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STIMULATION OF INNATE IMMUNITY LEADS TO CLEARANCE OF *C. NEOFORMANS* INFECTION IN ZEBRAFISH

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A thesis submitted to the University of Sheffield for fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Infection, Immunity and Cardiovascular Diseases

December 2017

Abstract

Cryptococcus neoformans is an opportunistic pathogen and a leading cause of life-threatening fungal infections in the immunocompromised. Individuals that are at greater risk are those with defective T-cell mediated immunity such as those with HIV/AIDS. Despite treatment with anti-fungal drugs, mortality remains excessively high and patients experience serious side effects hence the need to explore new therapeutic strategies. Activation of macrophages is essential for the control of cryptococcal infection. However, in the absence of T-cell mediated immunity, activation of macrophages is disrupted and clearance of cryptococcal infection is abrogated.

Herein I first report a zebrafish-*C. neoformans* model of infection and show that zebrafish can clear, control or fail to control cryptococcal infection. I then go on to test whether cytokines or PRR ligands are capable of stimulating innate immune resistance to *C. neoformans* in zebrafish. I demonstrate that concomitant injection of IFNγ with *C. neoformans* results in reduced fungal burden and increased fungal clearance. IFNγ increases the recruitment of phagocytes to the site of infection and enhances phagocytosis by macrophages. Macrophage deficient larvae fail to clear or suppress cryptococcal infection despite treatment with IFNγ. In addition, infected macrophages display low lysosomal pH and elevated expression of IL-1β in IFNγ-treated larvae. Although neutrophils take up the fungus, their depletion does not alter cryptococcal burden.

Secondly, I demonstrate that *S. aureus* CWP is a potent inducer of innate defences against *C. neoformans*. Using chemically digested *S. aureus* CWP to remove wall teichoic acid or mutants that do not produce wall teichoic acid (*tarO*) or lipoproteins (*lgt*), I establish that protective effects of *S. aureus* CWP require wall teichoic acid but not lipoproteins. Protection by *S. aureus* CWP are associated with increased recruitment of macrophages but not enhanced phagocytosis or TNFα expression.

This work provides new insights into cellular responses and effector molecules in the context of cryptococcal disease progression while identifying potential immunomodulatory targets.

Acknowledgements

Not just one or two, but many people have made a positive contribution to this piece of work and I express my sincere appreciation.

Firstly, I would like to express my heartfelt appreciation to my supervisor Simon Johnston. Simon, you have provided extraordinary support and guidance throughout my PhD. Your help was beyond expectation. You were so dedicated as if I was the only student you had. You have been very encouraging, for example, whenever I went to your office with my results I thought were not very good, I would come out smiling and feeling encouraged. I could not ask for a better supervisor, your approach is thrilling and I have learned a lot from you. You have been a blessing.

I am forever thankful to Jenny Wright for proofreading my thesis. I also thank Josie Gibson who selflessly provided a graph for my thesis. I would also like to thank Josh Hooker, a technician in Simon Foster's laboratory for making all the *S. aureus* cell wall preparations used in my project. My thanks also go to Victoriano Mulero at Department of Cell Biology and Histology, Faculty of Biology, University of Murcia, Spain for providing *Vibrio anguillarum* DNA.

I would also like to express my appreciation to my lab colleagues in the Johnston lab. Our socialising and laughter were comforts in my stressful moments, and your hard work and encouragement kept me focused on my work in those tough times. Words cannot articulate how grateful I am. Special thanks go to Robert Evans, Aleksandra Bojarczuk and Kate Pline.

I am very grateful to the Wellcome for funding my PhD.

Last, with love, extra special thanks go to all my family and friends both in Malawi and in the UK. Many thanks for the conversations, for texts and words of encouragement. I am eternally grateful. Truly, I am thankful to and humbled by all those who have contributed to my success. My prayers are with you always.

Above all, I thank God for bringing each and every individual mentioned here to support me.

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Abbreviations

AFLP – Amplified Fragment Length Polymorphism

AGM – aorta, gonad and mesonephros

AIDS – Acquired Immunodeficiency Syndrome

AP-1 – activator protein 1

App1 – Anti-phagocytic protein 1

Arg – Arginine

BAC - Bacterial Artificial Chromosome

BAL – Bronchoalveolar lavage

BBB – Blood brain barrier

CCL – C-C chemokine ligand

CCR – C-C chemokine receptor

CD – Cluster of differentiation

CFU – Colony forming units

CHT - caudal hematopoietic tissue

CLR – c-lectin receptor

CM – Cryptococcal meningoencephalitis/meningitis

CNS – Central nervous system

CR2 – Complement receptor two

CR3 – Complement receptor three

CrAg – Cryptococcal antigen

CSF – Cerebral spinal fluid

CWP – cell wall preparations

CXCL - C-X-C motif chemokine ligand

dH2O – Deionised water

dpf – day(s) post fertilisation

dsDNA – double-stranded DNA

dsRNA – double-stranded RNA

GFP – Green fluorescent protein

GlcNAc – N-Acetylglucosamine

GPI - glycosylphosphatidylinositol

GXM - glucuronoxylomannan

GXMGal - glucuronoxylomannogalactan

H2O2 – Hydrogen peroxide

HIV- Human Immunodeficiency Virus

hpi – hour(s) post infection

ICM - intermediate cell mass

IFNγ – Interferon gamma

IKK – inhibitor of NF-κB kinase

IL – interleukin

iNOS - Induced Nitric Oxide Synthases

IRAK – interleukin-1 receptor-associated kinase

IRF – Interferon regulatory factor

IRIS – Immune reconstitution inflammatory syndrome

IκBα – inhibitor of NF-κBα

JNK – JUN N-terminal kinase

LT – Leukotriene

LTA – Lipoteichoic acid

MAPK – Mitogen-activated protein kinases

MBL – Mannose Binding Lectin

MCP-1 – Macrophage chemotactic protein 1

MHC – Major histocompatibility complex

MKK – mitogen-activated protein kinase kinase

MLST - Multi-locus sequence typing

Mm- *Mycobacterium marinum*

MR – Mannose receptor

NaCl – Sodium chloride

NADPH- Nicotinamide adenine dinucleotide phosphate

NET – Neutrophil extracellular trap

NF-κB – nuclear factor kappa B

NLR – nucleotide-binding oligomerization domain (NOD)-like receptors

NO- Nitric Oxide

NOD – nucleotide-binding oligomerization domain

PAMP – Pathogen associated molecular pattern

PGN – Peptidoglycan

PRR - Pattern Recognition Receptors

RAPD - Random amplified polymorphic DNA

ROS - Reactive Oxygen Species

RT – room temperature

SR - Scavenger Receptors

SREC - Scavenger receptor class F member 1

ssRNA – single-stranded RNA

TAB – TAK1-binding protein

TAK1 – TGFβ-activated kinase 1

TBK1 – TANK-binding kinase 1

Th - T helper

TLR - Toll-Like Receptors

TNFα – Tumour necrosis factor alpha

TRAF – tumour necrosis factor receptor-associated factor

TRAM – TRIF-related adaptor molecule

VG – Variant Cryptococcus gattii

VN – Variant *Cryptococcus neoformans*

WHO – World Health Organisation

WTA – Wall teichoic acid

YPD – Yeast extract / peptone / dextrose media

CHAPTER 1

INTRODUCTION

1.1 *Cryptococcus* and cryptococcosis

Fungal pathogens have become of increasing medical importance in the last couple of decades due to the emergence of organ transplants, immunosuppressant treatments, and the HIV/AIDS pandemic. *Cryptococcus* is not only the most common human fungal pathogen causing life-threatening disease in the immunocompromised population, but it is also an important pathogen in the immunocompetent.

In this chapter, I will discuss the genus *Cryptococcus* with emphasis on the species *C. neoformans* and *C. gattii*. I will then describe the life cycle and genome followed by a detailed description of the *Cryptococcus* disease including clinical presentation, diagnosis, and current treatment options. Thereafter, I will describe the pathogenesis and the immune response to *C. neoformans* infection including its interactions with host immune cells. I will then introduce animal models that are used to study *C. neoformans* infection with emphasis on zebrafish larvae and its immune system. I will finish this chapter with the aims and strategies that were used.

1.2 Pathogenic *Cryptococcus* species

Cryptococcus is a basidiomycetous yeast, round or oval, and replicates by budding. There are over 80 species that belong to the genus *Cryptococcus* and most of which are nonpathogenic to humans (Arturo Casadevall 1988; Fell et al, 2000). Among these, *C. neoformans* and *C. gattii* are the most common species that cause human disease. However, there have been rare reports of *C. laurentii* (Johnson et al, 1998; Kordossis et al, 1998) Kunova & Krcmery, 1999), *C. curvatusi* (Dromer et al, 1995) and *C. albidus* (Burnik et al, 2007; de Castro et al, 2005; Kordossis et al, 1998; Loison et al, 1996) causing infections in humans. Interestingly *C. laurentii* is a well-known postharvest biocontrol yeast against blue mold rot caused by *Penicillium expansum* in apple fruit (Roberts, 1990; Yu et al, 2007).

Previously, *C. neoformans* and *C. gattii* species complex were classified into four serotypes (A - D) based on capsular agglutination reactions. According to the current classification, serotype A, D and AD (hybrid following mating of serotype

A and D) are now known as *C. neoformans* while serotype B and C bear the name *C. gattii* (Kwon-Chung et al, 2002; Kwon-Chung & Varma, 2006).

1.2.1 *Cryptococcus neoformans*

Discovery- Cryptococcus neoformans was first described in 1894 by Otto Busse, a professor of pathology at Greifswald in Germany, following isolation from a sarcoma-like lesion of a female patient's tibia (al-Doory, 1971; Mitchell & Perfect, 1995). Later in the same year, Francesco Sanfelice named the yeast *Saccharomyces neoformans* when he isolated it from fermenting peach juice (Reviewed in Srikanta et al, 2014). Jean-Paul Vuillemin first suggested the species' name, *Cryptococcus neoformans*, in 1901. However, it was not until the 1950's that the name was widely accepted and later published by Lodder and Kreger-van Rij in the first major nomenclature of known yeasts (Lodder & Kreger-Van Rij, 1952).

Ecology - Cryptococcus is a ubiquitous saprobic fungus and a free-living organism that is well adapted to a variety of environmental niches. *Cryptococcus neoformans* is specifically associated with avian guano (Agha Kuchak Afshari et al, 2012; Casali et al, 2003; Li et al, 1993; Nielsen et al, 2007; Sriburee et al, 2004) but has been isolated also from other sources including trees (Litvintseva et al, 2011), decaying wood (Lazera, 1993; Lazéra et al, 1996; Randhawa et al, 2006), and soil (Randhawa et al, 2008) usually contaminated with avian excreta or decayed wood (Ajello, 1958; Emmons, 1955). In relation to human disease or colonization, *C. neoformans* represents the most frequent fungal isolate from AIDS patients (Mitchell & Perfect, 1995). In very rare events, *C. neoformans* has been isolated from skin, saliva, oropharynges washings, toes and faecal specimens in healthy individuals posing as a transient commensal (Howard, 1973; Randhawa & Pal, 1977). Even though most patients with disseminated cryptococcosis are immunocompromised, *C. neoformans* can also cause disease in apparently immunocompetent individuals (Chen et al, 2008; Chen et al, 2000).

Classification - Classification of the genus *Cryptococcus* is based on molecular characterisation by PCR finger printing, Random amplified polymorphic DNA

(RAPD), Amplified Fragment Length Polymorphism (AFLP) and multi-locus sequence typing (MLST) analyses (Hagen et al, 2015). *Cryptococcus neoformans* is classified into two varieties: var. grubii (serotype A) and var. *neoformans* (serotype D). *C. neoformans* var. grubii, is the most virulent and most common cause of cryptococcosis worldwide, whereas *C. neoformans* var. neoformans is less virulent but more prevalent in Europe (Litvintseva et al, 2006; Dromer et al, 2007; Xu et al, 2000). *C. neoformans* comprises five distinct molecular types. *C. neoformans* var. grubii, serotype A molecular types include VNI, VNII and VNB. Whereas *C. neoformans* var. neoformans, serotype D comprises of VNIV while VNIII (serotype AD hybrid) is *C. neoformans* intervariety hybrid (Reviewed in Hagen et al, 2015; Figure 1). VNI isolates are prevalent in the environment globally and are responsible for most cryptococcal infections outside Africa. VNII are found worldwide but exceptionally rare in the environment and in causing human disease (Litvintseva et al, 2006; Meyer et al, 2003) whereas VNB isolates are primarily confined to sub-Saharan Africa. Conversely VNB isolates are unique to Bostwana (Cogliati, 2013; Litvintseva et al, 2006). The prevalence of VNIV isolates from the environment and patients are moderately comparable to VNI (Dromer et al, 2007). There are no recorded differences in the immunological status of the host infected by *C. neoformans* var. grubii and *C. neoformans* var. neoformans (Irokanulo et al, 1997; Jain & Fries, 2008).

1.2.2 *Cryptococcus gattii*

Cryptococcus gattii, formerly known as *Cryptococcus neoformans* var. gattii (serotype B and C), is currently classified as an independent species (Kwon-Chung et al, 2002). Unlike *C. neoformans*, *C. gatti* mostly causes disease in immunocompetent patients (Chen et al, 2008; Chen et al, 2000). Previously, *C. gattii* infection was thought to be prevalent only in tropical and subtropical climates and its presence in temperate regions was rare (Kwon-Chung & Bennett, 1984a; b). However, *C. gattii* has also been isolated in temperate regions of Europe (Baró et al, 1998; Colom et al, 2005). It is also known for the ongoing outbreaks in temperate regions including British Colombia and Vancouver islands in Canada (Hoang et al, 2004; MacDougall et al, 2007). *C. gattii* is associated

with the eucalyptus tree (*Eucalyptus camaldulensis* and *Eucalyptus tereticornis*) (Callejas et al, 1998; Ellis & Pfeiffer, 1990b; Fortes et al, 2001).

Cryptococcus gattii comprises four major distinct molecular types, which include VGI, VGII, VGIII, and VGIV (Meyer et al, 2009, Figure 1). Among environmental isolates, VGII is the most frequent molecular type followed by VGI, VGIII and VG4. However, VGI and VGII are more common in clinical isolates followed by VGIII and VGIV. VGIV is rarely seen in the environment (Chen et al, 2014). The molecular type VGII is highly virulent and is responsible for most infections in healthy hosts in an on-going outbreak in parts of Pacific Northwest USA and Canada (Byrnes et al, 2010; Byrnes et al, 2009a; Byrnes et al, 2011; Walraven et al, 2011). In human cryptococcal disease caused by *C. gattii*, molecular type-associated clinical and host features have been observed. For instance, VGI infections often present with meningitis, including granulomas and lung lesions (Chen et al, 2012) while VGII infections typically display pulmonary disease (Galanis et al, 2009; Harris et al, 2011). Both VGI and VGII have a predilection for immunocompetent individuals. In contrast, molecular type VGIII has a predilection for infecting immunocompromised HIV/AIDS patients in Southern California and Southwestern USA and isolated cases outside the USA have been reported (Choi et al, 2010; Firacative et al, 2011; Hagen et al, 2012; Trilles et al, 2008). VGIV has been seen to be associated with HIV/AIDS in Africa (Chen et al, 2000; Sorrell et al, 1996). Apart from these four distinct haploid molecular types, the PCR fingerprinting and AFLP typing have demonstrated diploid or aneuploid hybrids. These hybrids are thought to have originated from mating events between major molecular types *C. gattii* and *C. neoformans*. These include VGI/VNIV (Serotype B and D, AFLP8) hybrids (Bovers et al, 2006), the VNI/VGI hybrids (AFLP9) (Aminnejad et al, 2012; Bovers et al, 2008b), and the VNI/VGII hybrids (AFLP11) (Aminnejad et al, 2012; Figure 1).

Interestingly, there is an ongoing debate regarding the lineages and nomenclature of *C. neoformans*/*C. gattii* species complex mainly between the two-species concept proposed by Kwon-Chung and Varma (2006) and the sevens species concept by Bovers et al. (2008a) and Ngamskulrungroj et al.

(2009) and recently supported by Hagen et al. (2015). How this debate can have an impact on the care of patients with cryptococcal diseases fuels another debate.

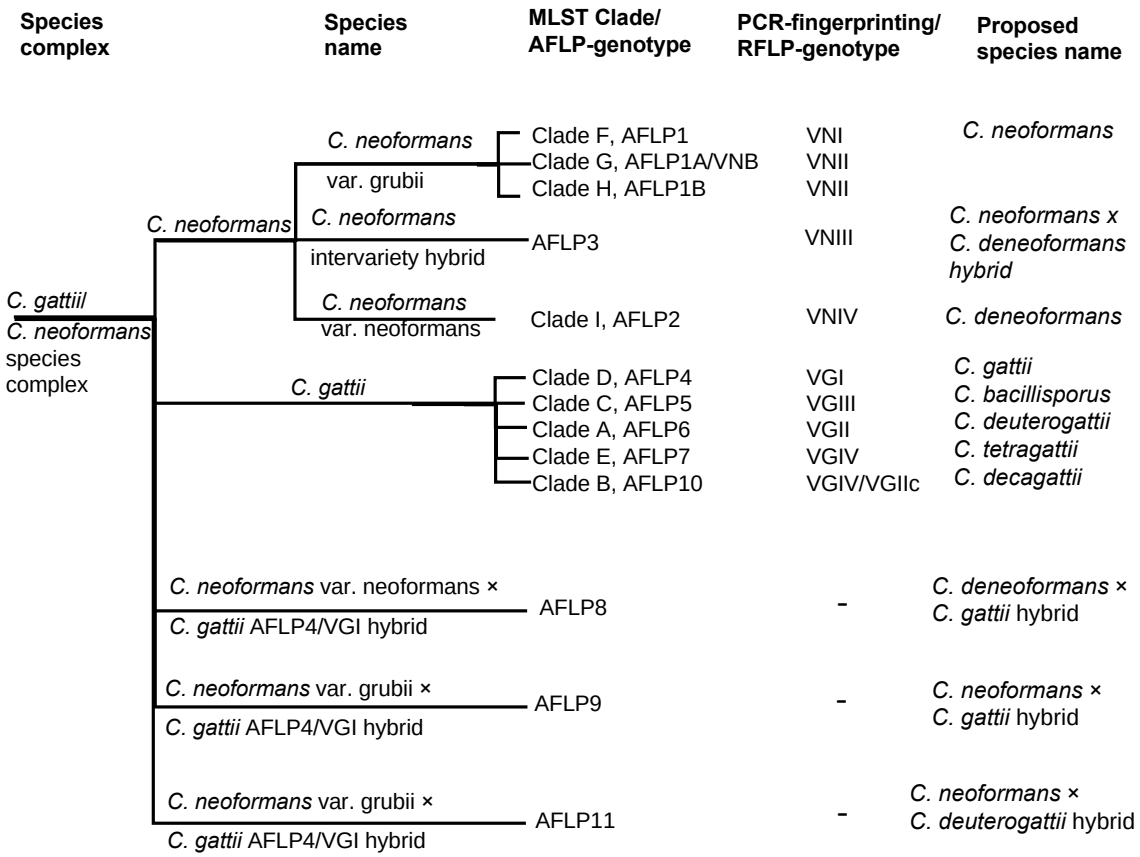

Figure 1: Species of *C. gattii*/*C. neoformans* complex

Classification of *C. gattii/C. neoformans* species complex and include species name, molecular types as described using MLST, AFLP, PCR fingerprinting and RFLP genotyping and proposed species names (Bovers et al, 2008a; Hagen et al, 2015; Ngamskulrungroj et al, 2009).

1.3 Life cycle of *Cryptococcus*

Cryptococcus is a basidiomycetous yeast that replicates by budding. The budding yeast is the most frequently isolated form of *C. neoformans* from both patients and environmental niches. However, *C. neoformans* can undergo a transition from the yeast form to filamentous growth form via two distinct pathways: mating (sexual cycle) and monokaryotic fruiting. Filamentation enables the fungus to acquire nutrients outside the confines of the colony and in the production of basidiospores. Basidiospores are believed to be the infectious propagules of *Cryptococcus*

Mating – *C. neoformans* is a heterothallic basidiomycete that means it can exist in two mating types, MAT**a** and MATα. The sexual cycle involves fusion of opposite mating types, MAT**a** and MATα haploid cells to produce dikaryotic filaments. Dikaryotic filaments eventually form a basidium. The two nuclei (from opposite mating types) of the basidium fuse and undergo meiosis to produce basidiospores comprised of MATα and MAT**a** type spores in equal numbers (Kwon-Chung, 1975; 1976; 1980).

There are significant differences in mating within the three *Cryptococcus* varieties. Mating in *C. neoformans* var. grubii is strain specific while *C. neoformans* var. neoformans is more robust and not confined to specific strains (Litvintseva et al, 2003; Nielsen et al, 2003). Mating has not been observed under laboratory conditions in *C. gattii* strains obtained from diverse sources and even in the environment where both MAT**a** and MATα strains coexist, no evidence of recombination has been seen (Fraser et al, 2003; Halliday & Carter, 2003). A significant exception are the strains from Vancouver outbreak, which appear clonal and entirely of the MATα and mate robustly (Fraser et al, 2003).

Mating type determines virulence in *C. neoformans*. MATα type is the most virulent mating type and consists most (96 % average) clinical and environment isolates (Kwon-Chung & Bennett, 1978; 1984a; Litvintseva et al, 2005) with one published exception of a *Cryptococcus* species from sub-Saharan Africa, which has almost equal ratios of MAT**a** and MATα cells (Litvintseva et al, 2003). It is

worthwhile to point out that mating of *C. neoformans* has never been reported occurring in nature, but *C. neoformans* readily mate under laboratory environment such as on media supplemented with pigeon excreta and live plants (Kwon-Chung, 1976; Nielsen et al, 2007; Xue et al, 2007). In less pathogenic *C. neoformans* var. neoformans, MATα type are most virulent whereas, co-infection of *C. neoformans* var. grubii with both mating types, results in MAT**a** and MATα strains reaching the lungs and spleen at equivalent levels (Nielsen et al, 2003). However, MATα readily disseminate to the CNS than congenic MAT**a** type (Nielsen et al, 2005).

Monokaryotic fruiting is thought to be a modified form of mating but involves the fusion of the same mating type (Lin et al, 2005). For example, MATα cell develops into diploid (α/α) cells, via either endoduplication or fusion of the nuclei after recombination of the two cells. The diploid monokaryotic hyphae form unfused clamp connections. This results in the formation basidium, followed by meiosis and production of haploid basidispores (Wickes et al, 1996). Mating and monokaryotic fruiting have similar features. However, the filaments that are produced during fruiting have unfused clamp connections and with a single nucleus while those produced following mating have fused clamp connections and contain two nuclei (Wickes et al, 1996). Monokaryotic fruiting is frequently observed in *MATα* type and formation of basidiospores and filaments has been observed under laboratory conditions in *C. neoformans* var. neoformans strains in response to environmental stimuli including nitrogen deficiency, desiccation, and darkness (Wickes et al, 1996). Monokaryotic fruiting was first thought to be strictly an asexual reproduction. However, Lin et al (2005) demonstrated that fruiting is a form of sexual reproduction between strains of the same mating type (Lin et al, 2005). Recently, Fu et al (2013) showed that monokaryotic hyphal production could be activated by arresting yeast cell cycle in the G2 stage with high temperature (Fu et al, 2013). It is still unknown whether monokaryotic fruiting occurs in *C. gattii*.

1.3.1 Infective forms of *C. neoformans*

Cryptococcus neoformans infection is acquired via inhalation of desiccated airborne yeast cells or sexually generated basidiospores into the lungs (Casadevall, 1998). However, size of the infectious propagule can pose as a restriction to pulmonary entry. For example, particles that are larger than 5µm such as encapsulate yeast are removed by the ciliary action of the lung epithelium (Hatch, 1961). Basidiospores have the potential to be deposited and penetrate the lung alveoli due to their small size (less than 2µm). Whereas yeast cells may lose their capsule upon exposure to conditions of low supply of moisture or deprivation of nutrients resulting in the reduction in size to less than 3µm (Ruiz et al, 1982; Wickes, 2002). Thus, desiccated yeast cells could also be small enough to get deposited and enter the lung alveoli following inhalation (Ellis & Pfeiffer, 1990b; Sukroongreung et al, 1998; Velagapudi et al, 2009). Basidiospores are thought to be the main infectious propagule-causing cryptococcosis. For example, one study has shown that basidiospores are more virulent and require very few numbers to cause cryptococcosis in mice than desiccated yeast cells (Sukroongreung et al, 1998). Moreover, basidiospores are less likely to undergo desiccation and can easily become aerosolised. However, encapsulated yeast cells struggle to survive in low supplies of moisture and deficiency of nutrients (Wickes et al, 1996). Using soluble components in receptor-ligand blocking experiments, Giles et al. (2009) identified Dectin-1 as a the most potential phagocytic receptor for *C. neoformans* spores (Giles et al, 2009). However, recent findings by the same group using a different approach, gain-of-function and loss-of-function assays with intact cells, demonstrated that Dectin-1 and other individual C-type lectins do not play critical roles in uptake and host innate defence by phagocytes against *C. neoformans* spores (Walsh et al, 2017) highlighting the need for further study into the pathogenesis of *C. neoformans* spores.

