibodies and complement,

and are able to use either or both methods of activation to enhance phagocytosis [10]. Complement activation occurs via one of three pathways: 1) the classical pathway following the formation of antibody-antigen complexes; 2) the alternative pathway upon sensing pathogen cell wall components; and 3) via the lectin pathway following binding of the mannose protein to microbial carbohydrates [11]. This culminates in the deposition of the complement component C3b on the surface of pathogens to initiate phagocytosis and trigger a series of inflammatory responses and the enhancement of the antimicrobial capabilities of innate immune cells [1].

Cytokines

Another soluble component of the innate immune system is cytokines. They act as chemical messengers that are released by cells to relay signals both internally and externally. There are more the 60 cytokines on record, released by a variety of cells [12]. A cytokine released by a particular cell type will initiate inflammatory responses in a cell expressing a receptor for that particular cytokine in order to bring about pathogen elimination [13]. The specificity of cytokine-releasing and cytokine receptor-expressing cells describes the complexity of immune responses to a variety of pathogens [12]. For example, certain classes of cytokines – the interferons- are released predominantly by virally infected cells, and have been shown to confer antimicrobial properties to surrounding cells. Cytokines are classified as being either pro-inflammatory or anti-inflammatory [12]. Well characterized pro-inflammatory cytokines include tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-1

(IL-1), IL-12, IL-18 and granulocyte-macrophage colony stimulating factor (GM-CSF); while anti-inflammatory cytokines include IL4, IL-10, IL-13, interferon-alpha (IFN-α) and transforming growth factor-beta (TGF-β) [14]. However, depending on the circumstances, a cytokine may behave as either pro- or anti-inflammatory [12, 14].

The adaptive immune system

The more specialized part of the immune system is known as the acquired (adaptive) immune system. This is a delayed response to pathogens represents the second line of defense. Adaptive immune responses are generated primarily in the secondary lymphoid tissues (spleen, lymph nodes and mucosa-associated lymph tissue), and involve the proliferation of lymphoid cells (T and B cells) that interact with components of the innate immune system to eliminate pathogens. This branch of immunity can further be divided into two types: humoral immunity (involves B cells), and cell-mediated immunity (driven by T-cells). Both B cells and T-cells develop from pluripotent stem cells in fetal liver tissue and in the bone marrow. Whilst B cells mature in the bone marrow, T-cells migrate towards the thymus where they mature and differentiate into effector T-cells (including T killer cells, regulatory T-cells and T helper cells) [12].

The adaptive immune system is also capable of developing an immunological memory to previous infections. During a primary infection, a subset of proliferating lymphoid cells differentiate into memory cells that lay dormant

until activated upon secondary infection. This allows a more rapid and stronger adaptive immune response to be mounted when circulating memory cells detect a pathogen during a subsequent exposure and differentiate into effector cells [12].

Interactions between innate and adaptive immunity

A typical immune response involves complex interactions between both the innate and adaptive immune responses. Whilst the innate immune response is mounted immediately, the adaptive response may take several days to come into effect. Therefore, innate immunity acts as a primary global response to infection, which is followed by an adaptive, more specialized immune response to an invading pathogen.

Upon infection, specialized cells known as antigen-presenting cells (which include dendritic cells, B cells, and activated macrophages) activate myeloid cells by presenting degraded antigens to T-cells via MHC molecules on the cell's surface, in order to initiate clearance of the pathogen (Fig. 1) [1]. MHC molecules are cell-surface glycoproteins encoded by a cluster of genes known as the major histocompatibility complex (MHC); they bind specific epitopes following degradation of an antigen by an antigen-presenting cell [12]. There are two predominant types of MHC molecules: Type 1 (MHC I) and Type 2 (MHC II). T-helper cell lineages are characterized by the set of markers and receptors they express; the two main classes of T-cells express either of two cell-surface

proteins known as CD8 and CD4, which act as co-receptors that confer functional diversity in T-cells, and determine the interactions between T-cells and other cells [12]. CD8 expressing T-cells (CD8+) bind MHC I, while CD4-expressing T-cells bind MHC II. In addition to T-cells' ability to clear pathogens, they are also involved in activating macrophages for the purpose of clearing intracellular pathogens and assisting B-cells to produce antigen-specific antibodies (Fig. 1) [1].

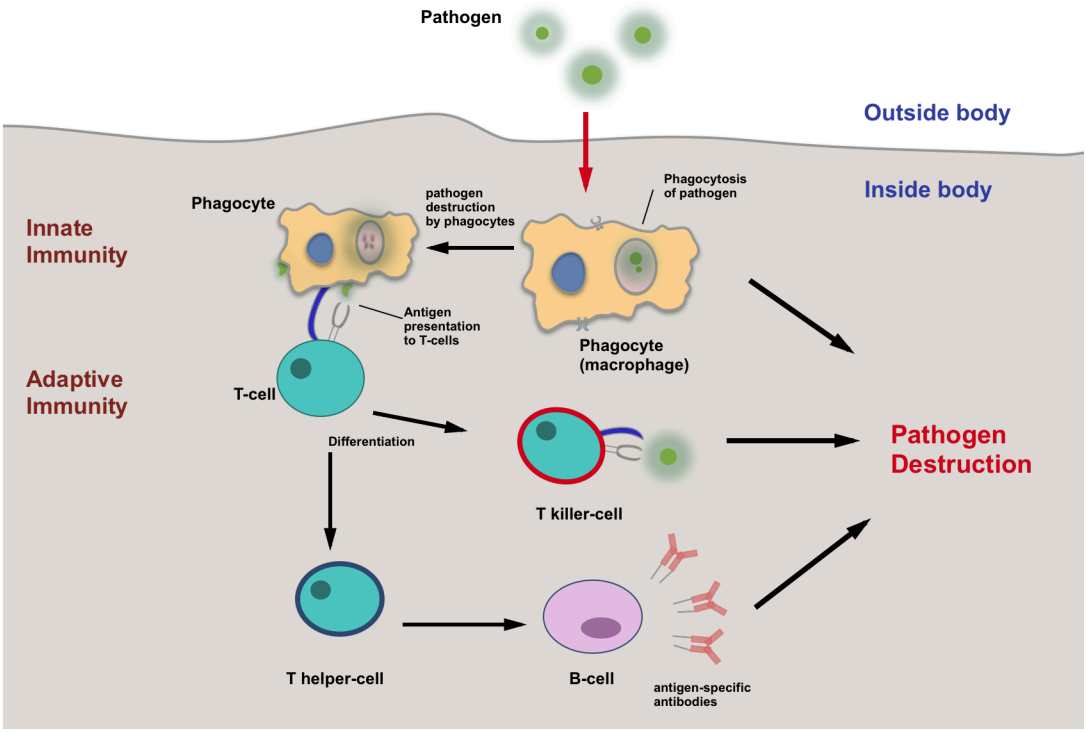


Figure 1. Illustration of the interaction between the innate and adaptive immune system. Upon entry into the body, a pathogen is first encountered by macrophages that phagocytose and degrade the pathogen. This is followed by antigen presentation to T-cells, which differentiate into T killer-cells that directly kill the pathogen, or into T helper-cells that initiate the production of antigen-specific antibodies by B-cells, also resulting in pathogen destruction.

T helper-cells also have diverse specificities: Type 1 (Th1) T helper cells secrete the cytokines interleukin-2 (IL-2) and interferon-γ and activate macrophages; while Type 2 (Th2) T helper cells secrete the IL-4, IL-5, IL-6, and IL-10 and inhibit Th1 responses [1]. Another T-cell lineage is the Type 17 (Th17) T helper-cells that are memory effector cells and have been associated with mounting inflammatory responses to pathogens by releasing the proinflammatory cytokines IL-17, IL-21, IL-22 and IL-26 [12].

Overall, depending on the nature of the pathogen, components in the innate and adaptive immune system will undergo complex interactions to a degree of high specificity towards a particular antigen, in able to orchestrate effective immune clearance of the pathogen.

CRYPTOCOCCOSIS

Cryptococcosis is a potentially fatal fungal disease caused by infectious members of the genus *Cryptococcus*, of which the two species *Cryptococcus neoformans* and *Cryptococcus gattii* are highly pathogenic towards humans [15, 16]. The severe form of the disease - Cryptococcal Meningitis (CM)- remains the leading cause of fungal meningitis worldwide, and the cause for 15% of AIDs-related deaths [17]. In 2014, up to 223,100 cases of CM were reported globally, with approximately 181,100 deaths resulting from severe disease [17, 18]. While clinical manifestations of the disease vary between species, the majority (80%) of cryptococcosis cases are HIV-associated, and are caused by *C. neoformans*. Over the last century, cryptococcosis has emerged as a fungal disease of significant clinical importance, placing it as the second most common cause of AIDs-related deaths after tuberculosis [17-19].

Prior to the 1950s, less than 300 clinical cases of cryptococcosis had been reported; the subsequent drastic rise in incidence was attributed to the emergence of the HIV/AIDS pandemic in the 1960s, and increased frequency of immunosuppressed patients [20]. However, *C. neoformans* also causes CM in non-HIV, immunocompromised patients and at a rising frequency in 'otherwise healthy' individuals [15, 21-23].

Previous assumptions that *C. gattii* was geographically restricted to tropical and subtropical regions were dismissed following a *C. gattii* outbreak that struck Vancouver Island, Canada in 1999 [24-26], followed by the subsequent spread of

the disease across North Western America in immunocompetent individuals [27, 28]. Clinical presentation, risk factors to the infection and response to treatments of cryptococcosis caused by either species vary between HIV/AIDs patients and non-HIV patients [29]. Because otherwise healthy cryptococcosis patients have been shown to present with less definitive symptoms than their immunocompromised counterparts, the disease is often misdiagnosed and can lead to fatalities, necessitating a deeper understanding of the etiology of the infection in both patient groups [22, 29].

Prior to the US Northwest Pacific/Canada British Columbia outbreak, *Cryptococcus gattii* had been localized to tropical and sub-tropical regions [30, 31]. This brought to light the expanding geographical limitations of the pathogen, suggesting a role for climate change in elevating the potential threat of disease to public health [32, 33].

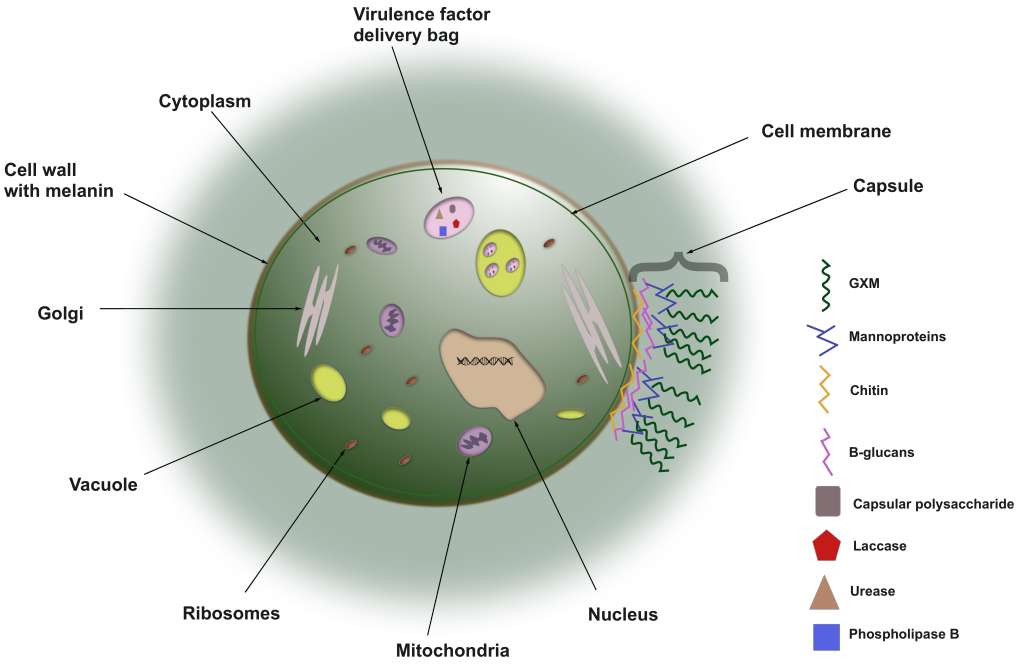
HISTORY, NOMENCLATURE AND TAXONOMY

The fungus was first described in 1894 by Otto Busse and Francesco Sanfelice in isolates from a bone infection in a female patient, and in fermenting peach juice, respectively [34, 35]. Sanfelice chose to name the yeast *Saccharomyces neoformans,* based on the form colonies took; however, the yeast was later renamed *Cryptococcus neoformans* in 1901 by Jean-Paul Vuillemin because it did not produce ascospores, a distinctive feature of members of the genus *Saccharomyces* [36]. In 1896, Ferdinand Curtis reported a 'yeast-like' fungus

from a patient's hip tumor, which he termed *Saccharomyces subcutaneous tumefaciens* due to variation in its culture compared to yeasts previously described by Busse and Sanfelice [37].

Both *C. neoformans and C. gattii* are basidiomycetes that are distinguishable from other yeasts such as *Candida* and *Saccharomyces* by the presence of an extensive polysaccharide capsule, melanin formation, and urease activity (Fig. 1a). These characteristics also function as virulence factors that enable the yeasts to invade and thrive within host systems.

a)



b) **c)**

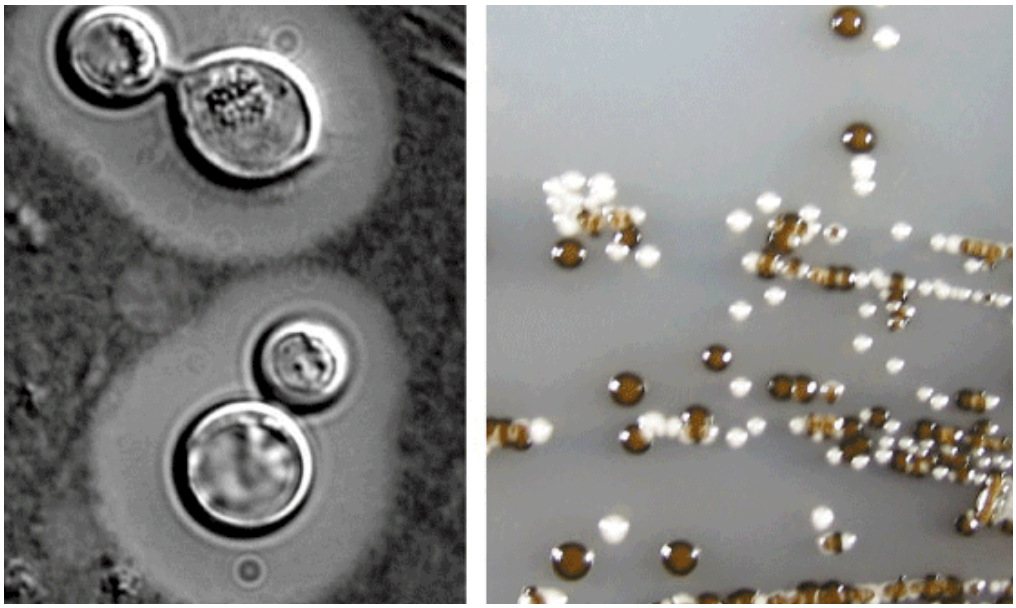


Figure 2- a) the structure of Cryptococcus; b) The Cryptococcus capsule when stained with India ink; c) Cryptococcal production of melanin in response to diphenols. b) and c) were adapted from [38].

Confusion surrounding the nomenclature of the etiological agents of cryptococcosis arose in the early twentieth century with subsequent isolation and identification of the yeast by other names such as *Cryptococcus hominis, Blastomyces neoformans* and *Torula histolytica.* This dispute was resolved by concluding that cryptococcosis was cased by the species *Cryptococcus neoformans* that consisted of two varieties based on their serological differences: *Cryptococcus neoformans* and *Cryptococcus gattii* [39-42]. The species was further characterized based on its capsular interactions with rabbit polyclonal sera, and divided into four serotypes: C. neoformans var. grubii (serotype A), C. neoformans var. gattii (serotypes B and C) and C. neoformans var. neoformans (serotype D) [43, 44].

The rising popularity of molecular approaches in the late twentieth and early twenty-first centuries allowed for further, more detailed taxonomic classification of *Cryptococcus neoformans* and *Cryptococcus gattii*. Molecular clock analysis, genotyping, and multilocus sequence typing (MLST) were used to categorize the two species into eight molecular subtypes: VNI, VNII, and VNB (C. neoformans var. grubbi); VNIII (serotype AD hybrid); VNIV (C. neoformans var. neoformans); and VGI-VGIV (*C. gattii*) [37, 45]. These studies revealed that the strain isolated by Curtis was the first clinical isolate of *Cryptococcus gattii* [46, 47], and disproved early taxonomic grouping of *C. neoformans* and *C. gattii* under a single species. It was also estimated that the different serotypes diverged from each other approximately forty million years ago [48]. Due to significant genetic and biological differences between serotypes A and D, and serotypes B and C, C. neoformans var. gattii was elevated to species level [47, 49].

Further taxonomic revisions followed, as further molecular and genetic characterization studies revealed more extensive differences within the two species complexes. In 2015, following phylogenetic analysis of 115 Cryptococcus spp. isolates, it was proposed that *C. neoformans* be divided into two species, and *C. gattii* into five species [50]. Hagen et *al*. described seven species within the *C. neoformans* and *C. gattii* complexes that were distinguishable from each other by MALDI-TOF, based on differences in their pathogenicity, biochemical and physiological features, and susceptibility to antifungals [50]. However, in 2017, Kwon-Chung et al. presented an argument for the adoption of a "species complex" system of nomenclature, in order to avoid further confusion around the taxonomy of clades within the *C. neoformans* and *C. gattii* groups [51]. The group believed that the proposal made in 2015 was premature due to predictions that the designation of species based on phylogenetics would change. They also discussed the current controversy surrounding species characterization based on lineage rather than phenotypic traits; and described the ensuing confusion that would arise in the published literature and in clinical practise [51]. In response, Hagen et *al*. expressed contrasting views to those of Kwon-Chung et *al.*, arguing that the "species complex" system would not eliminate the feared confusion, but rather delay the identification of clinically relevant differences between clades and ultimately cripple clinical approaches [52]. To-date, a consensus within the scientific community is yet to be reached.

ECOLOGY AND EPIDEMIOLOGY

Cryptococcus spp. are both pathogens and environmental yeasts that are able to adapt to changes in their internal and external environments through various survival mechanisms such as the control of signal transduction pathways and making changes to their physiology and morphology. Found in the soil, tree bark and in pigeon droppings (Fig. 2); *C. neoformans* is isolated mainly from bird guano [53], while the environmental habitat of *C. gattii* is tree bark [54]. The yeast interacts with organisms within the soil such as other fungi, bacteria, amoebae, insects and nematodes [2]; and one important ecological factor of clinical relevance is its interactions in the soil with phagocytic amoebae [55-57]. This predator-prey relationship is thought to have played a major role in the evolution of *Cryptococcus* as a human pathogen with the ability to survive within macrophages [37, 55-58]. In 2018, Watkins *et al.* showed that the social amoeba, *Dictyostelium discoideum*, interacted with *C. neoformans* in a manner similar to mammalian macrophages; in this report, the group described the phagocytosis of *C. neoformans* by the amoeba as "an apparently normal phagocytic transit" [57].

C. gattii is mainly prevalent in tropical to sub-tropical climates including South East Asia, Sub-Saharan Africa and Brazil [31]; whereas *C. neoformans* is up to eightfold more frequently isolated across the globe [59]. Analysis of the global geographical distribution of cryptococcosis revealed that C. neoformans var. grubii caused 82% of infections worldwide; while C. neoformans var. neoformans was attributed to 20%-30% of CM cases in HIV patients in northern Europe [60].

EPIDEMIOLOGY

Infection begins upon inhalation of basidiospores and encapsulated yeasT-cells from the environment (Fig. 2) [61], triggering the innate immune system. Macrophages play an integral role in anti-cryptococcal defense, with alveolar macrophages acting as first responders in the lungs where they detect and engulf cryptococcal spores [62, 63]. As intracellular pathogens, cryptococci are capable of survival and replication within host macrophages [64, 65]; the spores germinate and lead to a localized lung infection, which later disseminates to the Central Nervous System (CNS) [66], leading to the development of Cryptococcal Meningoencephalitis [67, 68]. It has been shown that non-engulfed cryptococci gain entry into the CNS by utilizing the virulence factor urease, and a secreted metalloprotease to transmigrate into the CNS [69, 70]; whereas the mechanism of transport of phagocytosed yeasts across the blood-brain barrier (BBB), is thought to occur via the "Trojan Horse Model" [66, 71]. Recent evidence in support of the latter route of transmigration has also revealed a higher expulsion rate of *C. neoformans* than *C. gattii* from human macrophage-like cell line (THP-1) across an *In vitro* model of the BBB [66]. This finding potentially describes the higher risk of developing CM due to *C. neoformans* infections rather than *C. gattii*. Thus, timing of recruitment and activation of inflammatory cells in the brain following *Cryptococcus neoformans* migration, plays a crucial role in determining the outcome of disease [72]. Hosts with intact immune systems mount an immune response that leads to clearance of the infection, or the establishment of a latent, asymptomatic infection accompanied by the formation of cryptococcomas. Patients with impaired cell-mediated immunity are unable to

effectively clear *C. neoformans.* Therefore, effective innate immune activation and a sufficient inflammatory response is key to the control of cryptococcosis.

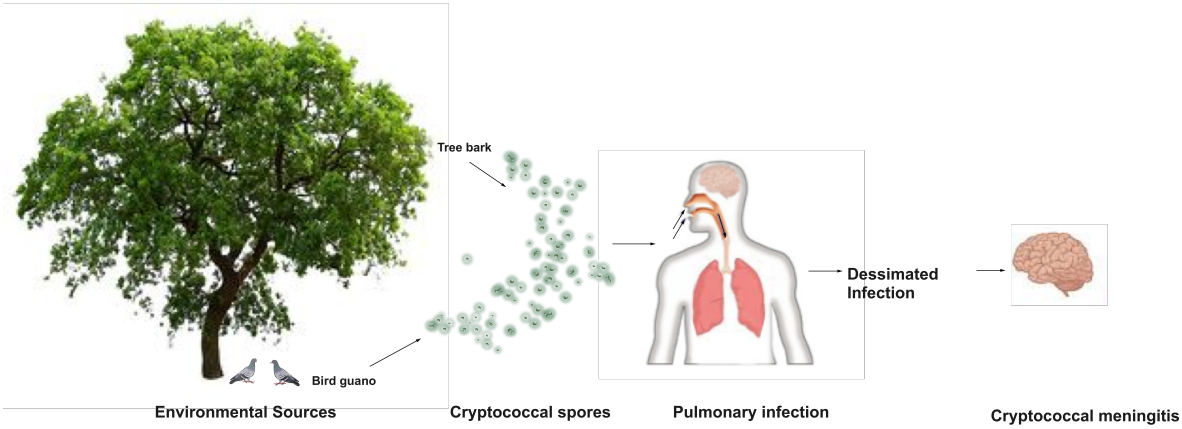


Figure 3- The epidemiology of cryptococcosis. Cryptococcal spores are inhaled from environmental sources such as tree bark and bird guano, causing a pulmonary infection. Disseminated infections in the central nervous system lead to Cryptococcal meningitis.

It is currently assumed that a primary cryptococcal infection may occur during childhood, followed by a period of dormancy; reactivation then occurs later in life during immunosuppression [73]. However, there is much debate surrounding latency and reactivation of cryptococcal infections.

LIFE CYCLE AND MATING TYPES

Cryptococcus is a bipolar heterothallic fungus that exists as two haploid mating types, MATa and MATα (Fig. 4) [37, 74]. The yeast is able to undergo sexual reproduction as well as haploid fruiting in order to produce basidiospores.

Both mating types undergo budding and propagate asexually. When they are within proximity of each other, MATα and MATa cells release the pheromones MFα and MFa respectively [37]. This exchange of pheromones induces the development of conjugation tubes in MATα cells, and isotropic growth in MATa cells. When the conjugation tubes fuse with the swollen MATa cells, heterokaryotic hyphae develop, followed by the formation of basidia at their tips. Meiotic division takes place after the fusion of the MATa and MATα nuclei to produce haploid spores [45].

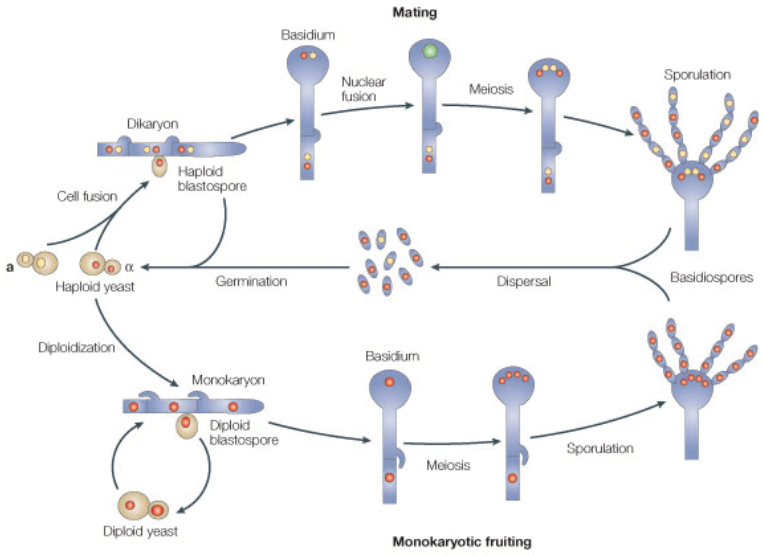


Figure 4- adapted from [38] The heterothallic life cycle of *Cryptococcus.*

The MATα mating type is more common in nature, and constitutes approximately 99.9% of all *Cryptococcus* clinical isolates; However, MATa has been found to occur at a higher frequency (up to 10% of isolates) in Southern Africa [75], which is currently considered the evolutionary home of *C. neoformans* [76]. Litvintseva et *al.*, first suggested an "out of Africa" model via which the global VNI population arose from an African VNI ancestor dispersed through pigeon migration [76]. This model was later supported by findings from [77] and [78] that showed lower genetic diversity of VNI populations in Southeast Asia and South America, respectively.

VIRULENCE FACTORS

THE CRYPTOCOCCUS CAPSULE

The *C. neoformans* capsule is the most extensively studied in comparison with other fungal capsules. Observable either by staining with India ink (Fig. 1b), scanning electron microscopy, or fluorescence; the capsule was the first distinguishable feature in identifying *Cryptococcus neoformans* isolates.

This structure plays a critical role in the establishment and progression of cryptococcosis [79], mediating host responses to infection and immune evasion and modulation through interactions with various components of the immune system [80, 81]. Capsule growth is coordinated with cell cycle progression [82, 83], and distal growth has been shown to occur in response to mammalian serum [84], triggering the formation of gianT-cells, large enough to escape

uptake by phagocytes [85]. The capsule also facilitates intracellular survival of *C. neoformans* by enabling it to evade killing by reactive oxygen species released by host immune cells [86].

The *C. neoformans* capsule is composed of polysaccharides, which make it highly hydrophilic [87]. These can be found mainly attached to the yeast's cell wall, or within the extracellular matrix, where they are actively being transported from the inside of the cells in vesicles (Fig.1a) [88]. The polysaccharides associated with capsular formation include glucuronoxylomannan (GXM), which comprises 90-95% of the entire capsule (Fig. 1a), and galactoxylomannan (GXMGal), in lower proportions of 5-8% [89, 90]. Recent evidence has shown that GXM in particular, plays a crucial role early on in the infection by acting as an anti-phagocytic cloak for *C. neoformans* [91]; and allows cryptococci to modulate host immune responses at different stages of infection, which will be discussed below. The capsule may also contain mannan, xylose, hyaluronic acid, sialic acid and glucoronic acid residues at varying ratios [37, 92, 93]. The glucoronic acid residues confer a strong negative charge on the capsule structure [94], while Glucosylceramides (GlcCer) are involved in the regulation of virulence in *C. neoformans* [95].

Capsule structure varies depending on strain, stage of disease, and molecular subtype. The existence of these polysaccharide components in different ratios within the cryptococcal capsule confers chemotype variability, a defining characteristic of each Cryptococcus serotype [96-98]. Acapsular *C. neoformans*

mutants have been shown to be less virulent than wild-type and capsule-reconstituted strains [99], however, non-encapsulated yeast cells have also been shown to successfully infiltrate immunocompromised individuals and bring about disease, suggesting the involvement of additional virulence factors in maintaining proliferation and dissemination within the host [2].

MELANIN FORMATION AND LACCASE PRODUCTION

A number of fungal species produce and deposit the pigment melanin within their cell walls as a form of protection from environmental stress. *C. neoformans* produces a particular type of melanin – eumelanin- in the presence of diphenolic and polyphenolic molecules such as L-DOPA [100]; and in response to host defenses during infection [101]. This phenotypic characteristic was also used as a diagnostic tool for identification of *Cryptococcus* species [102]. Melanin was identified as a virulence factor when melanin-lacking *C. neoformans* mutants were shown to be less virulent than their melanin producing counterparts [103]. This feature has since been reported to provide the yeast with protection from antifungal drugs [37].

Pigment production is reliant on the presence of the cell wall enzyme, laccase, which is responsible for oxidizing phenolic compounds and iron [104]. A number of additional genes, transcription factors, pathways and enzymes involved in copper transport, chitin synthesis and chromatin remodeling contribute to melanin formation [37, 105].

Aside from its role in melanin formation, laccase acts as an independent virulence factor [106], providing *C. neoformans* protection from killing by alveolar macrophages via its iron oxidase activity [107]. *C. neoformans* produces two copies of the laccase enzyme: CNLAC1 and CNLAC2; expressed by the LAC1 and LAC 2 genes, respectively. Of the two, CNLAC1 is the most abundantly expressed form of the enzyme and is solely involved in virulence and protection of *Cryptococcus neoformans* [104]. Laccase also plays a role in signaling [64]. Sabiiti et *al.* analyzed sixty-five *C. neoformans* isolates from clinical trial patients with matched clinical data were assayed *in vitro*, and found that isolates with greater laccase activity were more resistant to clearance following antifungal treatment, and more likely to survive *ex vivo* in purified CSF, highlighting a role for laccase-dependent melanin pathways in determining patient clinical outcomes [64].

OTHER VIRULENCE FACTORS

A number of other features allow *Cryptococcus neoformans* and *Cryptococcus gattii* to thrive as human pathogens, in additional to the virulence factors mentioned above. Unlike other cryptococcal species, *C. neoformans* and *C. gattii* are able to grow at 37 °C, rendering the mammalian host an ideal physiological environment for the yeasts [105]. *C. neoformans* produces degradation enzymes that support its pathogenicity.

The two best-established cryptococcal hydrolytic enzymes are urease and phospholipase B (PLB). Phospholipase B has been shown to enhance yeast adherence to lung epithelial cells during the early stages of infection [108]; increase uptake by phagocytes and survival within macrophages through the production of eicosanoids from macrophage-secreted arachidonic acid [109]; improves dissemination across the BBB[37, 71]; and improves intracellular proliferation and control titan cell morphology [110]. Cryptococcal urease also plays a role in virulence by promoting Type 2 (Th2) immune responses in the host, rather than Th1 fungicidal responses [111, 112] and facilitating fungal invasion of the CNS by increasing transmigration sites at the BBB [113, 114].

More recent molecular genetic studies have recognized genes that mediate mammalian infection [115]; Confer hypersensitive to fluconazole and oxidative stress [116]; express proteins essential for growth at high temperature [117]; and code for transcription factors that control complex developmental events, linking fungal development and virulence *[118].* A unique group of proteins involved in *Cryptococcus* virulence have also been identified in a study comparing the protein secretomes of virulent and non-virulent strains [119].

MACROPHAGE INTERACTIONS WITH CRYPTOCOCCUS SPP.

A number of human fungal pathogens such as *Candida albicans, Aspergillus fumigatus* and *Cryptococcus neoformans* have evolved various strategies of avoiding death including evasion of engulfment by phagocytes and intracellular parasitism [120]. Pathogenicity of *C. neoformans* toward the human host is thought to have evolved from the yeast's ability to protect itself from killing by its' ancestral predators and environmental phagocytes – amoebae such as *D. discoideum* [57, 58]. Recent findings by Watkin's *et al.* show that like macrophages, the amoeba was unable to kill *C. neoformans*; and that other processes such as vomocytosis (described below) of the yeast were conserved between hosts and amoebae [57]. This emphasizes the role of macrophages as a crucial factor in understanding the facultative intracellular abilities of *Cryptococcus neoformans* [121-124].