1.3.2 *C. neoformans* genome

The first genome of *C. neoformans* to be sequenced was that of laboratory strains JEC21 and B-3501A, which are closely related to VNIV

(*C. neoformans* var. neoformans). The 20 Mb genome is composed of 14 chromosomes and over 6500 genes. The chromosomes are extremely rich in genes. However, there is a sole large cluster on each made up of degenerating transposons that are thought to represent centromeres (Loftus et al, 2005). Very recently, the genome sequence of *C. neoformans* var. grubii has been published (Janbon et al, 2014). It is approximately 18.9 Mb in length, which is similar to *C. neoformans* var. neoformans and *C. gattii* (D'Souza et al, 2011; Loftus et al, 2005). It consists of 14 chromosomes and 6,962 protein-encoding genes that occupy 85% of the entire genome. Centromeres and intergenic regions make up the remaining 15%. Like other basidiomycetes, *C. neoformans* is richer in introns than many fungal species (Csuros et al, 2011). More than 40,000 introns have been identified in the genome, and most of these introns are located within the coding DNA sequence. Over 99 % of expressed genes have at least one intron (Janbon et al, 2014).

1.4 Cryptococcosis

1.4.1 Cause, transmission and affected organs, life cycle

Cryptococcosis is a disease caused by the fungi of the genus *Cryptococcus*, mostly by the species *C. neoformans* and *C. gattii*. *C. neoformans* is thought to be acquired through inhalation of small basidiospores (about 1-2µm) or desiccated yeast cells (found in the environment) into the lung alveoli (Ellis & Pfeiffer, 1990a; Sukroongreung et al, 1998; Velagapudi et al, 2009). Incidental findings of *C. neoformans* yeast cells in pulmonary foci and hilar lymph nodes at autopsy are consistent with this hypothesis (Baker, 1976; Baker & Haugen, 1955; Salyer et al, 1974). Inhalation of *C. neoformans* can result in pulmonary colonisation or disease and can also disseminate to other organs but tends to localise in the central nervous system causing meningitis (Mitchell & Perfect, 1995). Throughout life, humans are thought to be continuously exposed to *C. neoformans*. Individuals who have never been aware of having crytococcal infection have antibodies to *C. neoformans* (Chen et al, 1999). In fact, most children show antibody reactivity to *C. neoformans* before the age of five (Goldman et al, 2001). The outcome of *C. neoformans* infection depends on the

individuals' immune status. In the immunocompetent, an efficient immune response will eliminate most of the inhaled cryptococci. However, in the immunocompromised, such as those with HIV/AIDS, cryptococcal cells will proliferate and disseminate to the brain by crossing the blood brain barrier and cause meningoencephalitis. Although *C. neoformans* can invade any organ in the human body, infection of the CNS is the most commonly identified and serious clinical manifestation of cryptococcosis. Without treatment, CNS infection is fatal. HIV-associated cryptococcosis often presents with meningitis rather than respiratory disease. Cryptococcal meningitis is an AIDS-defining disease in HIV infected patients (Mitchell & Perfect, 1995). In some immunocompetent individuals, cryptococcal infection can remain dormant but viable in small lymph complex in the lung without causing any overt symptoms (Baker, 1976; Garcia-Hermoso et al, 1999; Salyer et al, 1974). If such individuals become immunocompromised later in life either due to HIV infection or corticosteroid treatment, the latent infection may become activated and begin to proliferate and disseminate into extrapulmonary sites (Dromer et al, 2011; Garcia-Hermoso et al, 1999). Other than pulmonary and brain involvement, *C. neoformans* has been reported to cause a distinct clinical entity involving the skin known as primary cutaneous cryptococcosis (Neuville et al, 2003).

Unlike *C. neoformans* which predominantly affects the immunocompromised and presents with meningoencephalitis, pulmonary cryptococcosis is considerably more common with *C. gattii* infections, and mostly the immunocompetent are affected (Chen et al, 2000; Galanis et al, 2010). Similary, mice infected with *C. neoformans* die due to CNS involvement, while mice infected with *C. gattii* succumb to pulmonary infection (Ngamskulrungroj et al, 2012). Interestingly, *C. gattii* VGIII has a predilection for infecting immunocompromised HIV/AIDS patients in Southern California and Southwestern USA (28 of 276 cryptococcal strains) (Byrnes et al, 2011; Chaturvedi et al, 2005). Isolated cases outside the USA have also been reported (Choi et al, 2010; Firacative et al, 2011; Hagen et al, 2012; Trilles et al, 2008). However, the prevalence of *C. gattii* in HIV/AIDS patients is not well documented as recovered isolates are rarely assigned species status.

1.4.2 Epidemiology

Although most people are exposed to *Cryptococcus* infectious propagules, they never become ill from it. *C. neoformans* infections are very rare in healthy individuals, and most cases occur in individuals with a pre-existing condition or disease. Predominantly, cryptococcosis due to *C. neoformans* affects individuals with advanced HIV/AIDS. However a growing number of cases are observed among solid organ transplant recipients, patients on prolonged immunosuppression therapy (glucocorticosteroids, cytotoxic chemotherapy, TNF-α inhibitors) and patients with hematological disorders such as chronic leukaemia and lymphoma (Pappas, 2001; 2013; Zhu et al, 2010). A very small proportion of cryptococcosis cases have been seen in clinically non-immunocompromised patients (Pappas, 2001; 2013; Zhu et al, 2010). On the other hand, *C. gattii* was mainly recognized as an emerging infection in healthy individuals in the northwest of North America (MacDougall et al, 2007). However, a subsequent report showed some of that patient population had underlying immunosuppression including HIV AIDS, solid organs transplant or corticosteroid treatment (Galanis et al, 2010; Harris et al, 2011). Below, I will describe the epidemiology of cryptococcosis categorised for HIV and non-HIV patients. µ

1.4.2.1 In HIV/AIDS patients

Cryptococcosis is the leading fungal cause of morbidity and mortality among HIV patients, and cryptococcal meningitis is an indication of the advanced stage of HIV infection (Mitchell & Perfect, 1995; Tenforde et al, 2017). The most recent estimates are that approximately 280 thousand cases and >180 thousand deaths occur each year due to cryptococcal meningitis among HIV/AIDS patients worldwide (Rajasingham et al, 2017). Cryptococcosis is the leading cause of meningitis among HIV/AIDS individuals in sub-Saharan Africa accounting for 26 - 60 % of all cases (Békondi et al, 2006; Gordon et al, 2000; Hakim et al, 2000; Rajasingham et al, 2015). About three-quarters of cryptococcal meningitis cases occur in sub-Saharan Africa followed by East, South and Southeast Asia (Rajasingham et al, 2017). Despite treatment with currently available anti-fungals, the mortality rate remains as high as 70 % in resource-limited settings

(Baldassarre et al, 2014; Gaskell et al, 2014; Jarvis et al, 2014; Kendi et al, 2013; Lessells et al, 2011; Letsou & Gusberg, 1990; Rothe et al, 2013), 40 % in middle-income countries (Jongwutiwes et al, 2007; Rajasingham et al, 2012; Vidal et al, 2013) and approximately 20 - 30 % in high-income countries (Lortholary et al, 2006; Pyrgos et al, 2013; Rajasingham et al, 2017). In North America, there has been a substantial decline in HIV-associated cryptococcal meningitis due to early diagnosis combined with antiretroviral therapy (ART) (Mirza et al, 2003; Pyrgos et al, 2013). In contrast, this has not been the case in sub-Saharan Africa despite the expansion of ART access programs. For example, studies in South Africa and Botswana showed no significant reduction in the cases of HIV-associated cryptococcosis (Jarvis et al, 2009; Tenforde et al, 2017). Recently, a clinical trial on HIV-associated cryptococcal meningitis reported that 49 % of the enrolled participants were already taking ART (Rhein et al, 2016), suggesting that the HIV-associated cryptococcosis remains a huge burden in developing countries despite mass ART programs in place. Interestingly, there is a huge discrepancy in the incidences of cryptococcosis between males and females including HIV- or organs transplant-associated cryptococcosis with males having higher incidences of *C. neoformans* infection than females (Hajjeh et al, 1995; Mitchell & Perfect, 1995; Tenforde et al, 2017). This difference was observed even in the pre-HIV era in which the incidence of cryptococcosis was 2-3:1 males:females (Edwards et al, 1970; Mitchell & Perfect, 1995). A recent study by Carvour et al. (2015) showed that females present with more severe disease and are less likely to survive than males when data was adjusted for potential confounders such as age, race, birth sex, healthcare facility type and opportunistic infections (Carvour et al, 2015). This was a small study, and large studies are required to establish a hypothesis. As for *C. gattii*, the prevalence in HIV/AIDS patients is not known mainly due to lack of routine species allocation of most *Cryptococcus* isolates. However, *C. gattii* VGIII strain is responsible for HIV/AIDS associated infections in Southern California and abroad (Choi et al, 2010; Firacative et al, 2011; Hagen et al, 2012; Trilles et al, 2008).

1.4.2.2 In HIV naïve patients

Solid organs transplant patients - It is difficult to establish the worldwide incidence of cryptococcosis in HIV naïve patients because the numbers are masked by HIV-associated cryptococcosis particularly in low- and middle- income countries. In the United States, cryptococcosis is the third most common fungal infection (after invasive candidiasis and aspergillosis) among recipients of solid organs transplant (SOT) (Pappas, 2001; Pappas et al, 2010). Cryptococcosis usually occurs late, between 16 to 21 months post-transplantation (Pappas et al, 2010). The recipients of liver transplants develop cryptococcosis as early as under 12 months-post-transplantation compared to kidney transplant recipients probably because of exacerbated immunosuppression in the former subgroup (Husain et al, 2001; Singh et al, 2007). The actual incidence of cryptococcosis in SOT recipients ranges from 0.2 – 5 % with an average of ~2.8 % (George et al, 2017; Person et al, 2010; Sun et al, 2009). The disease is confined to the lungs but may also spread to the CNS and other body organs. 39-55% of cryptococcosis cases in SOT recipients have CNS involvement (Husain et al, 2001; Pappas, 2001; Pappas et al, 2010; Singh et al, 2007). Data from one multinational study showed that 61% of the patients had disseminated cryptococcosis, about 32% of the patients had disease confined to the lungs, and 8.1% had skin, soft-tissue, or osteoarticular infections (Singh et al, 2007). Liver recipient patients are six-times more at risk of developing disseminated cryptococcosis than other types of SOT recipients. Fungemia is seen in up to 33% of SOT recipients with cryptococcal disease (Husain et al, 2001; Singh et al, 2007; Wu et al, 2002). The mortality rates in SOT recipients with cryptococcal disease have ranged from 14-27% and as high as 49% in those with CNS involvement (Neofytos et al, 2010; Sun et al, 2009; Wu et al, 2002).

Non-HIV patients, non-transplant patients - Other than affecting patients with HIV or SOT recipients, cryptococcosis also occurs in individuals with other predisposing factors or underlying disease including cancer, steroids, diabetes mellitus, kidney disease, cirrhosis and in individuals with no identifiable immunodeficiency (Fang et al, 2015; Jongwutiwes et al, 2008; Lin et al, 2015; Pappas et al, 2001; Shih et al, 2000; Singh et al, 2015; Thomas et al, 1998;

Yuchong et al, 2012). A US study by Brizendine et al. (2013) showed that out of 302 patients diagnosed with cryptococcal disease between 1996 and 2010, 36 % and 28 % were HIV positive and organs transplant recipients respectively while another 36 % were non-HIV, Non-transplant (NHNT) infected persons. Of the NHNT group, 36 % were phenotypically healthy patients, 25 % received steroid therapy, 28 % had cancer, and 11 % had chronic liver or renal disease while 12 % had diabetes mellitus (Brizendine et al, 2013). Of note, cases of *C. neoformans* among apparently immunocompetent patients occur across the globe with most cases reported in Asia (Zhu et al, 2010). Cryptococcal disease has also been associated with genetic polymorphism of phagocytic FCGR2A and 3A genes in HIV naïve Caucasians (Meletiadis et al, 2007), polymorphism of non-phagocytic FCGR2B but not FCGR3A in the Chinese population (Hu et al, 2012) and polymorphism of FCGR3A in HIV infected patients (Rohatgi et al, 2013). Recently, Panackal et al. (2015) reported a group of non-HIV with crytococcal meningitis that displayed activation and expansion of intrathecal cells including HLA-DR$^+$, CD4$^+$ and CD8$^+$ cells. The T cells expressed increased levels of IFNγ and reduced levels of IL-2 and IL-13. However, despite maintained recruitment of macrophages to the site of infection, they were characteristically M2 macrophages and demonstrated poor uptake of fungal cells suggesting that failure of macrophage activation risks susceptibility of cryptococcal disease (Panackal et al, 2015).

Meningoencephalitis caused by *C. gattii* in the immunocompetent has been reported throughout the tropics including Australasia (Speed & Dunt, 1995), South America and particularly in the ongoing outbreak in the Pacific Northwest of North America since 1999 (Phillips et al., 2015). *C. gattii* was thought to occur only in apparently immunocompetent individuals, however, of the 76 patients in US *C. gattii* outbreak between 2004 and 2010, 76 % of them had pre-existing predisposing conditions including steroid therapy, chronic lung, kidney or heart diseases and other conditions (Harris et al, 2011). Anti-GM-CSF has also been associated with cryptococcal meningitis caused by *C. gattii* in otherwise immunocompetent individuals (Rosen et al, 2013; Saijo et al, 2014).

Thus, the foregoing demonstrates the diversity of susceptible populations ranging from apparently healthy hosts to individuals with significant immunological impairments caused by chemotherapy or immunosuppressive therapy, organ dysfunction and those with immunodeficiencies, either innate or acquired. Given the heterogeneity of the host groups, it is difficult to draw conclusions regarding epidemiology, clinical presentation or outcomes of each group.

1.4.3 Clinical manifestations

1.4.3.1 CNS cryptococcosis

Clinical manifestations of cryptococcosis most commonly involve the lungs, central nervous system and rarely other body organs. Cryptococcal meningitis (CM) is a subacute meningoencephalitis and is the most common clinical presentation of cryptococcosis in HIV-infected patients (Mitchell & Perfect, 1995). Patients with CM usually present with neurological symptoms, including a headache and altered mental status which may be accompanied by fever, nausea and vomiting. The median incubation period from onset of symptoms to presentation is two weeks in HIV-associated CM and 6-12 weeks in HIV naïve patients. A significant number of patients develop visual problems, such as diplopia and the subsequent reduction in acuity as a result of high intracranial (CSF) pressure and/or optic nerve involvement (Moodley et al, 2012). In the absence of treatment, the patient may become confused, develop seizures and reduced level of consciousness resulting in coma (Bicanic & Harrison, 2004; Mitchell & Perfect, 1995). In non-HIV infected patients, there is a significant heterogeneity, and clinical manifestation can be influenced by the patient's immune competency (Pappas, 2001).

1.4.3.2 Pulmonary cryotococcosis

Pulmonary cryotococcosis is the most common manifestation of disease in HIV-negative (non-immunocompromised) patients (Kiertiburanakul et al, 2006). Pulmonary cryptococcosis is not commonly reported and is certainly misdiagnosed as other pulmonary infections such as tuberculosis in the immunocompromised (Batungwanayo et al, 1992; Bernicker et al, 1996; Jarvis et al, 2010; Murray et al, 1999; Wong et al, 2007). Approximately 10-55% of

HIV-associated cryptococcosis present as pulmonary disease (Reviewed in Jarvis & Harrison, 2008). The clinical pattern of cryptococcal pulmonary disease is significantly heterogeneous, ranging from asymptomatic colonisation to severe symptoms such as pneumonia and acute respiratory distress syndrome (Campbell, 1966; Nadrous et al, 2003; Perfect et al, 1983). In most cases, patients present with coughing with scanty sputum, chest pain, fever, malaise, dyspnoea, pleuritic chest pain and very rarely, haemoptysis and weight loss (Chechani & Kamholz, 1990; Meyohas et al, 1995; Rozenbaum & Gonçalves, 1994; Wasser & Talavera, 1987). Often, clinical presentations are indistinguishable from other causes of pneumonia in these patients (Loerinc et al, 1988; Visnegarwala et al, 1998). Pleural effusion, cavitation, and hilar lymphadenopathy have been described in patients with immune reconstitution inflammatory syndrome (IRIS) (Jenny-Avital & Abadi, 2002; Trevenzoli et al, 2002). The disease progresses rapidly in immunocompromised hosts and may require anti-fungal therapy. Pulmonary cryptococcosis may ensue in the absence of disseminated disease, and similarly, disseminated disease (i.e. meningitis) may develop in the absence of noticeable pulmonary pathology. In normal hosts, infection is confined to the lung and may not require active treatment (Aberg et al, 1999; Chang et al, 2006; Kerkering et al, 1981; Nadrous et al, 2003). However, symptoms when present are the same as in the immunocompromised and may be mild to severe (Emmons et al, 1995; Mitsuoka & Kanazawa, 2005; Nadrous et al, 2003).

1.4.3.3 Cryptococcosis in other sites

Other than the lung and CNS involvement, the next most common organs of disseminated cryptococcosis include the skin and medullary cavity of bones (Mitchell & Perfect, 1995). Cutaneous cryptococcosis usually presents in the form of papules, pustules, nodules, ulcers or draining sinuses (Neuville et al, 2003). Umbilicated papules that can be mistaken for molluscum contagiosum have been reported in HIV-positive patients (Gasiorowski et al, 2001; Picon et al, 1989). Cellulitis with necrotising vasculitis has been seen in SOT recipients (Baer et al, 2009; Horrevorts et al, 1994; Sun et al, 2010), patients with long-term corticosteroid treatment (Hafner et al, 2005) and a patient with rheumatoid

arthritis (Probst et al, 2010). Cryptococcal disease with bone involvement manifests osteolytic lesions that resemble cold abscesses and may be misdiagnosed as tuberculosis or neoplasm (Burch et al, 1975; Wood & Miedzinski, 1996). Very rare forms of cryptococcosis include myocarditis, hepatitis, renal disease, prostatitis, peritonitis and adrenal involvement (Joshi et al, 1989; Lewis et al, 1985; Siddiqui et al, 2005b).

1.4.4 Diagnosis

The physical findings to make a diagnosis in patients with cryptococcosis highly dependent on the immunocompetence of the patient prior to infection and the site or sites involved. For example, due to the poor inflammatory response to cryptococci, particularly in those patients with advanced HIV infection, the organs or tissue may be largely affected before patient present for medical evaluation thus obscuring diagnosis. Below I will describe steps taken for diagnosis of CNS and pulmonary cryptococcal disease.

1.4.4.1 Cryptococcal meningitis

There are several tests that are used for the diagnosis of cryptococcal disease involving the CNS (Boulware et al, 2014). The standard diagnostic methods focus on detection by microscopy, culture or antigen. The methods include India ink staining, CSF culture and cryptococcal antigen test (CrAG). India ink test remains the common diagnostic method for identifying *Cryptococcus* in CSF and yet has a sensitivity of less than 86% and is even less sensitive in patients with low fungal burden (Coovadia et al, 2015; Dominic et al, 2009). CSF culture is considered the gold standard; however, it requires laboratory infrastructure, trained personnel, can take longer (up to 10 days) and can also give false negative results when fungal burden is low (Barenfanger et al, 2004). However, despite the downsides of quantitative CSF culture, the method is used to measure response to treatment and is important in the diagnosis and differentiation of cryptococcal meningitis relapse and paradoxical cryptococcal immune reconstitution inflammatory syndrome (IRIS) (Boulware et al, 2010; Haddow et al, 2010). The other method that has become an important diagnostic method is the detection of cryptococcal antigen (CrAg) in CSF, serum or plasma. The WHO

guidelines recommend that lumbar puncture should be performed promptly and rapid CSF CrAg assay carried out on all patients with HIV with suspected cryptococcal meningitis (WHO, 2011). Several platforms for the detection of CrAg including latex agglutination or enzyme immunoassays that have been available for several years (Temstet et al, 1992). The recently developed CrAg lateral flow test detects the cryptococcal polysaccharide capsule by use of gold-conjugated anti-cryptococcal monoclonal antibodies impregnated onto an immunochromatographic test strip (Vidal & Boulware, 2015). This test is stable at room temperature, inexpensive and takes minutes to acquire results. A recent multi-national diagnostic study found that CrAg lateral flow assays (LFA) have 99.3 sensitivity and 99.1% specificity in CSF (Boulware et al, 2014). There is a semi-quantitative CrAg LTA that has been shown to correlate with quantitative cultures before treatment, but its usefulness drastically falls-short when monitoring treatment response (Kabanda et al, 2014).

1.4.4.2 Pulmonary cryptococcosis

Diagnosis of pulmonary cryptococcosis involves isolation of *Cryptococcus* from the pulmonary sample in addition to related clinical and radiological findings. In the immunocompetent, single or multiple pulmonary nodules are frequently found ranging from 5 to 30 mm in diameter. The immunocompromised display a wide spectrum of radiological abnormalities, with a reduced proportion of nodules but are associated with diffuse, spreading disease (Jarvis & Harrison, 2008). Other common abnormalities include lymphadenopathy, lesions and pleural effusions, which are indistinguishable from pulmonary tuberculosis (Chen et al, 2015; Dogbey et al, 2013; Young et al, 1980). Microscopic examination of respiratory specimens such as sputum, *Cryptococcus* appear as an encapsulated budding yeast. Culture on selective media takes 2 to 5 days for colonies to develop. However, whilst sputum culture has been widely described for diagnosis, it is not routinely used for diagnosis. Yeast cells observed in HIV positive patients may be assumed as *Candida* (Driver et al, 1995). Another diagnostic method that has been reported for cryptococcal pneumonia is through bronchoscopy and testing bronchoalveolar lavage samples with CrAg (Baughman et al, 1992; Chechani & Kamholz, 1990; Malabonga et al).

1.4.5 Management of cryptococcal disease

The management of cryptococcosis depends on the severity of the infection and the affected body organs.

1.4.5.1 Management of meningeal cryptococcosis

The management of meningitis is categorised into three phases: which includes induction, consolidation and maintenance treatment. The ultimate objective of the induction treatment is the rapid sterilisation of the cerebrospinal fluid which is measured by the rate of fungal clearance per millilitre of CSF per day. Early fungicidal activity (EFA) is a term used to describe the quantitative clearance. Slower rates of EFA are associated with high mortality at two and ten-week post initiation of therapy (Bicanic et al, 2009). The current guidelines by the Infectious Disease Society of America (IDSA) recommend two weeks of intravenous amphotericin B at 0.7-1.0mg/kg per day plus oral flucytosine (100mg/kg per day) for treatment of cryptococcal meningitis as an induction therapy. Amphotericin B is known to cause serious side effects including anaemia, kidney problems, hypokalaemia, and phlebitis. Amphotericin B is administered intravenously and thus requires inpatient hospitalisation and significant nursing care. Therefore, amphotericin B therapy should include pre-hydration, electrolyte replacement, monitoring and management of toxicity. In resource-limited settings where flucytosine is unavailable or where resources are not reliable or sustainable to monitor and prevent amphotericin B toxicity, the IDSA and the WHO recommends initial induction therapy for 1 week of intravenous amphotericin B (1mg/kg per day) in combination with 2 weeks of fluconazole (800mg/kg per day) (Perfect et al, 2010; WHO, 2011). High dose fluconazole (1200mg/kg per day) monotherapy for 10-12 weeks is recommended if amphotericin B and flucytosine are unavailable (Perfect et al, 2010; WHO, 2011).

The second phase, following two weeks of induction is consolidation therapy that consists of fluconazole 400-800mg/day for at least eight weeks (Perfect et al, 2010). However, in case induction monotherapy of fluconazole (1200mg/kg per day) is used or CSF sterility has not been achieved at end of 2 week-induction

therapy, the high-dose fluconazole should continue throughout the consolidation phase (Perfect et al, 2010).

Following satisfactory induction and consolidation treatment, CSF culture-negative patients should be given a maintenance therapy of fluconazole 200mg per day (Perfect et al, 2010; WHO, 2011). This maintenance therapy is recommended to avoid relapse as shown before in patients who discontinued treatment (Bozzette et al, 1991). IDSA recommends that maintenance therapy can be discontinued if the patient is on ART and has had over three months of undetectable HIV RNA levels with a $CD4^+$ cell count of ≥100 cells/µL (Perfect et al, 2010). In settings where viral load testing is not available, maintenance therapy must be continued for 12 months and discontinued only if $CD4^+$ cell counts are more than 200 cells/µL (WHO, 2011). If the patient shows immunological failure, poor adherence to ART, or reduction of the $CD4^+$ cell count to less than 100 cell/µL, then fluconazole maintenance therapy should be reinstituted (Perfect et al, 2010).

1.4.5.2 Management of cryptococcosis complications

Intracranial pressure - Approximately 50 % of HIV-infected patients with cryptococcal meningitis have higher baseline intracranial pressure of >25cm of CSF and is associated with high fungal burden in the CSF (Rhodes et al, 1980). IDSA and WHO guidelines recommend that a baseline CSF pressure is obtained and baseline lumbar puncture (LP) carried out promptly. If the intracranial pressure is ≥25 cm of CSF and there are symptoms associated with increased intracranial pressure, then an LP should be done to drain the CSF to reduce the pressure to normal (≤20 cm of CSF) or by half if it is extremely high. Additional LPs including daily LPs should be carried out if necessary until the normal pressure has been reached. IDSA further recommends that LP should wait if the patient has focal neurological signs or a compromised mentation pendant CT or MRI scan (Perfect et al, 2010). If the scan identifies an obstruction that needs decompression, then drainage can be performed safely using ventriculostomy of VP shunt (Woodworth et al, 2005).