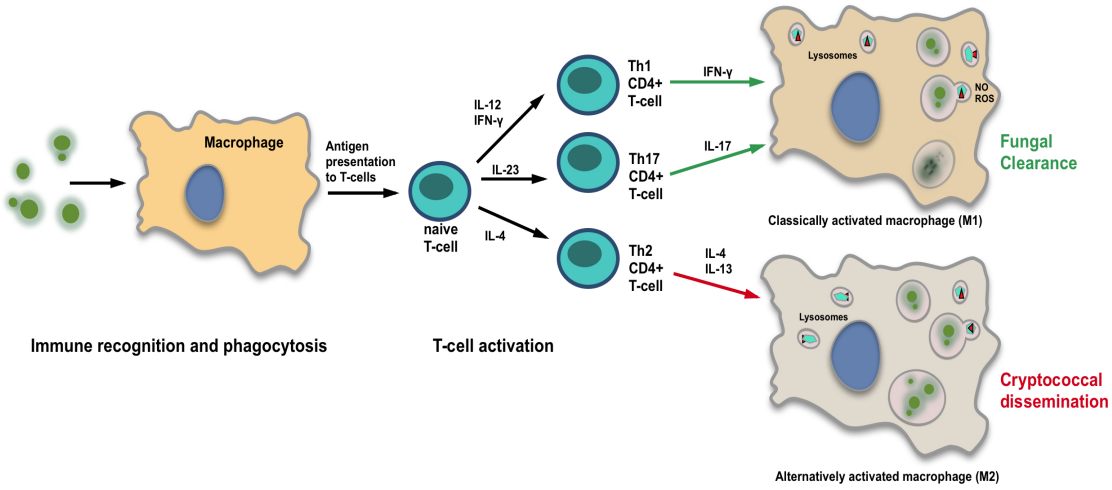


Figure 5. Immune responses leading to either fungal clearance, of disseminated cryptococcal disease. Macrophages recognize and engulf Cryptococcus spp. and present cryptococcal antigen to T-cells. This leads to the activation of naïve T-cells that develop into Th1, Th17 or Th2 CD4⁺ T-cells. Th1 and Th17 T-cells release IFN-γ and IL-17, respectively, to generate classically activated (M1) macrophages that aid in fungal clearance. Th2 T-cells release IL-4 and IL-13 to generate alternatively activated macrophages (M2) that enable cryptococcal dissemination.

Macrophage activation states directly influence the control of cryptococcosis. Classically activated macrophages are triggered by Th1-type CD4⁺ T-cells [125-127] that produce interferon-γ (IFN-γ), and direct macrophages to release proinflammatory cytokines and chemokines when activated by lipopolysaccharide (LPS) and IFN-γ [127-129]; concluding in the production of nitric oxide (NO), a crucial factor in macrophage elimination of intracellular pathogens [9]. M1 responses have been associated with increased fungal clearance (Fig. 5) and with enhanced signaling of the activation of signal transducer and activators of transcription1 (STAT1) of the Janus kinase (JAK)/

(STAT) pathway [130, 131], that mediates the release of pro-inflammatory cytokines, reactive oxygen (ROS) and nitrogen species such as NO [132]. Cytokines released by M1 macrophages include (TNF-α, IL-1α, IL-1β, and IL-6); Th1 cytokines (IFN-γ and IL-12); and Th17 cytokines (IL-17A and IL-22) [133].

Conversely, M2 macrophages are triggered by Th2-type $CD4^+$ T-cells [7, 12]. They produce anti-inflammatory, Th2 cytokines (IL-4 and IL-13) and have widely been associated with mediating immune responses to helminth infections, as well as tissue repair [12]. Alternatively activated macrophages are characterized by expressing cell surface markers like Fizz1 (Found in Inflammatory Zone 1), chitinase-like protein (Ym1), CD36 (fatty acid translocase), and CD206 [7]. In contrast to classically activated macrophages, M2 cells produce of low levels of IL-12 and iNOS, and culminate in the induction of arginase 1 (Arg1) [7, 12].

M2 macrophage responses have been associated with reduced cryptococcal clearance and increased intracellular yeast proliferation (Figure 4) [132, 134, 135]. There is evidence that suggests *C. neoformans* prompts the release of anti-inflammatory cytokines that aid survival within the host cell [134]. In a mouse model, pulmonary infection revealed that higher fungal burden and dissemination was associated with Th2 type responses and an M2 cytokine profile (including IL-4, IL-5, IL-10, and IL-13) that rendered hosts vulnerable to severe disease and mortality [134]. It has also been suggested that defects in the STAT1 signal transduction pathway may promote M2 macrophage activation and decreased killing of cryptococci. Infection of STAT1-/- mice with an IFN-γ

producing strain of *C. neoformans* showed decreased expression of M1 activation markers, along with an enlarged M2 macrophage population that correlated with decreased nitric oxide (NO) production and high mortality rates [131].

However, the broad categorization of macrophages into two distinct activation states is not representative of the plasticity in gene expression that regulates macrophage activation markers *in vivo* [9]. A number of studies have identified macrophage subpopulations that express both M1 and M2 markers of activation in different proportions; which influenced macrophage elimination of intracellular pathogens. Arora *et al.* analyzed cytokine profiles and compared macrophage activation status in *C. neoformans*-infected wild-type, IL-4 knockout (IL-4 -/-), and IFN-γ knockout C57BL/6 mice [135]. The group showed that cytokine expression in infected WT mice interchanged between a Th1 and Th2 bias based on the IFN-γ:IL-4 ratio that mediates macrophage polarization states. The group also identified macrophages that displayed both M1 and M2 markers of activation, as well as expressing iNOS and Arg-1. In addition, they showed that concurrent stimulation with both IL-4 and IFN-γ lead to the upregulation of both M1 and M2 cytokines in the same cells [135]. These results highlight the interplay between macrophage activation states, and how this plasticity may impact the progression and outcome of *C. neoformans* infections.

Among the PRRs, Toll-like receptors (TLRs) are the best characterized and are responsible for the regulation of signaling pathways including nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs)[136, 137]. The CD14 receptor in association with TLR4, and TLR2 [138] recognize cryptococcal GXM,

driving localized immune recognition and enhanced phagocytosis [62, 139-141]. Yauch et al. showed that MyD88-deficient mice showed significantly reduced survival rates in comparison to wild-type C57BL/6 mice following intranasal and intravenous infection with *C. neoformans*; while CD14-deficient mice only showed reduced survival following intravenous infection; and TLR2-deficient mice were susceptible to intranasal infection [141]. However, they observed similar mortality in TLR4-deficient C3H/HeJ mice and control C3H/HeOuJ mice; excluding the involvement of TLR4 in cryptococcal clearance. Further analysis of CD14−/−, TLR2−/−, and MyD88−/− mice showed no major differences in Th2 cytokine production in comparison to wild type mice. They also showed that MyD88-deficient mice exhibited increased numbers of lung CFU and serum and lung GXM levels in comparison with other knock out mice; which correlated with decreased survival of these mice [141]. Shoham et al. transfected Chinese hamster ovary fibroblasts with human TLR2, TLR4, and/or CD14 and showed that TLR4 and CD14 interacted with fluorescently labeled cryptococcal GXM [138]. They also constructed an NFκB-dependent reporter to evaluate nuclear translocation of NFκB via this interaction. The group showed that GXM activated Chinese hamster ovary fibroblasT-cells, PBMC and RAW 264.7 cells that had been transfected with TLR4 and CD14, and induced nuclear translocation of NFκB. However, this interaction was unable to activate MAPK pathways, nor induce TNF-α production [138].

The nuclear factor-κB (NFκB) is a family of transcription factors, capable of modulating the expression of cytokines. Members of this family include RelA (p65), NFκB 1 (p50), NFκB2 (p52), c-Rel and RelB [142]. The nuclear factor-κB

(NFκB)-dependent pathway is activated following triggering of TLRs, and results in the expression of inflammatory Th1 cytokines (such as TNF-α, IL-1β, and IFN-γ; Fig. 6) and the induction of iNOS expression in attempts to kill the pathogen [143, 144] [145, 146] . Upon activation of TLR4, the cytoplasmic adapter protein (MyD88) recruits the serine–threonine kinases IRAK1 and IRAK4, in association with tumor necrosis factor receptor-associated factor (TRAF6). Activation and phosphorylation of TRAF6 results in the activation of NFκB-inducing kinase and the subsequent activation of inhibitory protein kappa B (IκB) kinase. IκB is a cytoplasmic protein that constitutively binds to the NFκB, which is composed of two subunits, p50 and p65 [12]. Activation of IκB dissociates it from NFκB, allowing for the nuclear translocation of NFκB and initiating the expression of proinflammatory cytokines [147]. The p50 and p65 NFκB subunits have been shown to play different roles in the induction of anti-inflammatory and pro-inflammatory cytokines, respectively [142]. The p50 subunit has been shown to induce the expression of IL-10; mice lacking the p50 subunit were more susceptible to lethal endotoxemia, and macrophages derived from these mice produced reduced levels of IL-10, and increased production of TNF-α and IL-12 following exposure to LPS [142]. Conversely, the p65 subunit of NFκB has been associated with the expression of pro-inflammatory cytokines like TNF-α [148, 149]; and iNOS [150]. However, optimal production of TNF-α requires a cohort of pathways including mitogen-activated protein kinase (MAPK) cascades, the extracellular signal-regulated kinase (ERK), p38 MAPK, and stress-activated protein kinase (SAPK)/c-JUN N-terminal kinase (JNK)[151] [138].

Additionally, the NFκB pathway can be modulated by both shed GXM and live cryptococci via different mechanisms depending on the stage of infection [152]. Early on in pulmonary infection, GXM acts as an anti-phagocytic cloak for *C. neoformans* [91]. Following ingestion by macrophages, cryptococci continue to produce and shed the polysaccharide [91], which has been shown to reduce LPS-associated proinflammatory cytokine responses both *in vitro* [153] and in a murine infection model by inhibiting MyD88 activation following its interaction with the FcγRIIB receptor (Fig. 6) [153, 154]. Furthermore, Hayes et *al.* recently showed in RAW 264.7 murine macrophages that extracellular GXM inhibited LPS-induced nuclear translocation of the p65 protein, while intraphagosomal GXM increased the duration of nuclear translocation of the p65 and IκBα proteins, consequentially eliminating the expression of TNFα or inducible nitric oxide synthase (iNOS) [152].

Thus, the interplay between Th1 and Th2 host responses during cryptococcal infection is a major determinant of disease outcome [135, 155]. Host susceptibility studies in mice have shown that production of the Th1-type cytokines IFN-γ and IL-12, as well as the pro-inflammatory cytokine TNF-α confers protection against cryptococcosis [156-160]. GXM has also been shown to directly inhibit T-cell proliferation in mice, leading to dampened Th1 responses and diminished resolution of the infection [161].

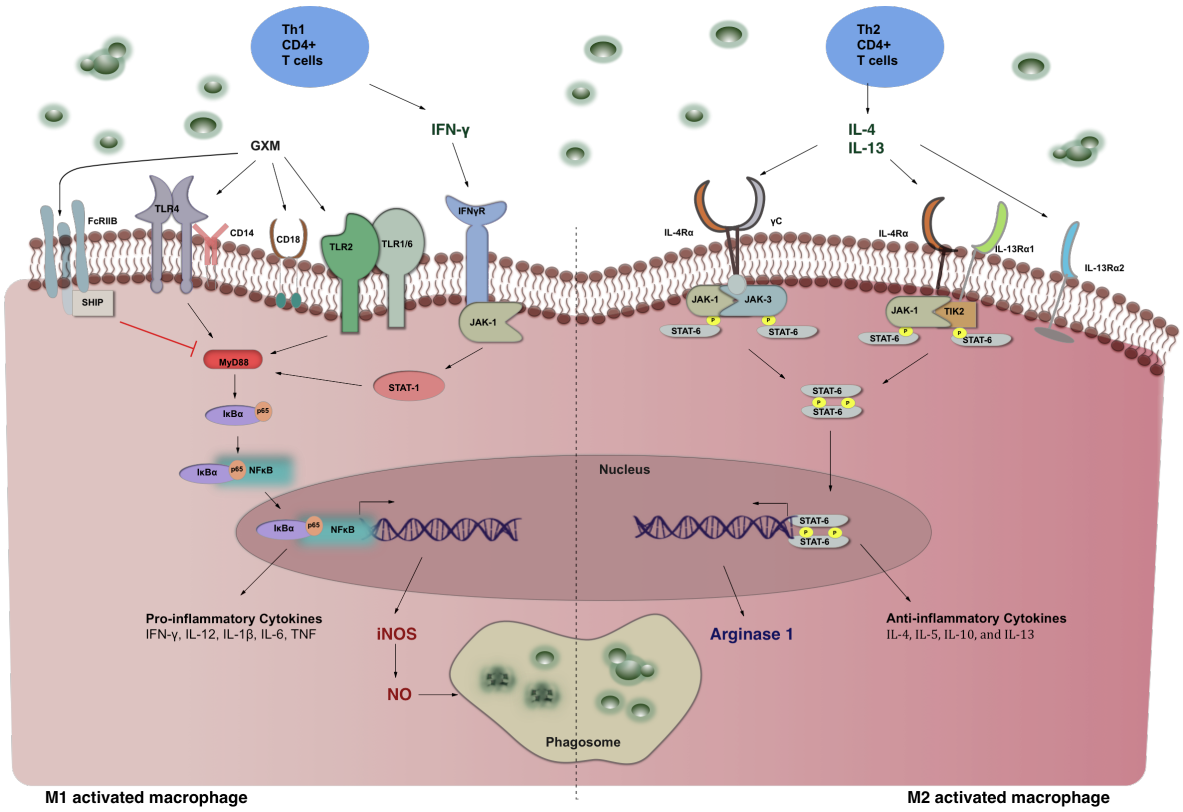


Figure 6. illustration of pathways leading to either M1 activated macrophages (left- lighly shaded), or M2 activated macrophages (right- dark shade) during pulmonary cryptococcal infection. M1 activated macrophages are marked by the production of pro-inflammatory cytokines and nitric oxide (NO) leading to pathogen destruction; while M2 activated macrophages are marked by the release of anti-inflammatory pathogens and Arginase 1, allowing cryptococcal cells to thrive and disseminate.

Fig. 6 (above) shows the classical and alternative activation pathways that lead to M1 and M2 macrophages, respectively, during pulmonary cryptococcal infection. Based on early interaction of the fungus with alveolar macrophages, cryptococci will also encounter antigen-specific antibodies, and T-cells in lung tissue; additionally, complement- and antibody-mediated phagocytosis will occur primarily in the blood.

However, it is important to note the difference in iNOS expression between human and mouse-derived macrophages both *in vivo* and *in vitro* [162-164], and

controversy has emerged following disparities in data disproving or supporting the ability of human macrophages to express iNOS. Murray and Teitelbaum showed that L-arginine-dependent generation of reactive nitrogen intermediates (RNI) was species-restricted to mice, and absent in human macrophages[164]. Schneemann *et al*. also showed that human macrophages failed to produce NO despite activation with endotoxin, IFN-γ, TNFα, G-CSF, bacteria nor with proliferating lymphocytes [165], suggesting a lack of NO activity in human macrophages. Furthermore, a review by Murray discussed species-specificity issues with iNOS, arginases, and NO production [9]. *Mycobacterium tuberculosis*, an intracellular pathogen of macrophages, has also been shown to induce the expression of iNOS in human macrophages [166]. Disparities arise between mouse and human macrophages in response to particular intracellular pathogens such as *M. tuberculosis.* Despite the establishment of iNOS induction and NO production in murine models of disease, *M. tuberculosis* is not pathogenic towards mice, suggesting that humans evolved pathways distinct from rodents as a selective pressure. This review emphasized the importance of understanding the differences and similarities in inflammatory responses between species, as this would affect the development of therapeutic compounds based on mouse models [9]. In support of the author's recommendations and in the context of cryptococcal disease; it would be paramount to establish variation in gene expression profiles between mouse and human macrophages both *in vitro* and *in vivo* in order to draw accurate and reliable conclusions.

VOMOCYTOSIS

Aside from its ability to escape killing by host macrophages [167], *Cryptococcus neoformans* is able to undergo 'lateral transfer' between phagocytes [168], and non-lytic escape (vomocytosis) [121], whilst maintaining its ability to propagate and disseminate further within the macrophages [169, 170]. This process has also been observed in other pathogenic fungi such as *Candida albicans* [171] and *Candida kruzei* [172], and from mammalian phagocytes [173], avian macrophages [174], fish [175] and amoebae [57], suggesting that this extrusion phenomenon is evolutionarily conserved.

Vomocytosis of *C. neoformans* has been confirmed *in vivo* within mice, and may possibly occur more frequently *in vivo* than *in vitro* [173]. Furthermore, comparison of vomocytosis in a variety of cell lines has shown that vomocytosis of cryptococci from primary human macrophages occurs more frequently than from phagocytic cell lines [170]. The current model for this non-lytic extrusion event involves the fusion of the phagosome and plasma membranes, sometimes associated with an actin flash that has been shown to prevent cryptococcal expulsion within an 'actin cage' that surrounds the phagolysosome [170, 176]. Host cell requires microtubule activity and the WASP (Wiskott–Aldrich syndrome protein)–Arp2/3 (actin-related protein 2/3) pathway are involved in the formation of actin flashes, a process which can last anywhere from seconds to hours in efforts by the host cell to prevent cryptococcal expulsion [176].

Interestingly, a number of studies have provided evidence that despite the formation of actin cages, host actin cytoskeleton is not required for vomocytosis [169, 170, 176]. For example, Watkins et al., recently showed that vomocytosis of *C. neoformans* from the phagocytic amoeba *D. discoideum* is an actin-independent process [57].

Whilst comprehension of the molecular mechanisms that govern the extrusion of *C. neoformans* from macrophages remains to be gained, a number of studies have made considerable progress in resolving this process.

The complement pathway plays a major role in initiating chemotaxis in phagocytic cells and boosting the engulfing abilities of macrophages [177]; however, monoclonal antibodies can affect complement deposition on the cryptococcal capsule by both classical pathway activation and steric hindrance [178]. Furthermore, complement-opsonized cryptococci have been shown to exit the macrophage individually, while antibody-opsonized yeasT-cells are expelled as a cluster [167, 179]. The mode of opsonization (either via complement or antibody) has been shown to affect the rate of vomocytosis [180]. Alvarez et al. showed that whilst phagocytosis of both complement- and antibody-opsonized *C. neoformans* and *C. gattii* was followed by intracellular replication; antibody-opsonised yeasT-cells were vomocytosed in 'biofilm-like microcolonies', whereas complement-opsonised *C. neoformans* dispersed as individual cells following vomocytosis [180]. The group found that antibody-mediated phagocytosis resulted in reduced dispersal of *C. neoformans* cells, suggesting a more complex role for antibodies in anticryptococcal defence. In addition, vomocytosed yeasT-

cells contained the capsule-bound antibody that was initially used to opsonize cryptococci, which showed that the mAb can remain bound following phagocytosis and phagosome maturation, and cause intracellular clustering of cryptococci. The authors speculated that antibody-mediated vomocytosis may also occur *in vivo* [180]. In cases of pulmonary infection, this event could reduce local dissemination and promote inflammatory responses against *C. neoformans* clusters. It is also likely that extracellular aggregated antibody-bound yeasT-cells may be phagocytosed by other cells following extrusion, or more readily accessible to antifungal drugs, enabling more effective clearance.

In addition, treating J774 and human primary monocyte-derived macrophages with either Th1 or Th2 cytokines has been shown to alter the rate of vomocytosis [132]. Voelz et al., showed that treatment of macrophages with Th1 cytokines IFN-γ and TNFα, and the Th17 cytokine IL-17, did not significantly affect the vomocytosis of *C. neoformans* in comparison to untreated cell [132]. In contrast treatment with Th2 cytokines (IL-4 and IL-13) considerably reduced the expulsion of the yeast from macrophages [132].

Investigations by Chayakulkeeree et al. provided evidence that the cryptococcal virulence factor phospholipase B1 (Plb1), and the protein required for Plb1 secretion, Sec14-1p, played an essential role in cryptococcal dissemination to the CNS and vomocytosis from murine macrophages [181]. *C. neoformans* mutants lacking either Plb1 or Sec14 showed reduced virulence, dissemination to the CNS and vomocytosis in mouse models [181]. In 2016, Stukes et al. suggested that the membrane phospholipid binding protein, annexin A2 (ANXA2) played a role

in the phagocytosis and vomocytosis of *C. neoformans*; where macrophages derived from ANXA2-deficient mice exhibited lower engulfment and expulsion of the yeast than wild type macrophages [182].

More recently, the vomocytosis of *C. neoformans* was shown be under the inhibition of a MAP kinase, extracellular receptor kinase 5 (ERK5) [120]. ERK5 belongs to the group of extracellular signal-regulated kinases (ERKs), also known as mitogen-activated protein kinases (MAPKs), are involved in cellular proliferation, differentiation, migration, and gene expression. ERK5 is activated through MEKK2/3 and MEK5 along the MAPK cascade. In macrophages, activation of the MAPK cascade occurs following induction with either bacterial LPS, or the proinflammatory cytokine TNFα [183]. Gilbert et *al.* exposed primary human macrophages and J774 cells to the ERK5 inhibitor XMD17-109, and noted significantly increased vomocytosis in both cell lines. They also showed that pharmacological inhibition or genetic manipulation significantly increased vomocytosis in primary human macrophages; and activation of the ERK5 signalling pathway resulted in significant reductions in vomocytosis [184]. These findings were also confirmed *in vivo*, were reducing ERK5 activity in a zebrafish model encouraged vomocytosis, and reduced dissemination of infection was observed [184].

Whilst the relevance of vomocytosis in disease progression is remains unclear; it is likely that vomocytosis of cryptococci from circulating monocytes would be left vulnerable to immune attack from other components of both innate and adaptive immunity. In cases of CNS involvement, reduced vomocytosis may

hinder cryptococcal dissemination into the brain via the Trojan Horse model. Hence, it is imperative that the molecular mechanisms that orchestrate the expulsion of this yeast from macrophages are characterized both *in vitro* and *in vivo*.

VARIATION IN HOST RESPONSES TO CRYPTOCOCCOSIS

EXPERIMENTAL MODELS OF CRYPTOCOCCOSIS

A number of *in vitro* and *in vivo* experimental models have been designed to aid the study of cryptococcosis. These models take into account the cutaneous, pulmonary, and meningococcal forms of disease presentation. [185]

In vitro cellular models for macrophages include the J774 and RAW cell lines and primary cells (including bone-marrow-derived murine cells and peripheral blood monocyte-derived human cells) [185]. However, interactions of cell lines with Cryptococcus spp. have been shown to differ from each other. Primary human macrophages show significantly increased vomocytosis in comparison to J774 cells [170].

Several *In vivo* models are also employed in cryptococcal research. Invertebrate models include *Caenorhabditis elegans*, amoeboid models, and insect models such as *Galleria mellonella* and *Drosophila melanogaster*; while vertebrate models include mice (*Mus musculus*), rats (*Rattus rattus*), guinea pigs (*Cavia porcellus*), rabbits (*Oryctolagus cuniculus*) and zebrafish (*Danio rerio*) [185]. The majority of investigations are carried out in mice, due to the establishment of this model in medical research and the availability of a variety of genetic backgrounds. Interestingly, different mouse strains show varying susceptibilities to cryptococcal disease. For example CBA/J mice were shown to be more susceptible to *C. neoformans* infections than BALB/c mice [186]. In contrast, rats

represent ideal candidates for the study of *C. gattii* infections due to their natural susceptibility to pulmonary cryptococcosis [187]. However, they are unable to clear *C. neoformans*, which causes more progressive and fatal disease in this model [188]. More recently, zebrafish have gained traction as a model system due to parallels with mammalian pathogenesis of cryptococcosis [175, 189], with the particular advantage of studying the establishment and maintenance of fungemia at a low cost, and large scale using mutagenesis, screening and live cell imaging methods [185, 190].

As discussed previously, species-specific attributes of macrophages would need to be taken into consideration when describing responses to *C. neoformans* infections *in vitro*, in addition to the variation in responses by different macrophage lineages and tissue-specificities. Additionaly, variation in the pathogenesis of cryptococcal disease in mice has been associated with differences in their innate and adaptive immune responses; hence, further characterization of intra-species differences of *in vivo* models of cryptococcosis is also warranted.

VARIATION IN HUMAN HOST RESPONSES TO CRYPTOCOCCOSIS

Whilst early studies of the manifestations of cryptococcosis have shown clinical and prognostic differences between the infecting species [191], more recent investigations into the pathogenesis of *C. neoformans* in particular have revealed a critical role for host immune status in conferring protection from, or controlling disease progression towards, meningitis [192]. This has lead to the division of *C. neoformans* patients into three groups: HIV-associated; Non-HIV immunocompromised patients; and 'otherwise healthy' immunocompetent individuals.

Immune responses in non-immunocompromised individuals

During the cryptococcosis outbreak in Vancouver 2003 that affected primarily non-immunocompromised individuals, *C. gattii* was identified as a primary pathogen of the healthy. However, increased recognition of cryptococcosis cases due to *C. neoformans* in other immunocompetent patients provided strong evidence that this species also harbors capabilities as a primary pathogen, despite early classification as a strictly opportunistic infection [193-195]. Despite the global distribution of this species, the highest frequency of CM in immunocompetent individuals appears to come from South East Asia, with mortality rates in China, Taiwan and Japan estimated to be at 17%, 70% and 35%, respectively [196-199]. Liu et al. conducted a retrospective analysis of 46 cases of cryptococcosis in adults, reported in Hong Kong over a 10-year period

from 1995–2005 [200]. The group identified that 43.5% of these patients were apparently immunocompetent, whilst 37.0% had non-HIV associated susceptibilities to cryptococcosis, and only 19.6% had HIV-associated cryptococcosis. Interestingly they found that 62.5% of cryptococcosis cases in immunocompetent patients were due to Cryptococcus neoformans var. grubii [200].

In 'otherwise healthy' individuals, pulmonary cryptococcal infection is generally asymptomatic. A retrospective study by Nadrous et *al.* of cryptococcosis in 42 immunocompetent hosts showed that 86% of patients had isolated pulmonary cryptococcosis with no evidence of fungal dissemination to the CNS, of which 33% were asymptomatic, and 92% were able to resolve the infection either with or without antifungal treatment or surgical abscission [201]. Whilst no quantitation of innate immune responses to infection at the individual level were performed, these findings show that the majority of cryptococcosis patients with previously intact immune systems and no evidence of disseminated disease are able to effectively eliminate cryptococcal infection. However, impaired macrophage differentiation was suggested to affect disease outcome in this patient group [202]. Macrophage differentiation is regulated by granulocyte-macrophage colony-stimulating factor (GM-CSF). Rosen et *al.* noted the presence of anti–GM-CSF autoantibodies in the spinal cerebral spinal fluid (CSF) of patients with no known immume defects prior to cryptococcosis, and associated the inhibition of the activity of GM-CSF with poor disease prognosis in cases of CM due to *C. neoformans* in otherwise healthy hosts [202].

Further attempts to characterize predispositions to CM in immunocompetent individuals have identified genetic background as a risk factor for developing severe cryptococcosis. Polymorphisms in the Fc gamma receptor (FcγR) [203, 204] and mannose-binding lectin (MBL) [205] have been shown to increase susceptibility to cryptococcal infections.

Immune responses in HIV patients

Cryptococcal meningitis is the second largest cause of mortality among HIV patients worldwide. Disease prevalence became more apparent among immunocompromised patients particularly during the HIV pandemic in the 1970s; where 80% of CM sufferers were HIV-seropositive. However, as of 2014, the frequency of cryptococcal disease among this group has dropped to a third [206, 207]; it was estimated that 223,100 cases of CM in HIV patients occur each year worldwide, causing 181,100 deaths annually [17]. This is considered to be due to widespread availability of antiretroviral (ART) drugs; analysis of cryptococcosis epidemiology in 2 parts of the United States showed a significant reduction in HIV-associated cryptococcal disease between 1992 and 2000 [207]. However, was noted that HIV patients in the USA who developed cryptococcosis had limited access to ARTs [207], as were HIV patients from the developing world where HIV prevalence remains high [208]. Even despite the provision of ARTs in Sub-Saharan Africa, mortality due to HIV-associated CM remains high [208, 209].

T-cell depletion in HIV patients is a determining immunological feature that renders this group susceptible to CM; where low T-cell infiltration of the CSF rather than alternative macrophage activation has been shown to be a major determinant of disease outcome in HIV-associated cryptococcosis [210].

However, in a recent study, Neal et *al*. demonstrated in a murine model that whilst $CD4^+$ T-cells assist in the clearance of the fungal disease, they also contribute to disease dissemination in the CNS as well as tissue damage as a result of immunotherapy, leading to Immune Reconstitution Inflammatory Syndrome (IRIS) [211]. IRIS develops as the balance between pro-inflammatory and anti-inflammatory responses is altered, and has been observed in HIV-infected patients following the initiation of ART, and in non-HIV, immunocompromised patients following the secession of immunosuppressive drugs [212]. Based on the model by Neal et al., depletion of $CD4^+$ T-cell in mice considerably impaired the production of IFN-γ, the accumulation of $CD8^+$ T-cells and myeloid cells, and cryptococcal clearance from the CNS, improved the clinical outcomes of disease and survival rates [211]. Hence, hyperpro-inflammatory responses in this patient group may lead to CM-associated fatalies.

Other reports have identified a number of protective immune traits and markers of susceptibility to acquiring CM in HIV-seropositive patients. Cerebral spinal fluid (CSF) cytokine profiles can reliably report disease progression and predict mortality in HIV patients. Data from several patient cohorts agree that inflammatory and Th1 cytokine responses confer protective advantages in HIV

patients [210, 213, 214]. HIV-seropositive hosts with high CSF levels of interleukin (IL)-6, interferon-γ (IFN-γ), IL-8, IL-12, TNF-α and CXCL10 show improved fungal clearance and survival rates; while increased levels of IL-4 and IL-10 have been associated with high serum levels of cryptococcal GXM that impairs monocyte activation and cripples cell-mediated responses to infection - effectively predicting mortality in this group of patients [214-216].

Interestingly, not all HIV patients develop cryptococcosis. This is thought to be largely due to the contribution of genetic factors to disease susceptibility. Following the association of polymorphisms in the FcγR with susceptibility to cryptococcosis in non-HIV patients [203-205], Rohatgi et *al.* showed that HIV patients who were either heterozygous or homozygous for the Fc gamma receptor 3A polymorphism (FCGR3A) 158V allele, were 2.1- and 21-fold at higher risk of developing cryptococcal disease than individuals without the allele, respectively [217]. IgG mediates phagocytosis via the Fc-gamma receptor in macrophages, where the FCGR subunit, FCGR3A, is crucial for signal transduction and initiation of macrophage effector functions towards a pathogen. A functional consequence of the 158V allele to the FCGR3A is increased affinity to IgG; resulting in increased phagocytosis of *C. neoformans* by host macrophages. However, increased uptake may result in impaired destruction of *C. neoformans* by macrophages, and increased dissemination to the CNS via the Trojan horse model resulting in cryptococcal disease as observed by Rohatgi et al. The group suggested that increased recognition of *C. neoformans* and immune activation leading to a rise in phagocytic uptake as the functional

implication of this allele. These findings provide an alternative approach for identification of at-risk individuals, and more personalized treatment strategies.

Immune responses in non-HIV, immunocompromised patients

This group of patients is highly heterogenous, encompassing a range of patients from solid organ transplant (SOT) recipients to those with underlying immune defects such as sarcoidosis, diabetes mellitus, and patients receiving anti-cancer treatments [218]. As cryptococcal infections in this group represents a small fraction of the overall disease burden and associated mortality, the majority of epidemiological data on susceptibility to cryptococcosis in non-HIV, immunocompromised patients is derived from clinical reports and retrospective studies.

Susceptibility to developing CM in this patient group arises due to weakened innate and adaptive immune responses. Cases of cryptococcal disease in sarcoidosis patients are rare, but are due to impaired T-cell-mediated immunity. Delayed identification of cryptococcal meningitis occurs in 43% of patients, as CNS involvement in sarcoidosis is often misdiagnosed as neurosarcoidosis [219]. A meta-analysis of 40 sarcoidosis patients with cryptococcal meningitis found that early and late diagnosis of CM in this patient group resulted in unfavourable outcomes in 21%, and 41% of patients, respectively; and CM-associated mortality was 19% [219].