Cryptococcal immune reconstitution inflammatory syndrome - Following treatment of cryptococcal meningitis and ART initiation, some patients may present with clinical manifestations of recurrent symptomatic cryptococcal meningitis. These patients are suspected to have developed a paradoxical cryptococcal immune reconstitution inflammatory syndrome (C-IRIS), treatment failure or relapse. IRIS is a condition observed in some AIDS or immunosuppressed patients on ART in which the immune system begins to recover but then overreacts to previously treated opportunistic infections or subclinical infections that paradoxically cause the symptoms of the severe infection (Cheng et al, 2000; Shelburne et al, 2002). Although treatment failure and C-IRIS are highly indistinguishable, patients with paradoxical C-IRIS usually present with symptoms of meningitis with negative CSF culture and higher CSF white blood cell (WBC) count whereas the former have a positive CSF culture and non-pronounced CSF inflammatory profile (Boulware et al, 2010; Haddow et al, 2010).

Management of C-IRIS - Management of C-IRIS includes management of increased intracranial pressures that involves drainage of large volumes of CSF using lumbar puncture. IDSA guidelines recommend administration of 0.5-1mg/kg of prednisone or dexamethasone to be spread over a period of 2-6 weeks. These recommendations are mainly based on opinions of experts and clinical experience and not on research findings (Abassi et al, 2015; Perfect et al, 2010).

1.4.5.3 Management of non-meningeal cryptococcosis

Pulmonary cryptococcosis - Cryptococcosis involving the lungs encompasses clinical manifestations ranging from asymptomatic pneumonia to severe acute respiratory distress syndrome (ARDS) (Pappas et al, 2001; Vilchez et al, 2001; Visnegarwala et al, 1998). The goal of the treatment regimen is to gain control of the signs and symptoms of cryptococcal pneumonia and prevent dissemination to the CNS. IDSA recommends firstly ruling out meningitis in immunosuppressed patients with pulmonary cryptococcosis because cryptococcal meningitis will require differing dosage and length of induction therapy. Cryptococcal pneumonia

associated with CNS disease or severe pneumonia should be treated like cryptococcal meningitis (Perfect et al, 2010). For moderate symptoms without the presence of pulmonary infiltrates and immunosuppression, patients should be treated with oral fluconazole 400mg per day for 6-12 months, however, if a patient is HIV-positive, on ART and has a $CD4^+$ count of >100 cells/µL, the fluconazole treatment should be longer than 12 months. For patients with ARDS and IRIS corticosteroids treatment should be considered to dampen the immune response. In addition, surgery should be considered in patients with persistent symptoms of pneumonia and radiographic abnormalities that are unresponsive to anti-fungal therapy (Perfect et al, 2010). In case fluconazole is unavailable or contraindicated, IDSA recommends the use of itraconazole, voriconazle or posaconazole as alternatives (Perfect et al, 2010).

Non-meningeal, non-pulmonary cryptococcosis - In the majority of patients, non-meningeal and non-pulmonary cryptococcosis represent a disseminated form whose clinical pattern is restricted to a single organ or tissue. Thus, IDSA recommends that a similar treatment regimen to that of CNS or disseminated cryptococcosis should be considered. The selection of anti-fungal drugs and duration should depend on the patient's immune status, responsiveness to treatment and disease severity. In patients with no obvious immunosuppressive risk factors, without CNS disease or fungemia but with localised infection, treatment similar to moderate pneumonia should be prescribed (Perfect et al, 2010).

1.4.6 *C. neoformans* virulence factors

1.4.6.1 The Capsule

C. neoformans is the only fungal pathogen with a capsule, a distinctive polysaccharide structure surrounding the cell body. The capsule is invisible by regular microscopy but can be made visible by suspending the yeast in an India ink preparation and is seen as a characteristic clear zone outside the cell wall (Figure 2). The capsule is composed of the two major types of polysaccharides, glucuronoxylomannan (GXM; 90-95%) and glucuronoxylomannogalactan (GXMGal; 5-10%) (Bose et al, 2003; Cherniak et al, 1982; Cherniak & Sundstrom,

1994). In addition, the capsule also contains 1 % of mannoproteins (MP) but its role in the capsule architecture remains unknown (Cherniak & Sundstrom, 1994; Jesus et al, 2010; Rodrigues & Nimrichter, 2012). Its diameter is roughly 4-6 μm but can reach up to 30 μm (Aksenov et al, 1973; Granger et al, 1985; Vartivarian et al, 1993). The function of the capsule in the environment is not known, however it is speculated that the capsule acts as a food source and protects the fungus from environmental desiccation and predators such as nematodes and amoebae (Chrisman et al, 2011; García-Rodas et al, 2011; Mylonakis et al, 2002; Steenbergen et al, 2003).

Virulence - The polysaccharide capsule is the main virulence factor of *C. neoformans* and in the host, it operates by disrupting normal immune cell responses at different levels during infection to evade killing by host cells. For example, the capsule demonstrates robust anti-phagocytic properties by providing a physical and chemical shield to fungal motifs that bind to macrophage receptors (Bolaños & Mitchell, 1989; Bulmer & Sans, 1967; Granger et al, 1985; Levitz & DiBenedetto, 1989; McGaw & Kozel, 1979), thus preventing recognition and inhibiting phagocytosis of the fungus. Acapsular mutants are readily phagocytosed, however, the addition of purified polysaccharide prevents uptake by phagocytes (Kozel, 1995). In addition, acapsular mutants fail to replicate inside phagocytes (Feldmesser et al, 2000) suggesting that the capsule is required for intracellular survival. The soluble polysaccharides are also thought to interfere with macrophage metabolism by binding to glycolytic enzymes such as phosphofructokinase following leakage into the cytoplasm due to permeable phagolysosomes (Grechi et al, 2011). One of the interesting characteristics of the capsule is its ability to change size in response to changes in the microenvironmental. For example, during infection and interaction with macrophages the capsule significantly increases in size (Feldmesser et al, 2001), presumably a virulent phenotype to deter phagocytosis. In the mammalian host, the capsule is involved in inhibition of phagocytosis and modulating immune response to its favour (Zaragoza et al, 2008). The capsule also protects the fungus against reactive oxygen species, which are vital antimicrobial molecules produced by macrophages (Zaragoza et al, 2008). In contrast to acapsular

mutants, ingested encapsulated *C. neoformans* cells do not induce nitric oxide synthase production in macrophages (Naslund et al, 1995).

Synthesis and regulation of capsular polysaccharide - Genes required for biosynthesis of the polysaccharide capsule include *cap59* (Chang & Kwon-Chung, 1994), *cap64* (Chang et al, 1996), *cap60* (Chang & Kwon-Chung, 1998) and *cap10* (Chang & Kwon-Chung, 1999). Disruption or deletion of any of these genes results in loss of virulence in mice models of cryptococcal infection (Chang & Kwon-Chung, 1994; 1998; 1999; Chang et al, 1996). In addition, the capsule of *C. neoformans* is highly regulated with regards to its size and complexity to enable survival of the fungus within the host. Depending on environmental conditions, the size of the capsule varies, becoming specifically large during mammalian infection (Rivera et al., 1998). *In vitro*, capsule expansion is possible under conditions similar to host environments such as low iron, serum, physiological concentrations of CO_2 and pH (pH7) (Bahn et al, 2005; Vartivarian et al, 1993; Zaragoza et al, 2003). The iron sensing mechanisms that regulate capsule formation include transcription factors Hap3 and Hap5 (Jung et al, 2010), the HOG pathways (Bahn et al, 2005) and chromatin remodelling involving histone acetyltransferase Gcn5 (O'Meara et al, 2010). In addition, there is a correlation between strain virulence and the size of the capsule *in vivo* (Clancy et al, 2006) indicating that regulation of capsular size is important in the pathogenesis of cryptococcal infection.

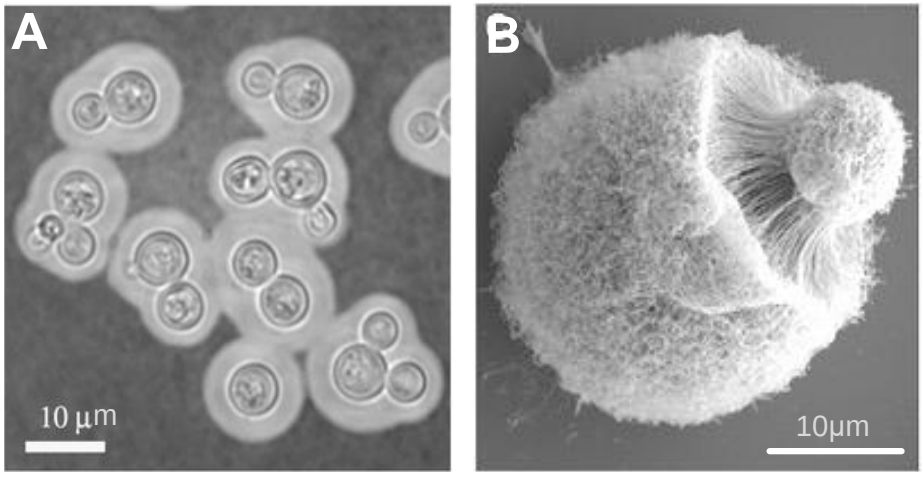

Figure 2: Cryptococcal capsule in India ink staining and electron microscopy micrograph

Micrographs were taken (A) under light and (B) scanning electron microscopy with *Cryptococcus* suspended in India ink (images obtained and modified from Zaragoza et al. (2009).

1.4.6.2 Melanin

Melanin is a brown-black pigment that is synthesised by a process catalysed by laccase in the presence of o-diphenolic compounds such as 3,4-dihydroxyphe-nylalanine (L-Dopa) and accumulates in the cell wall (Eisenman et al, 2007; Wang et al, 1996; Williamson, 1997; Williamson et al, 1998). It is believed that melanin protects *C. neoformans* from UV light and extremely high or low temperatures in the environment (Rosas & Casadevall, 1997; Wang et al, 1995; Wang & Casadevall, 1994). During infection, melanin protects *C. neoformans* from toxic oxygen- and nitrogen-derived oxidants released by host immune cells (Casadevall et al, 2000; Nosanchuk et al, 2000). Apart from its ability to absorb these free radicals, melanin protects *C. neoformans* from killing by anti-fungal drugs. For example, melanised *C. neoformans* are more resistant to amphotericin B and caspofungin, compared to non-melanised cryptococci (Ikeda et al, 2003; van Duin et al, 2002). Melanised cells are also resistant to phagocytosis, cell death and are more virulent than non-melanised cells (Casadevall et al, 2000; Huffnagle et al, 1995) suggesting that melanin is a key virulence factor of *C. neoformans*. Melanin may also inhibit T cell-mediated pulmonary inflammation and immunity (Huffnagle et al, 1995). It is thought that the predilection of *C. neoformans* for the CNS is associated with its ability to convert catecholamines such as dopamine, norepinephrine and epinephrine into melanin (Polacheck et al, 1990). Catecholamines function as neurotransmitters in the CNS. This hypothesis is consistent with the observation that catecholamine rich areas of the brain are often infiltrated with *C. neoformans* during cryptococcal meningitis, and fungal cells isolated from the brain appear pigmented upon microscopic examination (Lee et al, 1996). Progressive thickening and pigmentation of the fungal cell wall have also been observed during infection in a murine model of cryptococcal meningitis (Nosanchuk et al, 1999; Rosas et al, 2000).

1.4.6.3 Anti-phagocytic protein 1

Cryptococcus neoformans secretes anti-phagocytic protein (App1). App1 is downstream of the sphingolipid pathway and is present in the supernatant of

C. neoformans culture (Luberto et al, 2003). The Δ*app1 C. neoformans* mutant is readily phagocytosed by macrophages (Luberto et al, 2003). Addition of rApp1 protein blocks the complement-mediated phagocytosis of *C. neoformans* in a dose-dependent manner (Luberto et al, 2003). App1 binds to complement receptor, CR3 and anti-phagocytic activity of rApp1 is completely abrogated in alveolar macrophages in the absence of CR3 (Stano et al, 2009).

1.4.6.4 Urease

Fungi produce enzymes that are required for the breakdown of molecules to obtain nutrients from the surrounding environment. *C. neoformans* also secretes several enzymes one of which is urease. Since *C. neoformans* is often present in avian droppings (Patridge & Winner, 1965), the fungus must breakdown creatinine, xanthines and uric acid to survive and proliferate in this environment. Urease catalyses the hydrolysis of urea to ammonia and carbamate (Kwon-Chung et al, 1987; Vogel, 1969; Zimmer & Roberts, 1979). *C. neoformans* urease mutant strain has significantly reduced virulence than the wild-type in murine models of infection since mice infected with the mutant survive longer than mice infected with the wild-type strain (Cox et al, 2000; Olszewski et al, 2004). In addition, urease knockout *C. neoformans* strains cause meningitis but not pneumonia (Cox et al, 2000). Urease plays an important role in fungal transmigration through epithelial barriers and dissemination to the brain but is not essential for growth in the brain (Olszewski et al, 2004; Shi et al, 2010; Singh et al, 2013). Although urease activity has been demonstrated in most clinical isolates (Bava et al, 1993; Cox et al, 2000; Ruane et al, 1988), occasional urease-negative strains have also been recovered in clinical isolates (Bava et al, 1993). This indicates that urease may be dispensable for infection, provided other virulence factors compensate for its roles in virulence.

1.4.6.5 Thermotolerance

The ability of *C. neoformans* to grow at mammalian temperatures is essential for virulence (Martinez et al, 2001; Perfect, 2006; Robert & Casadevall, 2009). Since the ability to grow at physiological temperatures is a prerequisite for any pathogen, the thermotolerance of *C. neoformans* is essential over any other

virulence factor (Robert & Casadevall, 2009). Most *Cryptococcus* species possess other virulence factors including capsule and laccase but do not cause disease in mammals because they are unable to grow at physiological temperatures (Petter et al, 2001). Similar findings have been demonstrated in vertebrate and invertebrate models of infection (Garcia-Solache et al, 2013; McClelland et al, 2006).

1.4.6.6 Phospholipase

Phospholipase is an enzyme that breaks down phospholipids. An isoform of phospholipase B, Plb1, is produced during infection and promotes fungal viability within phagocytes (Cox et al, 2001). Deletion of Plb1 results in reduced virulence and decreased capacity to disseminate to the brain (Cox et al, 2001). *C. neoformans* culture supplemented with phospholipids induces the expression of Plb1 and increases capsule size. Break down of host phospholipids by Plb1 may facilitate damage to the phagosome enabling fungal cells to acquire nutrients from the cytoplasmic components (Chrisman et al, 2011). Interestingly, Plb1 mutant strains demonstrates decreased vomocytosis and intracellular proliferation compared to controls (Chayakulkeeree et al, 2011; Evans et al, 2015) suggesting that this enzyme is important for survival in the phagosome.

1.4.6.7 Other virulence factors

Apart from virulence factors described above, *C. neoformans* has others that have been discussed in this recent review (Coelho et al, 2014).

1.4.7 Pathogenesis of *C. neoformans*

This section will give a general overview of immune responses to cryptococcal infection and the subsequent sections will describe in detail the role of various immune components in cryptococcal infection.

Following inhalation of spores or desiccated yeast cells into the lungs, *C. neoformans* encounters alveolar macrophages or dendritic cells which will phagocytose the fungus and then initiate an appropriate immune response. The responses include secretion of cytokines, activation and recruitment of immune cells and presentation of antigen to the T cells (reviewed (Levitz, 1994). In most

immunocompetent individuals, *C. neoformans* is cleared, while in others, the fungus will be confined within a self-limiting granuloma in the hilar lymph nodes at the site of infection (Dromer et al, 1992; Goldman et al, 2001). This granuloma is characteristically composed of a compact aggregate of macrophages with multinucleated giant cells and histiocytes containing numerous yeasts within their cytoplasm (Levitz, 1994; Shibuya et al, 2005). $CD4^+$ lymphocytes are also seen at the site (Shibuya et al, 2002). The coincidental findings of cryptococcal granulomas in lungs at autopsy lead to the hypothesis that some immunocompetent individuals harbour the granuloma until death without developing overt disease (Baker, 1976; Haugen & Baker, 1954; Salyer et al, 1974). However, in the immunocompromised, such as those with AIDS, a totally different histology is seen in the lungs. There is a significantly reduced numbers of lymphocytes and histiocytes accompanied by extracellular and proliferating yeast cells (Shibuya et al, 2002; Shibuya et al, 2005). It is thought that the immunocompromised fail to control the pulmonary cryptococcal colonisation resulting in dissemination to other organs with a predilection for the CNS (Mitchell & Perfect, 1995; Figure 3).

It appears that *C. neoformans* infection occurs throughout the course of one's entire life. For example, serological studies have shown that over three-quarters of children aged between 0-10, living in urban areas have been infected with *C. neoformans*, although they did not present any obvious clinical manifestations. Moreover, their antibody titres increased with age (Chen et al, 1999; Goldman et al, 2001) suggesting an occurrence of repeated infections. It is thought that almost all cases of clinical cryptococcosis are a result of reactivation from latency in instances where severe defects in cell-mediated immunity (CMI) have occurred (Garcia-Hermoso et al, 1999; Mitchell & Perfect, 1995).

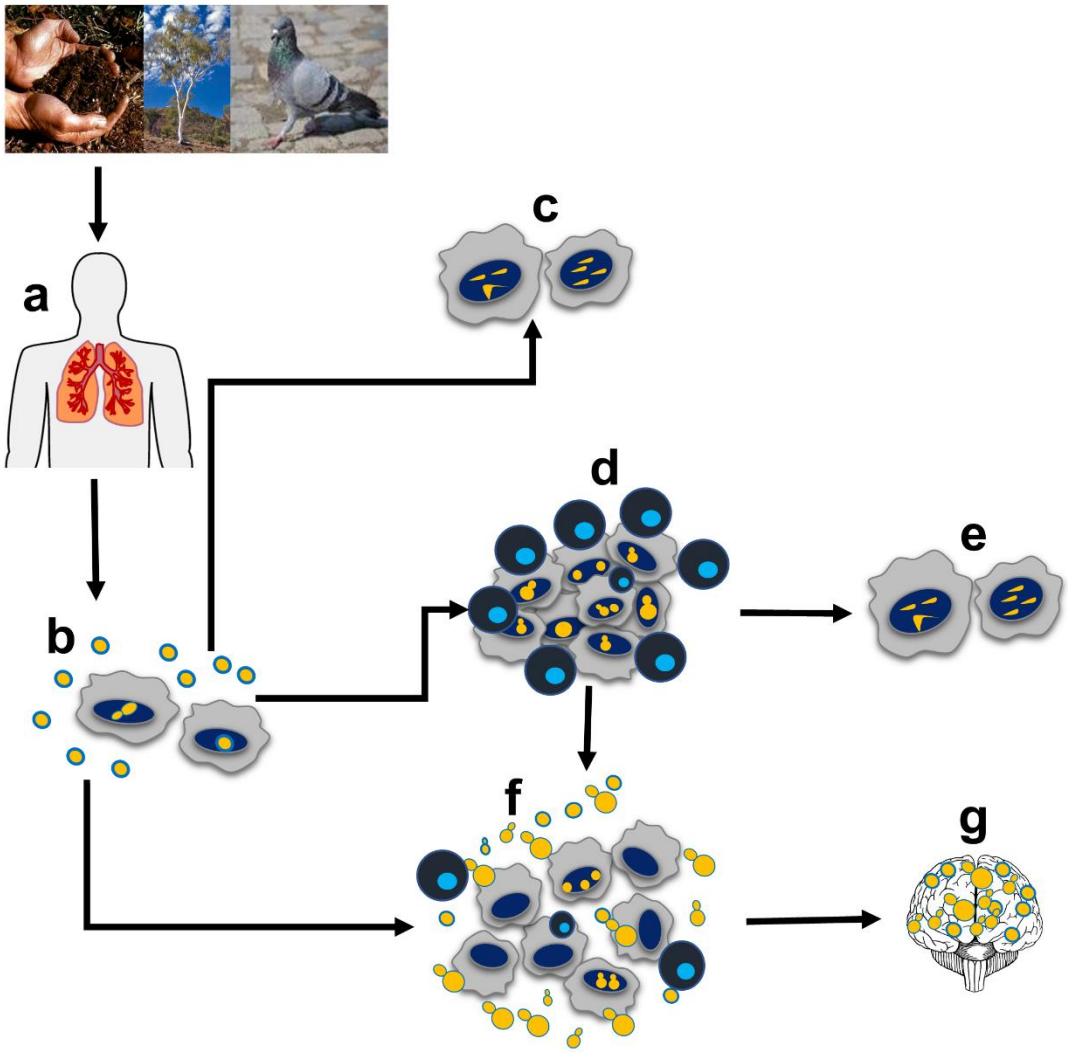

Figure 3: Pathogenesis of cryptococcosis

(a) Infection occurs when host inhales infectious propagule, usually desiccated yeast or basidiospores from the environment. (b) This is followed by recruitment of phagocytes and ingestion of the fungi. Three outcomes are expected and depend on the immunocompetence of the infected host. (c-e) Infection in the immunocompetent host may be (c) cleared or remain (d) dormant in a self-limiting granuloma which may lead to (e) clearance. (f-g) In the immunocompromised host (f) granuloma consist of large numbers of extracellular cryptococci (g) leading to dissemination to the CNS.

Images were taken and modified from

http://www.csus.edu/indiv/m/mckeoughd/aanatomyrev/cns/cns%20frontal/cnsfrontal.htm

1.4.7.1 Dissemination to the CNS

Dissemination of the fungus from the lungs via the circulatory system into the CNS compartment is the distinct feature of cryptococcal pathogenesis. Three hypothetical mechanisms of how *C. neoformans* makes its way through the blood brain (BBB) barrier have been proposed (Figure 4). First, through a process called paracytosis, in which the cryptococci penetrate between tight junctions of endothelial cells. A protease such Mpr1 is thought to enhance trans-endothelial migration (Vu et al, 2014). Interestingly, addition of *C. neoformans* MPR1 into *Saccharomyces cerevisiae* genome enabled the fungus to penetrate through endothelial cells in an *in vitro* transwell assay (Vu et al, 2014). *S. cerevisiae* does not normally cross the BBB. Furthermore, using intravital imaging, Shi et al. (2010) showed that *C. neoformans* cells get trapped in the mouse brain capillary and then cross the microvasculature and urease is required for transmigration into the brain (Shi et al, 2010). Second, transcytosis has been proposed to be another means *C. neoformans* access the brain (Chang et al, 2004). *C. neoformans* hyaluronic acid located on the cell surface attaches to CD44 on the brain microvascular endothelial cells (Jong et al, 2008). This attachment activates protein kinase C-dependent actin remodelling in the endothelial cells, resulting in uptake of the attached *Cryptococcus* cell (Jong et al, 2008). The third proposed hypothesis for *C. neoformans* crossing the BBB is by hiding inside macrophages, referred to as 'Trojan horse' (Charlier et al, 2009; Santiago-Tirado et al, 2017; Sorrell et al, 2016). This idea is held by the observation that deletion of alveolar macrophages in a murine model significantly attenuates cryptococcal dissemination to the brain (Kechichian et al, 2007). In addition, infecting bone marrow-derived monocytes with *C. neoformans in vitro* and transferring the cells to mice results in increased brain cryptococcal CFU compared to administration of free fungal cells alone (Charlier et al, 2009) suggesting that phagocytes act as fungal carriers into the brain.

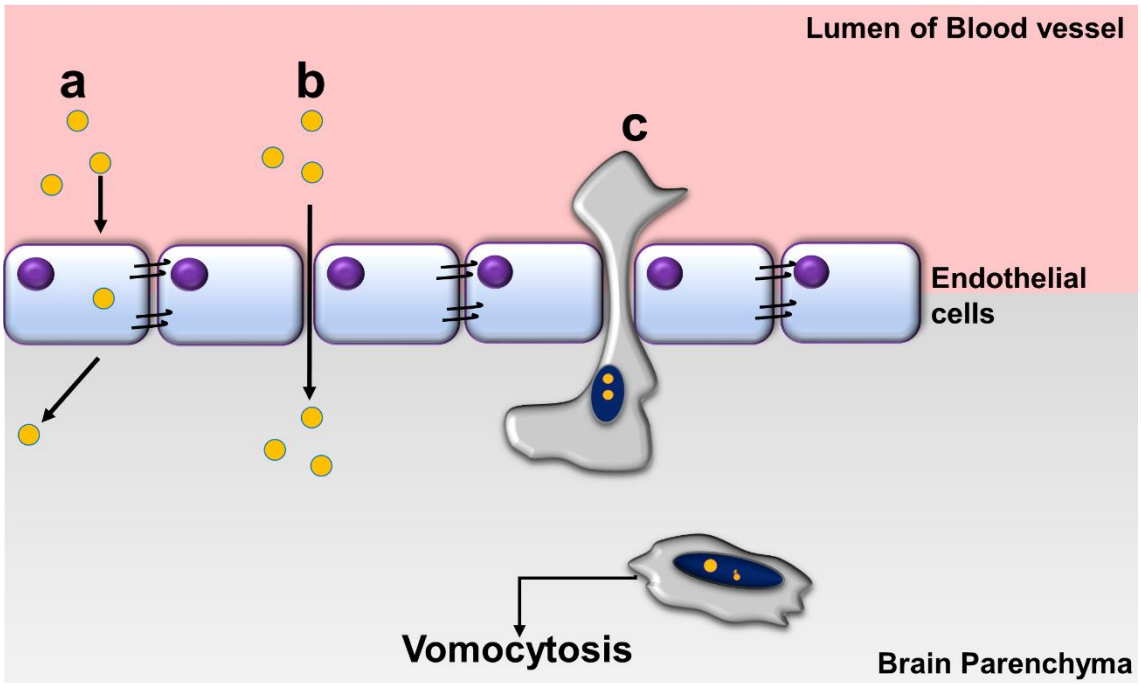

Figure 4: Crossing the blood-brain barrier

Illustration of three possible ways *C. neoformans* can penetrate the BBB into the CNS (a) Transcytosis (b) paracytosis (c) 'Trojan horse' (Liu et al, 2012; May et al, 2016).

1.5 Host immunity to *Cryptococcus neoformans*

1.5.1 Innate immune and cellular responses against *C. neoformans*

In this section, I will discuss the components of innate immune responses including complement and cellular responses to *C. neoformans*.

Physical barriers - Defence against inhaled *C. neoformans* basidiospores begins in the physical barriers of the respiratory tract. These include nasal mucosal and the mucociliary escalator of the respiratory epithelium. The epithelium provides protection by creating a semipermeable barrier that is permeable to exchange of nutrients, water, and gases while being impermeable to most inhaled pathogens. The pathogens are deposited against the airway surface fluid, allowing clearance through the mucociliary escalator (Knowles & Boucher, 2002). However, the small size of the *Cryptococcus* basidiospores (less than 2µm) allows some of the inhaled spores to evade these defence mechanisms and deposit in the lung alveoli (Ellis & Pfeiffer, 1990b; Sukroongreung et al, 1998; Velagapudi et al, 2009).

Complement- Complement is a vital constituent of the innate immune response that facilitates opsonisation of pathogens leading to enhanced uptake by phagocytic cells. Other roles include activation of inflammation, chemoattraction and pathogen lysis by formation of a membrane attack complex (Voelz & May, 2010). Very early in complement-cryptococci interaction research it was established that encapsulated cryptococci are potent activators of the complement system. Incubation of *C. neoformans* with normal serum results in activation and binding of complement fragments to the typical cryptococcal capsule (Goren & Warren, 1968). The capsule strongly activates the complement system via the alternative pathway leading to deposition of C3b on the cryptococcal capsule (Kozel & Pfrommer, 1986; Kozel et al, 1989). This causes phagocytosis via the complement receptor CR3 (Taborda & Casadevall, 2002; Zaragoza et al, 2003). Studies of human cryptococcal disease and animal models of cryptococcosis have demonstrated that complement activation also occurs *in vivo*. Macher et al. (1978) found that serum from patients presenting with crytococcemia had marked depletion of complement activity including loss of

haemolytic complement activity, inability to opsonise encapsulated cryptococci for phagocytosis by phagocytes. Very low levels of circulating C3,alternative complement factor B and loss of ability to deposit C3 fragment on yeast cells were also observed (Macher et al, 1978). The depletion of complement has also been observed in experimental guinea pigs and mice models of cryptococcosis. Intracardial injection of heat-killed *C. neoformans* in guinea pigs resulted in the loss of the haemolytic complement activity of serum (Gadebusch, 1961; Macher et al, 1978). Recently Sun et al. (2015) showed a significantly lower number of neutrophils attached to *C. neoformans* cells in the presence of $C5^{-/-}$ mice plasma than when incubated with plasma from the wild-type mice (Sun et al, 2015).

The role of complement system in host resistance to cryptococcal disease has been demonstrated using either genetic deletions or induced deficiencies of complement components in animal models. These animal models include a C4-deficient guinea pig which lacks a classical pathway but with a fully functional alternative pathway (Ellman et al, 1970), a C5-deficient mouse (Nilsson & Müller-Eberhard, 1967), a C3-defiecient mouse (Shapiro et al, 2002) and treatment of a mouse or guinea pig with cobra venom factor which depletes C3 and C5 (Vogel et al, 1996). Cryptococcal infection in C3-deficient mice or depletion of C3 using cobra venom in mice or guinea pigs results in shortened survival time and reduced ability to clear infection than C3-sufficient mice (Diamond et al, 1973; Graybill & Ahrens, 1981; Shapiro et al, 2002). Similarly, C5 deficient mice have a high fungal burden, develop acute, fatal cryptococcal pneumonia, have remarkably reduced ability to clear infection and die much sooner following intravenous inoculation with *C. neoformans* than C5-sufficient mice (Lovchik & Lipscomb, 1993; Rhodes, 1985; Rhodes et al, 1980). Tissue images of C5-deficient mice by electron microscopy showed less neutrophil infiltration in the pulmonary vessels and reduced internalised cryptococcal yeast cells than wild-type mice (Lovchik & Lipscomb, 1993). Vacchiaerelli et al. (1998) showed that C3a and C5a enhances IL-8 secretion by human neutrophils in response to GXM of *C. neoformans* (Vecchiarelli et al, 1998). Recently, Sun et al. (2015) has shown that swarming of neutrophils towards *C. neoformans* is absent in the presence of $C3^{-/-}$ and $CD11b^{-/-}$ mouse plasma (Sun et al, 2015) suggesting that

complement components C3 and CD11b are essential for the swarming of neutrophils towards *C. neoformans in vitro*. C5a-C5aR signalling directs migration of neutrophils towards *C. neoformans* resulting in phagocytosis and killing of the pathogen (Sun et al, 2015). In addition, mice treated with anti-C5aR mAb had abrogated recruitment of neutrophils to the lungs resulting in impaired intravascular clearance of the fungus (Sun et al, 2015). Interestingly, encapsulated *C. neoformans* or addition of GXM suppresses the expression of C5aR in neutrophils (Monari et al, 2002) suggesting that *C. neoformans* may modulate the function of neutrophils at the site of infection. In contrast, C4 protein, a vital component of the classical complement pathway is not needed for host immune resistance to cryptococcosis. Guinea pigs deficient in C4 have the same susceptibility to lethal cryptococcal infection as that of the wild-type, suggesting that the alternative complement system is the main protective complement pathway in cryptococcal disease (Diamond et al, 1974).

Deposition of C3 components on the surface of encapsulated cryptococci has been seen in histopathological tissue sections from patients and experimental animal models of cryptococcosis. Cryptococci isolated from lesions of patients with cutaneous cryptococcosis were found to have C3 fragments but not C1q on the surface of the yeast cells (Chiang et al, 1985). Truelsen at al., (1992) showed that a majority of cryptococci found in liver, lung and kidney tissues sections in mice were bound to C3 fragments in each tissue. There was absence of C3 binding to cryptococci in brain tissue sections (Truelsen et al, 1992). These findings are consistent with studies by Diamond et al. (1974) who found small to none detectable levels of C3 on the surface of cryptococci cells harvested from the fresh human cerebrospinal fluid of four patients with cryptococcal meningoencephalitis (Diamond et al, 1974). The absence of C3 deposition on the surface of yeast cells in brain tissue may provide an additional explanation for the predilection of *C. neoformans* for the central nervous system (Kozel, 1996).

The important functions of the complement system during cryptococcal infection are to attract phagocytic effector cells and enhance uptake of yeast cells by these phagocytic cells. The implication of complement in opsonisation of *C. neoformans* was demonstrated over three decades ago in phagocytosis

assays in which phagocytic indices by neutrophils and monocytes were reduced in heat-inactivated serum (Diamond & Erickson, 1982) whereas *C. neoformans* opsonised with normal human serum was readily phagocytosed by neutrophils (Kozel et al, 1987). Several other studies also revealed the involvement of the complement pathway in the uptake of cryptococci in which depletion of specific complement proteins resulted in abrogated phagocytosis by phagocytic cells (Davies et al, 1982; Diamond et al, 1974). Incubation of *C. neoformans* yeast cells in $C3^{-/-}$ or $C5^{-/-}$ mouse plasma completely abolishes phagocytosis by neutrophils (Sun & Shi, 2016). Kozel et al. (1988) examined binding of C3 fragments and subsequent phagocytosis in several capsulated *C. neoformans* strains. Capsule from strains tested did bind to C3 but some were phagocytosed, while others were not, suggesting that the complement cascade mediated opsonisation is necessary but not sufficient for phagocytosis to occur (Kozel et al, 1988).

Other components important for cryptococcal infection are surfactants which are complex fluids consisting of phospholipids and four proteins namely SP-A, SP-B, SP-C, and SP-D (Johansson et al, 1994). SP-A binds to both encapsulated and acapsular cryptococci in a calcium-dependent manner which can be affected by mannose and glucose (Walenkamp et al, 1999). However, SP-A does not influence cryptococcal disease progression. No significant difference in fungal burden or TNFα production was observed between SP-A deficient mice and wild-type (Giles et al, 2007). In contrast, SP-D binds more strongly to acapsular yeasts than SP-A (Schelenz et al, 1995; van de Wetering et al, 2004) and promotes uptake of thin capsular *C. neoformans* cells by mouse macrophages both *in vitro* and *in vivo* (Geunes-Boyer et al, 2012; Geunes-Boyer et al, 2009). In addition, SP-D enhances fungal survival (Geunes-Boyer et al, 2009) and shields *C. neoformans* against reactive oxygen species in the mouse model leading to disease progression (Geunes-Boyer et al, 2012).

1.5.2 Phagocytes

1.5.2.1 Neutrophils

Neutrophils are the first type of phagocytic effector cells that migrate to the site of infection to kill and eliminate pathogens but do not play a significant role in cryptococcal disease. *In vitro*, neutrophils have been shown to kill *Cryptococcus* by an oxidative-dependent mechanisms mediated by hydrogen peroxide, hypochlorous acid, hydroxide (Chaturvedi et al, 1996) and oxidative-independent activity arbitrated by calprotectin and defensins (Mambula et al, 2000; Qu & Wang, 1991). Treatment with inhibitors and scavengers of respiratory burst oxidants slightly reduce the anti-cryptococcal activity of human PMN (Mambula et al, 2000). Mice lacking myeloperoxidase, a neutrophil-specific enzyme of the reactive oxygen species cascade, have reduced ability to clear infection in the lungs and the spleen and succumb much earlier than wild-type upon intranasal or intravenous infection with *C. neoformans* (Aratani et al, 2006). Neutrophils are also capable of killing *C. neoformans* extracellularly. Qureshi et al. (2010) showed that inhibition of sphingomyelin synthase activity markedly abrogates extracellular killing of *C. neoformans* by preventing the secretion of extracellular anti-cryptococcal factors of neutrophils (Qureshi et al, 2010). Further studies have shown that the addition of G-CSF to human neutrophils significantly enhances the killing of *C. neoformans in vitro* (Chiller et al, 2002). In a mouse model of cryptococcal disease, treatment with fluconazole combined with G-CSF results in increased survival (Graybill et al, 1997). Treatment of HIV/AIDS-associated neutropaenia with G-CSF is associated with increased secretion of leukotrienes from neutrophils and restoration of fungistatic and fungicidal activity of neutrophils (Coffey et al, 1998; Vecchiarelli et al, 1995).

However, despite neutrophil ability to kill *C. neoformans*, the pathogen evades neutrophils by inhibiting neutrophil migration (Coenjaerts et al, 2001; Ellerbroek et al, 2004b; Monari et al, 2002), extracellular trap (NET) formation (Rocha et al, 2015), killing and respiratory burst (Chaturvedi et al, 1996; Qureshi et al, 2010) 2010). *C. neoformans* GXM prevents neutrophil migration by slightly inhibiting L-selectin, a molecule required for the initial step of neutrophil migration into

tissues (Dong & Murphy, 1996). GXM also inhibits adhesion of neutrophils to endothelium by interferering with E-selectin binding (Ellerbroek et al, 2002), IL-8 receptor (Lipovsky et al, 1998), CD14 or TLR4 (Ellerbroek et al, 2004) and CD18 (Dong & Murphy, 1997) on human neutrophils. Furthermore, Ellerbroek et al. (2004) found that O-acetylation of GXM blocks migration of neutrophils (Ellerbroek et al, 2004). Paradoxically, cryptococcal GXM elicits production of cytokines and chemokines (Lipovsky et al, 1998; Retini et al, 1996), thereby demonstrating indirect chemotactic activity on the PMNs (Dong & Murphy, 1993; 1995). Melanised *C. neoformans* inhibits sphingomyelin synthase that remarkably impairs the neutrophil killing activity against the fungi (Qureshi et al, 2010). The cryptococcal capsule also inhibits uptake by neutrophils (Richardson et al, 1993).

During early intranasal infection of *C. neoformans*, neutrophils migrate (are recruited) to the pulmonary vessels (Abe et al, 2000; Feldmesser et al, 2000) 2000; Herring et al, 2005). Neutrophil recruitment to the lungs is driven by chemokines including IL-8 (Guillot et al, 2008a), C-X-C chemokines, MIP-2 and KC (Kawakami et al, 1999a) which become elevated following *C. neoformans* infection. C5a-C5aR signalling also mediates migration of neutrophils towards *C. neoformans* (Lovchik & Lipscomb, 1993; Sun et al, 2015) leading to C3-mediated fungal internalisation (Kozel et al, 1984), increased expression of Erk, p38 MAPK and eventually killing of the cryptococci (Sun et al, 2015). Interestingly, p38 MAPK pathway inhibition results in a profound reduction of neutrophil migration and anti-cryptococcal activity (Sun et al, 2015). Further studies using real-time *in vivo* imaging have revealed that neutrophils can infiltrate the brain microvasculature, take up the cryptococcal cells and return to the circulation, thereby effectively eliminating the fungus from the brain (Zhang et al, 2016).

The importance of neutrophils in the protective role against cryptococcal disease remains uncertain. Depletion of neutrophils from previously infected (immunised) mice with an IFNγ-producing strain of *C. neoformans,* H99γ, did not affect the fungal burden (Wozniak et al, 2012). This data are consistent with previously published studies which showed that depletion of neutrophils in mice a day before

intratracheal infection with *C. neoformans*, results in prolonged survival with increased production of IL-10, TNF-α, IL-4 and IL-12 in the lungs (Mednick et al, 2003) suggesting that neutrophils are not essential for host resistance to cryptococcal disease. In addition, cryptococcosis is not associated with neutrophil deficient diseases or conditions characterised by defects in neutrophils (Casadevall, 1998). In fact, increased numbers of neutrophils in the lungs during cryptococcosis cause more damage to the lungs in response to the infection (Osterholzer et al, 2009). In contrast to the resistant role of neutrophil depletion, mice deficient in the neutrophil enzyme MPO are highly susceptible to *C. neoformans*, display increased IL-4 and decreased IL-12 and lFNγ levels in the lungs (Aratani et al, 2006) showing the complex nature of the role of neutrophils in cryptococcal disease.

1.5.2.2 Dendritic cells

Dendritic cells (DCs) are phagocytes and professional antigen presenting cells to naïve T cells that induce an adaptive immune response. DCs and alveolar macrophages are essential in the early host response. Depletion of $CD11c^+$ cells in CD11c-DTR mice one day before infection with a *C. neoformans* strain that is not lethal in many mouse strains resulted in rapid clinical deterioration and death within six days after infection (Osterholzer et al, 2009). Early studies on evaluating the role of DCs in cryptococcosis showed that classified DC subsets are required for the host resistance to cryptococcal infection. Mice were immunised with cryptococcal culture filtrate antigen (protective immunogen) or heat-killed cryptococcal filtrate antigen (non-protective immunogen) and the lymph node drainage of each mouse was examined for DC subsets. Myeloid DCs appeared to be essential for eliciting protective immunity against *C. neoformans* by activating $CD4^+$ T cell responses (Bauman et al, 2000) augmented by TNFα (Bauman et al, 2003). However, plasmacytoid DCs elicited non-protective immune responses (Bauman et al, 2000; Siegemund & Alber, 2008).

Recruitment - CCR2 mediates recruitment of DCs to the lungs. CCR2 deficient mice have markedly impaired DC recruitment and they develop a phenotype similar to a Th2-type immune response such as IL-4 production (Osterholzer et

al, 2008). The CCR2-dependent infiltration of $CD11c^+$ DCs in the lungs in mice with cryptococcal infection is associated with continuous migration and differentiation of Ly-$6C^{high}$ mononuclear cells into $CD11b^+$ DCs (Osterholzer et al, 2009a). Inhibition of TNFα reduces movement of mature DCs and results in chronic cryptococcal infection (Herring et al, 2005).

Antigen presentation - DCs are a link between innate and adaptive immunity, and their role is vital for host defence and survival. Syme et al. (2002) found that DCs are the most efficient antigen presenting cell types of *C. neoformans* antigens to T cells. Although many DCs bind and internalise *C. neoformans*, only a small proportion of these cells are required for antigen presentation to T cells (Syme et al, 2002). Studies *in vivo* showed that fluorescently labelled *C. neoformans* cells were taken up by DCs in the lungs within two-hours post infection (Wozniak et al, 2006). Seven days post-inoculation there was upregulation of CD80, CD86 and MHC class II on DC. *In vitro* culture of lung DCs from infected mice with *Cryptococcus*-specific T cells resulted in proliferation of T cells, shown by increased IL-12 secretion. These findings indicate that the DCs in the lungs can take up and present *C. neoformans* antigens to the T cells (Wozniak et al, 2006).

Inhibition of DCs by *C. neoformans* - Cryptococcal GXM profoundly inhibits immune response to cryptococcal infections (reviewed in Zaragoza et al, 2009). The capsule of *C. neoformans* greatly interferes with activation and maturation of DCs and prevents phagocytosis by DCs (Grijpstra et al, 2009; Lupo et al, 2008; Vecchiarelli et al, 2003). Acapsular *C. neoformans* strains are easily taken up by DCs without opsonisation and this leads to increased expression of MHC class II and other co-stimulatory molecules such as CD40 and CD83. However, encapsulated *C. neoformans* strains are not taken up by DCs (Kelly et al, 2005) and do not induce expression of these molecules except when opsonised by an anti-GXM antibody which is recognised by receptors CD32 and CD16 (Vecchiarelli et al, 2003). Grijpstra at al., (2009) also showed that non-capsulated *C. neoformans* strain ***cap56***Δ*,* but not ***cap10***Δ*,* elicit maturation and activation of DCs as evidenced by increased expression of CD86 and CD80 (Grijpstra et al, 2009). Furthermore, ***cap56***Δ induced upregulation of other genes involved in maturation of DCs such as cytokines IL-12, IL-1α, IL-1β, IL-6 and TNFα;

chemokines CCR7, CCL17, CCL22, and CXXL10; in addition to membrane proteins and genes involved in antigen processing and presentation. On the contrary, encapsulated *C. neoformans* strain reduced the expression of these genes and down-regulated several genes, particularly those coding for chemokines (Lupo et al, 2008).

Recognition and uptake of *C. neoformans* - DCs recognise and phagocytose *C. neoformans* through a mannose receptor and FCγR-II *in vitro* (Syme et al, 2002) but not via TLR2 or TLR4 (Nakamura et al, 2006). Several studies have shown that a mannose receptor is a cryptococcal mannoprotein (MP) receptor (Mansour et al, 2006; Mansour et al, 2002). MPs are present in the capsular polysaccharides and fungal cell wall. MPs are recognized by several mannose receptors including CD206 and DC-SIGN (CD209) in DCs (Mansour et al, 2006; Mansour et al, 2002). MPs elicit the activation and maturation of DCs shown by enhanced secretion of IL-12, TNFα and IκBα phosphorylation (Dan et al, 2008a; Pietrella et al, 2005). Co-incubation of DCs with purified MPs results in increased expression of co-stimulatory molecules including MHC class I, Class II, CD40, 80 and 86 (Pietrella et al, 2005). DCs carrying *C. neoformans* MPs are efficient inducers of T cell ($CD4^+$ and $CD8^+$) proliferation (Pietrella et al, 2005). Secretion of pro-inflammatory cytokines and chemokines by DCs and MP-specific MHC class II-restricted $CD4^+$ T cells responses is enhanced upon co-stimulation with *C. neoformans* MPs combined with TLR agonists such as PAM3CSK4, Poly(I:C) and imiquimod (Dan et al, 2008a).

Killing - Upon uptake by DCs, the fungi are taken to the endosomal compartment that fuses to the lysosome of the DC. The fungi are then killed by oxidative dependent and/or independent mechanisms (Wozniak & Levitz, 2008). Lysosomal extracts from BMDCs kill *C. neoformans in vitro* and are toxic to all cryptococcal serotypes (Hole et al, 2012; Wozniak & Levitz, 2008). Some DC lysosomal enzymes, particularly cathepsin B, inhibit growth of *C. neoformans* (Hole et al, 2012).

1.5.2.3 Macrophages

Importance of macrophages on *C. neoformans* infection - Alveolar macrophages are the first line of defence against pulmonary pathogens (Fels & Cohn, 1986). Histological samples from patients with pulmonary cryptococcosis display a predominant presence of alveolar macrophages (Shibuya et al, 2002; Shibuya et al, 2005). Murine studies to determine the role of macrophages in cryptococcal disease have yielded conflicting findings. Depletion of macrophages and dendritic cells with a diphtheria toxin one day before *C. neoformans* challenge in mice results in significantly decreased survival (Monga, 1981; Osterholzer et al, 2009). However, an intratracheal or intranasal treatment with chlodronate liposomes to deplete alveolar macrophages results in unaltered cryptococcal burden (Shao et al, 2005). The latter approach leaves macrophages and dendritic cells in other tissues undeleted and may have compensated the local loss (Osterholzer et al, 2009). Interestingly, studies in zebrafish have shown that depletion of macrophages results in high fungal burden and reduced survival (Bojarczuk et al, 2016; Tenor et al, 2015) underlining the critical role of macrophages during cryptococcosis.

Macrophage activation - Macrophages can undergo polarisation to a different activation phenotype following exposure to a specific stimulus (Davis et al, 2013; Leopold Wager & Wormley, 2014). Exposure of macrophages to Th1-type stimuli such as IFNγ or microbial ligands like LPS differentiate them into classically activated macrophages, also referred to as M1 macrophages (Hussell & Bell, 2014; Mantovani et al, 2004; Mosser & Edwards, 2008). M1 macrophages mediate antimicrobial activities through the generation of reactive oxygen species and nitrogen species (Ding & Vaziri, 1998). The M1 macrophages also shift their metabolism from mitochondrial oxidative phosphorylation to glycolysis in the presence of Th1-type stimuli (Rodríguez-Prados et al, 2010). Phenotypic signatures for identification of M1 macrophages include IL-12, iNOS, chemokine (CXC motif) ligand 9 (CXCLC9), CXCLC10, CXCLC11, and suppressor of cytokine signalling 3 (SOCCS3) (reviewed in Mosser & Edwards, 2008; Murray & Wynn, 2011).

On the other hand, exposure of macrophages to Th2-type cytokines such as IL-4 and IL-13 or parasite-derived products, fungal chitin or allergens differentiate them into alternatively activated macrophages, also known as M2 macrophages (Reviewed in Mantovani et al, 2013; Mosser & Edwards, 2008; Van Dyken & Locksley, 2013; Wiesner et al, 2015). M2 macrophages are efficient destroyers of parasites and play a critical role in repairing damaged tissue (Allen & Sutherland, 2014; Rückerl & Allen, 2014). The characteristic features associated with M2 macrophages include chitinase-like molecules such as Yml and YM2, found in an inflammatory zone (FIZZI, also called resistin-like-α or Relm-α), mannose receptor (CD206) and arginase-1 (Arg1) (Mosser & Edwards, 2008; Murray & Wynn, 2011; Stein et al, 1992).

A major difference between the two classes is that M2 macrophages produce Arg1 which catalyses the conversion of L-arginine to produce urea, polyamines, and ornithine (Bogdan et al, 2000) whereas M1 macrophages generate iNOS, which catalyses the formation of nitric oxide and citrulline from L-arginine. iNOS and Arg1 compete for the same substrate, L-arginine (McNeill et al, 2015) resulting in either hindering or promoting the production of NO in the macrophages.

Macrophage polarisation during *C. neoformans* infection - Experimental pulmonary cryptococcosis is associated with the induction of a strong Th2-type response and activation of alternative macrophages (Chen et al, 2008; Huffnagle et al, 1998; Milam et al, 2007; Müller et al, 2007; Osterholzer et al, 2009). Similarly, mice lacking Th1-type cytokine, IFNγ, have high levels of Th2-type cytokine production and alternatively activated macrophages in the lungs during cryptococcal infection (Arora et al, 2011). Muller at al., (2007) showed that Th2-type cytokine, IL-13, promotes M2 skewed macrophage differentiation, Th2-type cytokine phenotypes and allergic-type responses during experimental pulmonary *C. neoformans* infection in mice (Müller et al, 2007). In an experimental model to understand the relationship of Th1 and Th2 cytokines on macrophage activation, mice were infected with *C. neoformans* 52D strain. The immune response phenotypes showed plasticity, Th2-type cytokines appeared early in the infection followed by Th1-types cytokines later in the infection.

However, infection was not cleared despite the Th1-type responses, suggesting that early Th1-type immune responses are required for protection against cryptococcosis (Arora et al, 2011). Macrophages also demonstrated plasticity in activation depending on whether they were exposed to IFNγ and/or IL-4 during cryptococcosis. For example, the higher IL-4/IFNγ ratio skewed macrophages toward M2 while higher IFNγ/IL-4 skewed macrophages toward M1 phenotype (Arora et al, 2011). Some macrophages treated with IL-4 and IFNγ expressed both M1 and M2 hallmark markers showing that these cytokines have an additive effect (Arora et al, 2011). Indeed, macrophages that polarise to M1 phenotype when exposed to IFNγ can repolarise back to M2 when stimulated with IL-4 and vice versa (Davis et al, 2013; Gratchev et al, 2006; Stout et al, 2005) signifying the critical role of the local cytokine microenvironment in driving macrophage polarisation.