Cryptococcosis has also been shown to present as a lethal complication in patients with type-2 diabetes mellitus (DMII). These patients are considered susceptible to cryptococcosis as a result of persistent hyperglycemia that impairs innate and adaptive immune responses, providing a suitable environment for infectious pathogens to thrive in [220]. A retrospective study of 30 cryptococcosis cases in DMII Chinese patients found that 69% of patients received delayed diagnosis, while 60% received insufficient antifungal treatment, and fatalities due to CM in DMII patients were 33% [220].

Cryptococcal disease has long been identified in cancer patients, with the first cases being reported early in the 20th century [221]. Increased risk of developing CM in cancer patients is considered to arise following the induction of lymphocyte-depleting chemotherapeutic regimens and prolonged corticosteroid treatments that impair T-cell mediated immunity [221]. Spec et al. conducted a retrospective study to address the impact of infectious disease consultation on non-HIV, cryptococcosis patients [222]. The group analysed data from a cohort of 147 patients with cryptococcosis over 13 years, and reported that induction of chemotherapy in cancer patients was associated with a ~2.1 fold increase in risk of mortality due to cryptococcal infection. Furthermore, they showed that 68% of patients who had consulted with an infectious disease physician were more likely to receive early diagnosis of cryptococcal disease, received prolonged antifungal treatment regimens, and were at a lower risk of mortality [222].

Similar to HIV-sufferers, organ transplant recipients also present with defects in T-cell-mediated immune responses to cryptococcosis due to immunosuppression [193]. Approximately 2.8% of organ transplant recipients acquire cryptococcal infections, with an overall death rate of 42% [223]. In SOT patients, the severity of infection has been associated with the level of immunosuppression [223].

Studies have shown that the type of immunosuppressive treatment(s) administered to this group influences their susceptibility to CNS-associated cryptococcosis, responses to antifungal treatments, and likely fatality [193, 223]. Analysis of 127 transplant recipients who were treated with tacrolimus, cyclosporine, or a combination of cyclosporine and azathioprine revealed that SOT patients who were treated with tacrolimus as a primary immunosuppressive agent were significantly less likely to have CNS involvement than those who received non-tacrolimus treatments (78% versus 11%, p =0.001) [223].

Tacrolimus and cyclosporine act as selective inhibitors of the calcineurin pathway, impairing T-cell mediated immunity and proinflammatory cytokine production, while azathioprine inhibits purine and nucleic acid synthesis affecting cell metabolism. Interestingly, both Tacrolimus and cyclosporine are also toxic to *C. neoformans* via inhibition of fungal homologues of calcineurin *in vitro* may invoke antifungal effects *in vivo* [223]. Furthermore, administration of azathioprine as a sole immunosuppressant, delayed the onset of cryptococcosis in transplant recipients, and was associated with CNS involvement [223].

Another study by Saha et *al.* provided serological evidence that the majority of cryptococcal infections in these patients were due to reactivation of pre-transplantation infections [224]. Individuals with circulating antibodies against Cryptococcus prior to organ transplantation acquired cryptococcosis sooner than those without previous exposure to the pathogen, and were more likely to develop CM. Disease progression in this subset was also dependent on the type of organ transplanted, although transmission of infection from organ donor to recipient has rarely been observed [218].

DIAGNOSIS, TREATMENT AND VACCINE DEVELOPMENT

DIAGNOSIS

Cases of cryptococcosis are typically confirmed by the detection of cryptococcal antigen (CrAg) by either lateral flow immunoassay (LFA), or latex-agglutination test. A positive result in immunocompromised patients may be predictive of disease progression to Cryptococcal Meningitis, which is diagnosed via lumbar puncture (LP), examination of the Cerebral Spinal Fluid (CSF) opening pressure; staining with India ink; or CrAg screening [60].

TREATMENT

Due to its virulence mechanisms and cryptococcal resistance to various antifungal drugs [225], there is a limited range of effective antifungal therapies available for the treatment of *Cryptococcus neoformans* infections. Furthermore, the varieties of antifungal therapies are off-patent drugs, based on different classes of molecules that target distinct fungal attributes.

The current gold-standard treatment regimen for cryptococcosis patients includes a combination of Amphotericin B (AmB) and 5-flucytosine (5FC); however, due to the unavailability of AmB in resource-limited settings such as Asia and Africa [226], fluconazole (FLU) is used either as a sole treatment, or in combination with 5FU [227, 228]. Although the provision of this combination of treatments has greatly improved the 3-month prognosis of HIV patients with cryptococcal meningitis in developed nations [229], the mortality rates in developing countries remain high [228, 230].

Each drug presents with different limitations in the treatment of cryptococcal meningitis. Amphotericin B is a polyene macrolide antifungal that binds to ergosterol in the fungal cell membrane, resulting in increased permeability and cell death [231, 232]. Fluconazole also inhibits ergosterol synthesis and acts as a fungistatic agent against *C. neoformans* [233]. However, unlike AmB and 5FU, fluconazole is a cost-effective, widely available antifungal drug, that benefits from low toxicity and is better tolerated amongst HIV-associated CM patients

[233-235]. AmB has also been shown to act as an immunomodulator, influencing cytokine secretion [236], and promoting the release of nitric oxide by macrophages to eliminate engulfed cryptococci [237, 238]. Whilst AmB is currently regarded the most effective anticryptococcal drug, its use is limited by it's dose-related toxicity, as it is also able to bind cholesterol in mammalian cell membranes, leading to the development of nephrotoxicity (kidney toxicity), electrolyte imbalance, and anemia [231, 232]. Hence, monitoring of serum levels of potassium and creatinine, and electrolyte supplementation is key in limiting the effects of AmB toxicity in cryptococcosis patients.

Flucytosine acts indirectly as an antifungal following the conversion of flucytosine into 5-fluorouracil (5FU) within the cytosol of vulnerable fungal cells [226]. Once taken up by the fungal cell through the action of cytosine permease, flucytosine is converted into 5FU by cytosine deaminase (not present in humans). 5FU then excerts it's antifungal role by inhibiting DNA synthesis, and disrupting RNA translation [226]. While this drug has shown to be effective as a monotherapy against cryptococcosis with limited toxicity at low doses, cases of antimicrobial resistance have arisen [239]. Symptoms of toxicity due to FU include neutropenia; hepatotoxicity, diarrhoea and vomiting [226], where toxicity presents as problematic but not fatal in patients receiving high doses of the drug. At peak concentration, high doses in patient serum have been associated with drug-related reactivity. Currently, FU is administered in combination with AmB as the most effective treatment regimen for CM [240].

Tenforde et al. recently presented data from 13 randomized clinical trials that enrolled 2426 participants and compared antifungal induction therapies used for the first episode of HIV-associated cryptococcal meningitis was compiled and analysed, in efforts to identify the most effective therapy at reducing the risk of death from HIV-associated CM [241]. The group found that based on resource availability, one-week AmB- and 5FC-based therapy was the most superior in areas with access to AmB; while an all-oral regimen of two weeks 5FC and FLU was effective in resource-limited environments. They also found no reduction in mortality rates when combination treatments of AmB and FLU were administered, or when AmB was used as a stand-alone treatment [241].

Interestingly, Amphotericin B and Fluconazole have been shown to modulate immune responses in macrophages by bringing about reductions in cellular charge, and at sub-inhibitory levels, enhancing phagocytosis [242]. However, both fluconazole and flucytosine described above, lack anticryptococcal activity within the phagolysosome unlike lysosomal Amphotericin B, highlighting the need for the development of drugs that can effectively penetrate cells [243]. As drug development is a long and costly process, the repurposing of previously approved drug candidates provides an alternative route to identifying novel anticryptococcal drug targets.

VACCINE DEVELOPMENT

Given the poor prognosis HIV-patients receive following positive CrAg screening, in addition to the differential responses to treatments observed between patients and rise in antifungal drug resistance, there is an urgent need to find preventative measures that reduce the risk of acquiring cryptococcosis in this group of immunocompromised patients. HIV patients characteristically present with diminished $CD4^+$ T-cell counts; recommending T cell and B cell signaling as a potential target for vaccine development [126, 244]. Furthermore, cryptococcosis in non-HIV patients has been shown to present as acute or reactivated disease, Therefore, a potential anti-cryptococcal vaccine would need to prevent disease regardless of infection phase [244].

Kleinnijenhuis et *al.* have previously provided evidence for the concept of "trained immunity", where epigenetic reprogramming of innate immune cells induced adaptive features (namely in monocytes, macrophages and dendritic cells) that allow them to respond more effectively to secondary *C. albicans* infections [245]. The group demonstrated that bacille Calmette-Guerin (BCG) vaccination of severe combined immunodeficiency (SCID) mice conferred 100% protection from disseminated candidiasis, in comparison to the 30% protection observed in control mice [245]. It is likely that these innate immune cells could provide a strategic approach to preemptive protection from cryptococcosis, because of previous exposure to the β-glucan component of the fungal cell wall that is also present in Cryptococcus spp. [246].

Monoclonal antibodies against cryptococcal GXM have also been proposed as potentially effective treatments against cryptococcosis by reducing CNS fungal burdens in mice [128]. Anti-cryptococcal antibodies are able to enhance phagocytosis of Cryptococcus spp., regulate the subsequent innate immune response and alter the pathogen's gene expression [247]. This approach may be ineffective at conferring protection to HIV patients given their defective B cell populations, which are heavily relied upon by antibody-mediated immunity (AMI) [248]; but may be overcome by immunization. Wormley et al. previously showed that infecting $CD8^+$ and $CD4^+$ T cell depleted mice with a *C. neoformans* strain genetically engineered to produce IFN-γ, protected 100% of the population from acquiring a secondary *C. neoformans* infection [249, 250]. The short term protection provided by these approaches is encouraging (4 weeks for AMI approach, and up to 100 days for immunization route), although more enduring protection would be required of an ideal anti-cryptococcal vaccine [128]. In 2015, Wager and Wormley published an article discussing the feasibility of developing an anti-cryptococcal vaccine [128]. Given the impaired innate immune function in HIV positive patients, the authors speculated whether the provision of prophylactic anticryptococcal approach would predispose patients to cryptococcus-related Immune Reconstitution Inflammatory Syndrome (IRIS) [128], a fatal condition, thought to be caused by recovery of cryptococcus-specific immune responses [251]. Furthermore, whilst there is no conclusive evidence to suggest that AMI alone can confer protection from cryptococcal disease, monoclonal antibody treatments may reduce the severity of disease in cases of diminished but not depleted cell-mediated immunity [252]. There is evidence that $CD8^+$ T-cells display anticryptococcal functions in cases of

impaired $CD4^+$ T cell activity [253], releasing IFN-γ capable of enhancing macrophage responses to Cryptococcus spp. [253][252]. Efforts to develop an anticryptococcal vaccine are conducted in animal models of disease; despite the provision of evidence that immune responses in these models mirrors that observed in human hosts, it is imperative that species-specific responses and susceptibilities be taken into account. The growing incidence of cryptococcosis in non-HIV patients as well as the varied host responses to cryptococcal infection highlights the desperate need to develop an effective anti-cryptococcal vaccine.

Discussion

Whilst the reduction in global HIV-associated CM is testament to improved ART and antifungal treatment strategies, the rise in frequency of non-HIV and non-transplant associated cryptococcosis in the developed world represents a cause for concern. HIV-associated CM remains the most extensively investigated among the three groups. Whilst the majority of cryptococcal infections in previously healthy individuals are asymptomatic, clinical manifestations and prognoses vary greatly. Current clinical guidelines for this group are generated from immunocompromised cohorts and thus may not be appropriate for disease management in immunocompetent hosts. Whilst numerous case reports of 'unusual' cryptococcosis cases have been published, there is a scarcity of prospective data on the management of CM in previously healthy individuals.

A recent article by Pirofski and Casadevall discussed the effects of host-mediated damage on the progression of cryptococcosis [254]. Given the variety of host-microbe interactions that occur within the different patient groups discussed above, and the associated alteration of the inflammatory immune responses, this damage-response framework (DRF) model may be particularly helpful for understanding infections in otherwise healthy individuals. Clearly, however, there is currently a lack of characterization studies in non-HIV cryptococcal patients with which to inform this model.

It has previously been shown that cytokine expression by macrophages is not permanently biased to either M1 or M2 responses, and may be modulated [135]; hence, the fluidity in macrophage activation states instigates varied responses in individuals with robust immune systems [155]. Furthermore, the genetic contribution to phenotypic challenges in anti-cryptococcal responses among cryptococcosis sufferers provides an additional tool for early diagnosis in at-risk cohorts. However, difficulties arise in the case of non-HIV immunocompetent hosts who may not be considered at-risk and therefore subject to presumptive genetic testing in comparison to their immunocompromised counterparts. In addition, it was brought to light in a recent article by Netea, that the association of the above-mentioned polymorphisms with susceptibility to severe cryptococcosis was not descriptive of all patients across different cohorts as these polymorphisms only predisposed individuals of certain ethnic groups to CM [255].

Whilst therapeutic approaches for HIV-associated cryptococcal disease in combination with ARTs are successfully being established, efforts to determine protective features of the innate immunity in non-HIV-associated disease are still underway, as are attempts to develop an effective vaccine against cryptococcosis. Knowledge of cryptococcal pathogenesis remains minimal in patients with known immunological defects beside HIV, and treatment strategies remain unspecific. This may be at least partially due to the inappropriate clustering of these two groups together into a single "non-HIV-associated cryptococcosis" category, when in fact they represent at least two distinct cohorts. A better understanding of the sources of variation between and within patient groups is urgently needed in order to help inform strategies to appropriately modulate the immune responses at the level of the individual to improve disease outcomes.

Study aims and objectives

Above, we have reviewed current knowledge of *C. neoformans*' pathogenicity towards different groups of patients. As we highlighted the gaps in knowledge of cryptococcal interactions with macrophages derived from healthy human hosts, we aim to decipher the extent of variation of host macrophage responses to *in vitro* challenge with the intracellular pathogen. By deriving macrophages from a group of individuals with no reported immunocompromises, we hypothesize that phagocytic cells from each individual will exhibit consistent anti-cryptococcal responses over time. We also anticipate that whilst the intra-host rates of vomocytosis may be analogous, differences in vomocytosis between individuals, paired with cytokine profiles from each *in vitro* challenge will bring to light the molecular components involved in orchestrating cryptococcal expulsion from macrophages.

As previous studies have implicated the pattern recognition receptor (PRR), toll-like receptor 4 (TLR4) in the expulsion of bacteria from host epithelial cells, we aim to probe the involvement of this PRR in the vomocytosis of *C. neoformans* from host macrophages.

Furthermore, we hypothesize that cases of varied control of intracellular yeast proliferation and vomocytosis may be due to genetic factors. Single nucleotide polymorphisms (SNPs) that cause functional and structural alterations in host macrophage anti-cryptococcal machinery may result in either impaired or

improved fungal clearance. As the molecular mechanisms that determine vomocytosis of *C. neoformans* from macrophages remain unknown, we believe an understanding of host genetic contribution to PRRs and downstream regulatory and effector proteins involved in this process will present a novel target for therapeutic development. In addition, given the inability of current treatments to clear intracellular cryptococci from macrophages, we also aim to identify host macrophage pathways that may be augmented therapeutically to increase killing of phagocytosed yeasT-cells.

Altogether, we aim to establish the parameters of variation in intracellular control and expulsion of yeasT-cells from host macrophages; check the contribution of genetics to any observed variation; question the association of TLR4 with vomocytosis; and identify a anti-cryptococcal compound able to eliminate intracellular yeast. It is anticipated that this work will provide a better understanding of the interactions between healthy host macrophages and *C. neoformans* and ultimately improve the diagnosis and treatment of the 'otherwise healthy' group of cryptococcosis patients.

Chapter 2 – Variation in host macrophage responses to *Cryptococcus neoformans*

Parts of this chapter have been published in a manuscript titled: "Quantifying donor-to-donor variation in macrophage responses to the human fungal pathogen Cryptococcus neoformans" by Garelnabi et *al*.

Abstract

Cryptococcosis remains the leading cause of fungal meningitis worldwide, caused primarily by the pathogen *Cryptococcus neoformans*. Symptomatic cryptococcal infections typically affect immunocompromised patients. However, environmental exposure to cryptococci is ubiquitous and most healthy individuals are thought to harbor infections from early childhood onwards that are either resolved or become latent. Since macrophages are a key host cell for cryptococcal infection, we sought to quantify the extent of individual variation in this early phagocyte response within a small cohort of healthy volunteers. We compared the effects of complement-mediated opsonization, with those of antibody-mediated opsonization on the intracellular parasitism and expulsion of

C. neoformans from human monocyte-derived macrophages (MDMs). Our findings show that whilst the host's MDM killing of intracellular Cryptococci was not dependent on the method of opsinization; the rate of expulsion was enhanced by antibody-opsonization in some individuals. We also show that rates of both intracellular fungal proliferation and non-lytic expulsion (vomocytosis) are remarkably variable between individuals, irrespective of gender, and that *in vitro* host inflammatory cytokines profiles are not determinants of this variation. Despite our small sample size, we conducted post-hoc power calculations based on our data and determined that the sample size required to achieve reliable statistical value for IPR and vomocytosis to be 238 and 274, respectively.

Introduction

Host-Specific Variation In The Macrophage Response To Cryptococcal Infection In Healthy Individuals

Cryptococcosis is the leading cause of fungal meningitis worldwide, with the vast majority of clinical cases being caused by *Cryptococcus neoformans.* The last two decades have seen a decline in HIV-associated CM in developed countries due to improved access to a combination of antiretroviral therapies and effective antifungal treatments [194]. It has been estimated that 20% of global CM cases afflict non-HIV patients [193], with more recent reports indicating a growing concern over CM due to *C. neoformans* in otherwise healthy individuals [193-195].

Despite the global distribution of this species, the highest frequency of CM in immunocompetent individuals appears to come from South East Asia, with mortality rates in China, Taiwan and Japan estimated to be at 17%, 70% and 35%, respectively [196-198]. Whilst time to presentation following initial symptoms of infection does not differ between HIV positive and negative patients [195], one of the issues restricting effective disease management is that non-HIV CM patients represent a highly heterogenous group. In a retrospective study that assessed predictors of disease mortality in different groups of cryptococcosis patients, it was shown that it took an average of 68 days to diagnose otherwise healthy individuals with cryptococcosis, while HIV patients

were diagnosed within 22 days [256]. This may be due to differences in presentation of symptoms e.g. CM patients with HIV are more likely to present with fever whilst the non HIV group more frequently presents with abnormal mental status, lung involvement and lesions in the central nervous system (CNS) [195] or due to clinician awareness of cryptococcosis as a complication of HIV. Treatment regimens for HIV-negative cryptococcosis patients are primarily based on data from studies on HIV patients, due to the scarcity of data within a more relevant context [227]. Hence, an understanding of the immunological status that enables 'otherwise healthy' individuals to overcome cryptococcal disease would allow healthcare providers more robust treatment strategies in this group, and ultimately improve survival rates.

Macrophages represent a key target cell for *C. neoformans*. Mice with depleted macrophages infected with this pathogen showed enhanced survival over their wild type counterparts and significantly lower CNS involvement [257]. Most isolates of cryptococci are remarkably resistant to macrophage killing, and are able to adapt and survive within macrophages causing latent infections that may later disseminate upon immune suppression to cause cryptococcal meningoencephalitis (CM) [258, 259]. Evidence for latent infections is derived from the detection of anti-cryptococcal antibody in a large percentage of children by the age of 10 in different parts of the world [260, 261]; Also, reactivated cryptococcal disease has been shown to occur up to 60 years following the initial advent of infection by the same cryptococcal species [262].

Given that both latent and symptomatic infections likely arise from an early intracellular, macrophage-associated, fungal colonization, a key question is how variable this early macrophage response is to the fungus. Engulfed cryptococci are able to manipulate host phagosome maturation, enabling the yeast's intracellular survival [122, 143]. As described in Chapter 1, an interesting feature of *Cryptococcus's* intracellular parasitism is its ability to undergo vomocytosis (non-lytic expulsion) from the host macrophage either immediately or a few hours after engulfment [169, 170]. This rapid process has been suggested to facilitate cryptococcal dissemination throughout the host whilst evading immune detection, ultimately resulting in passage into the CNS via the Trojan horse model [66, 71]. Hence, we hypothesize that the mode of uptake of Cryptococci by human phagocytes could influence vomocytosis *in vitro*, we anticipate that this will provide an incite into how vomocytosis influences cryptococcal dissemination, and progression of cryptococcal disease depending of the site of infection *in vivo*.

As the cryptococcal capsule conveys anti-phagocytic advantages to the yeast, allowing for evasion of immune detection and dissemination towards the CNS, understanding how the host is able to overcome issues with pathogen detection is paramount. The complement system is a major component of the innate immune system, comprising of a number of plasma proteins that interact with each other to opsonize invading pathogens, and trigger inflammatory responses [263]. Opsonization essentially marks pathogens for engulfment and killing by leukocytes bearing receptors that detect opsonins on the pathogen's surface. Pathogens are able to activate complement via the classical (antibody-

dependent), alternative (antibody-independent) or the lectin pathways (antibody-independent) [263, 264]. These three pathways involve a series of proteolytic reactions that converge in the formation of the opsonin C3b [12]. The enzyme C3 convertase splits the complement component C3 into two components, C3a and C3b; C3b then covalently binds to the pathogen surface and is recognized by C3b receptors on the surface of phagocytes to initiate pathogen engulfment. C3b can also bind to C3 convertases to produce C5 convertase, which cleaves C5 to C5a and C5b [12]. Whilst these three pathways are initiated by different molecules, they ultimately converge to bring about phagocytosis of the C3b-opsonized pathogen; and increased recruitment of phagocytic effector cells, and facilitation of inflammatory responses by C3a and C5a to promote pathogen elimination [12, 263].

Early on, the alternative activation pathway was reported as a major protective component against cryptococcal infections [265]; However, it has since been shown that in the absence of complement proteins, anti-cryptococcal anti-GXM antibodies are able to act as effective opsonins that activate phagocytosis in the J774 murine macrophage-like cell line, and improve anti-cryptococcal activity [266-268]. In experiments by Mukherjee et al., various murine antibody subclasses (IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA) were shown to enhance the anticryptococcal activity of J774 cells which was measured by a reduction in cryptococcal colony-forming units (CFUs), where IgG2a monoclonal antibodies were the most effective at enhancing macrophage elimination of engulfed Cryptococci [267]. In subsequent experiments, the group showed that *in vitro* infection of J774 cells with *C. neoformans* in the absence or presence of the

monoclonal antibody 2H1, reduced CFU counts by 26.1% and >95%, respectively [268].

However, with regards to vomocytosis, Ma et *al*. also showed that expulsion of engulfed cryptococci was independent of the route of uptake [170]; interestingly, antibody-mediated phagocytosis has been associated with the expulsion of cryptococci as clumps of cells as opposed to the singular expulsion of yeast cells following complement-mediated uptake [180]. Since the effects of different complement activation pathways on fungal killing are still to be fully established, and given the observed variation in host responses to cryptococcosis, we were interested to find out whether the route of opsonization affected anti-cryptococcal activity of immunocompetent human MDMs *in vitro*.

In addition, the phospholipase B (Plb), an enzyme secreted by *C. neoformans* and crucial in cryptococcal virulence, is essential for dissemination to the CNS. Chayakulkeeree et al. showed that this enzyme was also shown to be essential for vomocytosis [181]. The group showed that deletion of Plb and Sec14-1p, the transfer protein essential for the secretion of Plb, reduced vomocytosis by ~60% from the murine macrophage-like J774 cell line; they also showed that vomocytosis fully restored in the reconstituted *C. neoformans* H99 strain [181].

CSF cytokine and chemokine profiles have been shown to be predictive of risk of early mortality and Immune Reconstitution Inflammatory Syndrome (IRIS) in HIV-associated Cryptococcal Meningitis [214]. Aside from high CSF fungal burden, rate of clearance of *Cryptococcus* and altered mental status, the host

cytokine profile has been identified as deterministic of fungal disease progression in HIV patients [213, 214]. Clinical studies to evaluate baseline CSF and sera cytokine profiles in HIV patients with CM, also showed that Th1- type immune responses (IL-8, IL-12p40, IL-17A, TNF-alpha, INF-gamma and sera TNF-alpha) played a role in reducing CSF fungal burden and improved survival in comparison to those with advanced immunodeficiency [215]. More recent in-depth analysis of the mechanisms regulating M1 and M2 activation states also revealed that macrophages are able to shift between the two polarization states, highlighting the potential impact of this fluidity on host susceptibility to disease [269, 270]. The ability of macrophages to be reprogrammed may explain the variation in host responses to cryptococcal infection.

Remarkably, it has been shown that IFN-γ levels were negatively correlated with fungal burden at the site of infection [271], despite the successful use of IFN-γ as an adjunctive treatment against cryptococcosis [272]. Siddiqui et al. analysed CSF fungal cultures in 62 HIV-associated cryptococcosis patients over time and found that levels of the pro-inflammatory cytokines IFN-γ, TNF-α, IL-6 and IL-8 were higher in survivors than those who succumbed to infection; however, the levels IFN-γ, TNF-α and IL-6 were negatively correlated with cryptococcal CFU counts [271]. In the context of macrophages, high levels of these pro-inflammatory cytokines may contribute to lowering CFU counts by encouraging vomocytosis of *C. neoformans* from within macrophages; exposing expelled cryptococci to killing by other immune mechanisms.

Despite the advances made in characterizing the pathogenic capabilities of *Cryptococcus* and the considerable body of data on fungal disease within the context of HIV positive patients, little is known about the innate immune characteristics in non-HIV patients that predispose them to developing cryptococcal meningitis (CM). Therefore, an understanding of the host-pathogen interactions outside the context of HIV would provide better-tailored treatment approaches to combat cryptococcal meningoencephalitis in this group.

The data presented here examines the extent to which macrophage heterogeneity contributes to innate immune control of infection in host macrophages taken from healthy individuals. We aimed to identify the determinants of host susceptibility cryptococcosis by studying variation in host macrophage responses to *Cryptococcus*, and characterizing how these interactions may either facilitate or impede *C. neoformans* invasion and progression to CM. Bases for variation under consideration include the ability of host macrophages to phagocytose and kill *Cryptococcus neoformans*, and the level to which they promote or restrict clearance of the fungal load, preventing dissemination to the central nervous system.

Below, we present quantitative data from the *in vitro* challenge of monocyte-derived macrophages (MDMs) from 15 healthy individuals with C. neoformans var. grubii. We compare the outcomes of *in vitro* infection of human MDMs with the *C. neoformans* Kn99α-GFP strain, opsonized with either the murine monoclonal antibody (MAb) 18B7 [273] to activate antibody-mediated immunity, or pooled human serum (PHS) to invoke alternative complement

activation. We also compare the cytokine profiles of each independent donor; and investigate how levels of secreted cytokines compare to intracellular cytokine production between individuals within our group of donors. Furthermore, we assess whether gender and seasonal variation play a role in promoting/impairing anticryptococcal defense in macrophages.

Despite the relatively small cohort, there is remarkable variation in macrophage ability to control both intracellular parasitism and vomocytosis of *C. neoformans*. Surprisingly, this variation in intracellular proliferation and expulsion of the yeast appears to be driven primarily by the local environment experienced by the monocyte prior to *in vitro* differentiation. We show that neither intra- and extra-cellular cytokine profiles, nor gender, play a significant role in describing the outcome of infection.

Materials and methods

Ethical approval for this study was obtained from the Science, Technology, Engineering and Mathematics Ethical Review Committee of the University of Birmingham (ERN_15-0804).

Sample collection and randomization

30-60ml venous blood samples were collected in lithium heparin VACUTAINER® tubes obtained from healthy volunteers with full informed consent. Immediately after donation, the identities of fifteen participants were randomized and assigned donor IDs (RG001 - RG016) that were maintained throughout the experimentation process. Donor RG008 was excluded from the study.

VACUTAINER® tubes with silica bead clot activator (CAT) were used to collect serum during each blood donation, and incubated at 37°C, 5% CO_2 for one hour prior to centrifugation at 800 x *g* for 10 minutes. Live serum aliquots were thereafter stored at -80°C for cytokine profiling, and the remaining serum was heat-inactivated in a water bath at 57°C for 30 minutes and stored at 4°C, for later use.

Monocyte isolation, differentiation and culture

Peripheral blood mononuclear cell (PBMCs) were isolated from fifteen healthy volunteers by double gradient centrifugation using Percoll® (Sigma-Aldrich, H4522). The dual gradient was created by layering 6 ml of 1.098 g/ml Percoll® (70.9% Percoll®, 19.1% H_2O, 10% 1.5M NaCl) underneath 1.079 g/ml Percoll® (56.3% Percoll®, 33.7% H_2O, 10% 1.5M NaCl); onto which 6 ml of undiluted donor blood was layered on top of, and centrifuged for 8 minutes at 150 x *g*, followed by 10 minutes at 1200 x *g* with no brake, or acceleration. The top layer of monocytes separated by the dual gradient were removed and added to Red Blood Cell (RBC) lysis Buffer (1L – 8.3g NH_4Cl, 1g $KHCO_3$, 0.04g Na_2 EDTA $2H_2O$, 2.5g BSA) at a ratio of 1:3 and incubated for 3 minutes at room temperature with gentle mixing then spun at 400 x *g* for 6 minutes. The buffer was removed, and monocytes washed twice with 50 ml of PBS (Sigma-Aldrich), and PBS supplemented with Ca^{+} and Mg^{++} at 4 °C (Sigma-Aldrich) to enhance cell adhesion.

Isolated monocytes were thereafter resuspended in 1 ml adhesion media (RPMI 1640 with L-glutamine; Thermo Fisher Scientific, supplemented with heat inactivated (56°C for 30 mins) 5% pooled human AB serum; Sigma-Aldrich, and 100 U/ml streptomycin, 100 U/ml penicillin; Sigma-Aldrich). Cells were counted on haemocytometer and then seeded into 48-well cell culture plates at a concentration of $1x10^6$ cells/well and incubated at 37°C at 5% CO_2. The

supernatant was removed 2 hours later, and replaced with differentiation media (adhesion media supplemented with 5% pooled human AB serum; Sigma-Aldrich and 20 ng/ml human M-CSF). Subsequent media replacements occurred on day 3 and day 6 post-isolation with adhesion media, and serum-free adhesion media, respectively. Human monocyte derived macrophages (HMDMs) were activated on day 7 with RPMI 1640 supplemented with 5% human AB serum (Sigma-Aldrich), 100 U/ml streptomycin, 100 U/ml penicillin, 0.5 μg/ml human IFN-γ; ImmunoTools; and 1 μg/ml *E. coli* LPS; Sigma-Aldrich) 24 hours before carrying out the phagocytosis assay. Cell culture media from donor MDMs was collected at each media replacement, divided into 3 aliquots and stored at -80°C for cytokine profiling.

Cryptococcus Infection of HMDMs

Green Fluorescent Protein (GFP) -labeled C. neoformans var. grubii serotype A, strain KN99α [199] was used in all macrophage challenge experiments.

An overnight culture of the yeast was started by inoculating 3 mL of YPD (10 g/L yeast extract, 20 g/L bacteriological peptone, and 20 g/L glucose (Sigma-Aldrich) media, and incubated on a rotor at 20 rpm at 25 °C, prior to conducting the phagocytosis assay. In preparation for phagocytosis, yeasT-cells were washed in PBS, counted on a haemocytometer, and opsonized with either mouse IgG anti-Crypto18B7 (18B7) antibody, or pooled human serum (PHS) in the initial phase of the study to establish the effects of opsonization on macrophage responses;

and thereafter opsonized solely with 5% heat-inactivated human AB serum (Sigma-Aldrich). Assuming 10% of the isolated monocytes had adhered and differentiated, human monocyte derived macrophages (hMDMs) where then infected with $1x10^6$ yeasT-cells per well (MOI 10:1), and incubated at 37°C, 5% CO_2 for 2 hours. At 0 (T0) and 18 (T18) hours post-infection, extracellular yeasT-cells were washed away using PBS and macrophages containing yeasT-cells were lysed in dH_2O at 37 °C, 5% CO_2 for 30 minutes. For live imaging to quantify vomocytosis, hMDMs were washed at T (0), fresh serum-free RPMI added to infection wells and taken for imaging. Infection media from T (0) and T (18) was also divided into 3 aliquots and stored at -80°C for cytokine profiling.