Fungicidal and fungistatic activity of macrophages – Human macrophages can arrest the growth of *C. neoformans in vitro* (Levitz & Farrell, 1990). In the phagolysosome, *C. neoformans* is exposed to low pH, reactive oxygen species, reactive nitrogen species and deficiency of nutrients (Shoham & Levitz, 2005). Mice deficient in phagocyte NADPH component $Phox^{-/-}$ have enhanced Th1-type responses accompanied by improved *Cryptococcus* containment in pulmonary granulomatous lesions and reduced fungal dissemination to the brain (Snelgrove et al, 2006). Therefore, counterintuitively, abrogation of this important antimicrobial activity, ROS, rendered mice more resistant to cryptococcal infection. On the other hand, exposure of *C. neoformans* to chemically generated antimicrobial nitric oxide inhibits fungal growth under acidic conditions (Alspaugh & Granger, 1991). The enzyme that catalyses the formation of NO, iNOS, is present in lung granulomas of mice infected with *C. neoformans* (Goldman et al, 1996; Goldman et al, 2000; Hardison & Brown, 2012a). In addition, $iNOS^{-/-}$ mice have reduced survival and fail to clear *Cryptococcus* infection (Aguirre & Gibson, 2000). These findings were confirmed recently by Wager et al. (2015) who showed that macrophages from $iNOS^{-/-}$ mice or WT mice treated with iNOS inhibitors fail to control intracellular replication of *C. neoformans* even in the presence of uninterrupted ROS secretion (Leopold Wager et al, 2015). This

suggests that NO, and not ROS, is required for control of *C. neoformans*. The question is whether these findings can be extrapolated to human disease because there is little evidence describing the production of NO in healthy human macrophages. Even in studies that have reported production of NO by human macrophages, the levels are extremely low (Albina, 1995; Amin et al, 1995; Schneemann et al, 1993). Recently, Gross et al. (2014) showed that the gene that mediates iNOS in humans is silenced by CpG methylation, histone modification and chromatin suppression, which is contrary to mouse macrophages (Gross et al., 2014). There is some evidence showing production of NO in chronic human lungs disease. For example, granulomas from lung tissue in humans and other primates contain iNOS positive macrophages (Facchetti et al., 1999, Mattila et al., 2013), indicating that human macrophages in appropriate chronic inflammatory contexts are capable of secreting NO, an aspect lacking in normal human macrophage physiology. This raises a need for further studies that would characterise human macrophage responses to *C. neoformans*.

Evading killing by macrophages - *C. neoformans* is known to inhibit the killing machinery of macrophages. *C. neoformans* suppress NO production in macrophages through a mechanism independent of capsular polysaccharide (Kawakami et al, 1997a; Xiao et al, 2008). Suppression of iNOS leads to M2 macrophage activation, abrogation of anti-fungal activity and progressive disease (Arora et al, 2005; Arora et al, 2011; Hardison et al, 2010; Naslund et al, 1995; Xiao et al, 2008). In addition, chitin indirectly induces the production of IL-5, IL-13 and IL-4, leading to M2 skewed macrophages (Van Dyken et al, 2014). In the macrophage phagosome, *C. neoformans* face low pH, ROS and NO. Early studies demonstrated that *C. neoformans* does not actively evade low pH of the macrophage phagolysosome, but in fact, replicates better in acidic pH rather than alkaline environments (Diamond & Bennett, 1973; Levitz et al, 1999). In contrast, Smith et al. (2015) have recently shown that *C. neoformans* can inhibit phagosome acidification, calcium flux, and protease activity, modulating the phagosome to allow cryptococcal replication. Interestingly, they also demonstrated that live, but not heat-killed, *C. neoformans* induces premature removal of early phagosome markers, Rab5 and Rab11 (Smith et al, 2015). Live

C. neoformans can damage the lysosome in BMDMs in a time-dependent manner. Activation of BMDM with IFNγ prevented the lysosomal damage and enhanced anti-cryptococcal activity of the macrophages (Davis et al, 2015).

Outcomes of *Cryptococcus*-macrophage interactions - *C. neoformans* is capable of replicating inside the macrophage phagosome (Tucker & Casadevall, 2002; Voelz et al, 2009) and can escape through non-lytic exocytosis or transfer laterally to another macrophage (Alvarez & Casadevall, 2006; Ma et al, 2006; Nicola et al, 2011). Non-lytic exocytosis, also known as vomocytosis is thought to be an immune evasion strategy that may facilitate *C. neoformans* dissemination from the lungs to other body organs including the brain (Johnston & May, 2013). A study recently examined the behaviour of clinical *C. neoformans* isolates *in vitro* in relation to disease severity. *C. neoformans* strains that were taken up quickly by macrophages had low intracellular proliferation rates *in vitro* and were associated with higher CSF cryptococcal burden and, unexpectedly, prolonged survival of HIV patients. In contrast, *C. neoformans* strains with a high phagocytic rate had a smaller capsule size, increased laccase activity and were less susceptible to anti-fungal therapy (Sabiiti et al, 2014). This study highlights that cryptococcal-macrophage interactions play a critical role in driving the outcomes of cryptococcosis in humans.

1.6 Experimental models for cryptococcosis

1.6.1 *In vitro* cell models

The interactions of *C. neoformans* with host cells including primary cells and cell lines such as macrophages, neutrophils, endothelial and epithelial cells and neutrophils have been widely studied. Most studied interactions have been those with macrophages. The uptake of unopsonised *C. neoformans* by macrophages is very poor, but readily are taken up following opsonisation with complement or antibody (Bolaños & Mitchell, 1989). Internalised *C. neoformans* cells can remain viable and proliferate within macrophages, and on some occasions, they can undergo non-lytic exocytosis, lateral transfer or death. It is worthwhile to mention that outcomes of these interactions differ depending on the type of macrophages used. For instance, vomocytosis rates are markedly higher in human primary

macrophages compared to mouse J774 cell lines (Ma et al, 2006; Voelz et al, 2009). Cells that are commonly used include J774, RAW and primary cells such as bone marrow-derived mouse cells and monocyte-derived human macrophage cells (Ma et al, 2006; Ralph et al, 1975; Srikanta et al, 2011; Tsuchiya et al, 1980).

1.7 Invertebrate models

1.7.1 *Galleria mellonella*

Galleria mellonella are the larvae of the greater wax moth and have been used to study virulence of a variety of pathogenic fungal species. The larvae can live at temperatures ranging from 25° C to 37° C, thus enabling investigation of temperature-depended pathogenesis (Mylonakis et al, 2005). Another advantage of *Galleria mellonella* is that inoculation is not complicated and can be done by either injection (Mylonakis et al, 2005) or topical application (Bouklas et al, 2015). Inoculation by injection provides the means to inoculate specific fungal doses. This is due to its larger size (about 1.5 -2.5 cm in length) compared to small sized insects such as *Drosophila*, in which maintaining a specific dose of inoculation can be difficult (Kavanagh & Fallon, 2010). Inoculation of fungi into the haemocoel is less damaging because injection does not require piercing of the haemocoel. Instead, exerting a gentle pressure on the side can open the pro-leg, and then a needle can be inserted. The crack will re-seal immediately following removal of the needle without forming a scar (Cotter et al, 2000). Piercing through the insect may activate immune responses aimed at repairing the wound and this may interfere with the experimental results (Kavanagh & Fallon, 2010). Haemocytes, which are equivalent to white blood cells in mammals, have been shown to contain proteins homologous to human neutrophil proteins p47phox and p67phox indicating that the haemocytes might be involved in NADPH oxidase activity (Bergin et al, 2005). *Galleria mellonella* is promoted as a model specifically for studying diseases that disseminate throughout the body via the circulatory system as in *C. neoformans* infections (Kavanagh & Fallon, 2010). The *G. mellonella-C. neoformans* model was first reported by Mylonakis et al, (2005). In this model, despite effective uptake of fungal cells by insect haemocytes, *Galleria* larvae were killed by all *C. neoformans* strains tested (Mylonakis et al,

2005). This suggests that *C. neoformans* can evade or manipulate *G*. *mellonella* immunity, as it does with phagocytes *in vitro*. Recently Bouklas et al. (2015) showed no correlation between survival of *G. mellonella* and that of mice following cryptococcal infection. *C. neoformans* strains that were highly virulent in mice were not in *G. mellonella* suggesting that the moth may not completely model host-pathogen interactions seen in mammalian models (Bouklas et al, 2015).

1.7.2 *Drosophila melanogaster* and *Drosophila* S2 cells

The fruit-fly *Drosophila melanogaster* offers a possibility to investigate local or systemic *C. neoformans* infection. Infection into the haemocoel involves an insertion of a needle into the thorax or abdomen as demonstrated by Luo et al. (2009). This method of infection generates a systemic infection since the fungus is inoculated directly into the haemolymph. The disadvantage of this method is that it is inconsistent with a natural route of infection as pathogens such as *Cryptococcus* would not be able to penetrate the insect cuticle unless a hole was already made. Alternatively, the pathogen can be incorporated into fly food. Use of *Drosophila melanogaster* to study pathogenic fungi is growing because immune signalling pathways in this organism are similar to that of mammals (De Gregorio et al, 2002; Hoffmann & Reichhart, 2002). For example, proteins involved in autophagy pathway mediate intracellular replication of *C. neoformans* in both *Drosophila* S2 cell and mammalian cell systems (Qin et al, 2011).

1.7.3 *Caenorhabditis elegans*

Caenorhabditis elegans is a non-parasitic transparent nematode used to study *C. neoformans* infection. Infections of *C. elegans* are administered via ingestion. *C. neoformans* are cultured on YPD agar overnight, and the nematodes are transferred to the plates and infects the nematode by being ingested, causing fatal disease (London et al, 2006). However, this mode of infection does not resonate with inoculation in mammals. Following ingestion by the nematode, *C. neoformans* are confined to the intestines, which is in contrasts to lung inhalation and subsequent dissemination in mammalian models. Also, it is difficult to enable *C. elegans* to ingest an exact dose of the pathogen, which can strictly

limit the scope of experiments. Furthermore, *C. elegans* lack phagocytes, therefore studying of definitive immune response like phagocytosis is not possible (Sabiiti et al, 2011). Despite these limitations, susceptibility to *C. neoformans* infection is similar to mammalian models. For instance, *C. neoformans* strains with defective virulence in mammalian hosts usually demonstrate the same attenuation in *C. elegans* (Mylonakis et al, 2002). *C. elegans* can also be used to identify *C. neoformans* genes required for virulence by screening large numbers of mutants. For example, Lee et al. (2010) identified certain mutations in *C. neoformans* that caused defective growth *in vitro* cerebrospinal fluid and had the corresponding attenuation in *C. elegans* (Lee et al, 2010). Interestingly, although an intact *C. neoformans* polysaccharide capsule is critical for pathogenesis in *Galleria* and *Acanthamoeba,* it does not cause killing of *C. elegans* (Mylonakis et al, 2002; Steenbergen et al, 2001).

1.7.4 *Dictyostelium discoideum* (Social amoebae)

Dictyostelium discoideum is a soil-living social amoeba and a useful model for studying the interactions between pathogen and host cells such as proliferation of pathogens within phagocytes. The *D. discoideum* genome has been sequenced (Eichinger et al, 2005) and is malleable to genetic manipulation (Hägele et al, 2000; Pradel & Ewbank, 2004). Some parallels have been drawn regarding virulence of *Cryptococcus* between *Dictyostelium* and human hosts. For instance, the *C. neoformans* polysaccharide capsule is vital for infection in *Dictyostelium* since non-encapsulated mutants fail to replicate in the amoeba (Steenbergen et al, 2003). Pre-incubation of *D. discoideum* with *C. neoformans* promotes virulence of the fungus in a murine model of infection (Steenbergen et al, 2003) possibly because the capsule of the fungus enlarges during co-incubation (Chrisman et al, 2011).

1.7.5 *Acanthamoeba castellanii*

Acanthamoeba castellanii is considered one of the protozoan predators of *C. neoformans* in the environment. *A. castellanii* can ingest yeast cells followed by intracellular replication and formation of vesicles carrying polysaccharides in

the cytoplasm of the amoeba. The vesicles in the amoeba are similar to those seen in *C. neoformans* infected macrophages (Steenbergen et al, 2003).

1.8 Vertebrate models

1.8.1 Mouse (*Mus musculus*)

Mice are the most common mammalian model systems used for *Cryptococcus*-related studies. This is not surprising since murine models are well established and characterised systems in many medical research laboratories and a variety of genetic backgrounds are widely available. A variety of inoculation routes to establish *C. neoformans* infection in mice include intranasal, intraperitoneal, intracerebral, intravenous, intratracheal, and via inhalation (Zaragoza et al, 2007). Due to the availability of vast data on the mouse immune system it means that parallels and gaps can be identified readily, enabling the design of robust experimental approaches. Susceptibility to *C. neoformans* between different inbred mouse strains varies significantly. For example, CBA/J mice are more susceptible to intratracheal *C. neoformans* infection than BALB/c mice (Zaragoza et al, 2007). Like cryptococcosis in humans, intrapharyngeal aspiration *C. neoformans* infection with H99 strains results in meningitis which is the major cause of fatality in animals (Ngamskulrungroj et al, 2012).

1.8.2 Rat (*Rattus rattus*)

Rats are comparable to mice but slightly bigger allowing the performance of complex experimental manipulations such as endotracheal intubation, bronchoalveolar lavage, consecutive venepunctures, CSF collection, radiography, computed tomography, and magnetic resonance imaging (Krockenberger et al, 2010). Rats in the wild have been documented to develop chronic pulmonary cryptococcal disease (Scrimgeour & Purohit, 1984) indicating that the rat is a potentially powerful disease model. A pulmonary cryptococcal disease rat model has been generated and infection is established following intratracheal inoculation (Krockenberger et al, 2010). A rat model that can survive long-term cryptococcal infection has been reported and is a useful tool for studying latency-of-infection (Goldman et al, 1996). In a rat model, arrested local cryptococcal growth is associated with granulomatous inflammation while

persistent pulmonary infection is sustained by downregulation of both cellular and humoral responses (Goldman et al, 2000).

1.8.3 Rabbit (*Oryctolagus cuniculus*)

The rabbit is relatively larger than mice or Guinea pigs. As such, the rabbit has been promoted as a model for cryptococcal meningitis. Stein et al. (2003) proposed that the rabbit is an exceptional model for cryptococcosis because infection can be studied at a localised site in a sequential manner such as the ability to repeatedly collect body fluid samples (Steen et al, 2003). Historically the rabbit has not been a model of choice for *C. neoformans* disease because it is naturally resistant to the pathogen (Perfect et al, 1980). However, it has been argued that the rabbit's body temperature of 39.5° C and use of steroids in this model closely resemble clinical situations in which patients with steroid-associated cryptococcal disease often present with fever (Perfect & Casadevall, 2002). Pre-treatment of the rabbit with steroid therapy followed by *Cryptococcus* infection successfully results in the development of meningoencephalitis (Perfect et al, 1980). *C. neoformans* mutants that demonstrated a significant reduction in viability when exposed to human CSF showed attenuation in virulence in a rabbit model too (Lee et al, 2010), displaying a powerful example of how a whole-organism model can confirm hypotheses of disease attenuation derived from findings *in vitro*.

1.8.4 Guinea pigs (*Cavia porcellus*)

Unlike other mammalian models, the Guinea pig is relatively one of the most recent mammals to be used to study cryptococcal disease (Kirkpatrick et al, 2007). Due to being larger than mice, Guinea pigs are ideal for more complex experimental manipulations. For instance, infection models requiring intravenous inoculation are relatively easier in Guinea pigs. In addition, the oral doses of anti-fungal drugs that are used in Guinea pig experiments to clear a fungal infection are similar to the doses administered in humans (Odds et al, 2000). For example, intravenous administration of itraconazole is effective against disseminated fungal infections including cryptococcosis as shown in a Guinea pig model (Odds et al, 2000).

However, the use animals larger than mice comes with higher costs and demanding husbandry requirements that need more intense observations during experimental infections. Moreover, availability of molecular tools such as antibodies and genetically defined strains are limited.

1.8.5 The zebrafish

The zebrafish (*Danio rerio*) belongs to the minnow family (Cyprinidae) of the order Cypriniformes. Zebrafish originates from the streams of Southeast Asia and are broadly spread across India, Bangladesh, Nepal, Myanmar and Pakistan, but now commonly found worldwide as an aquarium fish (Lawrence, 2007). The adult fish are approximately 4 cm long and have a lifespan of up to 5 years (Lawrence, 2007; Siccardi et al, 2009).

1.8.5.1 The zebrafish as a model organism.

George Streisinger first introduced the zebrafish model for the study of human disease in the late 1960s. He published his protocols on how to create homozygous diploid clones of zebrafish in a 1981 issue of Nature (Meeker & Trede, 2008). Much of the early research in zebrafish focused on studying vertebrate genetics and developmental biology (Fishman, 2001). Later, the study of human diseases and screening of therapeutic drugs were introduced (Barut & Zon, 2000; Penberthy et al, 2002). Attributes that favour the zebrafish rising popularity for biomedical research include small size (3-4cm long as an adult), rapid development and generation time (4 months), high fecundity (200 eggs per clutch), optical transparency allowing real-time tracking of the maturation processes at an early age of development, feasibility to perform large-scale genetic screens (Haffter et al, 1996; Lawrence, 2007), and genetic similarities to humans (for example see Lamason et al, [2005]). Zebrafish pairs produce approximately 300 eggs per week and eggs are externally fertilised. Development of the embryos therefore takes place outside the body of the female. To date, zebrafish study areas include cancer, cardiovascular diseases, ageing, infection and immunity (Davis et al, 2002; Menudier et al, 1996; Neely et al, 2002).

The zebrafish model for studying infection - The availability of a wide range of molecular tools, mutant resources and ease in generating transgenic reporter

lines have contributed to the versatility of the zebrafish model in the study infection. A growing list of bacterial, viral and fungal pathogens have been experimented on in zebrafish. Infections are usually initiated by inoculating the pathogen into zebrafish via different sites in accordance with the type of experiment to be carried out. The routes of infection include microinjection via the blood island, which is the most commonly used method. With systemic infection via blood islands, zebrafish are susceptible to bacterial infections including *Mycobacterium marinum*, *Salmonella typhimurium*, and *Escherichia coli* (Davis and Ramakrishnan, 2009, Volkman et al., 2010, Benard et al., 2012). Other routes of infection include injection into the hindbrain and yolk sac. The yolk sac is used for slow-growing pathogens such as *Mycobacterium marinum* (Pressley et al, 2005), and is suitable for large-scale screening because it is technically easier than other routes (Carvalho et al., 2011). Quantitation of the pathogen burden can be done by determination of CFU, pixel count using fluorescent images or high-throughput quantitation using COPAS systems (Carvalho et al., 2011, Veneman et al., 2014). There is a large volume of data describing zebrafish infection models of bacteria including *Staphylococcus aureus, Burkholderia cenocepacia, Salmonella typhimurium*, *Shigella flexneri, Mycobacterium marinum*, of fungi including *Candida albicans, Aspergillus fumigatus, Mucor circinelloides* and *Cryptococcus neoformans* and of viruses including infectious hematopoietic necrosis virus, infectious pancreatic necrosis virus, nervous necrosis virus and snakehead rhabdovirus (reviewed in Sullivan & Kim, 2008).

Zebrafish model of *C. neoformans* infection – There are currently three peer-reviewed papers that have reported zebrafish-*Cryptococcus* infection models. Most data resonate with previous findings, but there are also new discoveries. For example, macrophages are required for protection of zebrafish against cryptococcal disease. Ablation of macrophages by using either chrondonate liposomes or morpholino knockdown resulted in high fungal burden and increased mortality (Bojarczuk et al, 2016; Tenor et al, 2015) consistent with previously reported findings in mice (Monga, 1981; Osterholzer et al, 2009a). *C. neoformans* replicate intracellularly and undergo non-lytic exocytosis

(vomocytosis) in zebrafish macrophages *in vivo* (Bojarczuk et al, 2016; Tenor et al, 2015) which resonate with previous findings *in vitro* (Ma et al, 2006; Voelz et al, 2009). These findings suggest that zebrafish can be a powerful model to recapitulate observations that were somewhat only possible *ex vivo*. Also, Bojarczuk et al. (2016) and Tenor et al. (2015) have shown that the *C. neoformans* polysaccharide capsule enlarges during infection and inhibits phagocytosis by macrophages *in vivo* (Bojarczuk et al, 2016; Tenor et al, 2015). Recently another published paper has shown that *C. neoformans* can establish an infection in zebrafish that eventually disseminates to the brain (Davis et al, 2016; Tenor et al, 2015) suggesting that zebrafish can also be used to study brain invasion.

1.8.5.2 Zebrafish immune system

1.8.5.2.1 Haematopoiesis in zebrafish

As in all vertebrate embryos, haematopoiesis occurs in two temporally overlapping phases known as primitive and definitive haematopoiesis.

Primitive haematopoiesis - The primitive haematopoiesis in zebrafish, takes place in the embryo at two distinct locations: the anterior lateral mesoderm (ALM) and the posterior lateral mesoderm (PLM) (AL-Adhami, 1977; Detrich et al, 1995; Thompson et al, 1998). Collectively, the anterior and posterior mesoderm are similar to the primitive blood islands in the mammalian yolk sac. Erythroid progenitors which appear as bilateral stripes, originate from PLM which later merges along the midline to form a chord-shaped structure known as intermediate cell mass (ICM) (Table 1) (Davidson & Zon, 2004). By about 24hpf, the ICM is localised in the trunk dorsal to the yolk sac extension and the erythrocytes enter the circulation (Davidson & Zon, 2004). Myeloid progenitors originate from cells in the most anterior lateral plate mesoderm which later forms the rostral blood islands (RBI). The anterior and posterior mesoderm are similar to the primitive blood islands in the mammalian yolk sac. The myeloid progenitors differentiate into primitive macrophages in the rostral blood islands (anterior yolk sac blood sinus) of the cardiac region, in the area underneath the hatching grand (Herbomel et al, 1999; Lieschke et al, 2002). Primitive macrophages are first seen

in zebrafish embryos at approximately the same time as erythropoiesis take place, between the 13 to 30 somite stages (12-16 hours post fertilisation). From the anterior yolk sac, primitive macrophages migrate randomly to lateral sites then finally migrate to the mesenchyme of the head or invade various tissues through the blood circulation (Bennett et al, 2001; Herbomel et al, 1999; Lieschke et al, 2002). The development of myeloid precursors in the ALM is mediated by *spil* expression whereas the erythroid differentiation of the erythroid progenitors is mediated by *gata 1* expression in the PLM (Yamaguchi et al., 1998, Kulessa et al., 1995, Nerlov and Graf, 1998).

During primitive haematopoiesis, neutrophils and thrombocytes have also been observed. However, studies to determine the origin of neutrophils have shown contrasting findings. Using fate-mapping studies, Le Guyader et al. (2008) showed that neutrophils originate from a primitive macrophage lineage whereas Warga et al, (2009) showed that neutrophils come from a primitive erythrocyte lineage (Le Guyader et al, 2008; Warga et al, 2009). Thus, primitive haematopoiesis mainly involves generation of primitive erythrocytes, primitive macrophages and myeloid cells.

Since the primitive phase of haematopoiesis is transient and lasts for up to 24 hours and blood circulation initiates at approximately 24 hpf, macrophage precursors of definitive haematopoiesis are seen at this stage (Burns et al, 2002; Gering & Patient, 2005; Kalev-Zylinska et al, 2002).

Definitive haematopoiesis - Definitive haematopoiesis occurs at a later stage of development and predominantly involves maturation of erythroid, thrombocytic, myeloid and the lymphocytic lineages. Definitive haematopoiesis begins in the posterior blood island (PBI) at about 24hpi with the generation of erythroid-myeloid progenitor cells (EMPs) which are multipotent haematopoietic progenitor cells. The PBI later develops into the caudal hematopoietic tissue (Table 1) (Murayama et al, 2006). During this time, between 26-48 hpf, haemogenic endothelial cells in the ventral wall of the dorsal aorta differentiate into haematopoietic stem cells (HSC) as shown by HSC-associated genes including *c-myb* and *runx1* (Burns et al, 2002; Kalev-Zylinska et al, 2002;

Thompson et al, 1998). This dorsal aorta is analogous to the mammalian aorta gonad mesonephros (AGM). The HSC then migrate from the dorsal aorta to the caudal haematopoietic tissue (CHT) (Kissa et al, 2008). The HSCs in the CHT then replace the EMPs as they become the progenitors of haematopoietic lineages for the rest of the embryonic development (Bertrand et al, 2010; Kissa & Herbomel, 2010; Murayama et al, 2006). In the CHT, the HSC differentiate into erythroid, myeloid, and thrombotic cells at approximately 72hpf. The CHT represents an intermediate location of haematopoietic development like the foetal liver in mammals (Murayama et al, 2006). The HSC then migrate to the pronephros and thymus via the blood circulation at about 48-56hpf (Bertrand et al, 2010; Kissa & Herbomel, 2010). From about 4dpf to adulthood, the kidney becomes the primary location of haematopoiesis including erythropoiesis, myelopoiesis, and thrombopoiesis (Lin et al, 2005) (Table 1).

Table 1: Haematopoiesis sites in zebrafish and their human counterpart

Haematopoiesis stage	Site in zebrafish	Site in humans	Reference
Early (primary) haematopoiesis	Intermediate cell mass (ICM)	Yolk sac	(Detrich et al., 1995, Murayama et al., 2006, Thompson et al., 1998)
	Dorsal aorta	AGM	
	CHT	Foetal liver	
Adult haematopoiesis	Kidney marrow	Bone marrow	(AL-Adhami, 1977, Traver, 2004)

1.8.5.2.2 Innate immune system

The cell phenotypes present in both zebrafish and mammals include motility, phagocytic activity in neutrophils (Le Guyader et al, 2008) and macrophages (Herbomel et al, 1999), myeloperoxidase in neutrophils, macrophage respiratory burst (Hermann et al, 2004) and the ability to activate T/B cells (Traver et al, 2003). Unlike adult mammals, macrophages of early zebrafish embryos reside in tissues (Herbomel et al, 1999) rather than circulating in blood as monocytes. However, this is similar to the mammalian macrophages present in the developing embryo prior to definitive haematopoiesis (Naito, 2008).

Macrophage progenitors in the developing embryo have been observed before the development of circulation (Herbomel et al, 1999). Immature granulocytes begin to be seen in the circulation by 48hpf while eosinophils are first detected at 5dpf (Lieschke et al, 2001; Willett et al, 1999).

1.8.5.2.3 Adaptive immune system

The thymus in zebrafish is extremely important for adaptive immunity (Hansen & Zapata, 1998). The development of the thymus begins in zebrafish ontogeny at around 48hpf and the rudiment of the thymus is formed by 60hpf. T cell progenitors begin to invade the rudiment at 68hpf (Willett et al, 1999) and to populate the thymus by 3dpf. Thymocytes in the developing thymus express lymphoid genes such as *rag1*, 2 and ikaros (Willett et al, 1997). Moreover, mature lymphocytes have been observed in the thymic epithelium by 7dpf (Trede et al, 2001). However, functional T cells have not been observed during the first three weeks of development anywhere outside the thymus (Trede et al, 2004). The thymus remains morphologically immature until three weeks post feltilisation (Lam et al, 2002). The B lymphocytes are seen in the kidney and pancreas at about 19dpf (Danilova & Steiner, 2002; Langenau et al, 2004; Willett et al, 1999). Antibody production were detected by western blotting at four weeks post-fertilisation. Humoral responses to T cell-dependent and –independent antigens are not detected until 4-6 weeks post fertilisation (Lam et al, 2004). Zebrafish rely on the innate immunity for protection from environmental pathogens in the first four weeks until it acquires morphologically and functionally

mature cells of the adaptive immune system. Conversely, newborn mammals rely on innate immunity plus adaptive immunity (antibodies) from their mother (Tizard 2013) while they wait for their adaptive immune system to become fully functional. Thus, zebrafish larvae offer an opportunity to study the innate immune defence strategies without the interference of the adaptive immune response.

1.8.5.2.4 Pattern recognition receptors

TLR mediated signalling – Toll-like receptors (TLRs) are a family of type I transmembrane receptors that are highly similar from *Drosophila* to humans and share structural and functional characteristics (Lemaitre et al, 1995; Lemaitre et al, 1996; Medzhitov et al, 1997). There are at least 10 known TLRs in humans and 13 in mice. They differ mostly by ligand specificity, expression and activation of target genes (reviewed in Kumar et al, 2009). TLRs are integral glycoproteins which consist of an extracellular ligand-binding domain with Leucine-rich repeat (LRR) motifs and a Toll/Interleukin-1 (IL-1) receptors (TIL) homology domain located in the cytoplasm (Akira et al, 2006; O'Neill & Bowie, 2007). Some TLRs are localised on the cell surface (TLR1, 2, 4, 5, 6 and 10) while others are located in intracellular compartments such as endosomes and lysosomes (TLR3, -7, -8, -9, -11 and -13). In mammals, TLRs are predominantly expressed in antigen presenting cells (APCs) including macrophages, dendritic cells and B lymphocytes (Mogensen, 2009). However, many other cell types express TLRs too in response to localised infection (Miettinen et al, 2001). TLRs mainly recognise and bind to pathogen ligands such as lipoproteins (TLR2 ligand) of Gram-positive bacteria, lipid A motif of lipopolysaccharides (TLR4 ligand) of Gram-negative bacteria and flagellin(TLR5 ligand) which may be present on either gram type bacteria (Hayashi et al, 2001; Poltorak et al, 1998; Schwandner et al, 1999). The other TLRs detect nucleic acids present in endosomal and phagosomal compartments. For example, TLR3 recognises a double-stranded RNA (dsRNA) during viral replication, while TLR7 and TLR8 bind to single-stranded RNA (ssRNA) of RNA viruses and TLR9 recognises viral and bacterial unmethylated CpG DNA (Alexopoulou et al, 2001; Diebold et al, 2004; Heil et al, 2004; Hemmi et al, 2000; Ohto et al, 2015). The TLR signalling pathway in mammals involves TIR-domain-containing adaptor molecules including

MyD88, MAL/TIRAP, TRIF/TICAM1, TRAM/TICAM2, and SARM (Akira et al, 2006; Miettinen et al, 2001). All but TLR3 uses MyD88 adaptor molecule (Takeda & Akira, 2004). Downstream signalling leads to the activation of transcription factors such as ATF, NF-κB, AP-1, IRF and STAT families predominantly via central intermediate molecule TRAF6 (Takeda & Akira, 2004) (Figure 5).

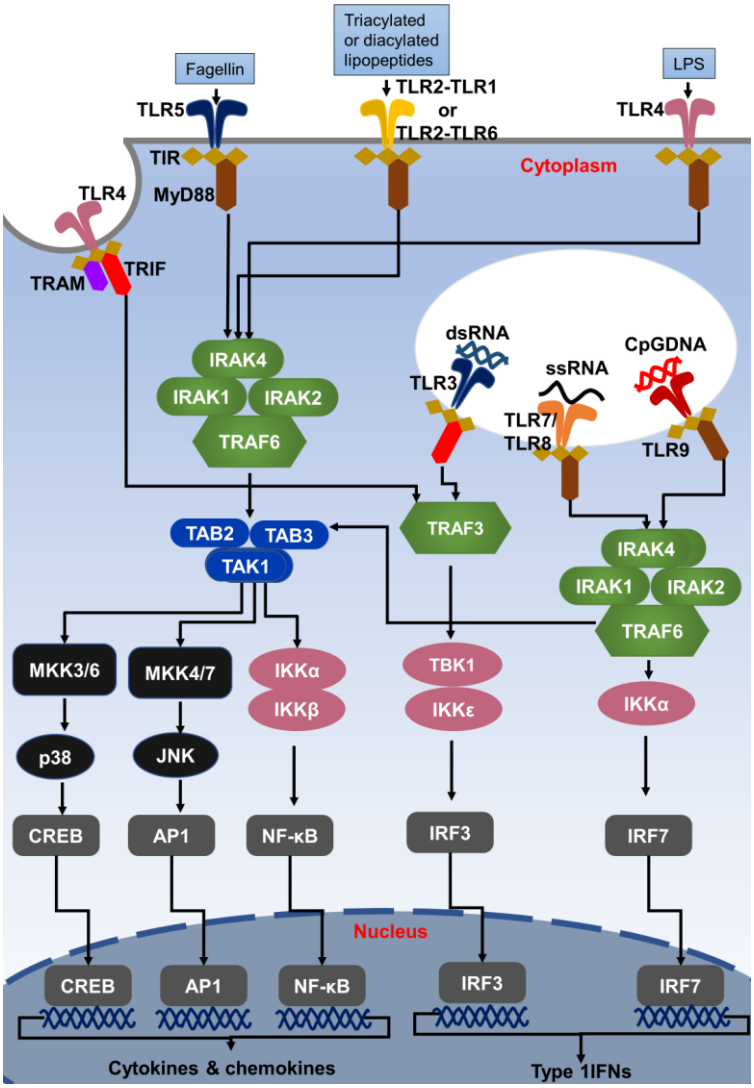

Figure 5: Mammalian TLR signalling pathways

Toll-like receptors are located on the cell surface and in the endosomes. They detect microbial signature components, foreign nucleic acids and danger associated self-molecules. TLR5, TLR4 and heterodimers of TLR2/1 or TLR2/6 are present on the cell surface while TLR3, TLR7/8 and TLR9 are located in the endosomes. TLR4 is present both on the cell surface and on the endosomes. Following stimulation, TLR signalling is initiated via either adaptor molecules myD88 or TRIF and TRAM. TLR4 migrate from the cell surface to the endosomes where it switches signalling from myD88 to TRIF. Upon engagement of signalling adaptor molecules, there is stimulation of downstream signalling pathways involving interactions between IRAKs and TRAFs resulting in activation of MAPKs, JNK and p38 and subsequent transcriptions factors NF-κB, IRFs and CREB and AP1. The important consequence of TLR signalling is the elicitation of pro-inflammatory cytokines and induction of type 1 IFNs when endosomal TLRs are involved.

TLR in zebrafish - Zebrafish have a set of 20 putative orthologs of mammalian TLRs (Jault et al, 2004; Meijer et al, 2004). Out of these, 10 are orthologs of human TLR family members (Table 2). The TLR22 is fish specific and closely related to a *toll9* gene of *Drosophila melanogaster* whereas the TLR21 is present in birds, amphibians and fish (Jault et al, 2004; Meijer et al, 2004). In zebrafish, TLR9 and TLR21 have been shown to recognise CpG-ODNs (Yeh et al, 2013). Zebrafish have gene duplicates for some of the corresponding mammalian TLRs. For example, TLR4 has *tlr4ba/tlr4bb*, TLR5 has *tlr5a/tlr5b* and TLR8 has *tlr8a/tlr8b*. However, it is not known whether having multiple receptors is associated with diversification in PAMP recognition (Meijer & Spaink, 2011). TLR18 in zebrafish is a homolog of human TLR1 (Meijer et al, 2004) while TLR20 zebrafish protein is similar to mouse TLR11 and TLR12 (Pietretti et al, 2014). Some zebrafish TLR ligands have been identified. For example, zebrafish TLR2 recognises bacterial lipoproteins or Pam3CSK4 (Meijer & Spaink, 2011; Yang et al, 2015) while TLR3, 5 and 9 bind to dsRNA, flagellin and unmethylated CpG DNA respectively (Alexopoulou et al, 2001; Hayashi et al, 2001; Matsuo et al, 2008; Ohto et al, 2015; Uematsu & Akira, 2008). However, zebrafish TLR4 does not recognise LPS stimulation (Sepulcre et al, 2009; Sullivan et al, 2009) although molecules (MD1 and Rp105) which mediate recognition have been identified in zebrafish. Candel et al. (2015) showed that MD1 bind to Rp105 and TLR4 in zebrafish, however accessory molecules including LBP, CD14 and MD2 have not been identified in zebrafish (Candel et al, 2015; Kaiser & Normile, 2015; Sullivan et al, 2007) suggesting that the TLR4 binding conformation is not conserved in zebrafish (Sullivan et al, 2009). In fact, deletion of tlr4a, tlr4b and myD88 did not affect zebrafish immune response to LPS stimulation (Sepulcre et al, 2009) suggesting that TLR4 play a different role in recognition of pathogens and recognition of LPS in zebrafish may be different from that of mammals. TLR22 is fish specific and recognises dsRNA or PolyI:C followed by engagement of the common adaptor molecule TRIF to elicit IFN expression. TLR22 is thought to be a homolog of mammalian TLR3 (Matsuo et al, 2008).

Homologs of signalling molecules downstream of mammalian TLR have been identified in zebrafish including MyD88, Mal/TIRAP, TRIF, IKAP, NEMO, SARM1

(Ordas et al, 2011) and TRAF family (Stein et al, 2007). Using knockdown studies, MyD88 and Traf6 have been shown to be required for pro-inflammatory innate immune response to microbial challenge in zebrafish (Bates et al, 2007; Stockhammer et al, 2010; Stockhammer et al, 2009; van der Sar et al, 2006). All of mammalian adaptor proteins except TICAM2 (TRAM) have been identified in zebrafish. However, zebrafish have TICAM1 and is located in the Golgi apparatus and can activate NF-κB enhancers and IRF3- and IRF7-mediated pathways but the N- and C-terminus domains which are present in mammals are absent in zebrafish (Fan et al, 2008; Poynter et al, 2015; Zhang & Gui, 2012).

Table 2: TLR that have been identified in zebrafish

Zebrafish	Mammals
TLR1	TLR1
TLR2	TLR2
TLR3	
TLR4b.a/b	TLR4
TLR5a/b	TLR5
	TLR6
TLR7	TLR7
TLR8a/b	TLR8
TLR9	TLR9
	TLR10
TLR14	
TLR18	
TLR19	
TLR20a	
TLR21	
TLR22	TLR3

Adapted from Li et al. (2017)

NLR mediated signalling - Several families of receptors to detect pathogens that evade recognition by endosomal or cell surface receptors perform cytoplasmic surveillance. Cytoplasmic receptors include NOD-like receptors (NLRs), RIG-like receptors (RLR) and AIM2-like receptors (ALRs). The NLRs recognise different PAMPS and DAMPs (Schroder et al, 2010) while RLRs recognise viral RNA (Wilkins & Gale, 2010) and ALRs detect cytosolic DNA (Fernandes-Alnemri et al, 2009; Hornung et al, 2009; Jin et al, 2012). The innate immune signalling pathways involving the NLRs and RLRs are similar across mammals and teleost fish. However, the signalling pathways associated with ALRs are not present in fish, suggesting that fish may have other mechanisms to deal with DNA viruses and intracellular bacteria (Cridland et al, 2012). There are almost 421 NLR family members in zebrafish (Howe et al, 2016). Molecular phylogeny data and expression studies of NLR subfamilies in zebrafish reveal three distinct NLR subfamilies which include NLR-A, NLR-B and NLR-C. NLR-A comprise eight genes that resemble mammalian NODs, whereas NLR-B, comprise nine genes that resemble mammalian NACHT-, LRR- and PYD-containing proteins (NLRP), and NLR-C comprise of 405 NLR genes that are unique to teleost fish (Cridland et al, 2012; Laing et al, 2008). Sha et al. 2009 has shown that mammalian NOD1, NOD2 and NLRC3 are conserved in zebrafish (Sha et al, 2009). NOD2 is associated with a dual oxidase enzyme that is essential for the production of antibacterial ROS in epithelial cells in mammals (Lipinski et al, 2009). Likewise, NRLs appear to be essential for innate antibacterial immunity in zebrafish. NOD1 or NOD2 zebrafish morpholino show significantly reduced DUOX expression resulting in impaired immune response to systemic *Salmonella* infection (Oehlers et al, 2011). On the other hand, NLRP3 inflammasome is not present in zebrafish (Boyle et al, 2013; Laing et al, 2008; Stein et al, 2007). Some components of the adaptor molecules in the inflammasome pathways are present in zebrafish including apoptosis-associated speck-like protein containing CARD (ASC) that connects the upstream receptors (such as NLRs/PYHINs) to the downstream signalling caspases (Broz et al, 2010; Fernandes-Alnemri et al, 2007).

C-type lectin signalling – The C-type lectin receptors (CLR) are part of a diverse superfamily of soluble and transmembrane proteins that bind carbohydrates. The

CLR are defined by a characteristic C-type lectin domain (Zelensky & Gready, 2005). CLR are comprised of 17 groups characterised based on structure. The most studied groups include calcium dependent lectins with single CRDs, calcium-independent receptors with single CTLDs and calcium dependent lectins with multiple CRDs (Zelensky & Gready, 2005; Hoving et al, 2014). Stimulation of the CLR can activate intracellular signalling in two ways: firstly, via direct signalling by Dectin-1 and DNGR-1 through ITA-like motifs found in the cytoplasmic leg of these receptors (Zelensky & Gready, 2005; Geijtenbeek & Gringhuis, 2009). The second mechanism is through indirect signalling involving receptors including macrophage-inducible C-type lectin (Mincle), Dectin-2 and C-type lectin domain family 5A (CLEC5A). The receptors bind to ITAM containing adaptor molecules such as FcRγ or DAP12 (Hoving et al, 2014). The downward signalling through both mechanisms involves the recruitment of syk leading to formation of CARD9, Bcl10 and Malt1 complex. This signalling pathway and others lead to induction of downstream molecules such NFκB and MAPK, that consequently activate cellular responses including phagocytosis, DC maturation, chemotaxis, the respiratory burst, inflammasome activation, and production of cytokines (Drummond et al, 2011; Strasser et al, 2012). The CLR are widely recognised to play a critical role in antifungal immunity, although they have been shown to orchestrate anti-viral, bacterial, helminth and protozoan immunity (Hoving et al, 2014). In antifungal immunity, Dectin-1 recognises β-glucans while the other receptors (the mannose receptor (MR), DC-SIGN, and Mincle) bind to undefined, mannose-based structures found in the mannan layer of the fungal cell wall (Hardison & Brown, 2012).

Research into in CLR in zebrafish is significantly sparse. CLR genes have been identified in zebrafish. Recently, Zheng et al, (2015) identified a zebrafish MR gene that is structurally similar to other MRs from other species. In addition, zebrafish lectin-like receptor, CD209 (DC-SIGN) homologue, has been shown to participate in the initiation and development of adaptive immunity (Zheng et al, 2015).

1.9 Hypothesis and aims

Cryptococcus neoformans remains the most common fungal agent causing morbidity and mortality in the immunocompromised. Macrophages are highly specialised for host defence against *C. neoformans.* The effector functions of macrophages are defective in the presence of very low $CD4^+$ T cells. However, the current antifungal drugs mainly target the pathogen and are unable to resolve macrophage inactivity. Activation immunotherapies that target the host, such as cytokines or agonists of PRRs, aimed at enhancement of innate immune resistance to pathogens are emerging. Currently several immunomodulatory therapies are licensed for medical use in the treatment and prevention of infectious diseases. The research presented in this thesis explores immunomodulation by activation of innate immune resistance as therapy for *C. neoformans* infections.

Hypothesis

I hypothesised that treatment that activates macrophages in the absence of T cell mediated immunity leads to clearance of *C. neoformans* infection

Aims

I aimed to establish a localised infection model in zebrafish to study infection outcomes following treatment with cytokines or TLR agonists

Objectives

To answer this hypothesis, the following objectives were employed.

1. Development a zebrafish-*C. neoformans* intramuscular infection model
2. Investigation of the effect of IFNγ on cellular and cytokine immune responses and clearance of infection.
    - Administration of IFNγ with anti-fungal drugs in HIV-associated cryptococcal meningitis results in increased clearance of infection from cerebrospinal fluid (CSF). This study is based on the hypothesis that IFNγ induces clearance of *Cryptococcus* by direct activation of effector cells including macrophages. Cellular and cytokine immune responses are analysed.

3. Investigation of the effect of TLR agonist on activation of innate immune resistance to *C. neoformans* and their underlying mechanisms.
    - TLRs are required for *C. neoformans* host immunity. However, manipulation of the receptors for the treatment of *C. neoformans* has not been investigated. The ability of several TLR agonist to improve containment of *C. neoformans* infection in zebrafish are analysed.

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CHAPTER 2

MATERIALS AND METHODS

2.1 Ethics statement

Handling of zebrafish lines was carried out in compliance with guidelines and legislation set out in UK law in the Animals (Scientific Procedures) Act 1986 and local animal welfare regulations. All animal work was approved by the University of Sheffield Local Ethical Review Panel.

2.2 Fish husbandry

The wild-type and transgenic zebrafish lines used in this work are listed in Table 3. Zebrafish strains were raised and maintained by following standard protocols (Joseph Fetcho, 2003). Adult fish were maintained in a continuous re-circulating closed aquarium system at 28° C on a 14-hour light, and 10-hour dark daily cycle in UK Home Office approved aquaria in the Bateson Centre at the University of Sheffield. Zebrafish at 5-days post fertilisation (dpf) were fed Tetra A-Z powdered fish feed, then live artemia twice daily from 13dpf onwards.

Embryos for experimentation were obtained either by mating individual pairs or marbling a tank for group mating. For mating pairs, two adult zebrafish male and female were put into breeding traps composed of two plastic containers that fit tightly into one another. The inner container held the fish and had a grid base to allow embryos to fall into the outer container. For collective mating, a container with marbles was placed into a fish tank containing up to 40 adult zebrafish. This container consisted of two plastic containers that were tightly fitted together. The inner container had a wire-mesh bottom which was filled with marbles. Zebrafish mate and spawn at dawn, thus in all cases, mating set-up was performed in the evening and embryos were harvested the following morning before noon. The embryos were collected by pouring the water from the outer container through a small strainer where they were collected in the mesh. They were then washed with system water to remove any dirt and transferred into a Petri dish (Scientific Laboratory Supplies Ltd. (SLS), Coatbridge, UK). Fertilised healthy embryos were sorted using a Pasteur pipette into Petri dishes with approximately 30 mL of 1x E3 medium with 0.1% (w/v) methylene blue (Stock- 10x E3: 5 mM NaCl+0.17 mM KCl+0.33 mM $CaCl_2$+0.33 mM $MgSO_4$+ dH_20 made up to 1L) at a density of approximately 60 viable embryos per dish. This solution aids in

distinguishing dead (which stain blue) from live embryos and also reduces bacterial growth. The embryos were maintained at 28.5º C in a designated incubator for further development. At the end of all experiments, and before the zebrafish reached 5.2dpf, they were killed by immersion in liquid bleach. All protocols were carried out following UK Home Office legislation, which allows the use of embryos up to 5.2dpf without the Animals (Scientific Procedures) Act.

Table 3: Zebrafish lines used for experimental work

Zebrafish line	Description	Reference
Nacre	Wild-type	(Lister et al., 1999))
*Tg(mpeg1:mCherryCAAX)*sh378	Macrophage fluorescent marker mCherry under the control of the macrophage-specific *mpeg1* promoter	(Bojarczuk et al., 2016)
Tg(*mpeg1*:Gal4-VP16/UAS:Kaede)	Zebrafish line expressing a photoconvertible protein kaede under control of the Gal4 UAS sequence in macrophages. Used as a macrophage line	(Ellett et al., 2011)
Tg(mpeg1:Gal4-UNM)sh256	Macrophage line expressing Gal4 driven by UNM promoter	
Tg(mpx:eGFP)i114	Neutrophil fluorescent GFP transgenic zebrafish	(Renshaw et al., 2006)
TgBAC(tnfa:GFP)pd1028	TNFα promoter BAC driving GFP line	(Marjoram et al., 2015)
Tg(cfms:GFP)sh377	Macrophages marker GFP driven by the fms promoter	(Dee et al., 2016)
Tg(il1:GFP)sh445	Il-1 promoter driving GFP	

2.3 *C. neoformans* strains and cultivation

Cryptococcus neoformans strains used in this study are listed in Table 4. The strains were stored long-term in Microbank™ vials (Pro-Lab Diagnostics) in a minus 80º C freezer (New Brunswick Scientific, Eppendorf, Hamburg, Germany). The strains from minus 80º C freezer were cultured on YPD agar (50g/L YPD + Agar 2g/L – both from Sigma-Aldrich) at 28º C for 48 hours and thereafter they were stored at 4º C until needed or for no longer than 1 month. The cultures were maintained by sub-culturing on fresh media periodically and checked for contamination before use.

Table 4: *C. neoformans* strains used in this study

***Cryptococcus neoformans* var. grubii**	**Description**	**Reference**
Kn99	*C. neoformans* serotype A mating type α. Congenic strain in the H99 genetic background	(Nielsen et al., 2003)
Kn99-GFP	Kn99 strain biolistically transformed with a plasmid pAG32_GFP	(Gibson et al., 2017)
Kn99-mCherry	Kn99 strain biolistically transformed with a plasmid pRS426H-CnmCherry	(Gibson et al., 2017)

2.4 Infection assay

2.4.1 Preparation of *C. neoformans* prior to injection

A day before infection, a colony of *C. neoformans* from YPD agar was inoculated into 2mL YPD broth (50g/L) in a 5mL T405 sterile plastic tube (Simport® Scientific, Beloeil, Canada) and incubated at 28º C overnight (approximately 18 hours) at 20 rotations per minute (rpm).

Preparing fungal suspension - The overnight 2mL liquid culture was mixed by pipetting up and down. Before the cryptococci had a chance to settle, 5µL of the culture was added in 95µL PBS (making 1:20 dilution) and mixed by pipetting up and down prior to counting.

Counting – 10µL of the diluted cryptococci suspension was added to a neubauer haemocytometer (Camlab, Cambridge, UK) and the cryptococci was counted using a 10x objective on all 4 sets of 16 corner squares.

Calculations – To calculate the number of cryptococci cells per mL, the average count of the all 4 sets of 16 corner squares was multiplied with 10^4 (volume correction to 1mL) and 20 (dilution factor).

i.e. if average of 301 cryptococci were counted

Then per mL = $301 \times 10^4 \times 20 = 6.02 \times 10^7$ cfu/mL

To prepare fungal suspension for injection, 1mL of well-mixed culture was transferred into a 1.5mL Eppendorf microtube (Sigma-Aldrich) and then pelleted at 3300g in GenFuge 24D centrifuge (Progen Scientific, London, UK) for 1 min. The supernatant was then decanted and the cryptococci cells were washed 3x in 1mL 1xPBS (1 tablet/100mL, Oxoid, Basingstoke, UK) and suspended in1mL 1xPBS after the last wash.

To calculate a volume that will give the required fungal inoculum per nanoliter, the following example calculation was used. This example was to give 1000 fungal cells/nanoliter based on a suspension containing 6.02×10^7 cells/mL.

$$\text{Volume to give} \frac{1000\text{cfu}}{\text{nL}} = \frac{1000\ x\ 10^{-6}}{6.02\ x\ 10^7} = 60.2\mu\text{L}$$

This volume (62µL for example) was taken from the washed suspension into another 1.5mL Eppendorf microtube and centrifuged at 3300g for 1 min and the pellet was re-suspended in sterile 10% (vol/vol) Polyvinylpyrrolidinone (PVP)-0.5% Phenol Red (Sigma-Aldrich) in PBS. PVP increases the viscosity of the injection solution and inhibits clumping of cells in the needle (Spaink et al., 2013).