CFU counts

Serial dilutions of lysates from the phagocytosis assay were prepared and plated onto 2% YPD, 1% agar (Sigma-Aldrich) plates then incubated for 48 hours at 25°C. Intracellular proliferation rates were measured by dividing the number of counting colony-forming units per milliliter at T (18) by those at T (0).

Live cell imaging

All time lapse images were captured on a Zeiss Axio Observer Live cell-imaging microscope enclosed within a humidified Okolab microscope chamber set at 37°C, 5% CO_2, a Hamamatsu digital camera, LD Plan-Neofluar 20x/0.4 Korr Ph 2 M27 objective, 38 HE Green Fluorescent reflector, using Zen software (Zeiss).

217 frames (1 frame, every 5 minutes for 18 hours) were taken from four different positions within each well to produce movies for manual analysis. Vomocytosis was measured as the percentage of intracellular cryptococci expelled from macrophages over the 18-hour period in all well positions.

Cytokine profiling

Extracellular cytokine profiling

Cell culture supernatants collected from donor MDMs prior to infection, at T0 and at T18 post-infection, along with live serum aliquots that had been previously stored at -80 °C were thawed on ice in preparation for cytokine profiling. The IFN-γ levels were quantified using a Human IFN- gamma DuoSet ELISA kit (R&D Systems, DY285); whilst a qualitative Human Th1 / Th2 / Th17 Cytokines Multi-Analyte ELISArray™ Kit (QIAGEN, MEH-003A) was used to detect increases or decreases in cytokine production relative to the positive controls provided in the kit.

Further, quantitative cytokine profiling was carried out using the Luminex Bio-plex (27-plex) (Bio-Rad, M500KCAF0Y). Initially, samples from 6 donors were probed for alternative or differential production of 27 human cytokines and chemokines using the Luminex Bio-plex (27-plex; Bio-Rad); which were then narrowed down to a panel of 7 cytokines that had previously been associated with cryptococcosis in HIV patients (IL-1β, IL-6, G-CSF, GM-CSF, IFN-γ, MCP-1 (MCAF), and TNF-α) due to unavailability of data in non HIV patients. We incorporated these cytokines into a custom-made Bio-plex Pro Human Cytokine

7-plex express assay for subsequent cytokine detection in 12 donors, in accordance with the manufacturer's protocol. The fold change (pg/mL) in cytokines released by donor MDMs was calculated by dividing the cytokine concentrations at T18 by those at T0 for each donor. Correlation with matched vomocytosis and IPR data was carried out using the Graphpad Prism 6 software.

Intracellular cytokine profiling

Flow Cytometry was used to measure intracellular cytokine produced by non-activated, M1 and M2 donor MDMs. During the 18-hour in vitro challenge with Kn99α-GFP, At T (13) post-infection, a 1:1000 dilution of Brefeldin A (Sigma-Aldrich, B7651) was added to the 48-well plate to block extracellular trafficking of cytokines, and reincubated at 37 °C, 5% CO_2 for 5 hours. At T (18), extracellular cryptococci were washed off, and 250 µl of Accutase (Sigma-Aldrich, A6964) was added to dislodge the MDMs. Cells were then transferred to v-bottom 96-well plates and spun at 1200 rpm for 3 mins. The cell pellet was fixed with 3.7% formaldehyde for 10 mins, washed with PBS, and then permeabilised with 0.1% Saponin for 10 mins, and washed again with PBS. Query MDMs were then stained for 20 mins in the dark with either IFN--gamma APC (BD biosciences, 554702) at a 1/167 dilution in 0.1% Saponin; or TNF-alpha eFluor450 (eBioscience, 48-7349-42) at a 1/50 dilution in 0.1% Saponin. Isotype controls were also stained for 20 mins in the dark with Mouse anti-IgG2b V450 (MBL International, FP20644010) at a 1/200 dilution, and Mouse anti-IgG1 APC (BD biosciences, 550854) at a 1/167 dilution. Data was collected using Beckman

Coulter CyAn ADP — Flow Cytometry Core Facility, and analyzed using FlowJo Version 10.0.7.

Power calculations

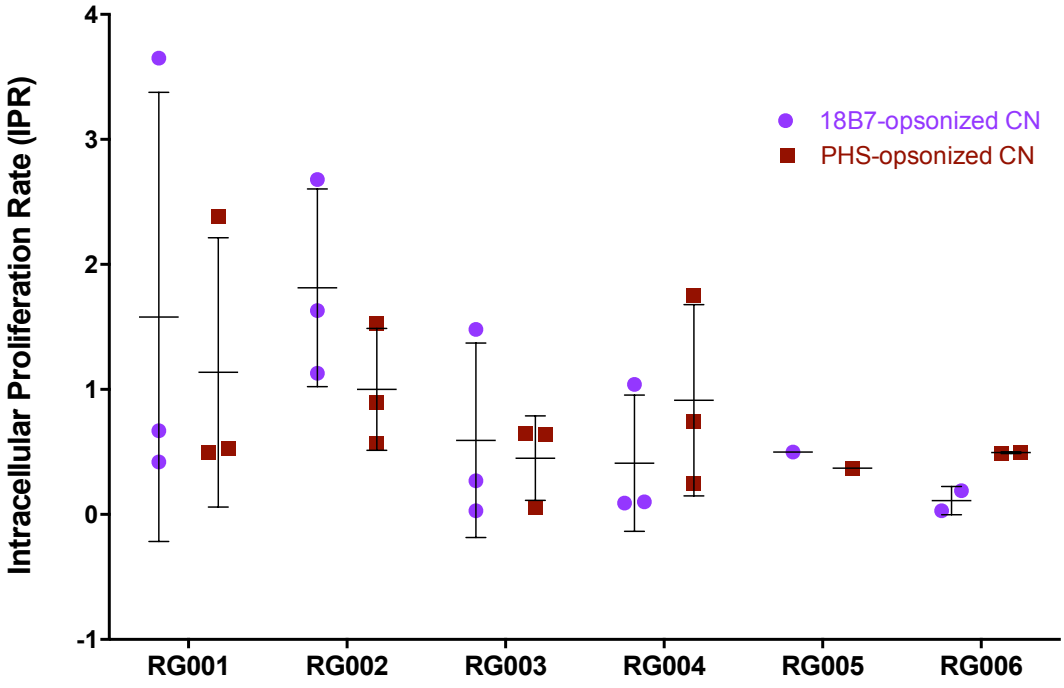
In order to assess the sample size that would generate statistically relevant results, we conducted a power calculation in order to identify the required population size "n", necessary for adequate power. In order to do this, we used the formula below; where "z" is the critical value of the normal distribution for a confidence level of 95% with a critical value of 1.96; and "e" is the margin of error of 0.05; and p is the sample proportion. The value of "p" was derived from the population the either had an IPR below 1, or vomocytosis rate above the 75% confidence interval (CI).

$$n = \frac{z^2 \times \hat{p}(1-\hat{p})}{\varepsilon^2}$$

Results

Effects of opsonization on host responses to *C. neoformans* infection

To assess the effects of complement-mediated immunity in comparison to antibody-mediated immunity on the intracellular parasitism of the yeast (Fig. 7) and on vomocytosis of cryptococci from macrophages (Fig. 8), we opsonized C. neoformans var. grubbi with either pooled human serum (PHS), or the anti-cryptococcal antibody 18B7, respectively. Opsonization lasted for 1 hour prior to infection of hMDMs from 6 of the cohort's participants. We thereafter measured the release of the Th1 cytokine IFN-γ into the cell culture supernatant by ELISA prior to activation of MDMs with LPS and IFN-γ, and at 0- and 18- hours post infection with cryptococci (Fig. 9). We also established baseline levels of serum IFN-γ for each individual at the point of blood donation (Fig. 9).

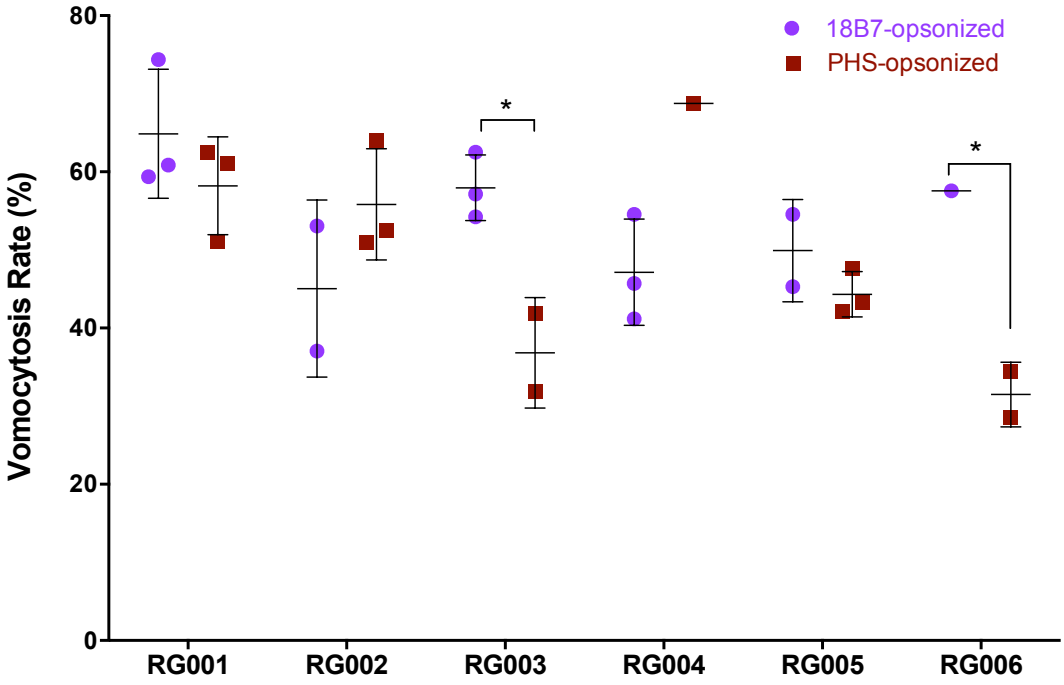


	RG001	RG002	RG003	RG004	RG005	RG006
18B7-opsonized CN	1.58	1.81	0.59	0.41	0.50	0.11
PHS-opsonized CN	1.14	1.00	0.45	0.91	0.37	0.50

Figure 7. Intracellular Proliferation Rates (IPRs) of 6 healthy donors' monocyte-derived macrophages (MDMs) challenged with C. neoformans Kn99 α -GFP opsonized with either the antibody 18B7, or Pooled Human Serum (PHS). Each point represents an independent blood donation where N=3 for RG001, RG002, RG003 and RG004; N=1 for RG005 and N=2 for RG006. Error bars are representative of means and standard deviations. Table of means is included below the graph.

Our findings show that opsonizing Cryptococci with either the anti-cryptococcal antibody (18B7) or pooled human serum (PHS), does not affect the intracellular parasitism of *C. neoformans* Kn99α-GFP in monocyte-derived macrophages (hMDMs) from 6 healthy donors (RG001-RG006). Two-way analysis of variance revealed no significant differences between the two methods of opsonization (P = 0.7611). We observed high intra-donor variability amongst donors RG001-RG004, and consistent IPRs for RG006 (Fig. 7). Whilst donor identity accounted

for 27.17% of the total variance, there were no significant differences in IPRs among the 6 donors (P = 0.2317).



	RG001	RG002	RG003	RG004	RG005	RG006
18B7-opsonized	64.87	45.05	57.96	47.15	49.91	57.58
PHS-opsonized	58.21	55.83	36.82	68.75	44.32	31.50

Figure 8. Vomocytosis Rates (%) of C. neoformans Kn99 α -GFP from monocyte-derived macrophages of 6 healthy donors following opsonization with either the antibody 18B7, or Pooled Human Serum (PHS). Each point represents an independent blood donation where N=3 for RG001 (18B7- and PHS-opsonized), N=2 for RG002 (PHS-opsonized), N=3 for RG002 (18B7-opsonized), N=3 for RG003 (18B7-opsonized), N=2 for RG003 (PHS-opsonized), N=3 for RG004 (18B7-opsonized), N=1 for RG004 (PHS-opsonized), N=2 for RG005 (18B7-opsonized), N=3 for RG005 (PHS-opsonized), N=1 for RG006 (18B7-opsonized), and N=2 for RG006 (PHS-opsonized). Error bars are representative of means and standard deviations. Methods of opsonization were significantly different for RG003, and RG006. Table of means is included below the graph.

We also measured yeast expulsion rates from each individual's MDMs following *in vitro* challenge with *C. neoformans* Kn99 α -GFP that had been opsonized with

either the 18B7 antibody or pooled human serum (PHS; Fig. 8). Two-way analysis of variance revealed that the method of opsonization accounted for approximately 3.58% of the total variance, and found no significant differences in vomocytosis between 18B7- and PHS- opsonized Cryptococci (P = 0.1138). However, donor identity accounted for approximately 30.11% of the total variance, and showed that vomocytosis varied significantly between donors (P = 0.0078). Bonferroni's multiple comparisons test showed that the method of opsonization significantly affected yeast expulsion from MDMs from donors RG003 and RG006 ($p < 0.05$). For both individuals, 18B7-opsonized Cryptococci were more likely to be expelled. These findings suggest that, depending on the individual; the method of opsonization would affect the extent of cryptococcal expulsion from macrophages.

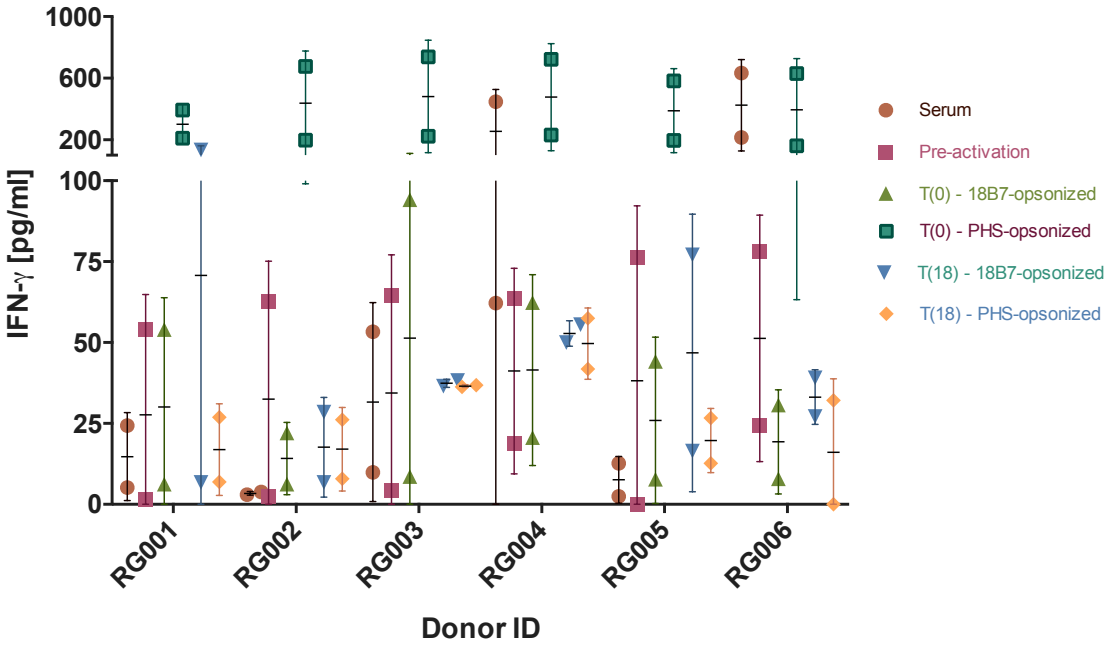


Figure 9. Human Gamma-interferon IFN-γ in donor sera at time of blood donation, and that released by donor MDMs before and after infection with *C. neoformans* Kn99 α -GFP opsonized with

either the antibody 18B7, or Pooled Human Serum (PHS). Error bars represent standard deviation; Each point represents an independent blood donation where N=2 for all donors.

We also measured the amounts of IFN-γ in donor sera, as well as that released by MDMs prior to activation with IFN-γ and LPS; and at 0-hours and 18-hours after infection with either PHS- of 18B7- opsonized *C. neoformans* (Fig. 9). Two-way ANOVA revealed that a significant interaction between donor identity and method of opsonization (P = 0.0152) that accounted for 11.62% of the total variance. Whilst the opsonization method accounted for 55.16% of the total variance (after adjusting for matching), this variance was not significant (P = 0.1268). In addition, even though donor identity accounted for only 2.91% of the total variance (after adjusting for matching); donor identity significantly contributed to the observed variance (P = 0.0309). We found that serum levels of IFN-γ varied significantly between donors, but no significant differences in IFN-γ release were found in all other culture supernatant samples. RG004 and RG006 sera contained significantly higher levels of IFN-γ compared to the rest of the group (Bonferroni's multiple comparisons test; RG004, $p < 0.05$; and RG006, $p < 0.0001$). Whilst were detected high sera levels of IFN-γ for RG004 and RG006 that may be residual, these were within normal limits for healthy adults, as baseline IFN-γ levels can vary greatly between individuals.

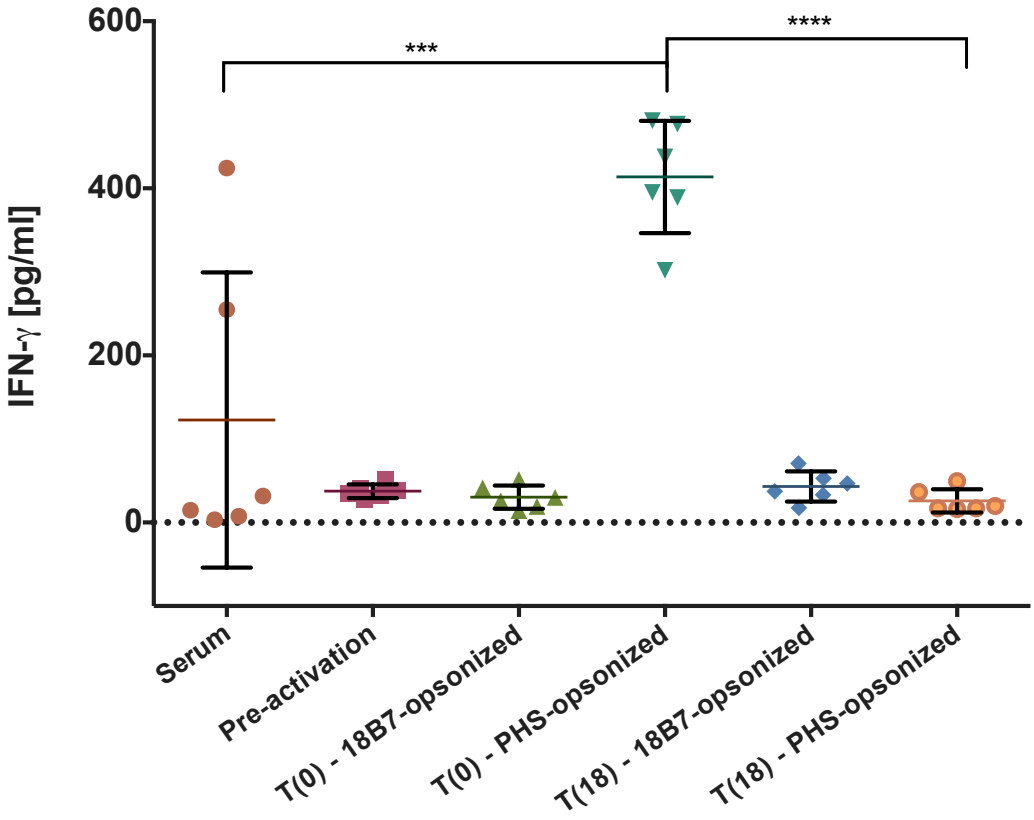


Figure 10. Average amounts of IFN-γ in sera from 6 participants, and that released by MDMs prior to activation, and following infection with either Antibody/18B7- or Pooled Human Serum (PHS)-opsonized C. neoformans.

Additionally, we analysed group differences at each time point (Fig. 10) using 2-way ANOVA. Bonferroni's multiple comparisons test revealed significant differences between Serum and T(0) - PHS-opsonized ($p < 0.001$); Pre-activation and T(0) - PHS-opsonized ($p < 0.0001$); T(0) - 18B7-opsonized and T(0) - PHS-opsonized ($p < 0.0001$); T(0) - PHS-opsonized and T(18) - 18B7-opsonized ($p < 0.0001$); and between T(0) - PHS-opsonized and T(18) - PHS-opsonized ($p < 0.0001$).

Furthermore, grouped analysis of the 6 donors proved there was no significant difference in IFN-γ levels between serum and pre-activation ($p > 0.9999$); pre-activation and 18B7-opsonized ($p > 0.9999$) nor PHS-opsonized ($p = 0.1445$). At T (0) and T (18) there were no significant differences between MDM responses to either 18B7-opsonized or PHS-opsonized cryptococci (at T (0), $p=0.0610$; at T (18), $p=0.8598$). For the 18B7-opsonized cryptococcal challenge, there was no significant difference in amounts of IFN-γ between T (0) and T (18) ($p > 0.9999$).

Overall, all donors showed heightened IFN-γ production in response to complement-opsonized cryptococci at T (0), but not to antibody-opsonized yeast; that subsequently reduced by the end of the cryptococcal challenge at T (18).

Host responses to C. neoformans infections vary within and between donors

To compare intra-donor and inter-donor variation in macrophage responses to infection with *C. neoformans*, we took repeated blood samples from a cohort of 15 healthy volunteers over a period of 2-3 years, taking into account seasonal effects on host immunity. We derived macrophages *in vitro* with macrophage colony-stimulating factor (M-CSF), and then analyzed both intracellular fungal proliferation rates (IPR; Fig. 11A) and the rate of non-lytic yeast expulsion from within macrophages (Fig. 11B). Intracellular proliferation rate varied dramatically between and within donors (Fig. 11A and Table 1; 2) and there was no significant difference in mean IPR between individual donors, suggesting that environmental variation (e.g. in the inflammatory status of the donor at the time of donation) is a more significant driver of variation in IPR than donor genetic background. In contrast, vomocytosis rate (calculated as the proportion of cryptococcal cells expelled from donor MDMs over an 18-hour period) was much less variable between repeat samples with no significant inter-donor difference in expulsion ($p = 0.0820$, one-way ANOVA; Fig. 11B).

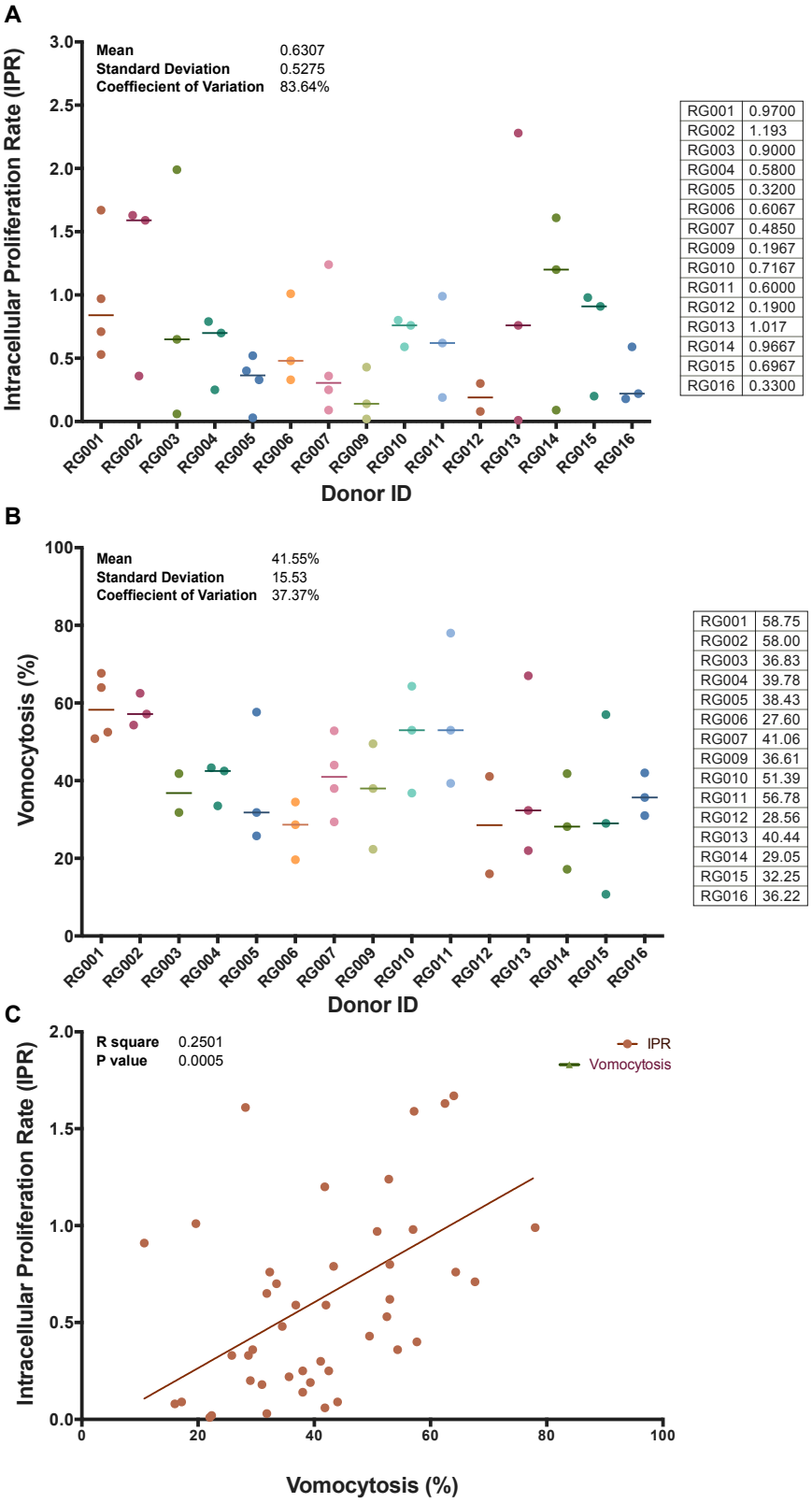


Figure 11. Variation in host responses to C. neoformans infections; A) measured intracellular proliferation rate (IPR) for each donor showing median of at least 2 biological repeats each (mean= 0.6307, SD= 0.5275 and Coefficient of variation= 83.64%); B) Variable rates of vomocytosis

observed between and within donors showing median of at least 2 biological repeats each (mean= 41.55%, SD= 15.53 and Coefficient of variation= 37.37%); C) Correlation between intracellular parasitism and non-lytic expulsion events (R square= 0.2501, P-value= 0.0005).

We thereafter analyzed our donor-specific matched data to check for correlation between IPR and vomocytosis rates (Fig. 11C). Whilst low intracellular proliferation rates corresponded with low vomocytosis rates, IPRs above 0.8 could not be associated with either low or high vomocytosis rates; leading to an overall weak correlation between the two variables (R square= 0.2501, P-value= 0.0005).

Donor ID	Intracellular Proliferation Rate				Vomocytosis (%)			
	Mean	Median	Standard Deviation	Coefficient of variation	Mean	Median	Standard Deviation	Coefficient of variation
RG001	0.97	0.84	0.50	51.59%	58.75	58.25	8.35	14.20%
RG002	1.19	1.59	0.72	60.50%	58.00	57.17	4.15	7.15%
RG003	0.90	0.65	0.99	109.89%	36.83	36.83	7.07	19.20%
RG004	0.58	0.70	0.29	49.88%	39.78	42.50	5.45	13.71%
RG005	0.32	0.37	0.21	65.20%	38.43	31.83	16.93	44.05%
RG006	0.61	0.48	0.36	58.89%	27.60	28.67	7.49	27.15%
RG007	0.49	0.31	0.52	106.27%	41.06	41.00	9.87	24.05%
RG009	0.20	0.14	0.21	107.18%	36.61	38.00	13.64	37.25%
RG010	0.72	0.76	0.11	15.56%	51.39	53.00	13.82	26.90%
RG011	0.60	0.62	0.40	66.73%	56.78	53.00	19.61	34.54%
RG012	0.19	0.19	0.16	81.88%	28.56	28.56	17.76	62.18%
RG013	1.02	0.76	1.16	113.76%	40.44	32.33	23.57	58.28%
RG014	0.97	1.20	0.79	81.35%	29.05	28.17	12.34	42.49%
RG015	0.70	0.91	0.43	61.94%	32.25	29.00	23.30	72.23%
RG016	0.33	0.22	0.23	68.50%	36.22	35.67	5.52	15.24%

Table 1. Intra-donor variation in monocyte-derived macrophage (MDM) responses to C. neoformans infections *in vitro*. Means and medians shown are of at least 2 biological repeats per donor extrapolated from at least 2 technical repeats.

	Intracellular Proliferation	Vomocytosis
Mean	0.6307	41.55
Std. Deviation	0.5275	15.53
Std. Error of Mean	0.07863	2.315
Coefficient of Variation	83.64%	37.37%

Table 2. Quantitation of variation in intracellular proliferation rates and vomocytosis rates (%) across 15 donors.

Cytokine profiles are not predictive of varied responses to *C. neoformans* infections

Host cytokine profile has previously been shown to impact strongly on the response to cryptococcocal infection [210, 214]. We therefore wondered whether differences in the secreted cytokine and chemokine milieu during *in vitro* culture might influence their subsequent response to cryptococci.

To assess this, we examined cell culture supernatants for T (0) and T (18) from 6 donors for increases or decreases in the release of Th1, Th2 and Th17 cytokines (Table 3). No consistent cytokine profile was observed; IL2 and TGF-β was undetected in RG005, while only RG012 showed decreased production of IL2 in comparison to the rest of the group. An increase in IL6 and G-CSF production was observed in 3 individuals, and decreased in the other 3 donors. An increase in IL10 and IFN-γ release was only observed in RG006 and RG001, respectively; while TNF-α production was lowered in only two of the donors (RG013 and RG014). Except for RG005, TGF-β production decreased over the 18-hour infection period (Table 3).

Cytokine	RG001	RG013	RG014	RG005	RG006	RG012
IL2	↑	↑	↑	–	↑	↓
IL6	↓	↑	↓	↓	↑	↑
IL10	↓	↓	↓	↓	↑	↓
IFN-γ	↑	↓	↓	↓	↓	↓
TNF-α	↑	↓	↓	↑	↑	↑
G-CSF	↓	↓	↓	↑	↑	↑
TGF-β	↓	↓	↓	–	↓	↓

Table 3. Th1, Th2 and Th17 qualitative cytokine release by MDMs from 6 donors showing inconsistent cytokine profiles. Cells highlighted purple indicate a varied profile of a each donor in comparison to other donors. Upward arrows indicate an increase in cytokine release over an 18-hour period, while downward arrows indicate a reduction.

We thereafter quantified levels of seven cytokines and chemokines (interferon-γ (IFN-γ), TNF-α, interleukin-1b (IL-1b), interleukin-6 (IL-6), MCP-1, G-CSF and GM-CSF) in the media immediately following cryptococcal infection and again 18 hours later. We correlated them with measured IPRs (Figure 12; Table 4) and vomocytosis rates for each donor (Figure 13; Table 4).

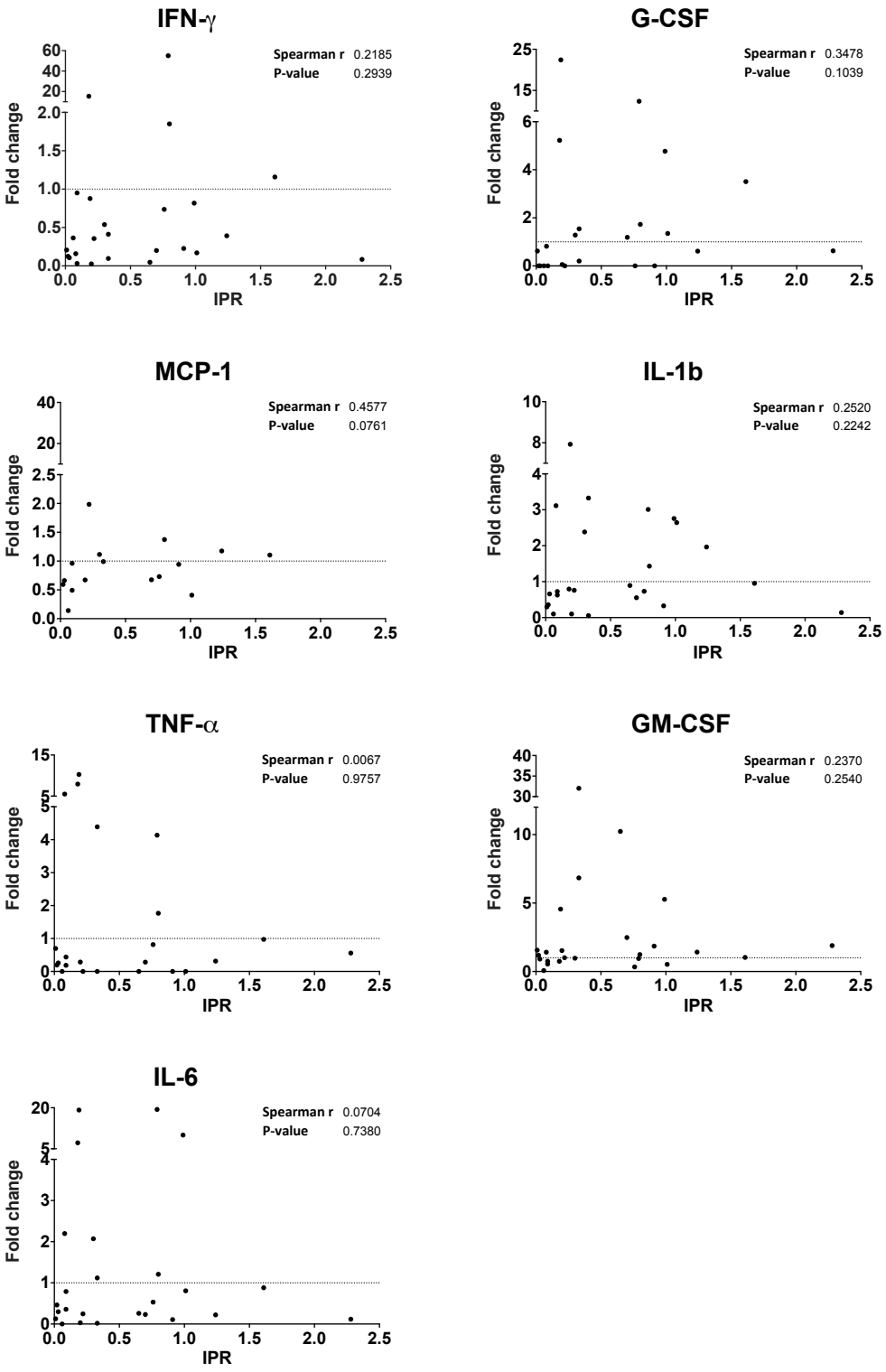


Figure 12. Correlations of fold changes in pro-inflammatory cytokines with IPR. Cytokines released over 18 hours were correlated with intracellular proliferation rates of KN99 α-GFP from donor MDMs for 13 donors. No significant associations were found between IPR and with a) IFN-γ; b) TNF-α; c) IL-1b; d) IL-6; f) G-CSF; nor g) GM-CSF. Note that only data points that crossed the detection threshold are shown hence not all graphs contain all data points.

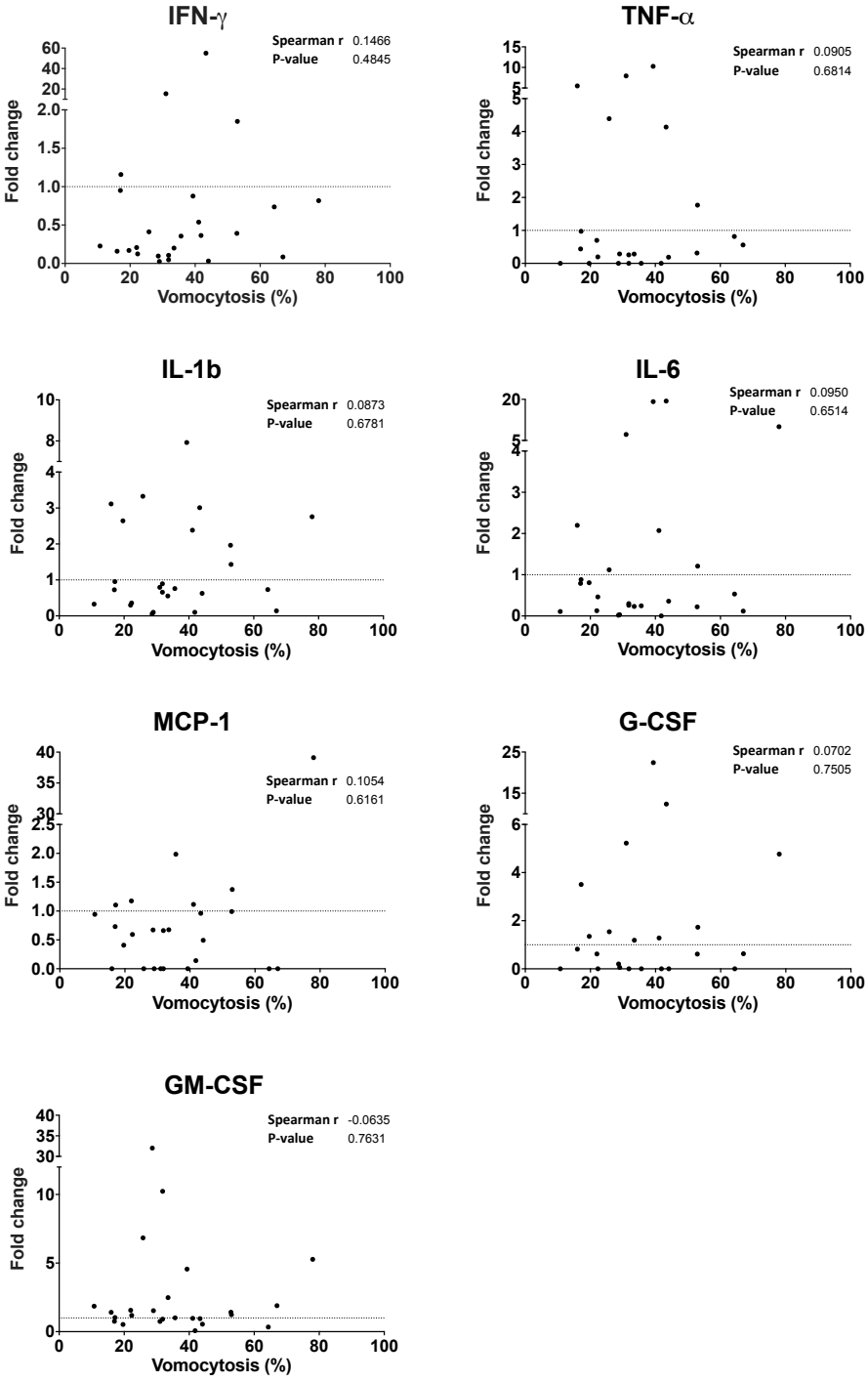


Figure 13. Correlation of pro-inflammatory cytokine profile from donor MDMs over 18-hours with non-lytic expulsion (vomocytosis) of KN99 α -GFP. No significant correlations were observed vomocytosis data from 13 donors with a) IFN- γ ; b) TNF- α ; c) IL-1b; d) IL-6; e) MCP-1; f) G-CSF; nor g) GM-CSF. Note that only data points that crossed the detection threshold are shown hence not all graphs contain all data points.

Cytokine/chemokine	IPR		Vomocytosis	
	Spearman r	P-value	Spearman r	P-value
IL-1b	0.2520	0.2242	0.0873	0.6781
IL-5	-0.2008	0.5015	-0.5989	0.0340
IL-6	0.0704	0.7380	0.0950	0.6514
IL-17	0.0332	0.9201	-0.3923	0.1795
IFN-γ	0.2185	0.2939	0.1466	0.4845
TNF-α	0.0067	0.9757	0.0905	0.6814
G-CSF	0.3478	0.1039	0.0702	0.7505
GM-CSF	0.2370	0.2540	-0.0635	0.7631
MCP-1	0.4917	0.0467	0.1054	0.6161

Table 4. Correlation data for fold changes in detected cytokines with Intracellular Proliferation Rate (IPR) and vomocytosis of KN99α-GFP from MDMs from 13 healthy donors.

We found that cytokine levels varied dramatically between samples there was no significant correlation with either vomocytosis or IPR.

To test whether vomocytosis was dependent on intracellular cytokine signaling as opposed to the above measurements of extracellular cytokines in the cell culture supernatants, we measured the amount of intracellular IFN-γ and TNF-α in two of the study participants (RG002 and RG006) between 13 and 18- hours post-infection with *C. neoformans* Kn99α-GFP (Fig. 15) as their MDMs showed relatively varied average vomocytosis rates of 58.00% and 27.60%, respectively (Fig.11).

The amounts of each proinflammatory cytokine released by each donor's infected M1 and M2 macrophages were compared to their own control MDMs that had not been activated, nor infected. For RG002, whilst similar amounts of intracellular IFN-γ were detected in test and control sample, *C. neoformans*–

infected macrophages released higher amounts of TNF-α than uninfected macrophages with M2-activated macrophages releasing the highest amounts of the cytokine (Fig. 15). In opposition, we found no differences between infected M1, M2 and uninfected macrophages in intracellular IFN-γ and TNF-α released by MDMs from RG006 (Fig. 15). Thus, donor variation in autocrine inflammatory signaling during culture contributes to, but cannot fully explain, the observed differences in vomocytosis.

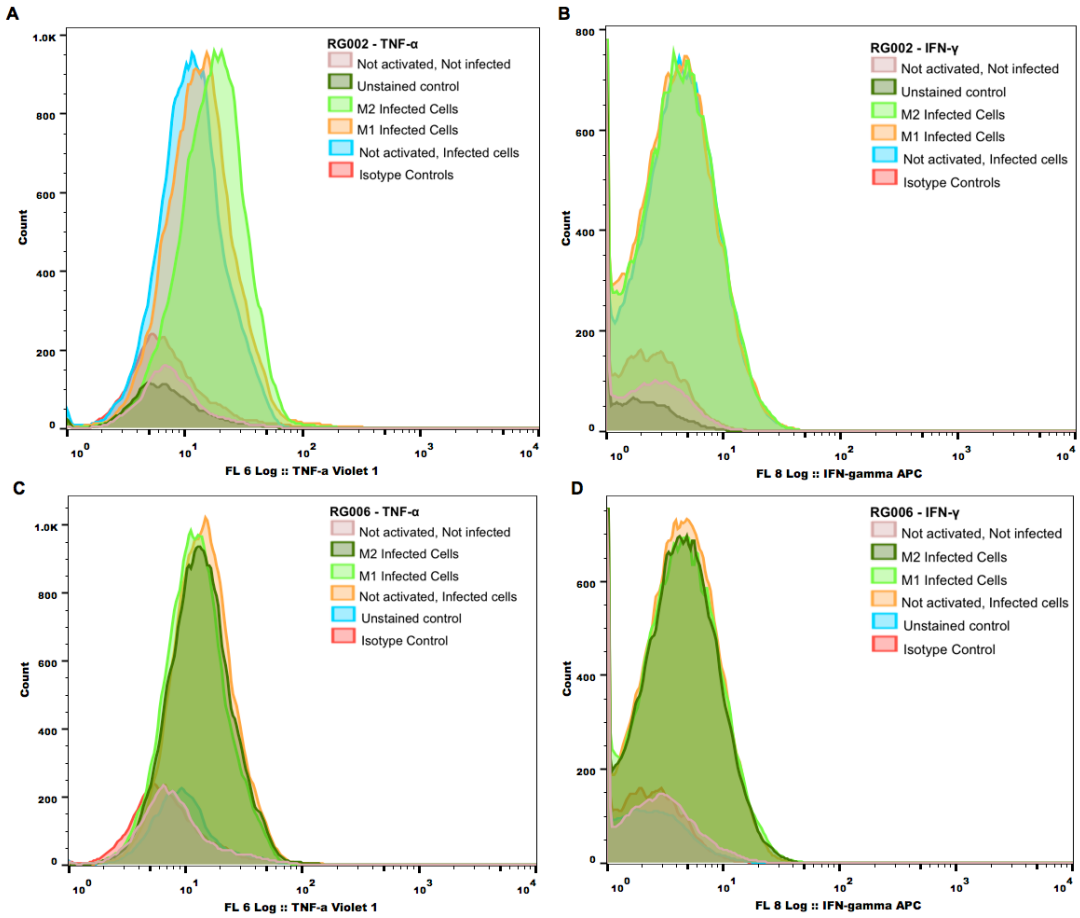


Figure 14. Intracellular cytokine measurements for M1 and M2 activated macrophages from 2 healthy donors between 13 and 18- hours post-infection with *C. neoformans* Kn99α-GFP: A) RG002 TNF-α; B) RG002 IFN-γ; C) RG006 TNF-α; and D) RG006 IFN-γ.

Variation in immunocompetent host macrophage responses to *C. neoformans* is not driven by gender

Given the established enhanced risk of cryptococcosis in men with HIV [274], but lack of gender bias in previously immunocompetent cryptococcosis patients [201] we tested whether gender impacts on these macrophage responses *in vitro*. Consistent with the latter, neither IPR nor vomocytosis rate showed variation with donor gender in this cohort (Fig. 12).

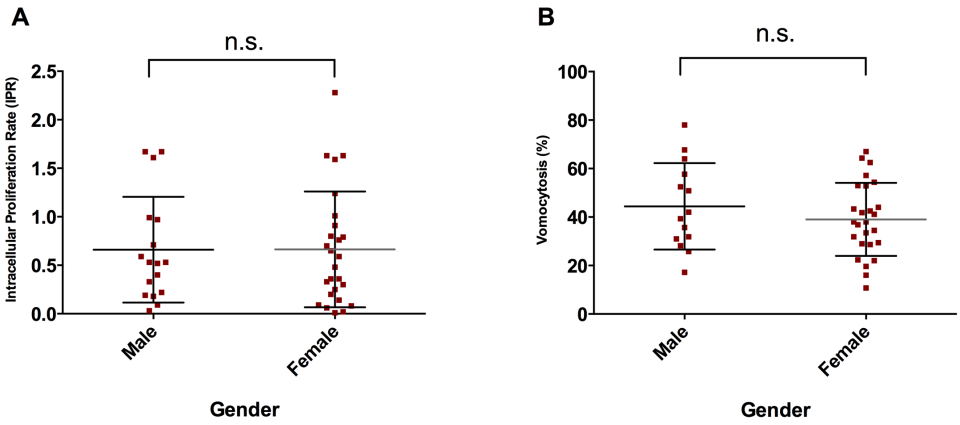


Figure 15. Analysis of gender contribution to observed variation. Two-tailed t-tests revealed no significant differences in a) intracellular virulence of KN99α-GFP (P-value = 0.9856); and b) non-lytic yeast expulsion from donor MDMs (P-value = 0.3181). Plot shows means, error bars are representative of standard deviations and n.s. states for non significant.

Power calculations

Based on the formula described in the Materials and methods section, we calculated that the sample size required to achieve a magnitude of significance for IPR and Vomocytosis was 238 and 274, respectively.

Discussion

Studies comparing clinical outcomes of different groups of cryptococcosis patients show that HIV negative patients suffer higher mortality rates than those with HIV. The high death rates in otherwise healthy individuals have been attributed to delayed diagnosis, inadequate treatment strategies, and exacerbated responses to cryptococcal infections that arise from otherwise 'intact' immune systems. Previous attempts to characterize the differences in disease presentation and outcomes between HIV positive and negative CM patients suggest that exacerbated immune responses in otherwise healthy individuals contribute to the high mortality rates in non-HIV CM patients [275].

We have compared primary immune responses to Cryptococcosis by monitoring the intracellular pathogenicity of cryptococci in host monocyte-derived macrophages – a critical cell type in anti-cryptococcal defense - from 15 healthy individuals. We have documented reproducible person-to-person variability in the rates of intracellular proliferation and vomocytosis, or non-lytic extrusion, of the pathogen; and aimed to relate these effects to varying M1/M2 cytokine responses.

In cryptococcosis patients, the ability of macrophages to phagocytose infecting cryptococci plays a crucial role in determining the outcome of the infection. The varied clinical outcomes in immunocompetent individuals raises the question of whether the immune responses mounted against cryptococci were dependent on

which opsonization strategy was utilized by macrophages in the presence of differentially opsonized *C. neoformans*. Ma et *al*. previously showed that expulsion of engulfed cryptococci was independent of the route of uptake [170]. However, whilst our data set suggest that vomocytosis may differ based on the opsonization route in some individuals. We show that there is a marked difference in individual responses to cryptococcal infection, and overall improved clearance of serum-opsonized *Cryptococcus*; however, this preliminary dataset is too small to provide statistical deductions relative to the wider population. Given that intracellular parasitism of *C. neoformans* was similar in of serum-opsonized and antibody-opsonized yeast cells (Fig. 7), and despite the similar expulsion rates of antibody- and serum- opsonized yeast cells in 4 of the 6 donors (Fig. 8); we would recommend repetition of these experiments, as we did not quantify the pooled human serum used in our investigations for anti-cryptococcal antibodies. As a majority of individuals from different geographical regions test positively for anti-cryptococcal antibodies by the age of 10 (discussed in chapter 1); it is likely that the serum used to opsonized cryptococci in our study constituted said antibodies. In order to eliminate donor-specific opsonization biases, we tested the reliability of standardized human AB serum. We found no differences in phagocytosis rates between cryptococci opsonized with autologous donor serum, and those opsonized with standardized human AB Serum. Hence, we chose to proceed with the latter in subsequent phagocytosis experiments.

We recently showed that the rate of vomocytosis may be increased via the inhibition of a MAP kinase, ERK5 [184]. However, the molecular mechanisms

that orchestrate the expulsion of this yeast from macrophages are yet to be deciphered [257]. Even within the small cohort characterized here, it is clear that vomocytosis rates show significant, intra-donor variation. Whilst there is no observable correlation with vomocytosis and IPRs at the individual level, matched, singlet data points are positively correlated (P-value= 0.0167, R^2= 0.2544). However, the results from our study depart from previous observations by Chayakulkeeree et al. that the deletion of the cryptococcal virulence factor Phosoplipase B (Plb), reduced the rate of cryptococcal expulsion from macrophages [181]. These findings suggest an inverse correlation between the two measures, and extensive, more details investigations of the relationship between intracellular parasitism and vomocytosis would need to be carried out.

In contrast, the ability of macrophages to control cryptococcal intracellular proliferation is highly variable even between samples from the same donor, suggesting that thus-far unidentified environmental factors such as allergies, seasonal flu and smoking may impact strongly on this phenotype.

Levels of key inflammatory cytokines have been shown to impact strongly on cryptococcal disease progression in HIV patients [214]. However, the results obtained from the qualitative cytokine profiling showed inconsistent cytokine profiles that were validated using the quantitative Luminex system. The data we present here shows that varying cytokine profile does not explain the *in vitro* variation in vomocytosis or IPR. The results from extracellular cytokine detection appear to be consistent with data suggesting the involvement of IFN-γ in anticryptococcal responses, but are not consistent with data showing that IFN-

γ and vomocytosis are negatively correlated [271]. These disparities may be attributed to the small sample of donors in this study that we propose is expanded to provide more reliable statistical conclusions. Hence, we conducted post-hoc power calculations based on our data and determined that the sample size required to achieve said statistical validity for IPR and vomocytosis to be 238 and 274, respectively.

Of the donors with average IPRs lower than 1 (RG003, RG004, RG005 and RG006), higher amounts of IFN-γ were released in comparison to RG001 and RG002. Although the high concentration of IFN-γ detected in the sera of RG004 and RG006 were significantly high in comparison to the rest of the group (Fig. 6), these results are representative of two independent MDM isolations (taken two or more weeks apart), and may be reflective of underlying asymptomatic inflammatory responses. The serum IFN-γ levels detected for these two individuals were high on both occasions, but did not deviate outside the range of serum IFN-γ in healthy adults.

Analysis of intracellular cytokine release of TNF-α and IFN-γ by M1 and M2 macrophages from 2 donors showed no significant differences between macrophage activation states when compared to non-activated macrophages. One particular reason for this might be the delayed addition of Brefeldin A (added at T (13)). Since macrophage responses to pathogens are rapid and occur within the first few hours of infection, it is likely that cytokine release had tapered off over the course of 13 hours. As Brefeldin A induces macrophage lysis at prolonged exposure, further optimization of this assay would be required by

blocking extracellular trafficking within 1-hour post exposure to Brefeldin A, and not more than 5 hours following exposure to the fungal pathogen.

Thus the *in vivo* impact of cytokine profile on cryptococcosis most likely does not act at the level of single macrophage/fungus interactions, and together, these data suggest that, even in immunocompetent individuals, intrinsic differences due to underlying immunogenetic defects or infections in macrophage responses may play an important role in regulating susceptibility to this fungal infection.

Vomocytosis respresents an essential mechanism in the study of cryptococcal disease as, depending on the site of infection, may serve as a vehicle of disease progression. Expulsion of cryptococci from alveolar macrophages may allow *C. neoformans* escape into the blood stream and CNS invasion. Alternatively, cryptococci may 'hitch-hike' within macrophages, and undergo vomocytosis once they have crossed the blood-brain barrier (BBB) via the Trojan horse model. Alternatively, vomocytosis may also serve as a potential therapeutic target, as induced expulsion of cryptococci from M2 macrophages following failed destruction of the fungus within the phagolysosome, may render vomocytosed fungal cells vulnerable to killing by antifungal drugs. Hence, a more in-depth understanding of this process in various models of disease pathology is necessary. Ultimately, further understanding of the basis of varied expulsion rates between 'otherwise healthy' individuals may help explain disease heterogeneity in non-HIV cryptococcosis patients and shed light on how disease progression to cryptococcal meningitis occurs in this patient group.

Chapter 3 – The role of TLR4 in the expulsion of *Cryptococcus neoformans* from host macrophages

Abstract

Among the 10 Toll-like receptors (TLRs) discovered in humans to date, TLR2 and TLR4 have been associated with the detection of fungal pathogens. During cryptococcal infections, TLR2 and TLR4 detect the cryptococcal capsular polysaccharide galactoxylomannan (GXM) in association with CD14 and CD18. The recent involvement of TLR4 in the expulsion of intracellular uropathogenic *Escherichia coli* (UPEC) within bacteria-containing vesicles (BCVs) from bladder epithelial cells (BECs) has prompted questions surrounding TLR4's association with the vomocytosis of cryptococci from macrophages. Below, we provide data associating TLR4 with the vomocytosis of *C. neoformans* from mouse bone marrow derived macrophages (bMDMs) deficient in TLR4, in comparison to their wild type Balb/c counterparts. We also investigated the effects of TLR4 on the rate of intracellular parasitism (IPR) of cryptococci within macrophages. Whilst our results showed no significant IPR differences between knockout and wild type mice, we also found that gender of wild type mice did not affect IPRs

nor vomocytosis of *C. neoformans*, corroborating previous results observed in human monocyte derived macrophages (hMDMs).

Introduction

Toll-like receptors (TLRs) are a major class of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) to activate signaling cascades and mount innate immune responses against pathogens [276, 277]. The role of these type 1 transmembrane receptors in innate immunity was first reported when Lemaitre et *al*. showed that *Drosophila melanogaster* mutants with aberrations along the Toll signaling pathway were more likely to succumb to fungal and bacterial infections such as *Aspergillus fumigatus* and Gram-positive bacteria, respectively [278]. The discovery of structural and functional similarities between the Toll signaling pathway in *Drosophila* and the intracellular pathways initiated by IL-1RI signaling in humans [279], highlighted the importance of Toll-like proteins in innate immune responses to pathogens and inspired more extensive study [277]. Currently, 10 toll-like receptors are known in humans (TLR 1-10), each responsible for the recognition of specific ligands [277, 280].

These receptors carry out their function either as homodimers, or through cis- or trans- heterodimerization with other TLRs or cell surface receptors. Whilst TLR4 homodimerizes [282]; TLR2 is able to form dimers with either TLR1 or TLR6

[276, 281, 282]. Their ability to homodimerize or hetrodimerize influences the outcome and intensity of immune reactions [282]. Studies of knockout mice have revealed that the main TLRs associated with fungal pathogen recognition are TLR4 (previously shown to detect LPS of Gram-negative bacteria), and TLR2 (binds lipoteichoic acid, bacterial lipoproteins and zymosan) [276, 281].

However, other TLRs may be involved in the detection and engulfment of *C. neoformans*. Redlich et al. showed that stimulation of TLR1/2, TLR3, TLR4 and TLR9 with their agonists in murine microglial cells – the resident macrophages in the CNS- increased the phagocytosis of *C. neoformans* [283]. Induction of these receptors activated microglial cells and increased the release of TNF-α, CXCL1 (KC), IL-6, IL-10 and MIP-2, and increased the elimination of intracellular cryptococci. In line with other investigations, MyD88-deficienT-cells showed impaired ability to phagocytose *C. neoformans* than wild-type cells. These findings provide an insight to CNS-associated cryptococcosis, and how infection may be eliminated in the brain in cases of disseminated disease [283].

Further investigations into the immune recognition of Cryptococcus spp. have shown that both TLR2 and TLR4 associate with CD14 [138] and CD18 [284] to bind the cryptococcal capsular polysaccharide glucuronoxylomannan (GXM), driving localized immune recognition and enhanced phagocytosis [62, 139-141, 284]. Activation of TLR2 and TLR4 leads to the activation of the NF-κB signaling pathway (described in chapter 1), which modulates the expression of proinflammatory cytokines by phagocytes to induce killing of the pathogen. Shoham et *al.* transfected Chinese hamster ovary fibroblasts with human TLR2,

TLR4, and/or CD14; and showed that the NF-κB pathway was activated when these fibroblasts, as well as PBMC and RAW 264.7 cells transfected with both CD14 and TLR4 bound fluorescently labeled cryptococcal Galactoxylomannan (GXM) [138], resulting in nuclear translocation of NF-κB. However, the mitogen-activated protein kinase pathways that leads to the production of the proinflammatory cytokine TNF-α was not induced in these experiments [138], suggesting more complex signaling modifications along the pathway.

Following the association of these toll-like receptors with GXM recognition, Yauch et *al.* Showed that the TLR-associated adaptor protein MyD88 plays a crucial role in response to cryptococcal infection [141]. MYD88 knockout (-/-) mice were significantly less likely to survive intranasal (IN) and intravenous (IV) infection with *C. neoformans* in comparison to wild-type C57BL/6 mice [141].

Activation of TLR4 elicits the production of either pro-inflammatory cytokines or Type I interferons and, more recently, has been associated with modulating the intracellular trafficking system [285]. Miao et *al*. showed that TLR4 was able to enhance the expulsion of intracellular uropathogenic *E. coli* (UPEC) within bacteria-containing vesicles (BCVs) from bladder epithelial cells (BECs); and demonstrated that this process occurs via a unique modification of the downstream element TRAF3 – a member of the TNF receptor associated factor (TRAF) protein family – that led to the activation of exocyst complex assembly [285]. The group also showed that the TIR-domain-containing adapter-inducing interferon-β (TRIF), and the TRIF-related adaptor molecule (TRAM) adapter proteins were also involved in TLR4-mediated expulsion of UPEC from bladder

epithelial cells or mouse bladders, as they were able to localise to UPEC-containing vacuoles [285].

Cryptococcus spp. have been described to undergo extrusion from within phagosomes in a process known as vomocytosis [169, 170]; however, it is unknown if the above mechanism is also involved in the expulsion of cryptococci from macrophages. Therefore, we aimed to investigate whether TLR4 plays a role in *C. neoformans* vomocytosis, with a hope that further understanding of this mechanism will inspire the design of new therapeutic approaches to cryptococcal infections.

Materials and Methods

Maintenance and euthanasia of TLR4 knockout animals and wildtype controls was done in accordance with the Animals (Scientific Procedures) Act of the UK and under the licence and oversight of the Biomedical Services Unit (BMSU) at the University of Birmingham. All the work reported here was conducted on ex vivo tissues obtained from uninfected animals killed by an approved Schedule 1 method.

Isolation of bone Marrow Derived Macrophages

Bone Marrow Derived Macrophages (bMDMs) were extracted from 16 age-matched homozygous TLR4 knockout (-/-) mice, as well as from Balb/c wild type mice (4 wild type males, 4 wild type females, 4 TLR4 knockout -/- males and 4 TLR4 knockout -/- females). Following extraction, cells were counted and seeded at a concentration of $1x10^6$ cells/well in RPMI 1640 (with 100 U/ml streptomycin, 100 U/ml penicillin, 10% FBS and 10% L929 cell culture media) and incubated at 37 °C, 5% CO_2 . At day 3 and 6 post-extraction, cells were maintained in RPMI 1640 (with 100 U/ml streptomycin, 100 U/ml penicillin, and 10% FBS). bMDMs were challenged as described in the phagocytosis assay (Chapter 2).

Cryptococcus Infection of bMDMs

C. neoformans var. grubii serotype A, strain KN99α expressing GFP [286] was used in all macrophage challenge experiments.

An overnight culture of the yeast was started by inoculating 3 mL of YPD (10 g/L yeast extract, 20 g/L bacteriological peptone, and 20 g/L glucose (Sigma-Aldrich) media, and incubated on a rotor at 20 rpm at 25 °C, prior to conducting the phagocytosis assay. In preparation for phagocytosis, yeasT-cells were washed in PBS, counted on a haemocytometer, and opsonized with mouse IgG anti-Crypto18B7 (18B7) antibody. bMDMs where then infected with $1x10^6$ yeasT-cells per well (MOI 10:1), and incubated at 37°C, 5% CO_2 for 2 hours. At 0 (T0) and 18 (T18) hours post-infection, extracellular yeasT-cells were washed away using PBS and macrophages containing yeasT-cells were lysed in dH_2O at 37 °C, 5% CO_2 for 30 minutes. For live imaging to quantify vomocytosis, bMDMs were washed at T0, fresh serum-free RPMI added to infection wells and taken for imaging.

CFU counts

Serial dilutions of the lysate from the phagocytosis assay were prepared and plated onto 2% YPD, 1% agar (Sigma-Aldrich) plates then incubated for 48 hours at 25°C. Intracellular proliferation rates were measured by dividing the number of counting colony-forming units per milliliter at T18 by those at T0.

Live cell imaging

Time-lapse imaging was performed on bMDMs from 8 mice (2 wild type males, 2 wild type females, 2 TLR4 knockout -/- males and 2 TLR4 knockout -/- females). All time lapse images were captured on a Zeiss Axio Observer Live cell-imaging microscope enclosed within a humidified Okolab microscope chamber set at 37°C, 5% CO_2, a Hamamatsu digital camera, LD Plan-Neofluar 20x/0.4 Korr Ph 2 M27 objective, 38 HE Green Fluorescent reflector, using Zen software (Zeiss). 217 frames (1 frame, every 5 minutes for 18 hours) were taken from four different positions within each well to produce movies for manual analysis.

Power calculations

In order to assess the sample size that would generate statistically relevant results, we conducted a power calculation in order to identify the required population size “n”, necessary for adequate power. In order to do this, we used the formula below; where “z” is the critical value of the normal distribution for a confidence level of 95% with a critical value of 1.96; and “e” is the margin of

error of 0.05; and p is the sample proportion. The value of "p" was derived from the population the either had an IPR below 1, or vomocytosis rate above the 75% confidence interval (CI).

$$n = \frac{z^2 \times \hat{p}(1-\hat{p})}{\varepsilon^2}$$

Results

Bone Marrow Derived Macrophages (bMDMs) from 5 wild type Balb/c mice (1:1 male: female ratio), and 5 TLR4 knockout -/- Balb/c mice (1:1 male: female ratio) were challenged *in vitro* with the cryptococcal strain KN99α-GFP. CFU counts were used to extrapolate the rate of intracellular parasitism (IPRs) of KN99α-GFP within bMDMs (Fig. 16, Fig. 18); and vomocytosis was measured as the percentage of intracellular cryptococci expelled from macrophages over the 18-hour period in all well positions (Fig. 17, Fig. 19). To evaluate the impact of gender on the above data, we divided our test group of mice into male and female to assess where hormonal differences influenced cryptococcal intracellular parasitism (IPR) and vomocytosis. Vomocytosis data is only available for 8 mice (2 male controls, 2 female controls, 2 male TLR4 -/-, and 2 female TLR4 -/-).