2.4.2 Intramuscular injection

To perform injections, 3dpf zebrafish larvae were anesthetised by immersion in 0.168 mg/mL tricaine (Stock solution: 400 mg Tricaine methanesulfonate or MS222, Sigma-Aldrich; + 97.9 mL dH_20 + 2.1 mL 1 M Tris–HCl, pH 9.0) in E3 for at least 15 min. The injection solution (*C. neoformans* and/or immunomodulatory compounds) were loaded into a Kwik-Fil borosilicate glass capillary (World Precision Instruments, Hitchin, UK) pulled into 51-gauge needle using P-1000 Next Generation Micropipette Puller (Sutter Instrument, CA, USA). Using a graticule slide, the micromanipulator was set to dispense a droplet size equivalent to 0.5 nl. The larvae were laid on 10g/L agarose (Sigma Aldrich) in petri dish. Two 0.5 nl droplets were injected into either 15, 16 or 17 myotomes (Figure 9). After injections, larvae were transferred into fresh E3 to recover from the anaesthetic and maintained at 28º C. Any larvae that were visibly damaged or had solution accidentally injected into the bloodstream were excluded from the experiment.

2.4.3 High content imaging method

Before imaging, infected larvae were anesthetised by immersion in 0.168 mg/mL tricaine in E3 and transferred into plastic 96-wells-F plates (VWR, Pennsylvania, USA) with a single larva per well. For time-lapse imaging, larvae were mounted in 5g/L low gelling agarose (Sigma Aldrich) in E3 containing 0.168 mg/mL tricaine into glass-bottomed 96-well plates (Porvair sciences, Wrexham, UK). The larvae were imaged on a Nikon Ti-E with either a CFI Plan Apochromat λ 10X N.A.0.45 or 20X N.A. 0.75 or 40X N.A. 0.95 objective lens, a custom built 500 µm Piezo Z-stage (Mad City Labs, Madison, WI, USA) and using SPECTRA X light engine with 7 bandpass filters (Lumencor, Beaverton, Oregon, USA). Images were captured with Neo sCMOS, 16.6 mm x 14.0 mm Sensor Size, 6.5 µm pixel size

camera (Andor, Belfast, UK) and NIS-Elements (Nikon, Richmond, UK). Settings used and imaging sections used will be described in each section below.

2.5 Effects of immunomodulation on infection outcomes

2.5.1 Preparation of immunomodulatory compounds for injection

Chemical compounds were dissolved in dH_20 or as indicated (Table 5). The required volume of stock solution was added to cryptococci suspension in 10% PVP-0.5% Phenol Red to make a working concentration. For example, to make a concentration of 100cells/nl in 5µg/mL IFNγ, 2µL *C. neoformans* (1000cells/nl) + 2µL IFNγ (50µg/mL) + 16µL PVP were mixed. Testing of immunomodulatory compounds was always carried out alongside appropriate vehicle control as indicated. The immunomodulatory compounds used in this study are listed in Table 5.

2.5.2 Inoculation and imaging

Three dpf zebrafish larvae were co-injected with *C. neoformans* and an immunomodulatory compound prepared as described in section 2.5.1. Injections were performed as described above (Section 2.4.2). Because of the technical difficulty to inject the same number of fungal cells in each larva, the fungal inoculum was set at 1 and 25 cryptococci per larva and cryptococci numbers outside this limit were discarded.

To quantitate the initial fungal inoculum, the larvae were imaged immediately (zero-hour post infection) in 96-well plate. Imaging was performed as described in above except a CFI Plan Achromat UW 10X N.A. 0.45 objective lens was used and each larva was imaged on the infected region (between 15-17 myotome) taking 21 z-sections, 5µm apart, captured in GFP channel. Following imaging, tricaine E3 was replaced with fresh E3 to recover the fish from the anaesthetic. The larvae were maintained at 28º C until 48hpi. At 48hpi, larvae were anaesthetised in 0.168 mg/mL tricaine E3 and imaged with the same settings at before (0 hpi).

2.5.3 Measurement of fungal burden

The fungal burden at zero and forty-eight hours-post infection was determined in NIS-Elements by manually counting individual fungal cells per fish. Larvae with a fungal inoculum of <1 or >25 were removed from the analysis. Each biological repeat contained 30-50 infected larvae.

Table 5: Immunomodulatory compounds used in this study

Condition	Manufacturer	Stock [C]	Solvent	Working [C]
Interferon-gamma from Kingfisher, Biotech, MN, USA Protein Sequence: SVPENLDKSI EELKAYYIKE DSQLHNAHPI FLRILKDLKV NLEESEQNLL MSIVMDTYSR IFTRMQNDSV DEATKERLAH VQEHLKKLQE SYFPGKSAEL RTYAETLWAI KENDPIVQRK ALFELKRVYR EATLLKNLKN KERKRRQAKA SRSKSLNRG (159)		50 µg/mL	dH_20	5 µg/mL
Lipopolysaccharide (E. coli serotype 055:K59[B5]H)	Invivogen, CA, USA	100 µg/mL	dH_20	10 µg/mL
Imiquimod (R837)	Invivogen, CA, USA	100 µg/mL	dH_20	10 µg/mL
Pam2SCK4	Invivogen, CA, USA	100 µg/mL	dH_20	10 µg/mL
CpG ODN 2395	Invivogen, CA, USA	25 µg/mL	dH_20	2.5 µg/mL
***S. aureus* CWP**	Generous gift from Simon Foster, UOS	10mg/mL	dH_20	5 mg/mL
***Vibrio* DNA**	Generous gift from Victoriano Mulero University of Murcia, Spain	50 µg/ml	dH_20	5 µg/ml

2.5.4 Effects of immunomodulatory compounds of fungal growth

To examine if the compounds at this concentration (shown in Table 5) are not toxic to the fungal cells, *C. neoformans* was prepared with the immunomodulatory compounds as described in section 2.5.1. The solutions were left for two hours and 10µl was transferred to YPD agar, incubated at 28.5º C overnight and colonies counted and calculated per mL.

2.6 Assays to analyse macrophage and neutrophil response during cryptococcal infection in zebrafish

2.6.1 Macrophage and neutrophil recruitment and phagocytosis assay

To assess the effect of *C. neoformans* or immunomodulatory compounds on recruitment of immune cells to the site of infection, *C. neoformans* and/or immunomodulatory compounds were prepared as described above (section 2.4.1 and 2.5.1). Three dpf Tg(*mpeg1*:Gal4-VP16/UAS:Kaede) larvae for macrophages, and *Tg(mpx:*GFP)i114 larvae for neutrophils, were inoculated with the solutions as described above (Section 2.4.2). At 2, 12 and 24 hpi, larvae were anaesthetised and imaged using Nikon Ti-E with a CFI Plan Apochromat λ 20X N.A. 0.75 objective lens taking 21 z-sections, 5µm apart, captured in GFP, Red channels. The other settings were as described above (Section 2.4.3). The numbers of neutrophils and macrophages at the infection site were counted as describe in Figure 6.

For phagocytosis assay, the number of extracellular cryptococci, proportion of intracellular cryptococci and phagocytic index in both neutrophils and macrophages were obtained at time-points. The formula in Figure 7 were used to obtain these numbers. The numbers were calculated separately for neutrophils and macrophages. Where feasible, blind counting to experimental conditions was carried out.

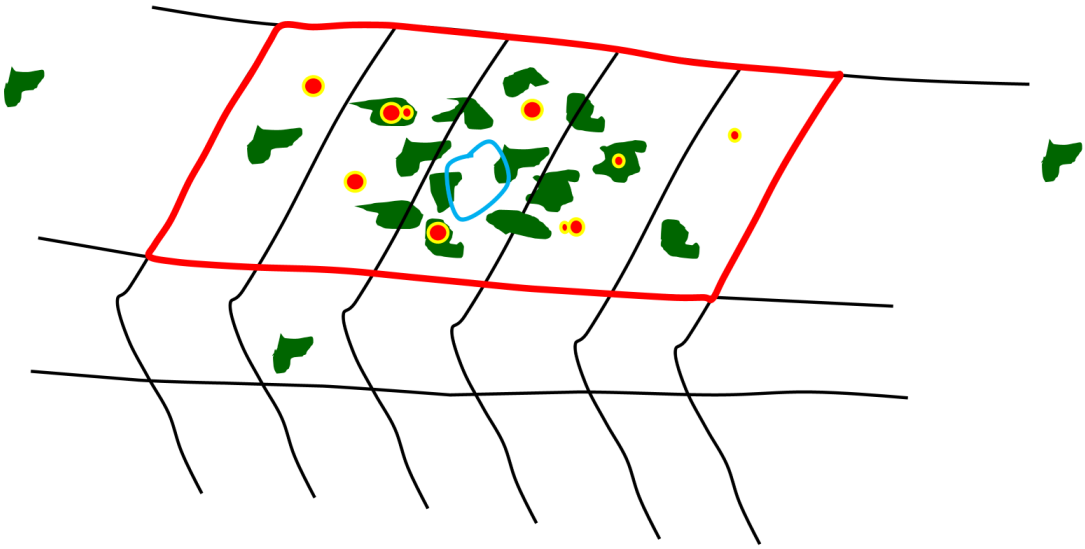

Figure 6. Schematic diagram of enumeration of recruited macrophages or neutrophils.

Enumeration of recruited innate cells was carried out on a region surrounding the wound site. A region (red box) was drawn on a wound (blue circle) site covering 2-3 somites on both sides of the wound site. The same criteria were applied to all fish analysed in the experiment.

$$\text{No. of extracellular cryptococci} = a - b$$

$$\text{Proportion of intracellular cryptococci} = \frac{b}{a} \; x \; 100$$

$$\text{Phagocytic index} = \frac{b}{c}$$

Figure 7: Formulas used to calculate number and proportion of extracellular cryptococci and phagocytic index.

a = total number of cryptococci; *b* = number of intracellular cryptococci; *c* = number of infected macrophages

2.6.2 Tumour necrosis factor-α GFP expression assay

To investigate the effect of immunomodulatory compounds on the expression of TNFα GFP, 3dpf *TgBAC(tnfa:GFP)pd1028* crossed to *Tg(mpeg1:mCherryCAAX)sh*378 larvae were co-injected intramuscularly with *C. neoformans* and immunomodulatory compounds. At 12, 24 and 36 hpi, larvae were mounted in 5g/L low gelling agarose in E3 containing 0.168g/mL tricaine and imaging was performed as described above with the following adjustments: Images were captured with CFI Plan Apochromat λ 40X N.A. 0.95 objective lens, 45vz-sections, 2µm apart, using the following settings: GFP, filter 49002, 700 ms exposure, gain 1; mCherry, filter 49008, 900 ms exposure, gain 1; and bright field, 30ms, gain 1. The image analysis was performed using NIS-elements. A line was drawn around the infected macrophages and values of GFP mean fluorescent intensity were obtained. Background GFP mean fluorescent intensity values were also obtained. Actual GFP mean fluorescent intensity of a macrophage was obtained by subtracting background GFP mean fluorescent intensity value. GFP TNFα expression per fish was obtained by adding up all actual GFP mean fluorescent intensity values of each macrophage.

2.6.3 Interleukin-1 (IL-1) expression assay

To investigate the effect of immunomodulatory compounds on the expression of IL-1 GFP, 3dpf *Tg(il1:GFP)sh445* crossed to nacre were co-injected intramuscularly with *C. neoformans* and immunomodulatory compounds. Larvae were mounted and time lapse imaging was performed as described above (Section 2.4.3) with the following adjustments: Images were captured with CFI Plan Apochromat λ 20X N.A. 0.75 objective lens, 21 z-sections, 5µm apart, with Perfect Focus system, every 15 minutes for 12 hours. A line was drawn around the injection site, and values of GFP mean fluorescent intensity were obtained.

2.7 Lysosomal pH

To investigate if IFNγ enhances acidification of lysosomal pH, cresyl violet was employed as a fluorescent lysosomal marker as shown by Ostrowski et al. (2016). Cresyl violet is an acidotropic compound in that it can cross the membrane in its

unprotonated state. Once protonated, cresyl violet is trapped and eventually accumulates in acidic organelles such as lysosomes. Cresyl violet excitation and emission wavelengths are 585 and 627 respectively. Cresyl violet has higher emission wavelength than most conventional red fluorescent dyes and is more photostable than lysotracker red (Ostrowski et al, 2016). A solution comprising *C. neoformans*, 5µg/mL IFNγ and 0.5mM cresyl violet (Sigma Aldrich) was injected in 3dpf *Tg(fms:Gal4.VP16)i186* as described above (Section 2.4.2). To make a concentration of 100cells/nl in 5µg/mL IFNγ and 0.5mM cresyl violet for example, 2µL *C. neoformans* (1000cells/nl) + 2µL IFNγ (50µg/mL) + 2ul cresyl violet (5mM) +14µL PVP were mixed. Images were captured with CFI Plan Apochromat λ 20X N.A. 0.75 objective lens, 45vz-sections, 2µm apart. Analysis was performed using NIS-elements. A line was drawn around the infected macrophages and values of Cy7 mean fluorescent intensity were obtained. Background Cy7 mean fluorescent intensity values were also obtained from macrophages of infected larvae that were not treated with cresyl violet. The actual Cy7 mean fluorescent intensity of a macrophage was obtained by subtracting background Cy7 mean fluorescent intensity value. Phagosome acidification per fish was obtained by adding up all actual Cy7 mean fluorescent intensity values of each macrophage.

2.7.1 Proliferation rate assay in macrophages

To assess the ability of immunomodulatory compounds to inhibit proliferation of *C. neoformans* within macrophages in zebrafish, 3dpf Tg(*mpeg1*:Gal4-VP16/UAS:Kaede) larvae were co-injected with *C. neoformans* and immunomodulatory compounds as described above (Section 2.4.2). At 12hpi, at which almost all cryptococci were internalised, zebrafish larvae were mounted in 5g/L low gelling agarose in E3 containing 0.168g/mL tricaine. Time-lapse imaging was carried out as described above with the following adjustments: images were captured with CFI Plan Apochromat λ 20X N.A. 0.75 objective lens, 21 z-sections, 5µm apart, with Perfect Focus system, every 15 minutes for 12 hours.

For calculation of the proliferation rate (PR) within macrophages, the number of internalised cryptococci in each macrophage were recorded every 15 min until the end of imaging.

To obtain PR per hour, the following calculations were performed: PR = (total number of cryptococci within a macrophage at the end of time-lapse imaging minus the total number of cryptococci within the same macrophage at the beginning of the time-lapse imaging) divide by total number of time-lapse hours.

2.8 Macrophage ablation assay using metronidazole

Ablation of macrophages was performed as previously described (Curado et al., 2008). Zero-dpf *Tg(mpeg1:Gal4-UNM)sh256* zebrafish transgenic embryos were immersed in freshly prepared 5mM Metronidazole (Sigma Aldrich) in E3 with 0.2% (vol/vol) DMSO (sigma-Aldrich). Control embryos were immersed in E3 with 0.2% DMSO. Embryos were transferred into freshly prepared 5mM Metronidazole in E3 with 0.2% DMSO daily until ready for infection (at 3dpf). Larvae were inoculated with *C. neoformans* and immunomodulatory compounds as described above (Section.2.4.2). Following infection, larvae were transferred to freshly prepared 2.5mM metronidazole in 0.2% DMSO and E3. The zebrafish were incubated at 28º C and wrapped with aluminium foil throughout the experiment. Imaging was carried out as described above except that CFI Plan Achromat UW 10X N.A. 0.45 objective lens was used and three adjacent fields of view that were assigned from bright-field images were taken. Using a custom built 500µm Piezo Z-stage, 80 z-sections, 5 µm apart, were captured in GFP and Red channels and each position in that order. The fungal burden at the site of infection was obtained as described above. To confirm ablation, the total body macrophage count was performed by manually counting the macrophages in images in NIS-elements. As a control, metronidazole had no significant effect on the fungal burden upon ablation of neutrophils (Data provided by Josie Gibson).

2.9 α-nitrotyrosine staining

To investigate the effect of IFNγ on the production of nitrotyrosine, 3dpf *Tg(cfms:GFP)sh377* larvae were injected with wild type *C. neoformans* as described above and incubated at 28.5° C for 6, 12 and 24hrs post infection.

Following incubation, the fish were anaesthetised with tricaine and transferred into a microfuge tube. The tricaineted E3 was removed and immediately 4% Paraformaldehyde (PFA) + 0.4% TritonX-100 (TX) was added and incubated overnight at 4° C to fix the fish. The fish were then washed 4x in 0.4% TX:PBS at 5min each wash. Then 1mL of 10µg/mL proteinase-K:0.4%TX:PBS was added and left at room temperature for 30min. Since a protease enzyme can degrade the proteins of interest, the proteinase-K was not left longer than 30min. The fish were then washed again 4x at 10min each wash on a Rocker in 0.4%TX:PBS. Following the final wash, 1mL sheep serum (5% SS:0.4%TX:PBS) was added and left for 2h at room temperature on a Rocker. Then 100-200µl α-nitrotyrosine antibody (diluted 1:250 in SS:0.4%TX:PBS) was added and incubated at 4° C on orbital shaker overnight. The antibody was then washed off 4x at 10min each wash on a Rocker in 0.4%TX:PBS. Then 500µl sheep serum (5% SS:0.4%TX:PBS) was added and left for 1h at room temperature on a Rocker. Then added 200µl Alexa Fluor 657 (red) Goat Anti-Rabbit secondary antibody (diluted 1:500 in 5% SS:0.4%TX:PBS) for 2h at room temperature in the dark (wrapped with aluminium foil) then washed off 4x at 20 min each wash on a Rocker in 0.4%TX:PBS, in the dark. The fish were then washed again in 4%PFA:0.4TX:PBS for 20 min at room temperature and then rinsed briefly in 0.4%TX:PBS and store in the same at 4° C in aluminium foil until (maximum of 1 week) ready to image.

2.10 Statistics

Statistical analysis was performed using GraphPad Software (La Jolla, CA, USA). To compare if the mean of two groups (that are normally distributed) are significantly different from each other, the unpaired t test was used. However, if the two groups are not normally distributed, Mann-Whitney test was used instead. To compared multiple comparison, one-way or two-way analysis of variance (ANOVA) with Bonferroni test was used to compare every mean to every other mean whereas Dunnett's test was used to compare every mean to the control mean. However, for non-parametric data, ANOVA with Dunn's test was employed. P-values were adjusted to correct for multiple comparisons. Fisher's

exact test was employed to compare categorical variables such as intracellular and extracellular fungi. In all analyses, statistical significance was set at $P < 0.05$.

CHAPTER 3

ZEBRAFISH CAN CLEAR, CONTROL OR FAIL TO CONTROL *C. NEOFORMANS* INFECTION AT A LOCALISED SITE

3.1 Introduction

3.1.1 Animal models of *C. neoformans*

Cryptococcus neoformans remains the leading fungal pathogen of the life-threatening illness in individuals with immunocompromised immunity. A comprehensive understanding of *C. neoformans* pathogenesis should help to facilitate the development of new therapeutic strategies. Therefore, establishing an efficient animal model system to study and investigate new therapies for *C. neoformans* is critical. Mice are the predominant animal models used in *C. neoformans* infection research. However, mice are difficult to use in large-scale studies because of the limited number of progeny produced, exorbitant costs and ethical issues. As discussed in Chapter 1, several invertebrate organisms have been used to study *C. neoformans* infection. They include *Galleria mellonella (wax moth), Acanthamoeba castellanii, Dictyostelium discoideum* (social amoeba) and *Caenorhabditis elegans* (nematode) (reviewed in Sabiiti et al., 2012). The advantages of using some of these hosts include a well-developed innate immunity, widely available molecular tools, affordable care systems and the possibility of enabling high-throughput infection and drug screening assays (Mylonakis et al., 2007). However, some of these hosts have some limitation regarding genetic tools, physiology and anatomical structure, highlighting the necessity for other models to study the complex host-pathogen interactions.

The use of zebrafish (*Danio rerio*) in biomedical research has been increasing, mainly due to its ability to produce a large numbers of offspring, transparency of the larva, genetic malleability and cheaper running costs (Novoa and Figueras, 2012). Since scientists began using zebrafish to study *C. neoformans* infection, to date, there have been three papers published (Tenor et al., 2015, Davis et al., 2016, Bojarczuk et al., 2016). See Chapter 1 for a summary of findings from these studies.

3.1.2 Routes of infection

When developing an animal model to study fungal infection, a route of infection must be considered to achieve the desired outcome. In mouse models, routes of

infection that have been used include pulmonary, intraperitoneal, intravenous, intracerebral and footpad inoculations. Each of these routes is used to study specific objectives. For example, to examine BBB crossing of *C. neoformans* in mice, fungal cells are inoculated intravenously whereas to study the progression of cryptococcal meningitis, an intracerebral inoculation model is used (Liu et al., 2012).

Because *C. neoformans* infections are acquired through inhalation of infectious propagules, most studies use an intranasal inhalation model because it simulates the natural entry into the human host. The establishment and consequent outcomes of cryptococcal infection in animal models do not always mimic all forms of human cryptococcosis. For example, pulmonary inoculation with *C. neoformans* in immunocompetent mice results in dissemination of the fungus to the brain (Zaragoza et al., 2007). However, in some patients, particularly in the non-HIV infected, cryptococcal infection may remain localised in the lungs or other body organs (Pappas, 2013). In consideration of these differences lies the objective of animal modelling. The objective is to simulate an infectious process in humans by varying the animal models or routes of infection. Thus, localised inoculations have been established throughout animal modelling research to mimic the human pathology of the targeted organs. This usually involves inoculating the pathogen into the organs of interest. The advantage of localised infection is that it allows the study of cellular responses such as chemotaxis at the infection site.

In zebrafish, various routes of infection have been established, and each route or site of microinjection determines whether infection will develop systemically or will remain localised. For example, intravenous injection into the caudal vein at the posterior blood island or Duct of Cuvier, (which is a large circulation vessel adjacent to the yolk sac linking the heart to the trunk vasculature), will establish a rapid systemic infection. Microinjection into the hindbrain ventricle, tail muscle, otic vesicle or somite (intramuscular) will create a localised infection (Zakrzewska et al., 2010, Le Guyader et al., 2008, Herbomel et al., 1999). However, inoculation into the notochord will generate an infection that is inaccessible to phagocytes (Alibaud et al., 2011, Benard et al., 2012) (Figure 8). The caudal vein at the

posterior blood island is mainly performed in 1dpf embryos but in the later stages of embryonic development, penetration of this site becomes more difficult. Therefore, the Duct of Cuvier is the preferred site of injection in 2-3 days post fertilisation (dpf) zebrafish larvae (Benard et al., 2012).

Previous zebrafish cryptococcal infection models have used systemic infection via the Duct of Cuvier microinjection to model mammalian systemic infection and subsequent brain invasion (Tenor et al., 2015). This method is equivalent to the tail vein inoculation in mice. However, my aim was to develop a localised *C. neoformans* infection model with an objective of stimulating local host immune cells to study their phenotypic behaviour toward *C. neoformans in vivo*. I selected an intramuscular route of infection to be able to characterise migration of phagocytes and their interaction with *C. neoformans*. Zebrafish muscles display high stimulation of pro-inflammatory cytokines, including IL-1β and TNFα, which is thought to be due to muscle-resident macrophages (Chatterjee et al., 2016). suggesting that the zebrafish muscle can be an excellent model to study the cellular immune responses.

First, I investigated outcomes of *C. neoformans* infection following intramuscular injection in zebrafish larvae.

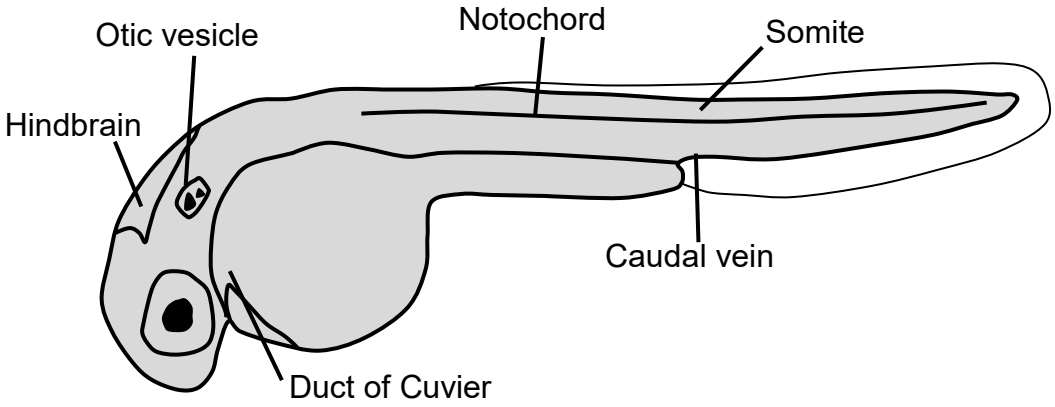

Figure 8: Microinjection sites

Schematic diagram of 2dpf zebrafish larva showing various routes of infection. Each of these routes are used for a specific purpose.

3.2 Results

3.2.1 Infection model of *C. neoformans* in zebrafish

Deciding the right age of larvae to perform intramuscular infection was one of the challenging aspects of developing this model. I first started infecting 2dpf larvae so I could follow them up for up to 72-hours post infection before the 5.2dpf. However, because of their size and fragility, most fish died or developed an uncontrolled infection. I then switched to 3dpf larvae, found that they were easier to handle and were not significantly harmed by microinjection.