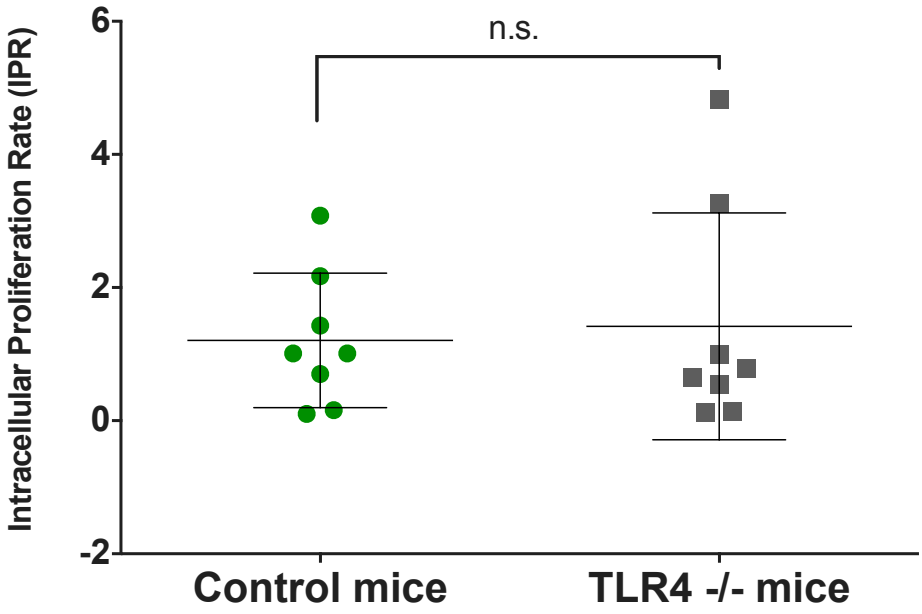


Figure 16. Comparison of cryptococcal intracellar parasitist in control and knockout TLR4 mice within Bone Marrow Derived Macrophages (bMDMs). No significant differences in IPRs observed (2-way ANOVA; p = 0.7345). Error bars represent mean and standard deviations where for control mice N=8 and TLR4 -/- mice N=8.

Two-way analysis of variance to determine whether genetic background or gender had an influence on the intracellular parasitism of *C. neoformans* within murine bMDMs was performed. Our results show no significant difference between IPRs recorded for TLR4 -/- mice in comparison to control mice (Fig. 16; p = 0.7345) where genetic background contributed to 0.64% of the variation. However, we found that gender significantly contributed to the variation in IPR values (Fig. 17; 2-way ANOVA; p = 0.0255) and accounted for 34.55% of the variation in our data.

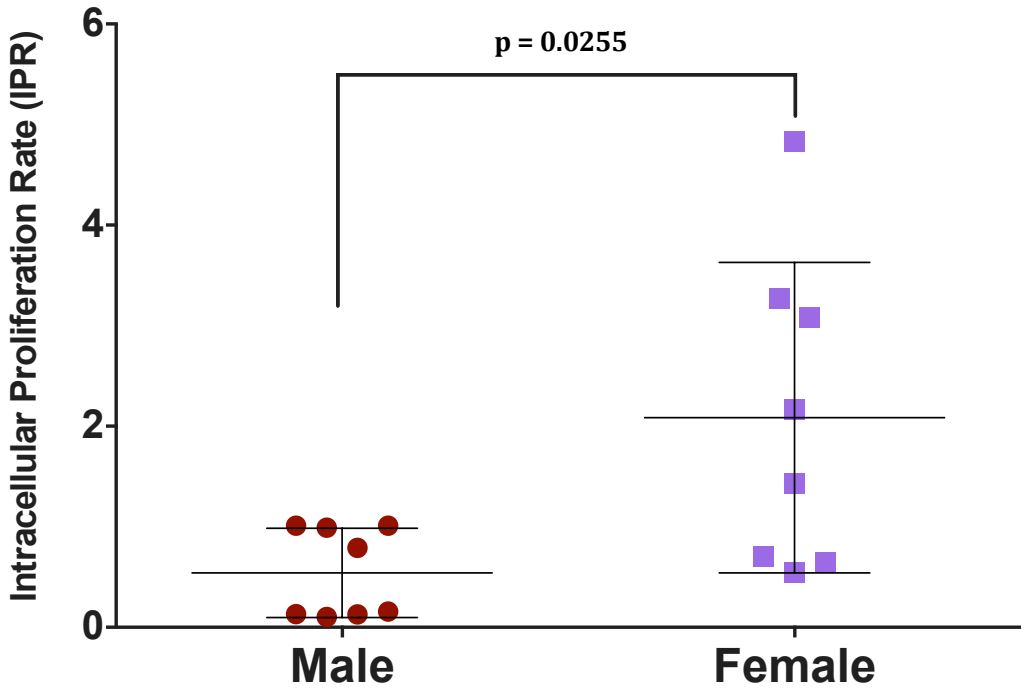


Figure 17. Comparison of cryptococcal intracellar parasitism in Bone Marrow Derived Macrophages (bMDMs) from male and female Balb/c mice. Both populations consist of a 1:1 ratio of wild-type and TLR4 knockout -/- Balb/c mice. Two-way ANOVA revealed significant differences between male and female control mice (p = 0.0255). Error bars represent standard deviations where N=8 for both sets of data.

Conversely, we found that vomocytosis of Cryptococci from TLR4 -/- bMDMs was significantly reduced in comparison to control bMDMs (Fig. 18; 2-way ANOVA; p = 0.0198) and genetic background contributed to 72.66% of the variation. Interestingly, gender did not contribute to any of the observed variation and no significant differences were observed between male and female mice (Fig. 19; 2-way ANOVA: P> 0.9999).

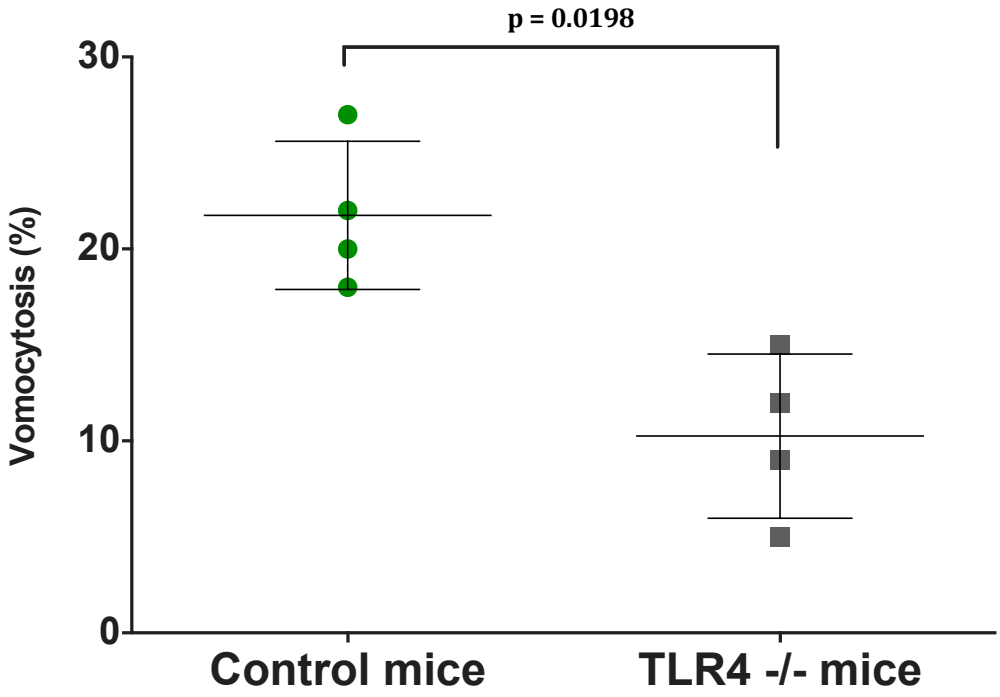


Figure 18. Vomocytosis of *C. neoformans* from Bone Marrow Derived Macrophages (bMDMs) from wild type and TLR4 knockout -/- Balb/c mice. Results show a significant difference between both groups (2-way ANOVA; p = 0.0198). Error bars represent means and standard deviations. Where N=4 for both control and TLR4 -/- mice.

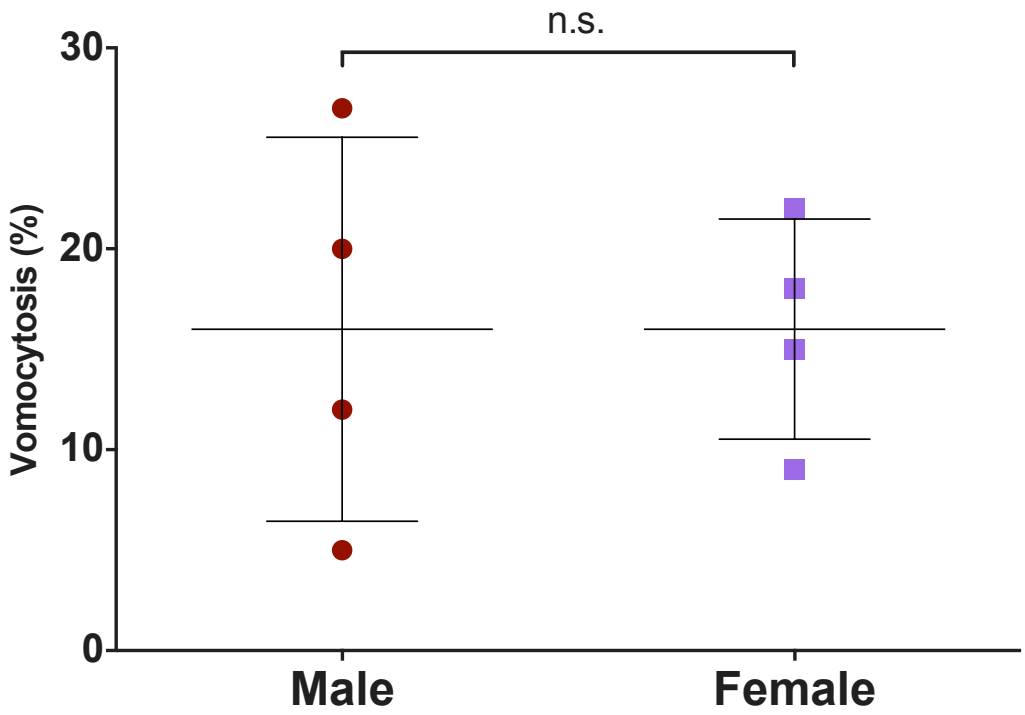


Figure 19. Vomocytosis of C. neoformans from Bone Marrow Derived Macrophages (bMDMs) from wild type Balb/c mice and TLR4 knockout -/- Balb/c mice. Two-way ANOVA showed no significant differences between male and female control mice (P>0.9999). Error bars represent standard deviations.

Elimination of TLR4 reduced the rate of cryptococcal extrusion from within murine bMDMs by approximately 50%, suggesting a role for this toll-like receptor in the vomocytosis of *C. neoformans*.

Power calculations

Based on the formula described in the Materials and methods section, we calculated that the sample size required to achieve a magnitude of significance for IPR and Vomocytosis for control mice was 360 and 288, respectively; while that required for IPR and Vomocytosis for knockout mice was 288 and 341, respectively.

Discussion

We have probed the involvement of TLR4 in controlling the intracellular parasitism and vomocytosis of *C. neoformans* KN99α-GFP in bMDMs from wild type Balb/c mice and TLR4 knockout (-/-) Balb/c mice. Whilst our data suggest that elimination of TLR4 bears no effects on IPRs between control and test mice bMDMs, we have found a significant difference in the effects of gender on IPR. Conversely, gender did not influence vomocytosis, but we show that the elimination of TLR4 reduces the rate of cryptococcal extrusion from bMDMs by ~2-fold.

Our finding that a gender bias exists in the control of intracellular parasitism is not novel. However, it is contrary to what is currently known of gender susceptibility to cryptoccocosis. Men have been shown to be more prone to fatal cryptococcal disease than women. Given the small sample size of male (N=8) and female (N=8) mice, we strongly recommend increasing the number of test subjects in order to provide more reliable results.

A recent review by Campuzano and Wormley discussed the contribution of PRRs to cryptococcal disease elimination in innate immune cells [287]. The authors referred to studies using TLR4-knockout mice, which showed that TLR4 was not an essential component in anti-cryptococcal immunity [138, 141, 288]. Conversely, Shoham et *al.* showed that both CD14 and TLR4 bound fluorescently labeled GXM [138], resulting in nuclear translocation of NF-κB but no activation

of the mitogen-activated protein kinase pathways that lead to the production of the proinflammatory cytokine TNF-α [138]. In 2005, Yauch et *al.* showed that whilst TLR2, TLR4, CD14 and CD18 were able to detect cryptococcal GXM *in vitro*, these PRRs were not required for serum clearance *in vivo* [289]. The disparities in data surrounding the involvement of TLR4 in the detection and expulsion of *C. neoformans* from macrophages will therefore need to be settled through further experimentation using both *in vitro* and *in vivo* models of disease.

Whilst out data is in line with previous studies by Miao et al., the sample size used to evaluate differences in vomocytosis of *C. neoformans* from macrophages is too small to conclude the involvement of TLR4 in cryptococcal expulsion. Our power calculations predicted that the sample size required to achieve a magnitude of significance for both IPR and Vomocytosis for control mice was 360 and 288, respectively; while that required for IPR and Vomocytosis for TLR4 knockout mice was 288 and 341, respectively. In addition, Balb/c mice have previously shown to be resistant to *C. neoformans* [186]. Hence, we recommend repetition of these results in alternative mouse strains to confirm our findings.

A report by Redlich et *al.* where murine microglial cells were stimulated with TLR agonists for TLR1/2, TLR3, TLR4 and TLR9 for 24 h showed that phagocytosis and pro-inflammatory cytokines secretion (TNF-alpha, CXCL1 (KC), IL-6, IL-10 and MIP-2) were increased [283]. Interestingly, they also found that Intracellular killing of cryptococci was also increased in TLR-stimulated cells compared to unstimulated microglial cells [283]. Whilst TLR4 may not be required for serum clearance of *C. neoformans in vivo*, intracellular TLR4 may play a different role following phagocytosis of the yeast that culminates in either cryptococcal killing or expulsion from macrophages. The increased proinflammatory response mounted following TLR4 stimulation with an agonist recommends it as a potential supplementary treatment, particularly in cryptococcosis patients receiving immunosuppressive therapies.

Furthermore, two polymorphisms within the TLR4 gene (rs4986790 and rs4986791) have been implicated as predispositions for acquiring bloodstream *Candida* infections in humans (Discussed in Chapter 4) [290]. We hypothesize that individuals facing the phenotypic consequences of these polymorphisms would present with impaired phagocytosis and intracellular killing of *C. neoformans*, as well as reduced anti-cryptococcal inflammatory responses.

Given the complexity of immune response pathways associated with cryptococcal infection, and the compensatory responses to sensory defects observed in knockout experiments, further, more detailed study of the molecular patterns that orchestrate the extrusion of *C. neoformans* from macrophages

would be required as TLR4 can transduce signals via either the MyD88-dependent or TRIF-dependent pathways [291].

Altogether, our data advocate TLR4 as a component in the vomocytosis of cryptococci; however, further investigation of this receptor's involvement in this process is highly warranted. Our data has been generated from a small group of test mice (16 for IPR; 8 for vomocytosis) that would need to be expanded for more reliable statistical inferences to be made. We also recommend that further *in vitro* experimentation be carried out on alternative macrophage cell lines; as well as *in vivo* study of infection progression in TLR4 knockout model organisms compared to wild type individuals. We anticipate these findings will lend to personalizing treatment strategies for the diverse range of cryptococcosis patients worldwide.

Chapter 4 – Contribution of single nucleotide polymorphisms to variation in host macrophage responses

Abstract

Cryptococcosis is a fungal disease that affects immunocompromised and immunocompetent individuals following infection with *Cryptococcus neoformans.* Macrophages play a critical role in anticryptococcal defense by either eliminating or disseminating the pathogen. We have previously established that macrophage responses to *in vitro* infection with *C. neoformans* vary between and within individuals; however, the determinants of the observed variance remain unknown. GWAS studies have demonstrated a genetic basis for variation in many parameters of innate immune cells in healthy adults; and susceptibility studies have identified single nucleotide polymorphisms (SNPs) that predispose otherwise healthy individuals to invasive fungal diseases including cryptococcosis. Consequently, we hypothesized that genetic variation in known innate immune genes may underlie some of the person-to-person variation were previously reported. Below, we assess the contribution of SNPs in

some of these genes (Toll-like receptor 2 (TLR2); TLR4; the C-type Lectin, Dectin-1; the mitogen-activated protein (MAP) kinase, ERK5; and the autophagy protein, Galectin-8 (GAL8)) to regulating variation in macrophage control of intracellular parasitism and vomocytosis of *C. neoformans*. Although we found one SNP (TLR4-rs4986791) among 9 healthy adults, we conclude that larger GWAS studies would be required to verify the association of these polymorphisms with susceptibility to cryptococcosis in immunocompetent hosts.

Introduction

To date, cryptococcosis has largely been described as an HIV-associated infection that leads to cryptococcal meningitis and subsequent death in patients. However, the rising frequency of this fungal disease due to *C. neoformans* in otherwise healthy individuals is cause for concern. Efforts to understand disease predispositions in immunocompetent individuals are underway.

We have previously sought to quantify the varied responses to cryptococcosis in healthy adults by assessing *in vitro* macrophage responses to cryptococcal infections including control of intracellular parasitism, rate of vomocytosis, cytokine responses and gender bias. However, our findings suggest that genetic factors along with the host's immune environment, rather than individual cellular responses may confer susceptibility or protection from acquiring CM. Findings from Patin et *al.* support this concept by showing that natural variation in the parameters of innate immune cells is predominantly driven by genetic factors rather than environmental determinants [292].

The majority of studies that have attempted to decipher the genetic predisposition to cryptococcosis have focused mainly on HIV patients [255], and data describing risk factors in healthy individuals is rare and non definitive. A review by Lanternier et al., suggested that inborn genetic defects of the IFN-γ and/or GM-CSF signaling pathways played a larger role in predisposition to fungal infections than is currently perceived. Some of the immune deficiencies noted in non-HIV cryptococcosis patients included idiopathic CD4 lymphopenia, AD GATA2 deficiency, and in a few cases X-linked CD40L deficiency [293]. More recent studies have attempted to identify small nucleotide polymorphisms that may predispose individuals to cryptococcosis [294]. To-date, defects in the Fc gamma receptor (FCGR) [217, 295]; IL-12 signaling and Interleukin 12 Receptor Subunit Beta 1 (IL-12RB1) cell surface expression [296]; and Mannose-binding lectin (MBL) [297] have been associated with CM.

We previously showed that the rate of vomocytosis could be modulated via the inhibition of the mitogen-activated protein (MAP) kinase, ERK5 [184] that is encoded by the MAPK7 gene; and that TLR4 plays a role in limiting the expulsion of engulfed cryptococci from within macrophages (Chapter 3). Genome wide studies have provided evidence that that two synonymous mutations (rs2233083 and rs3866958) within MAPK7 are associated with increased risk of lung cancer amongst smokers [298]; and that two polymorphisms within the TLR4 gene (rs4986790 and rs4986791) present as risk factors for *Candida* bloodstream infections [290], and were shown to increase susceptibility to invasive aspergillosis in donors and recipients of hematopoietic cell transplants

[299]. Hence, we hypothesized that the presence of these SNPs may influence the rate of vomocytosis observed between individuals and explain the observed variance (Fig. 20).

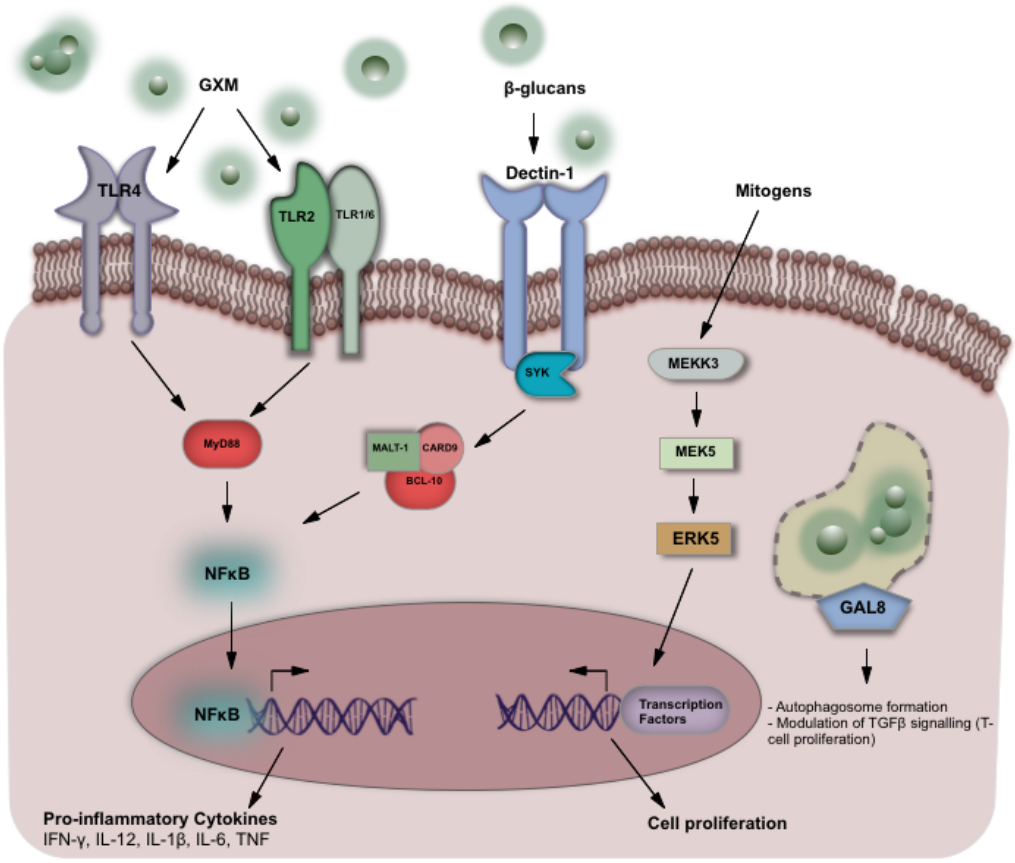


Figure 20. Illustration of pattern recognition receptors (PRRs) and proteins previously associated with cryptococcosis with known small nucleotide polymorphisms (SNPs).

Macrophage cell surface receptors interact with cryptococcal pathogen associated molecular patterns (PAMPs) to bring about phagocytosis and antifungal responses. The C-type lectin receptor (CLR) Dectin-1, encoded by CLEC7A, along with the CD11b receptor, interact with cryptococcal β-glucans to induce phagocytosis and increased elimination of the fungal pathogen[62]. In addition, the toll-like receptors TLR2 and TLR4 detect cryptococcal galactoxylomannan (GXM) embedded in the capsule, triggering intracellular

signaling and inflammatory responses in macrophages to eliminate engulfed cryptococci [138, 276, 300]. Despite previous findings that Dectin-1 was not required for protection against cryptococcosis in mice [301], SNPs in Dectin-1 (rs16910526) and in TLR2 (rs121917864, and rs5743708) have been shown to predispose patients with Acute Myeloid Leukemia (AML) to invasive fungal disease [302].

Galectins are a class of soluble proteins that bind beta-galactoside sugars (β-galactoside) and function both intra- and inter-cellularly [303, 304]. Galectin-8 (GAL8), encoded by the LGALS8 gene, is a secreted protein that associates with integrin-bound glycans to activate integrin-specific signaling pathways and ultimately modulate cell adhesion [305]. This galectin also plays a role in autophagy where it has been shown to act as a 'danger' receptor that restricts *Salmonella* proliferation [306] and detects damages in lysosomes and endosomes caused by engulfed pathogens [306]. Given that *C. neoformans* is able to manipulate host phagosome maturation and bring about phagosomal permeabilization [122, 143], allowing for pathogen proliferation and evasion of killing via vomocytosis [169, 170, 176]; we pondered the effects of the insertion/deletion variants (rs35962423 and rs60283046) in LGALS8 on intraphagosomal cryptococcal survival (Fig. 17).

Therefore, we questioned the extent to which the above nucleotide changes may influence or limit macrophage control of the intracellular parasitism and non-lytic expulsion of Cryptococcus spp. and contribute to the varied vomocytosis rates observed within and between our group of healthy volunteers (Chapter 2).

To assess this, we analyzed DNA taken from 9 consenting healthy donors for the presence of the above polymorphisms. Our search revealed that all 9 participants carried the major alleles within the regions of interest, in exception of one non-synonymous missense mutation in TLR4 (rs4986791) in one individual; and conclude that there was no association between the above polymorphisms and observed variation.

Materials and Methods

Consent for DNA analysis was obtained from 9 of the donors in this study (RG001-RG007, RG015 and RG016).

GENOMIC DNA EXTRACTION

The Wizard Genomic DNA Purification kit (Promega, A1120) was used to extract genomic DNA from 3, 300 µl whole blood aliquots of 9 donors (RG001-RG007, RG015 and RG016) in accordance with the manufacturer's protocol. A 1% agarose gel was used to confirm successful DNA extraction.

Primer design and PCR amplification

All primer sequences used to assess TLR2, TLR4, Galectin-8, Dectin-1 and ERK5 SNPs were designed by using the NCBI/ Primer-BLAST tool and are presented in Table 1 below. The regions encompassing the SNPs described above were obtained from the NCBI via a blast of the Human nucleotide databases. Due to the large number of base pairs spanning the polymorphisms for ERK5, TLR2 and GAL8 we wished to sequence, two sets of Forward and Reverse primers (Table 1.) were designed in order to amplify donor SNP regions as two smaller DNA fragments for each respective gene. The Phusion High-Fidelity DNA Polymerase kit (New England BioLabs, M0530S), and DreamTaq Green PCR Master Mix (2X) (Life Technologies, K1082) were used to amplify the two regions of interest following the manufacturer's recipe and cycle steps, and a 1% agarose gel was

used to check for correct amplification. The PCR products for each primer set were analyzed on a 1% agarose gel stained with Sybr Safe (Invivogen), and purified using Qiagen's QIAquick PCR Purification Kit.

Fragment sequencing and analysis

For sequencing, we used the cycle sequencing technology provided by Eurofins Genomics on an ABI 3730XL sequencer. Sequence data was visualized using 4Peaks software, version 1.8; and the Clustal X software version 2.1 was used to generate multiple sequence alignments. We thereafter used Seaview, version 4.6.1 to identify if any of the donors expressed any or all of the polymorphisms in question.

Pattern Recognition Receptor	SNP ID	SNP Details	Location	Global Minor Allelle Frequency	Primer	Sequence
TLR2	rs121917864	C>T missense	Intron	0.2849	F'	5'- CAGATGCTTTCTTCCCCTTTGAGA -3'
					R'	5'- CGGAAATGGGAGAAGTCCAGT -3'
TLR2	rs5743708	A>G missense	5' Near Gene	0.0068	F'	5'- TACAGTGAGCGGGATGCCT -3'
					R'	5'- TATCGCAGCTCTCAGATTTACCC -3'
Dectin-1	rs16910526	T>G missense	3' Near Gene	0.0409	F'	5'- TGCAGCTAGTAGCAGTTCTTG -3'
					R'	5'-CTCCACCCTTCCTCTTACATTGA -3'
ERK5	rs2233083	C>T Synonymous	Exon	0.0272	F'	5'- GTTCTCAGGCACACCAAAGG -3'
					R'	5'- AAGGATTGTTCAGGGGGAGTTG -3'
ERK5	rs3866958	G>T	Promoter	0.1837	F'	5'- CGAACCCTCCACTGACTTCC -3'
					R'	5'- CCTTTCCTTCCAGCTCACAGT -3'
TLR4	rs4986790	A>G missense	Exon	0.0599	F'	5'- GCACAGACTTGCGGGTTCTA -3'
					R'	5'- CCTGGAAAGAATTGCCAGCC -3'
TLR4	rs4986791	C>T missense	Exon	0.0407	F'	5'- GCACAGACTTGCGGGTTCTA -3'
					R'	5'- CCTGGAAAGAATTGCCAGCC -3'
GAL8	rs35962423	C>T indel	5' Near Gene	0.4938	F'	5'- TGTTTTTCCTGCTTCCCCCA -3'
					R'	5'- CTGTAGTGGGAAACCTCGCA -3'
GAL8	rs60283046	G>T indel	5' Near Gene	0.4918	F'	5'- GGAGTTCTTTAGGAGCCAGGT -3'
					R'	5'- AGAAGCCACTAGGCCAAACC -3'

Table 5. Details of small nucleotide polymorphisms in pattern recognition receptors under investigation for association with cryptococcosis in healthy individuals.

Results

GENOMIC DNA EXTRACTION

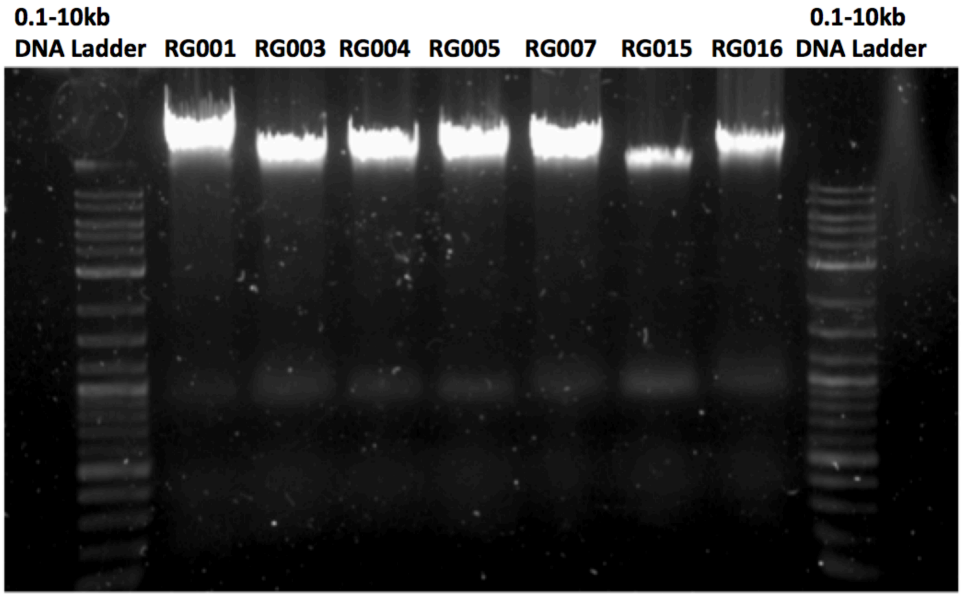


Figure 21. Determination of successful genomic DNA extraction for 8 healthy donors on 1% agarose gel. Genomic DNA for RG002 not shown.

We successfully obtained genomic DNA from 8 consenting donors (Fig. 18) initially; genomic DNA for RG002 (not shown in Fig. 18) was obtained later.

SNP Fragment Amplification

To assess whether we had obtained the correct PCR fragments for each SNP, we ran the purified PCR products on 1% agarose gel stained with Sybr Safe (Invivogen). Predicted fragments for all SNPs under investigation were obtained; Fig. 19-20 show representative gel images for ERK5 rs2233083 and ERK5 rs3866958, respectively.

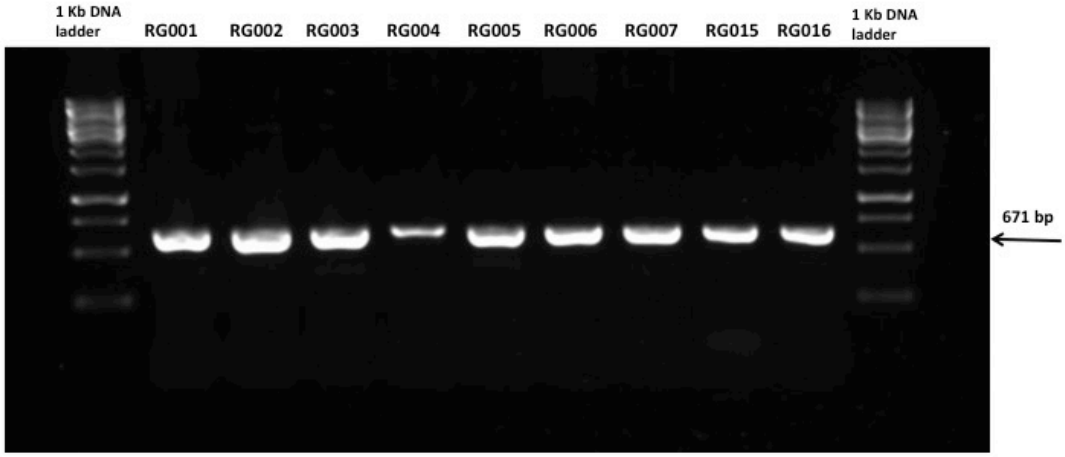


Figure 22. Purified PCR amplicons for ERK5 rs2233083 from 9 healthy donors.

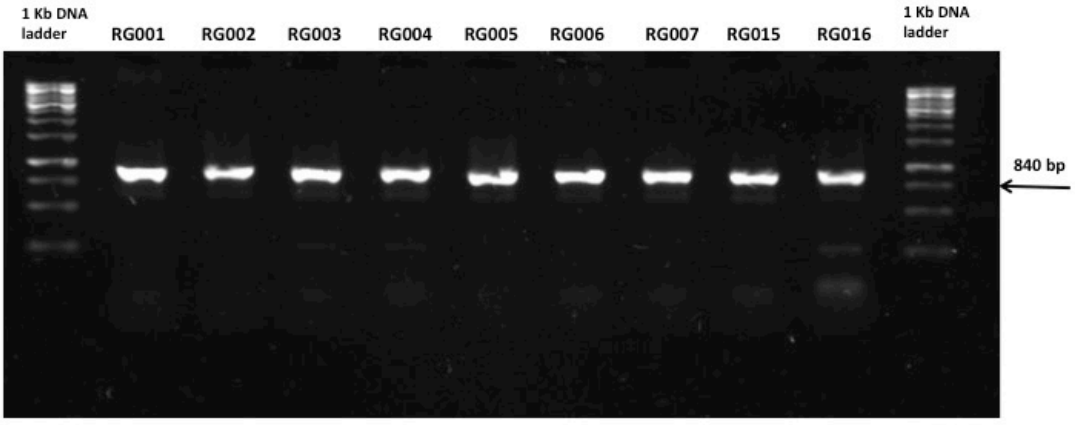


Figure 23. Purified PCR amplicons for ERK5 rs3866958 from 9 healthy donors.

SNP DETECTION

To check whether each of the 9 donors in our study carried any of the above SNPs, we generated multiple sequence alignments using Clustal X (Fig. 21-25).

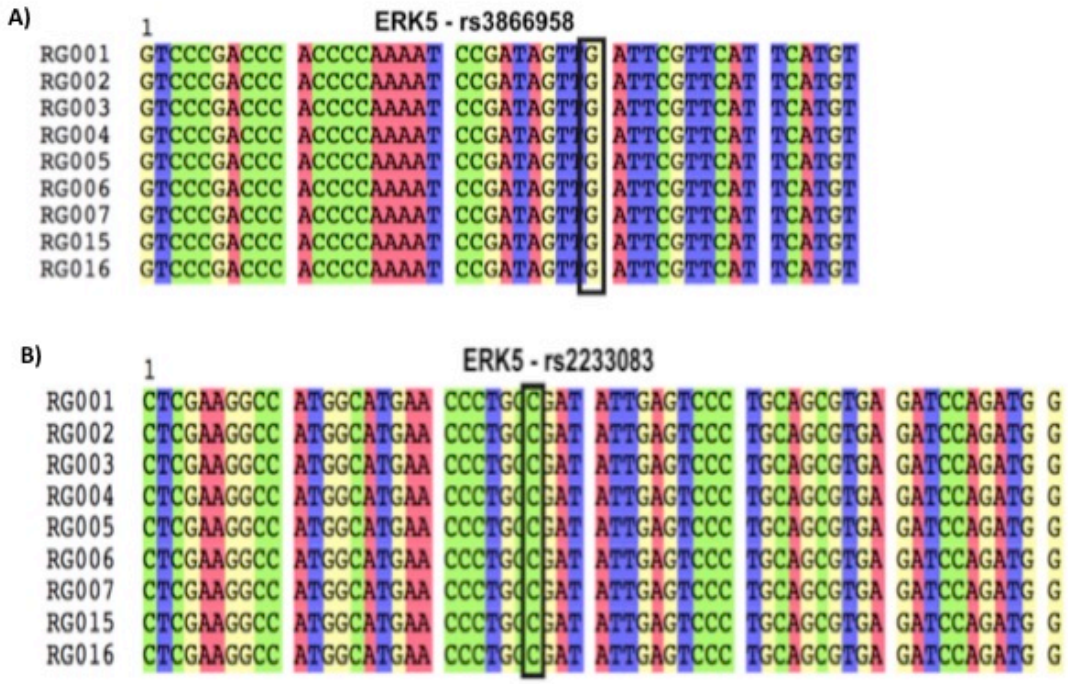


Figure 24. Multiple sequence alignment analysis for 9 healthy donors to detect the presence of: A) ERK5-rs3866958; and B) ERK5- rs2233083. SNP regions of interest are highlighted.

Our results show that donors possessed major alleles for both ERK5 SNP regions (Fig. 21); both TLR2 SNPs (Fig. 22); both TLR4 SNPs (Fig. 23); Dectin-1 (Fig. 24); and in both Galectin-8 SNPs (Fig. 25). The TLR4 SNP rs4986791 was only detected one donor (RG002; Fig. 23). However, our findings are inconsistent with the relatively low minor allele frequencies based on the 100,000 genomes project described above (Table 5), suggesting that our data departs from statistical projections due to the small number of donors tested.

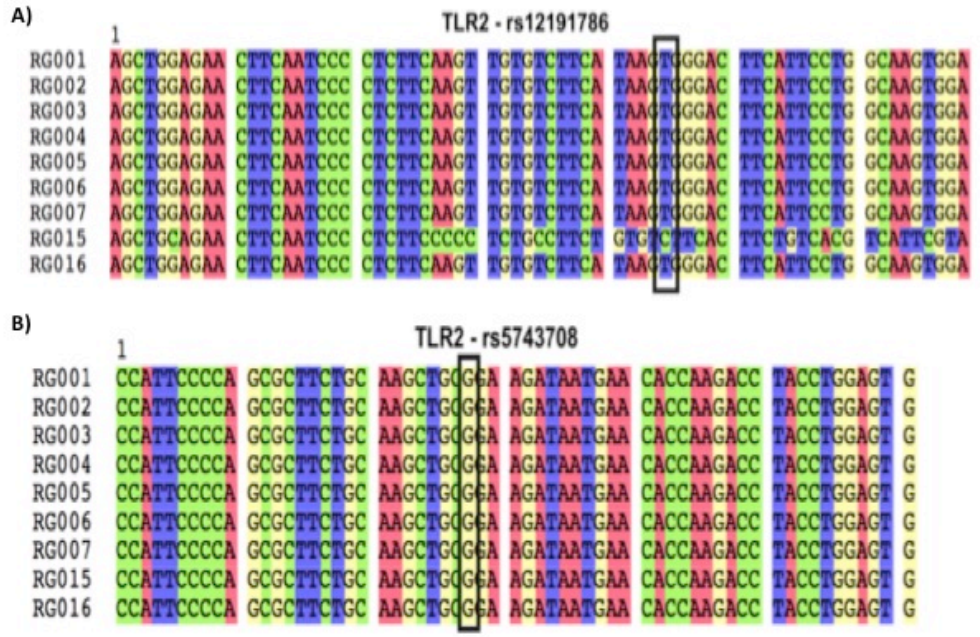


Figure 25. Multiple sequence alignment analysis for 9 healthy donors to detect the presence of: A) TLR2-rs12191786; and B) TLR2-rs5743708. SNP regions of interest are highlighted.

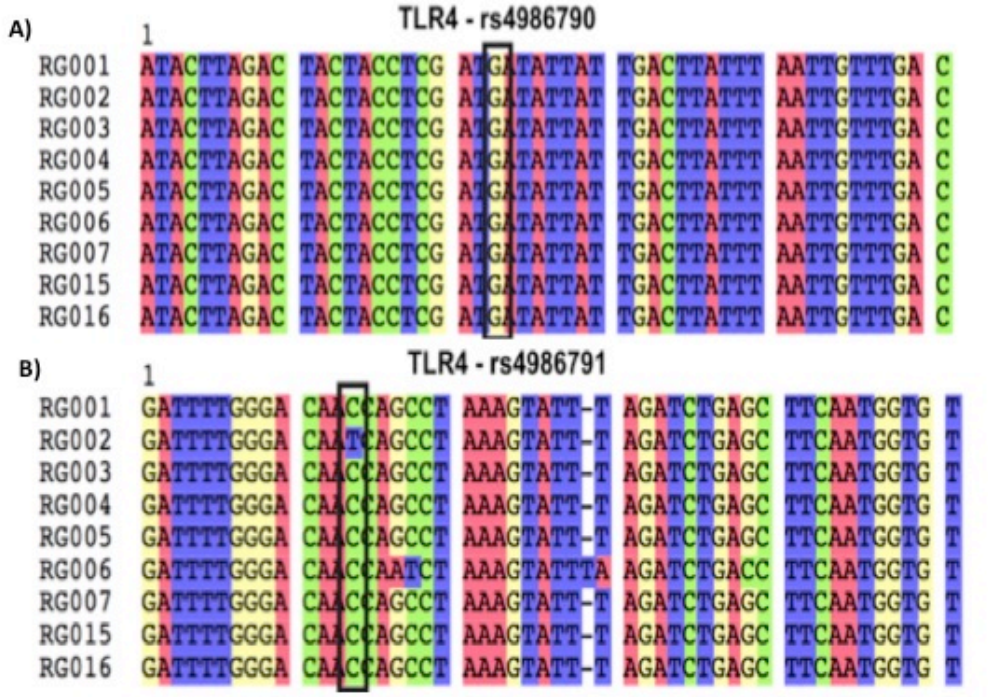


Figure 26. Multiple sequence alignment analysis for 9 healthy donors to detect the presence of: A) TLR4-rs4986790; and B) TLR4-rs4986791. SNP regions of interest are highlighted.

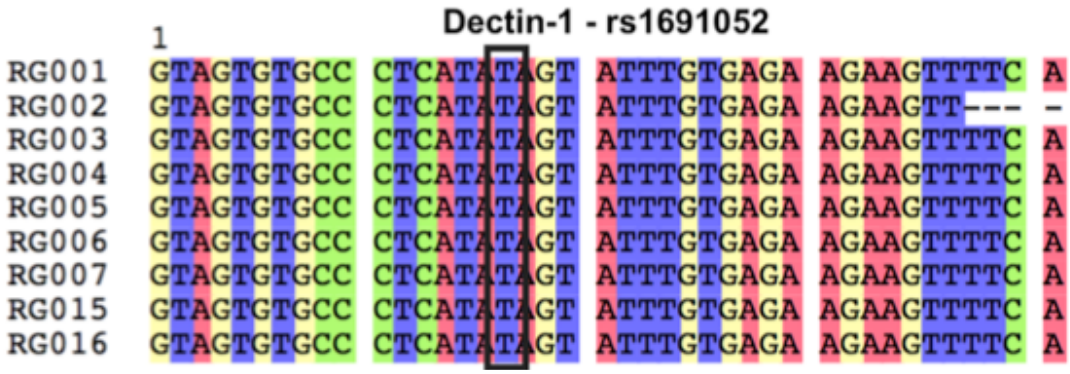


Figure 27. Multiple sequence alignment analysis for 9 healthy donors to detect the presence of Dectin-1-rs1691052. SNP regions of interest are highlighted.

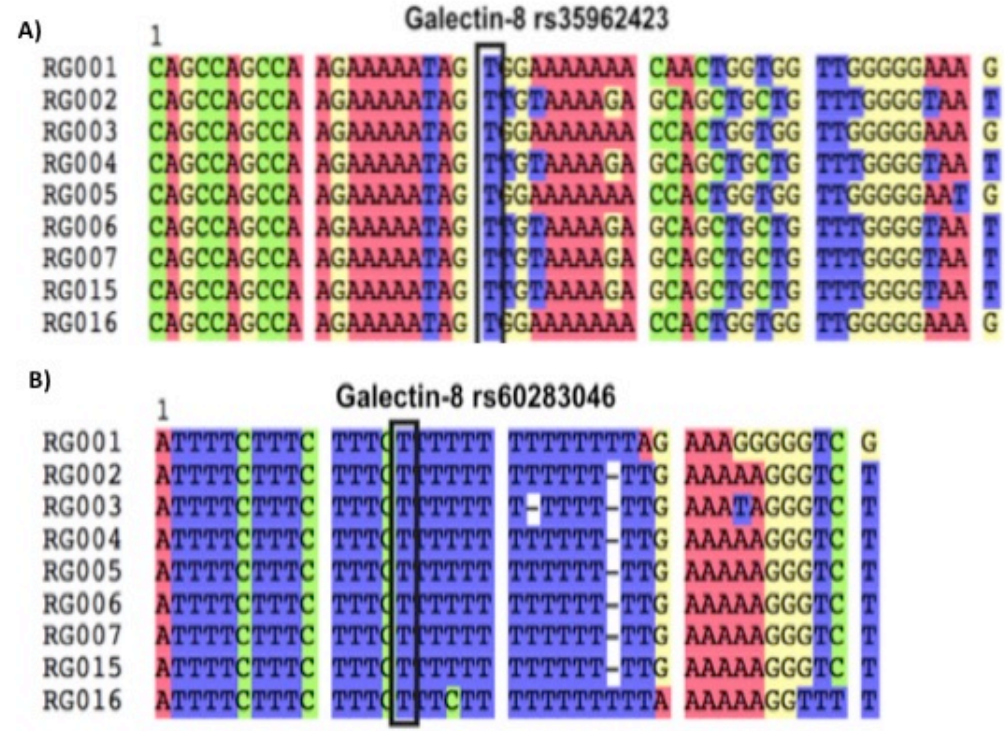


Figure 28. Multiple sequence alignment analysis for 9 healthy donors to detect the presence of: A) GAL8-rs35962423; and B) GAL8- rs60283046. SNP regions of interest are highlighted.

Discussion

To investigate the contribution of small nucleotide polymorphisms to previously observed variation in host macrophage responses to *in vitro* cryptococcal infection, we isolated and sequenced DNA fragments from 9 healthy donors. Genetic variants of functional consequence in TLR2, TLR4, Dectin-1, ERK5 and GAL8 were absent in all donors except for one TLR4 SNP (rs4986791) in donor RG002, suggesting an absence of association of these polymorphisms with observed variation in host macrophage responses to *C. neoformans* infections. It is likely that alternative genetic or environmental determinants of variation may be at play.

To confirm our sequencing results and confirm the validity of frameshifts (such as Fig. 25A and Fig. 26B), we conducted a BLASTX search of all sequencing data that confirmed the identity of our fragments. However, we identified errors in protein sequence predictions rendering these fragments unreliable. We believe these errors may have arisen as a result of gel compression that generated insertions and deletions of bases. As the number of donors (N=9) was too small to make statistical conclusions associating the SNPs tested in this study; we recommend expansion of the study group and more scrutinized methodology in generating the required fragments.

Add text material on homo/heterozygous influence here; add interpretations of sequencing to discussion (what would be likely phenotypic outcomes if sequencing correct?)

Bioinformatic approaches by Aguirre-Gamboa et *al.* to assess the influence of environmental and genetic factors on immune cell populations in peripheral blood of ~500 healthy volunteers revealed that intrinsic factors such as age and gender and season strongly effect immune traits [307]. Whilst genetic factors contributed to approximately 50% of the observed variance in immune traits, aging was associated with increases in myeloid cell levels, and that due to seasonal variation; monocyte levels were significantly higher in winter [307]. These findings were later reiterated in a study by Patin et *al.* to establish the composition of myeloid cells from 1,000 healthy people of Western European decent. It was found that age, gender, smoking and previous exposure to cytomegalovirus (CMV) independently affected the constraints of innate and adaptive immune cells [292]. An interesting finding from this investigation was that innate immune cell counts were driven 'preferentially' by genetic determinants, as opposed to adaptive immune traits that we shown to be under the control of non-genetic, environmental determinants.

The TLR4 missense SNP, rs4986791, causes an amino acid change +1196C/T that lowers TLR4's affinity for its ligands, and has previously been associated with susceptibility to age-related disorders such as late-onset Alzheimer's Disease (LOAD) in Han Chinese people [308]. Interestingly, donor RG002 did not carry the missense rs4986790 TLR4 SNP, which had previously been found to be

co-segregated and form haplotypes with rs4986791. We speculate that the presence of both these polymorphisms would be required to affect macrophage detection of cryptococcal GXM by further disrupting the PRR's binding to the capsular element.

Activation of TLR2 and TLR4 leads to the recruitment of the intracellular adapter MyD88, that orchestrates downstream signaling cascades and bring about inflammatory responses in macrophages. Interestingly, variants in TLR2 and TLR4 have been associated with monocyte activation markers that Bielinski et al. showed to differ between individuals of different ethnicities [309]. While TLR4 has been shown to respond to *Cryptococcus neoformans* GXM, there is conflicting data pertaining to whether it is TLR2 or TLR4 that is involved in the detection of cryptococcal PAMPs, as both TLRs activate the downstream MYD88, and illicit pro-inflammatory responses [138, 141, 300]. This adapter plays a crucial role in conferring protection from invasive fungal disease. An investigation by Biondo et *al.* revealed that whilst control mice survived infection with *C. neoformans;* only 38% of mice lacking TLR2 survived, and all of the MyD88 KO mice succumbed to the fungal disease [300]. Conversely, Nakamura et *al.* presented data describing the weak contribution of TLR2 and TLR4 to protection against *C. neoformans* [288]. In these experiments, TLR2 and TLR4 Knockout mice produced similar levels of inflammatory cytokines to control mice, as did their respective bone marrow-derived dendritic cells (BM-DCs) in the production of IL-12p40 and TNF-α [288]. Whilst the compensatory behaviors of other TLRs in the absence of either TLR2 or TLR4 remain unknown, along with previous findings that MyD88 also played a role as an adapter for IL-1R, IL-18R and TLR9 [310, 311], it was

speculated that protection against cryptococcal infection was driven by a TLR-independent and MyD88-dependent mechanism. Wang et *al.* went on to describe that multiple signaling pathways contributed to MyD88-dependent protection against *C. neoformans in vivo* where the elimination of the IL-18R caused a shift in the survival curves of mice in comparison to wild type and IL-1RKO mice [311]. Thus, an understanding of MyD88-dependent defenses, along with the genetic determinants affecting immune responses may highlight the components of this signaling pathway that modulate antifungal macrophage responses.

Given the small size of the cohort in our study, these results rule neither in nor out a role for genetic variation in modifying the macrophage/Cryptococcus interaction. However, at least for the data we present elsewhere in this thesis, the variation in intracellular proliferation of the yeast that we observe is likely driven primarily by variation in the local environment or by other, untested genetic loci, rather than by genetic variation within the donors in the key immune genes tested here. Given the complexity of the interactions between components of innate immune cell pathways, and the extensive regulation of associated molecular pathways, further investigation of genetic variants is warranted.

Chapter 5 – Identifying novel anti-cryptococcal treatments

Parts of this chapter have been published in an article by Samantaray et *al.* [312]

Abstract

Cryptococcal meningitis is a fatal fungal infection of both immunocompromised and immunocompetent hosts. The causative agent, *C. neoformans*, is an intracellular pathogen that has evolved mechanisms to evade killing by macrophages, including manipulating phagosome maturation. This trait also renders current anticryptococcal treatments ineffective, emphasizing a need for identification of compounds able to traverse host cell compartments and kill intracellular cryptococci. Below, we present data assessing the potential of 13 compounds, previously identified from an intracellular screen of 1,200 off-patent drugs from the Prestwick Chemical Library® of FDA-approved small molecules, for activity against intracellular *C. neoformans*. We carried out infection experiments on J774 macrophages using a GFP-expressing C. neoformans var. grubii strain H99 to study the effects of the selected drug hits on macrophage viability and intracellular proliferation of *C. neoformans*, as well as the ability to kill extracellular fungi. Our results highlight Fendiline Hydrochloride –an anti-angina drug- as a potential anticryptococcal drug by virtue of its ability to eliminate intracellular fungi, but not extracellular cryptococci. However, further

characterization of this compound is required to establish the efficacy of this drug in clinical settings. We propose a role for calcium channel blockers in the inhibition of intracellular survival of *C. neoformans* within the host and anticipate that further understanding of this process will facilitate the development of novel antifungal compounds against intracellular *C. neoformans*.

Introduction

There is currently a limited range of effective therapies available for the treatment of *C. neoformans* infections [225]. Current treatment regimens for cryptococcosis include a combination of Amphotericin B (AmB), flucytosine, and fluconazole [227]. Although the provision of this combination of treatments has greatly improved the 3-month prognosis of HIV patients with cryptococcal meningitis in developed nations [229], the mortality rates in developing countries remain high [230]. In addition, both fluconazole and flucytosine lack anticryptococcal activity within the phagolysosome, unlike liposomal Amphotericin B, highlighting the need for the development of drugs that can effectively penetrate cells [243].

Despite extensive research, the processes by which *C. neoformans* manipulates phagosome maturation remain unknown [143]. The molecular mechanisms pertaining to the ability of this fungal pathogen to survive and thrive in macrophages, in the presence or absence of a concurrent HIV infection also remain to be understood. Furthermore, the high costs associated with delivering current antifungal treatments against cryptococcosis along with anti-retroviral therapeutics in resource-limited settings highlights a need to develop novel antifungal drugs that are more effective, affordable and accessible to HIV patients.

A study conducted by Butts et *al.* [313] used a high-throughput screening assay to test the effects of 1,120 off-patent drug candidates from the Prestwick library for direct antifungal activity against cryptococci. Among the screened drugs, a collection of 31 were identified as having fungicidal activity against *Cryptococcus*, and 8 potential adjunctive agents were identified based on their interaction with fluconazole treatment; two further drugs were shown to have fungicidal activity against intracellular *C. neoformans*, and seven drugs confirmed to bind *C. neoformans* calmodulin, which was presented as an alternative molecular target for antifungal drugs [313].

Based on the above protocol, Samantaray et *al*. developed a high throughput fluorescence-based drug screening method to screen 1,200 US Food and Drug Administration (FDA)-approved small molecules for potential inhibition of intracellular proliferation of *C. neoformans* [312]. This assessment shortlisted 19 potential off-patent, anticryptococcal compounds that were reduced to 13 compounds following further characterization by eliminating compounds with previously known antimicrobial activity, significant macrophage toxicity and those that showed potential extracellular fungicidal traits [312].

Below, we present data (not included in Samantaray et *al*., 2016) of these 13 compounds (denoted as D2-D14) generated from *in vitro* intracellular killing assays in the study by Samantaray et *al.* [312]. The findings from these host cell cytotoxicity and fungal growth inhibition experiments highlight Fendiline Hydrochloride as a potential anticryptococcal drug, capable of killing engulfed cryptococci. Subsequent phagosomal maturation studies (not shown here) by the

group to characterize the mode of action of Fendiline revealed that it's activity as a Ca^{2+} channel blocker strongly enhanced phagosome maturation in macrophages leading to improved killing of cryptococci and reduced intracellular replication. However, due to the high doses of this drug required for effective antifungal clearance that render it unfit for further clinical development; we propose a role for calcium-channel blockers as potential inhibitors of intracellular replication of *C. neoformans*.

Materials and Methods

Yeast strains and growth conditions

C. neoformans var. grubii serotype A strain (H99) expressing Green fluorescence protein (GFP) was used for this study [314]. Overnight cultures were grown in YPD medium (2% glucose, 1% peptone and 1% yeast extract) at 25 °C.

Macrophage cell lines and culture

The murine macrophage-like cell line J774A.1 was used for cryptococcal challenge experiments and cytotoxicity tests between passages 4 and 14. Dulbecco's modified Eagle's medium (DMEM) (supplemented with 2 mM l-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin and 10% Fetal bovine serum (FBS)) was used to culture cells at 37 °C and 5% CO_2.

Drug preparation

13 drugs (denoted D2-D14), previously short-listed for anti-cryptococcal activity from the Prestwick Chemical Library® of FDA-approved small molecules were used in this study (Table 1). These compounds were dissolved in dimethyl sulphoxide (DMSO) at a final assay concentration of 10 µM. Amphotericin B

(AmB) was used as a positive control at a final concentration of 1.25 μg/mL; while DMSO served as a negative control.

Drug code	Name	Published antifungal	Clinical use
D2	Niflumic acid	Yes	inhibitor of cyclooxygenase-2, inhibits chloride channels, acts on GABA-A and NMDA channels and blocks T-type clacium channels
D3	Diethylcarbamazine citrate	No	inhibitor of arachidonic acid
D4	Doxofylline	No	antitussive, bronchodilator effects, and acts as a phosphodiesterase inhibitor
D5	Dequalinium dichloride	Yes	antiseptic
D6	Chlorhexidine	Yes	antiseptic
D7	Dimethadione	No	antiepileptic agent. Can be used for in vivo measurement of intracellular pH
D8	Scopolamine hydrochloride	No	competitive antagonist at muscarinic acetylcholine receptors,
D9	Fendiline hydrochloride	No	calcium channel blocker, LuxR QSM inhibitor
D10	Tolazamide	Yes	oral blood glucose lowering drug
D11	Amrinone	No	pyridine phosphodiesterase 3 inhibitor
D12	Gliquidone	No	anti-diabetic drug
D13	Morantel tartrate	No	anthelmintic drug
D14	Halofantrine hydrochloride	No	anti-malarial drug

Table 6. Details of compounds short-listed for anti-cryptococcal activity from the Prestwick Chemical Library® of FDA-approved small molecules.

Macrophage infection assay

Macrophages were seeded at a concentration of 0.25×10^5 cells/well in a glass-bottom 96-well plate (Greiner Bio One Ltd., Stonehouse, UK) up to 18 h before infection. An overnight culture of H99-GFP was harvested by centrifugation at 6500 rpm for 2.5 min, washed three times with phosphate-buffered saline (PBS) and opsonized with 5% pooled live human serum for 1 h at room temperature. Macrophages were activated with 150 ng/mL phorbol myristate acetate (PMA) for 1 h in DMEM without FBS and were infected with opsonized yeasT-cells [multiplicity of infection (MOI) 10:1] for 2 h at 37 °C. The medium was then removed and cells were washed with PBS to remove extracellular yeasts.

CFU counts

To measure intracellular proliferation rates of yeast following infection with H99-GFP, macrophages were lysed at T(0) and at T(18) with H2O for 15 mins. Serial dilutions of the lysate from the phagocytosis assay were prepared and plated onto 2% YPD, 1% agar (Sigma-Aldrich) plates then incubated for 48 hours at 25°C. Intracellular proliferation rates were measured by dividing the number of counting colony-forming units per milliliter at T(18) by those of untreated macrophages at T(0). Mann–Whitney U-test was used to compare the effectiveness of each drug in reducing IPRs with that of Amphotericin B.

Cytotoxicity assay

To test the toxicity of the drugs towards macrophages, cells were seeded into 96-well plates at a density of either 0.25×10^5 cells/well or 0.5×10^5 cells/well in triplicate for each treatment and cultured overnight. Medium was replaced with serum-free DMEM containing either 5 μM or 10 μM of drug and cells were incubated at 37 °C in 5% CO2 for a further 18 h. The LDH Cytotoxicity Detection Kit (Takara Bio Inc., Kusatsu, Japan) was used in accordance with the manufacturer's protocol to measure the release of Lactate dehydrogenase (LDH). The plate was read at 490 nm using a FLUOstar Omega Microplate Reader (BMG Labtech). 1% Triton X-100 was used as a positive control to represent 100% cell death. The mean absorbance was calculated and data was presented as the percentage of cells that survived each treatment relative to the positive control.

To validate the cell survival rates extrapolated from the LDH cytotoxicity assay, we also measured cell death by counting the proportion of cells that stained positively with 0.2% Trypan Blue.

Fungal growth inhibition assay

To test the inhibitory effects of the drugs in this study on *in vitro* cryptococcal growth, 10^5 yeasT-cells suspended in YPD broth were added to a 96-well plate (Greiner Bio One) and supplemented with 5 µM of drug or the respective control (1.25 µg/mL AmB or 0.1% DMSO). Fungal growth at 25 °C over a 24-h period was measured using a FLUOstar Omega Microplate Reader at 600 nm every 30 min.

Results

Ability to eliminate intracellular *C. neoformans*

To test the ability of each drug to kill intracellular *C. neoformans* H99-GFP, we counted the number of viable yeast cells in macrophage lysates following treatment with the drug during the phagocytosis assay in order to deduce intracellular proliferation rates relative to untreated, yeast-infected cells at T(0) (Fig. 26). All drugs were used at a working concentration of 10 μM unless stated otherwise.

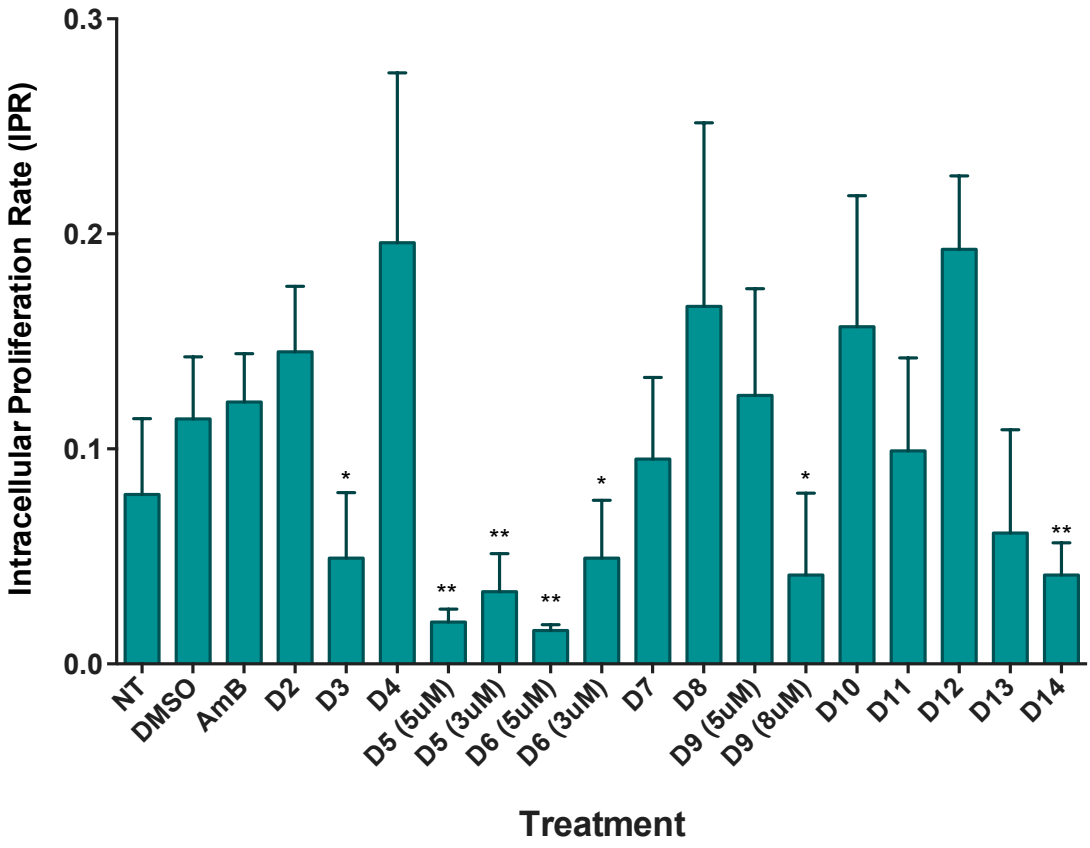


Figure 29. Intracellular proliferation rates (IPR) of phagocytosed cryptococci following treatment with drug hits (D2-D14) for 18 hours relative to untreated (NT) cells at T(0). Different concentrations (μ M) of D5, D6 and D9 were used in these experients. All other drugs were at 10 μ

M. Data provided is of means of 3 technical replicates; Error bars represent Standard Deviations. IPR analysed by Mann–Whitney U-test, *P < 0.05, ** P < 0.01.

Mean IPRs for each drug were compared with the positive control in order to identify compounds more effective at lowering IPR than AmB. Analysis using the Mann–Whitney U-test showed significant reductions in IPR following treatment with D3 (p= 0.0294); D5 at 5 μM (p= 0.0016) and at 3 μM (p= 0.0060); D6 at 5 μM (p= 0.0013) and at 3 μM (p= 0.0233); D9 (8 μM; p= 0.0346) and D14 (p= 0.0068).

Despite the significantly reduced IPRs by action of D3 (Diethylcarbamazine citrate), an inhibitor of arachidonic acid (AA), we chose to exclude this compound as a potential anticryptococcal drug as the method via which AA is incorporated into cryptococcal eicosanoids during infection remains unknown [109]. In addition, the anti-malarial drug, D14 (Halofantrine hydrochloride) was eliminated due to high cytotoxicity towards macrophages *in vitro*.

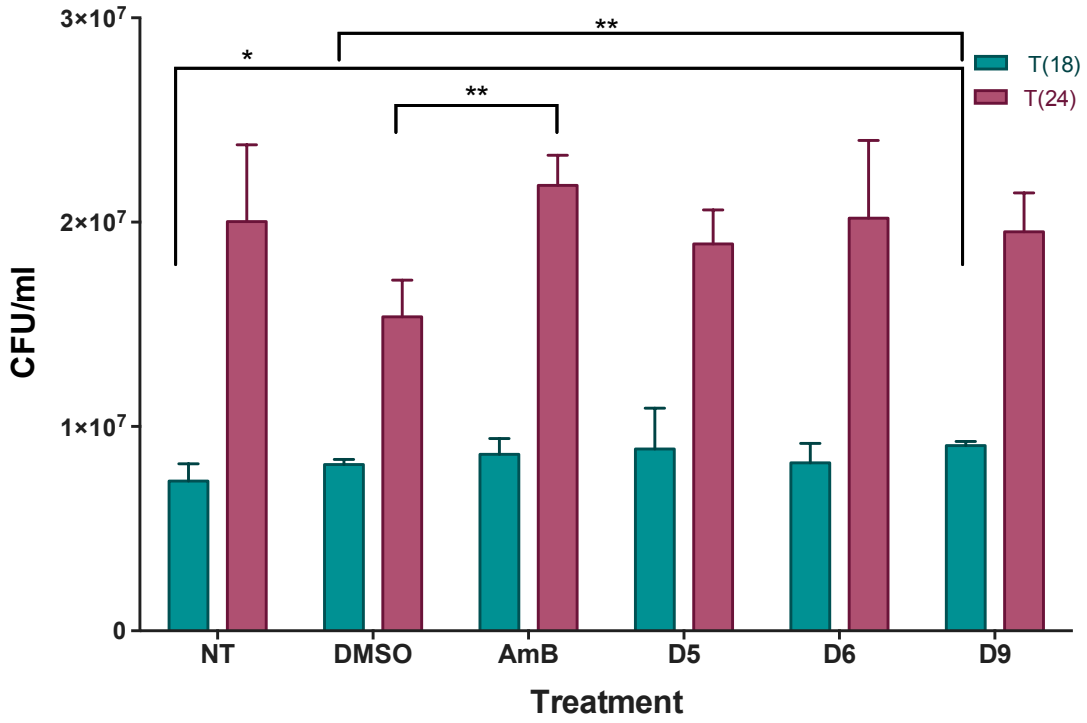


Figure 30. Activity of drug hits D5, D6 and D9 against intracellular *C. neoformans* over 18- and 24-hours following infection. Data provided shows means of 3 technical replicates; Error bars represent Standard Deviations. Analysed by Mann–Whitney U-test, *P < 0.05, ** P < 0.01.

We further investigated the abilities of D5, D6 and D9 to effectively lower intracellular proliferation of *C. neoformans* -18 and -24 hours following *in vitro* infection of J774.1 macrophages (Fig. 27). Though we observed approximately 2-fold increases in intracellular fungal loads between T (18) and T (24) for all treatments, analysis of variance revealed no significant differences in mean CFU counts. Mann–Whitney U-tests show that 18 hours post-infection, CFU counts were significantly different between untreated and D9-treated macrophages (p= 0.02541), and between DMSO-treated and D9-treated samples (p= 0.0072). At T(24), CFU counts following treatment with the positive control (AmB) were significantly higher than DMSO-treated macrophages (p= 0.0087). DMSO has previously been reported to bind the plasma membrane of fungal cells and

increase membrane permeability, influencing the efficacy of water-insoluble antifungal agents [315], in comparison to the control (AmB), which is water-soluble.

Given that D5 (Dequalinium dichloride) and D6 (Chlorhexidine) had previously been shown to possess anti-fungal abilities, in addition to the associated cytotoxicity towards macrophages (D5, data not shown; D6, Fig. 29-31); only D9 (Fendiline Hydrochloride) remained as a potential anticryptococcal compound during the secondary screening. Hence, we tested the ability of this drug to kill intracellular fungi at different concentrations (0.5, 1, 2, 4, 5 and 6 µM), -0, -6 and -24 hours following the phagocytosis assay (Fig. 28). Again, DMSO was used as a negative control to eliminate solvent contribution to fungal killing.

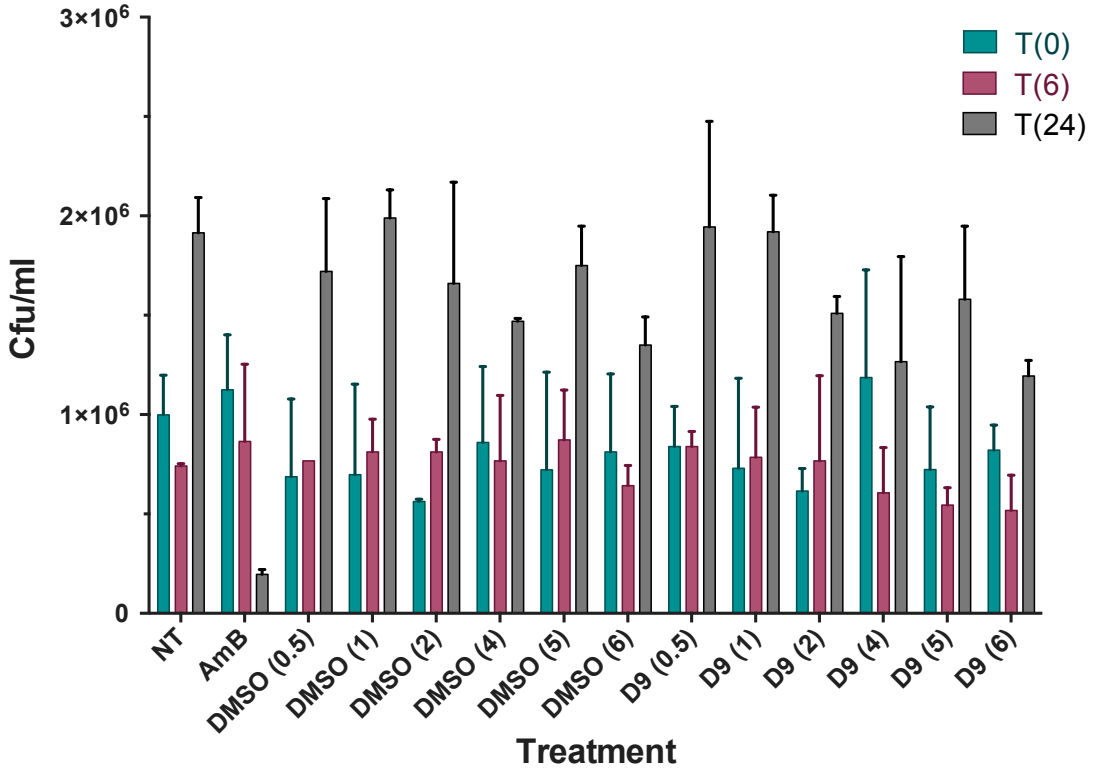


Figure 31. Establishment of dose-associated antifungal activity of D9 (0.5, 1, 2, 4, 5 and 6 µM). Means of 3 technical replicates are shown with standard deviation error bars.

We observed no significant differences in CFU counts between all treatments in the first 6 hours of the intracellular infection assay. However, we noted significant rises in intracellular fungal loads between T (6) and T (24) in untreated samples (P= 0.0001) and in those treated with 0.5 μM DMSO (P= 0.0012), 1 μM DMSO (P= 0.0001), 2 μM DMSO (P= 0.0033) and 5 μM DMSO (P= 0.0025) (Fig. 18). There were also significant increases between T (6) and T (24) following treatment with 0.5 μM D9 (P= 0.0003), 1 μM D9 (P= 0.0002), and 5 μM D9 (P= 0.0005) (Fig. 18). Whilst the positive control (AmB) effectively reduced intracellular *C. neoformans*, there were no significant differences in CFU counts between T (6) and T (24) (T-test; P= 0.0174).

We also compared the efficacy of D9 at killing macrophage-engulfed cryptococci with that of AmB. Mann–Whitney U-tests showed that CFU counts following treatment with different concentrations of D9 were significantly higher than treatment with the positive control (0.5 μM, p= 0.0012; 1 μM, p= 0.0012; 2 μM, p= 0.0027; and 5 μM, p= 0.0026). However, we also observed a significant difference in intracellular *C. neoformans* CFU counts following treatment with AmB and the negative control, DMSO (0.5 μM, p= 0.0020; 1 μM, p= 0.0007; 2 μM, p= 0.0022; 4 μM, p= 0.0042; 5 μM, p= 0.0024; and 6 μM, p= 0.0046), suggesting and overwhelming solvent contribution to the observed effects of D9.

Cytotoxic effects on host macrophages

To assess the toxicity of the drug hits (D6, D9-14) towards host macrophages, we measured cell death following exposure to either low dose (5 µM; Fig. 29) or high dose (10 µM; Fig. 30) treatments for 24 hours. These measurements were made by either manually counting the proportion of hosT-cells stained with Trypan blue (TB) at each time point, or by measuring the release of the LDH enzyme - a marker of cell death- using the LDH cytotoxicity kit (LDH). Results are presented as percentages of cell survival.

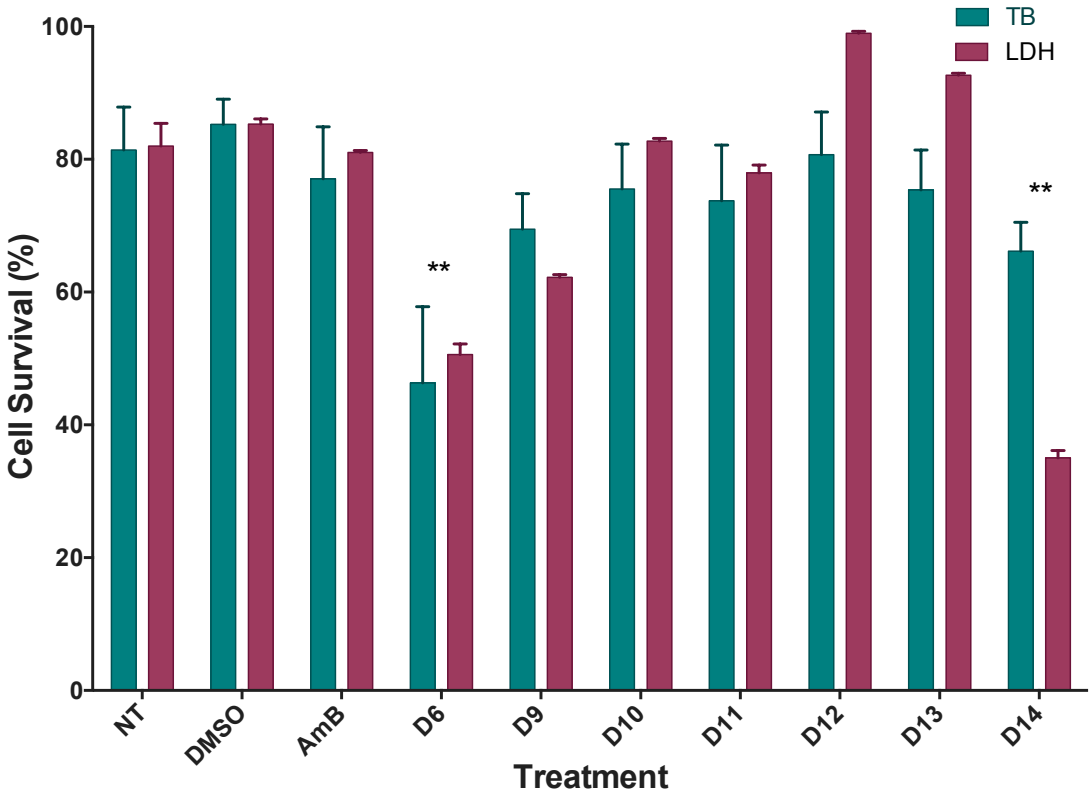


Figure 32. Cell survival following treatment with low doses (5 μ M) of potential anti-cryptococcal drugs measured either by staining with Trypan Blue (TB) or by measuring LDH release (LDH). Means of 3 technical replicates are provided with Standard deviation error bars. Analysed by One–way ANOVA showing significant difference of mean cell survival to untreated cells, *P < 0.05, ** P < 0.01.

Whilst assessing the cytotoxicity of each drug towards macrophages, we also assessed the efficacy of each cytotoxicity detection method. At both low and high treatment doses, Two-Way Anova analysis shows that there are no significant differences in the overall ability for each method to detect cytotoxicity (low dose: p= 0.1865; high dose: p= 0.8301). However, the type of treatment macrophages were exposed to contributed to 75.31% and 66.93% of the variation in cytotoxicity readouts from each method following macrophage exposure to low and high doses of test compounds, respectively.

We found significant disparities in results from treatments with 5 μM D12, D13 and D14 (t-test; p= 0.0079, 0.0077 and 0.0003, respectively); and from treatment with 10 μM DMSO and D6 (t-test; p= 0.0059 and 0.0031, respectively). Coefficients of variation between each cytotoxicity detection method for each treatment at low and high doses are shown below (Table. 7-8).

	Low Dose			
Treatment	**Median**	**Mean**	**Std. Deviation**	**Coefficient of Variation**
NT	81.70	81.70	0.43	0.53%
DMSO	85.28	85.28	0.03	0.03%
AmB	79.04	79.04	2.81	3.56%
D6	48.46	48.46	3.03	6.25%
D9	65.84	65.84	5.12	7.78%
D10	79.14	79.14	5.10	6.44%
D11	75.85	75.85	2.97	3.92%
D12	89.83	89.83	12.93	14.39%
D13	84.01	84.01	12.22	14.54%
D14	50.60	50.60	22.01	43.49%

Table 7. Descriptive statistics of variation in cytotoxicity detection by Trypan Blue (TB) and LDH methods following treatment with low doses (5 μM) of potential anti-cryptococcal drugs.

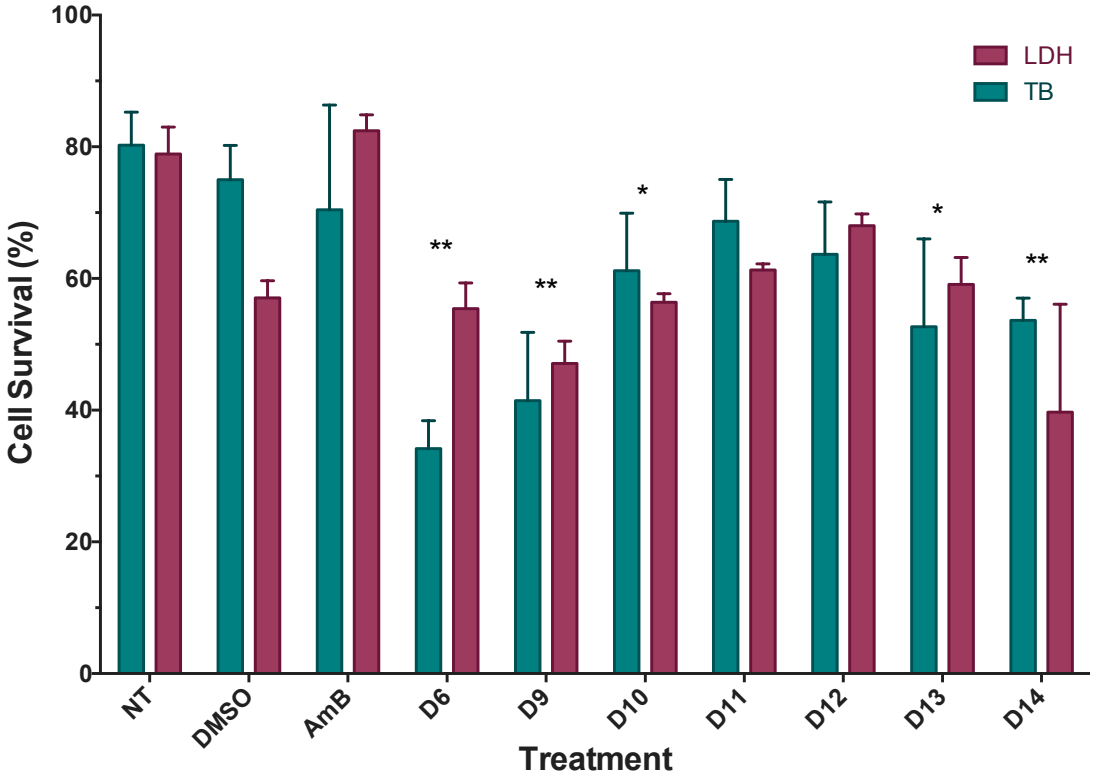


Figure 33. Cell survival following treatment with high doses (10 µM) of potential anti-cryptococcal drugs measured either by staining with Trypan Blue (TB) or by measuring LDH release (LDH). Means of 3 technical replicates are provided with Standard deviation error bars. Analysed by One–way ANOVA showing significant difference of mean cell survival to untreated cells, *P < 0.05, ** P < 0.01.

	High Dose			
Treatment	**Median**	**Mean**	**Std. Deviation**	**Coefficient of Variation**
NT	79.58	79.58	0.95	1.20%
DMSO	66.03	66.03	12.69	19.21%
AmB	76.45	76.45	8.49	11.11%
D6	44.80	44.80	15.02	33.52%
D9	44.29	44.29	3.98	8.99%
D10	58.78	58.78	3.39	5.77%
D11	65.00	65.00	5.22	8.03%
D12	65.86	65.86	3.06	4.65%
D13	55.89	55.89	4.54	8.12%
D14	46.68	46.68	9.86	21.12%

Table 8. Descriptive statistics of variation in cytotoxicity detection by Trypan Blue (TB) and LDH methods following treatment with high doses (10 μM) of potential anti-cryptococcal drugs.

We thereafter compared the percentages of macrophages surviving each treatment with those of untreated cells (NT). One-way ANOVA analysis found significant declines in cell survival following treatment with both low (Fig. 29) and high doses (Fig. 30) of D6 (5 μM, p= 0.0053; 10 μM, p= 0.0015) and D14 (5 μM, p= 0.0078; 10 μM, p= 0.0021); and high dose treatments (Fig. 30) of D9 (p= 0.0013), D10 (p= 0.0266) and D13 (p= 0.0143).

Both cytotoxicity measurements show that on average, ~80% of untreated J774.1 macrophages will survive over a 24-hour period. As results from the LDH assay showed a substantial effect of increasing DMSO concentration on cell death; we wondered whether increased concentrations of DMSO, the solvent into which all drugs except AmB were dissolved, notably reduced cell survival. Solvent contribution to the observed cytotoxicity was eliminated as we found a significant difference in cytotoxicity between DMSO and 5 μM treatments of D6

and D14 (One-way ANOVA; p=0.0028 and 0.0041, respectively); and with higher doses of D6, D9 and D14 (One-way ANOVA; p= 0.0243, 0.0217 and 0.0363, respectively).

Toxicity towards *C. neoformans*

Finally, we compared the effects of each drug hit (at a concentration of 5 µM) on limiting cryptococcal expansion over a 24-hour period (Fig. 31-32) in YPD. While AmB effectively controlled yeast growth over the 24 hours, no yeast growth was observed following treatment with D5 and D6.

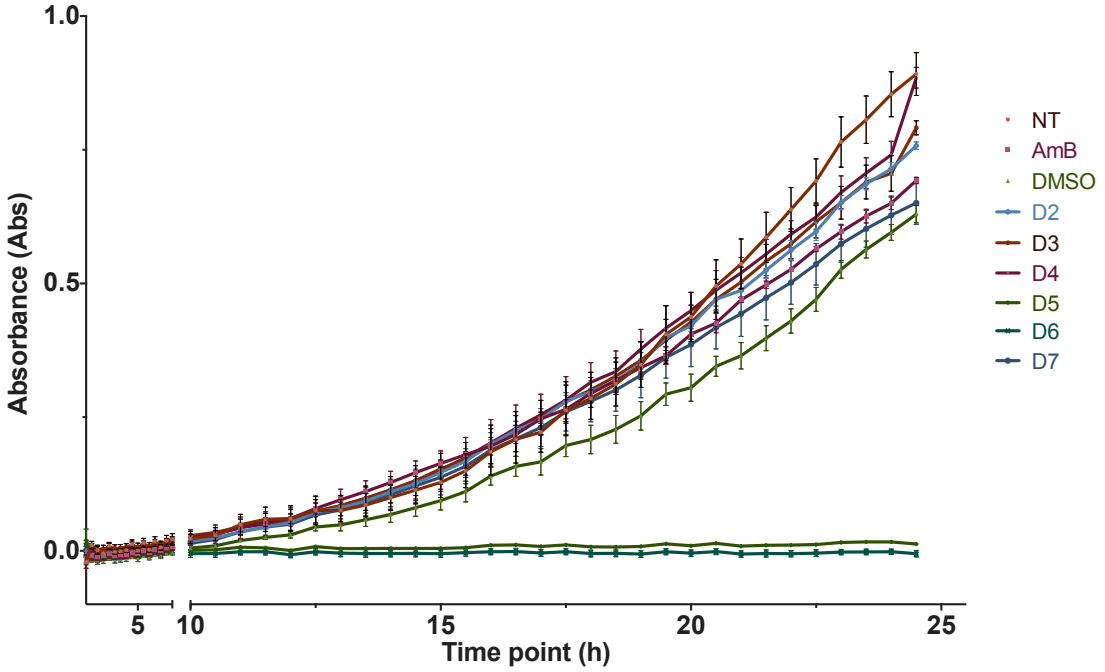


Figure 34. Growth curves for *C. neoformans* H99 in YPD following treatment with the potential anti-cryptococcal compounds D2-D7 (5 µM). Error bars represent standard error of the mean (SEM).

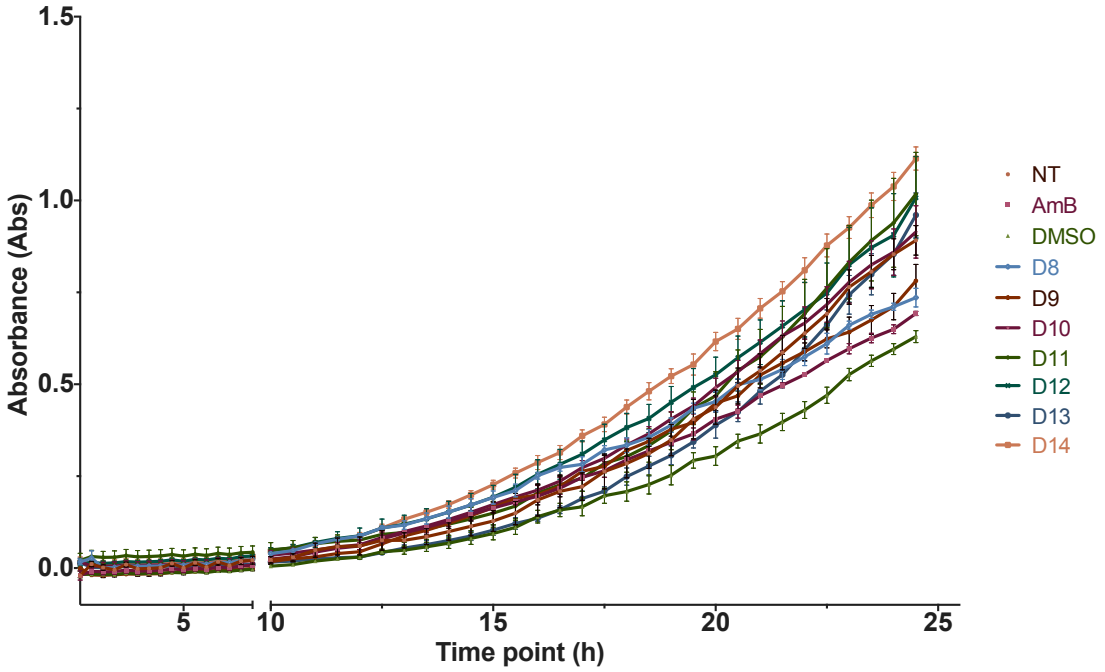


Figure 35. Growth curves for *C. neoformans* H99 in YPD following treatment with the potential anti-cryptococcal compounds D8-D14 (5 µM). Error bars represent standard error of the mean (SEM).

Aside from DMSO control, only D7 showed improved inhibition of fungal growth in comparison to AmB. All other drugs (D2-6, Fig. 31; D8-14 Fig. 32) were less effective than AmB at controlling cryptococcal expansion in YPD, suggesting no direct fungal killing by these drugs.

Discussion

Given the rise in resistance to anticryptococcal treatments, there is urgent need for the identification of new drug targets. Whilst previous screens have identified drugs with direct anticryptococcal activity [313, 316, 317], we sought to identify compounds capable of killing intracellular fungi. Samantaray et *al.* developed a method to screen for such drugs that uses GFP fluorescence as an index for intracellular proliferation [312], that was used to explore the Prestwick library of FDA-approved molecules identified 13 off-patent drugs as potential anticryptococcal treatments.

Above, we treated *C. neoformans*-infected macrophages with each drug to test their ability to kill intracellular yeast, and assessed drug cytotoxicity towards macrophages and direct antifungal capabilities.

As the compounds under investigation were dissolved in dimethyl sulphoxide (DMSO) at a final assay concentration of 10 μM, we chose to use this solvent as a negative control in our experiments. Amphotericin B (AmB) was used at a final concentration of 1.25 μg/mL, as a positive control as it has previously been used as an anticryptococcal drug. However, results from our experiments show that AmB failed to eliminate both extracellular and intracellular Cryptococci, rendering the data acquired unreliable. We believe the concentration of AmB used was insufficient, as this drug has been used at higher concentrations for *in vitro* experiments at concentrations ranging from 1-100 μg/mL. Alternatively, errors in serial dilutions during drug preparation may have resulted in failure to

kill *C. neoformans in vitro*. This was not the case for data presented in Samantaray et al., where AmB behaved as predicted suggesting a user-generated error.

Despite the negation of our positive control, our findings suggest D9 (Fendeline Hydrochloride) as a potential anticryptococcal compound based on the above attributes. This compound was able to effectively kill intracellular cryptococci at concentrations above 5 μM, over a 24-hour period. Following treatment with low (5 μM) and high (10 μM) doses of D9, macrophages survival was ~60% and ~40%, respectively. However, growth curves reveal that D9 does not kill extracellular yeast.

Fendeline Hydrochloride is used in the treatment of angina due to its activity as an l-type calcium-channel blocker [318]. Whilst the drug did not demonstrate direct antifungal traits, we speculated that this drug promoted macrophage killing of intracellular cryptococci by inducing phagosomal acidification. Further characterization experiments presented by Samantaray et *al.* showed that at concentrations of fendiline above 5 μM, an increase in [Ca^{2+}] was observed; a process inhibited by blocking calcium release from the endoplasmic reticulum with thapsigargin [312]. These findings support the previous hypothesis that Fendiline interacts with intracellular endoplasmic reticulum calcium stores to transiently elevate [Ca^{2+}] in certain cell types [319, 320].

Whilst Fendiline Hydrochloride was identified a novel anticryptococcal drug, the authors highlighted the limitations of intracellular screens and potential false-

negative rates associated with identifying anticryptococcal drugs. Therefore, it is likely that the initial screening of the Prestwick library may have excluded additional potential anti-cryptococcal compounds. Knowledge of *C. neoformans*' ability to manipulate phagosome maturation [143] instigated the hypothesis that fendiline reversed the effects of cryptococcal influences, effectively reinstating phagosome maturation, and consequently fungal killing [312]. The group recommended further investigation of calcium flux in phagosomes, and testing of this hypothesis in animal models. They also discussed the pharmacological advantages of using Fendiline as an anticryptococcal drug, as it is trafficked into the phagosome [321, 322], and is able to cross the blood brain barrier (BBB) [323].

However, further characterization of this drug for clinical use would be required as the concentration of Fendiline required to eliminate *C. neoformans in vitro* was more that 10-fold higher than typical serum Fendiline levels in patients receiving this drug for cardiovascular complaints [324].

In conclusion, our results highlight Fendiline Hydrochloride as a potential anticryptococcal drug by virtue of its ability to eliminate intracellular fungi. Furthermore, in line with additional data presented by Samantaray et *al.*, we propose a role for calcium channel blockers in the inhibition of intracellular survival of *C. neoformans* within host macrophages based on previous association with *M. tuberculosis* [312]. We anticipate that further understanding of this process will facilitate the development of novel antifungal compounds against intracellular *C. neoformans*.

SYNOPSIS

Cryptoccosis remains the leading cause of fungal meningitis worldwide. Whilst one of the causative agents, *C. neoformans* has been described as an opportunistic pathogen of the immunocompromised, there is growing evidence of its' abilities as a primary pathogen towards otherwise healthy individuals. This necessitates further understanding of the epidemiology and pathology to cryptococcosis in the immunocompetent patient group, as the majority of research has been conducted within the context of HIV.

In this thesis, we discuss how underlying host innate immune responses vary between human hosts in response to cryptococcal disease caused by *C. neoformans*. We also assess current understanding of how immune responses in different hosts may be predictive of protection from, or susceptibility to cryptococcal meningitis (CM).

As macrophages play a crucial role in the innate immune response to *C. neoformans*, we have presented quantitative data from the *in vitro* challenge of monocyte-derived macrophages (MDMs) from 15 healthy individuals, and described the variability in macrophage responses to cryptococcal infections. We have shown that phagocytic cells from each individual do not exhibit consistent anti-cryptococcal responses over time, with varied control of intracellular proliferation rates and yeast expulsion (vomocytosis) from macrophages. Cytokine profiles play an important part in the anticryptococcal response, and

have been shown to be predictive of disease progression in HIV-positive patients. However, we are yet to establish markers of susceptibility in HIV-negative, immunocompetent patients. We compared the cytokine profiles of each independent donor; and investigated how levels of secreted cytokines compare to intracellular cytokine production between individuals within our group of donors. Understanding the effects of cytokine signalling is also pertinent to determining the role that different macrophage activation states play at different stages of infection. Whilst mainly two macrophage activation states (M1 and M2) are considered in the context of cryptococcal disease, a plethora of investigations have described a 'gradient' of macrophage activation states that may interact variably with the intracellular pathogen to either, hinder or promote dissemination.

We probed the association of Toll-like receptor 4 (TLR4) with the vomocytosis of *C. neoformans* from murine bone marrow-derived macrophages (bMDMs), based on findings from previous research. Despite the small sample size of mice used in these experiments, we have shown that elimination of TLR4 reduces the expulsion of yeast from macrophages. Our suggestion is that further experimentation be carried out to confirm these findings and characterize the signaling cascades that orchestrate this phenomenon.

We also assessed the contribution of SNPs in TLR2; TLR4; the C-type Lectin, Dectin-1; the mitogen-activated protein (MAP) kinase, ERK5; and the autophagy protein, Galectin-8 (GAL8). However, due to the small cohort of individuals, we recommend that larger GWAS studies be carried out to verify the association of

these polymorphisms with susceptibility to cryptococcosis in immunocompetent hosts.

Lastly, we contributed to experiments that highlighted the off-patent drug, Fendiline Hydrochloride, as a potential anticryptococcal treatment. While this drug was able to eliminate intracellular Cryptococci, it was not able to kill extracellular fungi and showed relatively high toxicity towards macrophages *in vitro*. However, we proposed a role for calcium channel blockers in restricting intracellular proliferation of *C. neoformans* and anticipate that further understanding of this process will lead to the development of novel anticryptococcal drugs.

We anticipate that the findings from this research will provide indications of variation in macrophage responses to cryptococcosis that may provide greater insight to disease progression, and pave way to understanding the mechanisms that drive intra- and inter- host variation. We hope that our findings will inspire larger cohorts of immunocompetent participants, and more specifically, a more detailed understanding of the parameters of macrophage responses to cryptococcal disease both *in vitro* and *in vivo*. This will allow for development of more effective screening and personalized treatment strategies for patients with previously robust immune systems.

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