Therefore, to determine the outcomes of *C. neoformans* infection in the zebrafish larvae, three dpf zebrafish larvae were intramuscularly infected (Figure 9a) with a fungal inoculum of 1-25 yeast cells and outcomes of infection were determined at 48-hours post infection (hpi). The following infection outcomes were observed: Cleared infection – defined as complete clearance of fungal infection at the site of infection; controlled infection – defined as of up to 10-fold of the initial fungal inoculum; and uncontrolled infection – defined as fungal burden over 10-fold to that of the initial fungal inoculum.

At 48hpi, *C. neoformans* cells remained at the site of infection. The outcomes were as follows: of the 145 zebrafish larvae infected, 28 (19%) cleared the infection, 90 (62%) controlled the infection, 23 (16%) failed to control the infection and 4 (3%) died (Figure 9b&c). Although mortality was observed, it was not significantly different from uninfected fish (P =0.317). There was a direct correlation between the number of injected cryptococci and the percentage of cleared infection (R^2 = 0.611; P = 0.0075) and uncontrolled infection (R^2 = 0.698; P = 0.0026) but not with controlled infection (R^2 = 0.255; P = 0.14; Figure 9e).

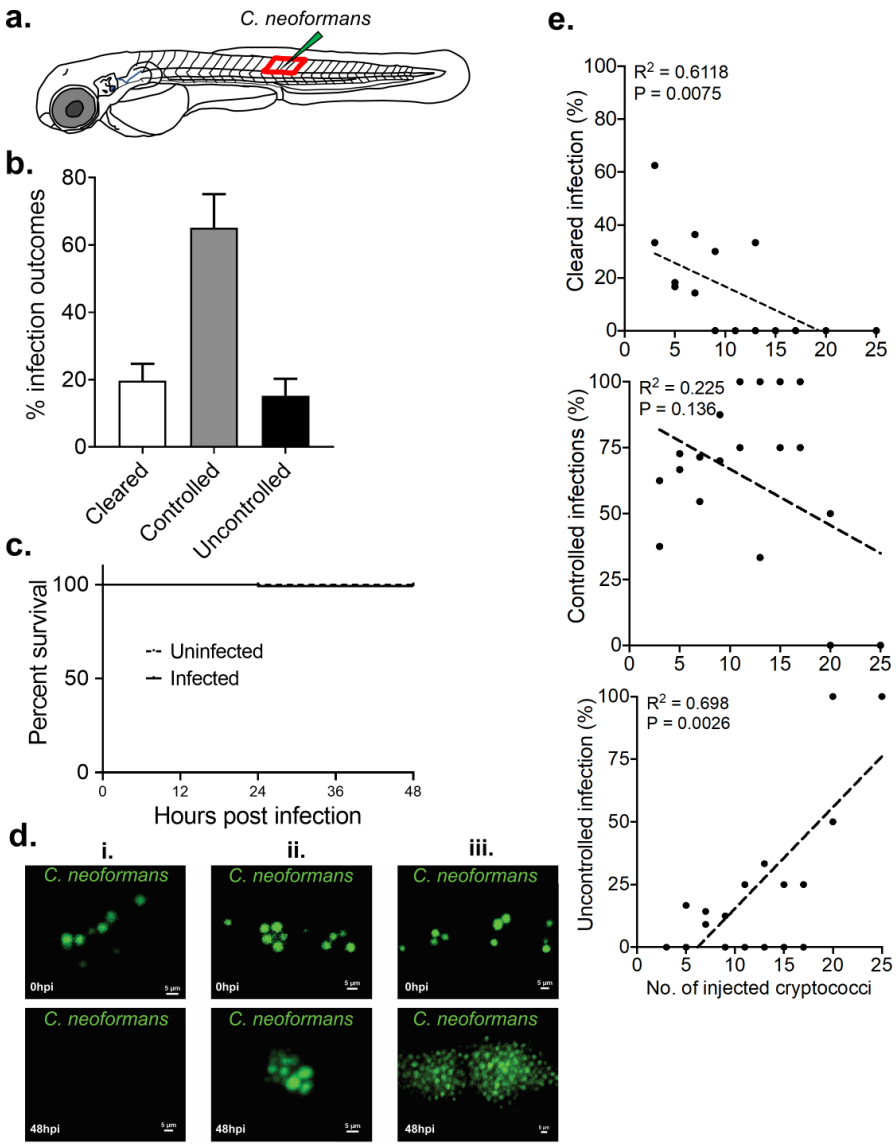

Figure 9: Infection model of *C. neoformans* in zebrafish

(a) Schematic diagram of larva showing the site (Red box, myotome 15-17) of microinjection with *C. neoformans*. (b-d) Three dpf nacre larvae were injected intramuscularly with a range of 1 to 2.5x10 *C. neoformans* and infection outcomes determined at 48-hours post infection (hpi). (b) Percentage infection outcomes. Data is representative of 3 independent experiments (n=145) (c) Percent survival analysed using Log-rank (Manted-Cox) test, hazard ratio = 2.5 (logrank; 95% confidence interval 0.4, 2.3; infected vs uninfected larvae, P = 317. (d) Representative time-point fluorescent micrographs of *C. neoformans* initial inoculum at 0hpi and fungal burden or infection outcome at 48hpi of (i) cleared infection (ii) controlled infection and (iii) uncontrolled infection (e) Correlation, number of injected cryptococci vs cleared infection (%) **P = 0.0075; vs controlled infection (%) P=0.14; vs uncontrolled infection (%) **P = 0.0026. Data was analysed using correlation and slop was constructed using linear regression.

3.3 Discussion

The intramuscular zebrafish model described here is the first to be used in the study of cryptococcal infection. Previous studies of the zebrafish-*C. neoformans* infection model employed intravenous microinjection method (Tenor et al., 2015, Davis et al., 2016, Bojarczuk et al., 2016). To date, zebrafish have been used to study other fungal infections including *Candida albicans*, *Mucor circinelloides* and *Aspergillus fumigatus* and were microinjected into the hindbrain ventricles (Chao et al., 2010, Voelz et al., 2015, Knox et al., 2014).

My findings show that intramuscular inoculation of *C. neoformans* in zebrafish larvae results in infection being completely cleared, controlled or uncontrolled. Cleared and uncontrolled, but not controlled infection outcomes were dependent on the number of injected cryptococci. There was a higher proportion of zebrafish larvae that controlled the infection than those that cleared or failed to control the infection. The fact that controlled infection outcomes did not correlate with the number of injected cryptococci requires further investigation. One explanation for this is that the zebrafish used are not clonal, hence variable in their genetic and/or phenotypic makeup and response to infection. Clonal lines are important for attaining stable and reproducible data in biological studies. Techniques to generate zebrafish clonal lines are emerging. Recently, Hou et al. (2015) demonstrated the generation of clonal zebrafish line using cold-shock androgenesis without egg irradiation (Hou et al., 2015).

The susceptibility of the host to *C. neoformans* infection depends on the route of infection. For example, both mice and zebrafish larvae easily succumb to cryptococcal infection when inoculated intravenously (Tenor et al., 2015, Bojarczuk et al., 2016, Zaragoza et al., 2007). In contrast, mortality in my intramuscular zebrafish model is significantly low despite the high fungal burden. Interestingly, the mouse model is invariably fatal, even following localised infection. CBA/J mice have only a 20 % survival rate after intratracheal inoculation even though the mice are considered immunocompetent (Zaragoza et al., 2007). Susceptibility to *C. neoformans* infection might involve both host and pathogen factors. Mice that are more susceptible to *C. neoformans* infection display a

Th2-type response (Carroll et al., 2012). On the other hand, *C. neoformans* does not induce robust pro-inflammatory responses both *in vitro* and *in vivo* experiments. *C. neoformans* fails to activate monocytes and macrophages to produce pro-inflammatory cytokines such as TNFα and IL-1β (Levitz et al., 1994, Naslund et al., 1995, Vecchiarelli et al., 1995). It also induces low levels of IFNγ in T cells (Vecchiarelli et al., 1994) and limited leukocyte infiltration in organs harbouring *C. neoformans* (Murphy, 1989, Clemons et al., 2000). This has been attributable to the presence of circulating cryptococcal polysaccharide capsule that stimulates shedding of L-selectin from neutrophil surfaces, thus preventing first step of extravasation (Clemons et al., 2000). Thus, *C. neoformans* is able to hide and protect itself from being detected and eliminated by host immunity. This reflects the complex interplay between host and pathogen attributes that drive infection outcomes.

Zebrafish have a physiological temperature of 28° C that is lower than murine and human hosts' body temperatures. The ability of *C. neoformans* to grow at 37° C is considered a virulence factor because 38 other cryptococcal species fail to grow at temperatures above 30° C. *C. neoformans* can grow and be pathogenic in a wide range of temperatures (Casadevall, 1998; Fuchs and Mylonakis, 2006). Apart from causing infection inside the human host, *C. neoformans* can also cause infection at low-temperature environments, such as the skin, suggesting that temperature is not a hindrance to its pathogenicity. Besides, genes that are tolerant to higher temperatures are studied at mammalian physiological temperature. However, not all temperature-tolerant genes can be studied at mammalian physiological temperature. For instance, an *eca1 C. neoformans* mutant displayed growth defects at 37° C. As such it was difficult to associate the mutant with virulence in mammalian models, *Galleria* or amoeba being incubated at 37° C. The mutant showed virulence using *Galleria* at 30° C and *C. elegans* at 25° C (Fan et al., 2007). Moreover, it was shown that *C. neoformans* genes that mediate the trehalose pathways are required for growth at 37° C. The trehalose mutant is avirulent in rabbits and mice. Interestingly, the mutant is also attenuated in virulence in *C. elegans* where the

in vivo high-temperature environment is not present, suggesting that the trehalose pathways are involved in other host survival activities apart from simply mediating protection from high-temperature pressures (Petzold et al., 2006). Recently, *C. neoformans* has been shown to be virulent in zebrafish larvae that are maintained at 28° C (Tenor et al., 2015, Bojarczuk et al., 2016). Thus, studying *C. neoformans* at different temperatures can give valuable findings that would have otherwise be undiscovered.

In summary, this is novel promising model capable of enabling a detailed dissection of the pathogenesis of *C. neoformans* and its interactions with the host immunity. I have established a zebrafish-*C. neoformans* intramuscular infection model and show outcomes of infection. Transparency of the zebrafish larva enables significant benefits in research because it allows *in vivo* imaging assays that are difficult to perform in other vertebrate models. Interactions of *C. neoformans* with fluorescently labelled immune cells can also be examined. Thus, in the next two chapters, I will show the effects of modulation of innate immunity by Th1-type cytokine IFNγ and TLR agonists on cryptococcal burden and cellular responses.

CHAPTER 4

SINGLE DOSE INTERFERON-GAMMA IS SUFFICIENT TO INCREASES MACROPHAGE RECRUITMENT, PHAGOCYTOSIS AND KILLING FOR THE CLEARANCE OF *CRYPTOCOCCUS NEOFORMANS* INFECTION

4.1 Introduction

The Th1-type $CD4^+$ T cells mediated cellular immunity is the main host defence mechanism against cryptococcal disease as evidenced by high incidences of cryptococcosis in individuals with compromised cell-mediated immunity (CMI) (Chuck & Sande, 1989; Collins et al, 1951; Dismukes, 1988; Eng et al, 1986; Kovacs et al, 1985). Protective anti-cryptococcal host immunity by $CD4^+$ T cells involves the generation of Th1-type cytokines such as IL-12, IFNγ and the pro-inflammatory cytokine, TNFα (Bauman et al, 2003; Decken et al, 1998; Herring et al, 2002; Kawakami et al, 1996a; Kawakami et al, 1996b; Kawakami et al, 1996c; Zhou et al, 2007). Neutralisation of IL-12, IFNγ or TNFα results in increased susceptibility to cryptococcal infection in murine models (Decken et al, 1998; Herring et al, 2002; Huffnagle et al, 1996; Kawakami et al, 1996a). Administration of recombinant Th1-type cytokines have been shown to enhance anti-cryptococcal chemotherapy as shown in experimental and clinical studies (Clemons et al, 1994; Clemons et al, 2001; Jarvis et al, 2012; Kawakami et al, 1997b; Kawakami et al, 1996c). In contrast, generation of Th2-type cytokines including IL-4 and IL-13, results in diminished control of infection (Arora et al, 2011; Blackstock et al, 1999; Hardison et al, 2012b; Müller et al, 2012; Müller et al, 2007; Müller et al, 2013; Qiu et al, 2012).

Experimental studies with the Th1-type cytokine IFNγ have generated promising findings that it may be a potential adjunct immunotherapy to anti-fungal chemotherapy (Joly et al, 1994; Lutz et al, 2000). For example, in experimental pulmonary and peritoneal cryptococcosis, the administration of IFNγ prolongs mice survival and significantly reduces fungal burden (Joly et al., 1994, Kawakami et al., 1996b). Although treatment with IFNγ alone has little efficacy in an experimental systemic cryptococcosis, when administered in combination with amphotericin B, IFNγ displays an additive effect over amphotericin B alone in clearing brain infections (Clemons et al, 2001; Lutz et al, 2000). Neutralisation of IFNγ by anti-IFNγ antibody in an experimental murine cryptococcal infection results in increased fungal burden and shortened survival time (Aguirre et al., 1995, Hoag et al., 1997, Kawakami et al., 1996b). Besides, IFNγ mediates

induction of anti-cryptococcal activity by other pro-inflammatory cytokines. For example, the protective effects induced by anti-CD40/IL-2, or IL-12 are abrogated in IFNγ and IFNγR knockout mice (Kawakami et al, 1996c; Zhou et al, 2006). In patients with cryptococcal meningitis, levels of IFNγ in the cerebrospinal fluid (CSF) correlated to the rates of infection clearance following anti-fungal treatment (Siddiqui et al., 2005, Bicanic et al., 2009). In a randomised controlled trial, the adjunct of exogenous IFNγ to standard anti-fungal therapy in patients with HIV-associated cryptococcal meningitis increased the rates of cryptococcal clearance from the CSF (Jarvis et al, 2012). Another clinical trial demonstrated a trend towards more rapid sterilisation of the CSF in IFNγ-treated patients with cryptococcal meningitis (Pappas et al, 2004). Taken together these data suggest that IFNγ is a potential adjunctive therapy for cryptococcosis in the immunocompromised host.

4.1.1 Mechanisms of IFNγ protection

Interferon-γ belongs to a group of cytokines known as interferons. There are three types of interferons, Type I, Type II and Type III. IFNγ is the only type II interferon and differs from type I and III interferons by structure, receptor and chromosome locus that encode them (Platanias, 2005). IFNγ was first thought to be secreted by $CD4^+$ Th1, $CD8^+$ cytotoxic T cells and NK cells (Bach et al, 1997; Young, 1996). However, there is evidence that prove that other cells including B cells, NKT cell and APC also produce IFNγ (Carnaud et al, 1999; Flaishon et al, 2000; Frucht et al, 2001; Gessani & Belardelli, 1998). IFNγ was at first described as an antiviral agent. However, IFNγ has since been shown to promote cellular immune responses including activation of macrophages, NK cells, enhancement of MHC expression and leukocyte migration. IFNγ is an effector cytokine of Th1-type immune responses (Billiau & Matthys, 2009).

The mechanisms by which IFNγ enhances resistance to cryptococcal infection have not been completely described. During infection, inhaled *C. neoformans* basidiospores are taken up by the innate immune cells such as alveolar macrophages. Studies in an experimental pulmonary cryptococcosis suggest that IFNγ produced by the Th1-type $CD4^+$ T cells directly activates effector cells such

as alveolar macrophages. This leads to phagocytosis of the cryptococci (Kawakami et al, 1995), intracellular killing and skewing to the Th1-type immune responses (Hardison et al, 2010). The effect of IFNγ in *in vitro* experiments have produced contrasting findings between human and mouse macrophages. The mouse bone marrow-derived macrophages treated with IFNγ display increased fungicidal activity and increased uptake of *C. neoformans* (Flesch et al, 1989; Ikeda-Dantsuji et al, 2015). On the other hand, the addition of IFNγ to the human alveolar macrophages or the human monocyte-derived macrophages results in either reduced (Levitz & Farrell, 1990; Reardon et al, 1996) or no change in fungistatic capacity (Voelz et al, 2009). Infection with IFNγ-producing *C. neoformans* strain, H99γ, results in increased leukocyte recruitment to the lungs (Wormley et al, 2007), increased classically activated macrophages (Hardison et al, 2010) and increased macrophage fungistatic activity when cultured *in vitro* (Hardison et al, 2012b). In contrast, IFNγ knockout mice display the alternatively activated macrophage phenotypes with increased levels of intracellular cryptococci (Arora et al, 2005). Similarly, administration of anti-IFNγ results in reduced accumulation of the inflammatory cells and iNOS expressing macrophages in the lungs (Aguirre et al., 1995, Hoag et al., 1997, Kawakami et al., 1996b).

Despite these observations, little work has been done *in vivo* on the effects of IFNγ on the interactions between phagocytes and *C. neoformans*. This chapter reports a systemic investigation of the influence of IFNγ on cellular responses to cryptococcal infection. My findings will help to add knowledge to the mechanism of protection associated with IFNγ.

4.2 Results

4.2.1 A single dose of interferon-γ markedly reduces fungal burden and improves fungal clearance in zebrafish challenged with *C. neoformans*

I first measured the capacity IFNγ to protect zebrafish from cryptococcal infection. The objective was to describe the infection outcomes with a single dose of IFNγ. This was also done to mimic a short course of IFNγ that is as effective as a two-week course in patients with cryptococcal meningitis (Jarvis 20102). In addition, a single dose was chosen because multiple doses would introduce multiple wounds that could interfere with immune responses to the cryptococcal infection.

The concentration of IFNγ (5µg/ml) used has previously been demonstrated to reduce fungal burden in an experimental murine model of cryptococcosis (Lutz et al, 2000). Three dpf *nacre* zebrafish larvae were intramuscularly challenged with *C. neoformans* in phenol red (vehicle control) alone or supplemented with 5µg/mL IFNγ. I found that at 48hpi, IFNγ-treated larvae had significantly reduced fungal burden compared to those larvae treated with phenol red (Figure 10a, $P < 0.0001$). Further analysis showed that the percentage of larvae that completely cleared the infection was significantly higher in IFNγ-treated larvae compared to larvae in control group (Figure 10b, $P = 0.003$). These results corroborate previous studies in mice and humans that demonstrated that administration of IFNγ in combination with anti-fungal chemotherapy can stimulate protective immunity against *C. neoformans* leading to a reduction in fungal load (Jarvis et al, 2012; Joly et al, 1994; Kawakami et al, 1996b). No significant difference in mortality was observed between the groups (Figure 10c, $P = 0.65$), and was extremely low in both groups (3 and 3.6 %, phenol red and IFNγ respectively). To detect any IFNγ-evoked toxic effects on *C. neoformans*, growth was monitored over 24 hours *in vitro*. *C. neoformans* exhibited no growth defects following treatment with IFNγ compared to controls (Figure 10d, $P = 0.3168$).

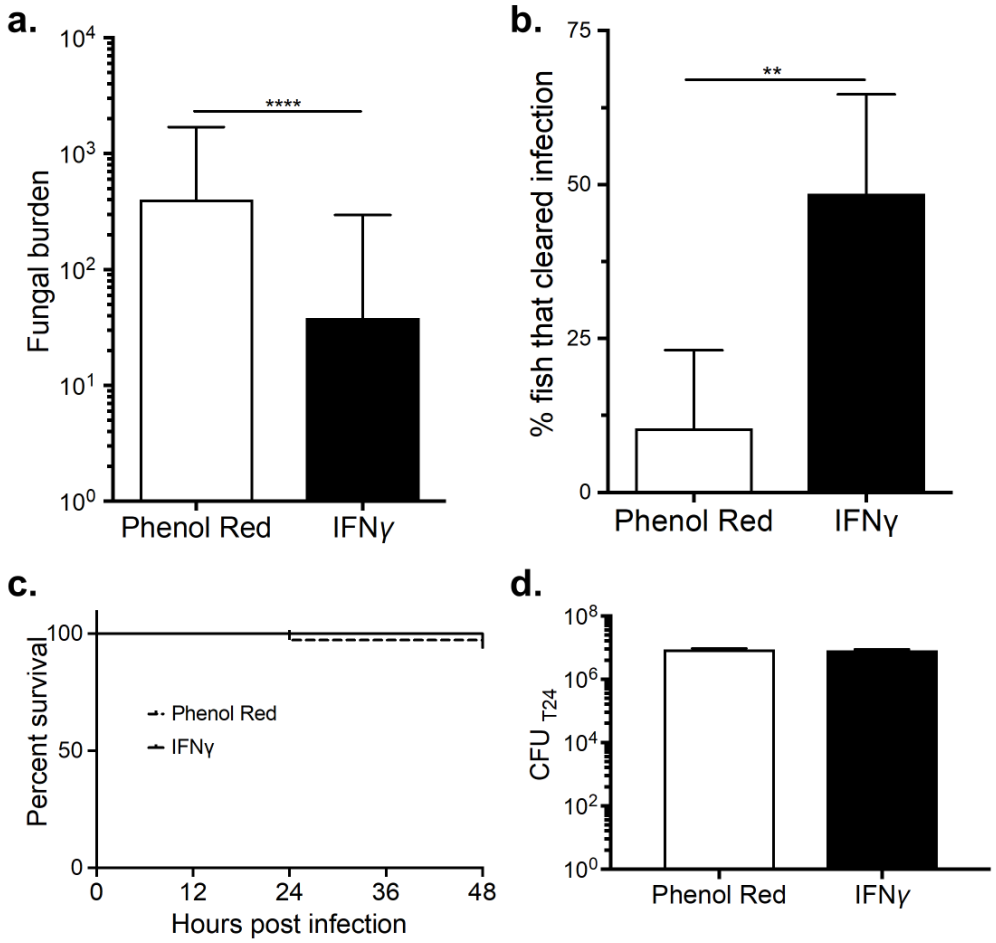

Figure 10: Interferon-γ markedly reduces fungal burden and improves fungal clearance in zebrafish challenged with *C. neoformans*.

(a) Fungal burden at 48hpi of nacre larvae challenged with *C. neoformans* in phenol red alone or supplemented with 5ug/mL IFNγ: data are a combination of three independent experiments (n=140-156 per group) and expressed as a mean ±standard deviation analysed using Mann Whitney test; Phenol red vs IFNγ, ****P < 0.0001. (b) Percentage of larvae that cleared infection per group: data presented as %, however, statistical analysis was performed on raw data (total number of larvae that cleared infection vs total number of larvae that did not clear infection) using Fisher's exact test; results are expressed as a percent mean ± standard deviation, Phenol red vs IFNγ, **P = 0.003. (c) Survival of 3dpf nacre larvae (n=64 per group) challenged with *C. neoformans* with Phenol red alone or supplemented with 5ug/mL IFNγ and analysed using Log-rank (Manted-Cox) test, hazard ratio = 2.5 (logrank; 95% confidence interval 0.4, 2.3; Phenol red or IFNγ, P = 0.25. (d) IFNγ treatment does not influence *C. neoformans* survival: data presented as a mean ± standard deviation representative of three independent experiments analysed using unpaired t-test, Phenol red or IFNγ, P = 0.31

4.2.2 Interferon-γ promotes phagocytes infiltration to infection site during *C. neoformans* infection in zebrafish

I then investigated the possible mechanisms that initiated the improved fungal clearance in IFNγ-treated larvae. I first determined the effect of IFNγ to recruit phagocytes to the site of infection. The transgenic zebrafish, with marked macrophages, Tg(*mpeg1*:Gal4-VP16/UAS:Kaede), were used to determine influx of macrophages. Larvae that were challenged with IFNγ+*C. neoformans* induced an increase in the influx of macrophages at the site of infection at 2hrs ($P < 0.0001$) and 24hrs ($P = 0.002$) post inoculation than phenol red+*C. neoformans* challenged larvae (Figure 11a). However, *C. neoformans* inhibited the recruitment of macrophages. The influx of macrophages at 24hpi was more pronounced in larvae treated with Phenol red or IFNγ alone compared with larvae co-injected with phenol red+*C. neoformans* ($P < 0.0001$) or IFNγ+*C. neoformans* ($P < 0.0001$) respectively. These differences were not observed early during infection at 2hpi ($P > 0.999$). The transgenic zebrafish with marked neutrophils *Tg(mpx:*GFP)i114 larvae were used to examine infiltration of neutrophils. I found that the larvae that were inoculated with IFNγ+*C. neoformans* had increased infiltration of neutrophils at 12 and 24hpi than the larvae treated with phenol red+*C. neoformans* ($P = 0.038$ and $P=0.027$ respectively). This difference was not observed at 2hpi ($P=0.18$). Infiltration of neutrophils was significantly increased in larvae treated with phenol red alone compared to phenol red+*C. neoformans* challenged larvae at 2 ($P = 0.007$), but not at 12 ($P = 0.418$) and 24 ($P > 0.999$) hpi (Figure 11b). However, *C. neoformans* inhibition of neutrophil infiltration was rescued by IFNγ treatment. The number of recruited neutrophils between larvae treated with IFNγ alone and larvae challenged with IFNγ+*C. neoformans* was not significantly different at 2hpi ($P = 0.313$), 12 ($P = 0.171$) and 24 ($P = 0.999$) hpi (Figure 11b).

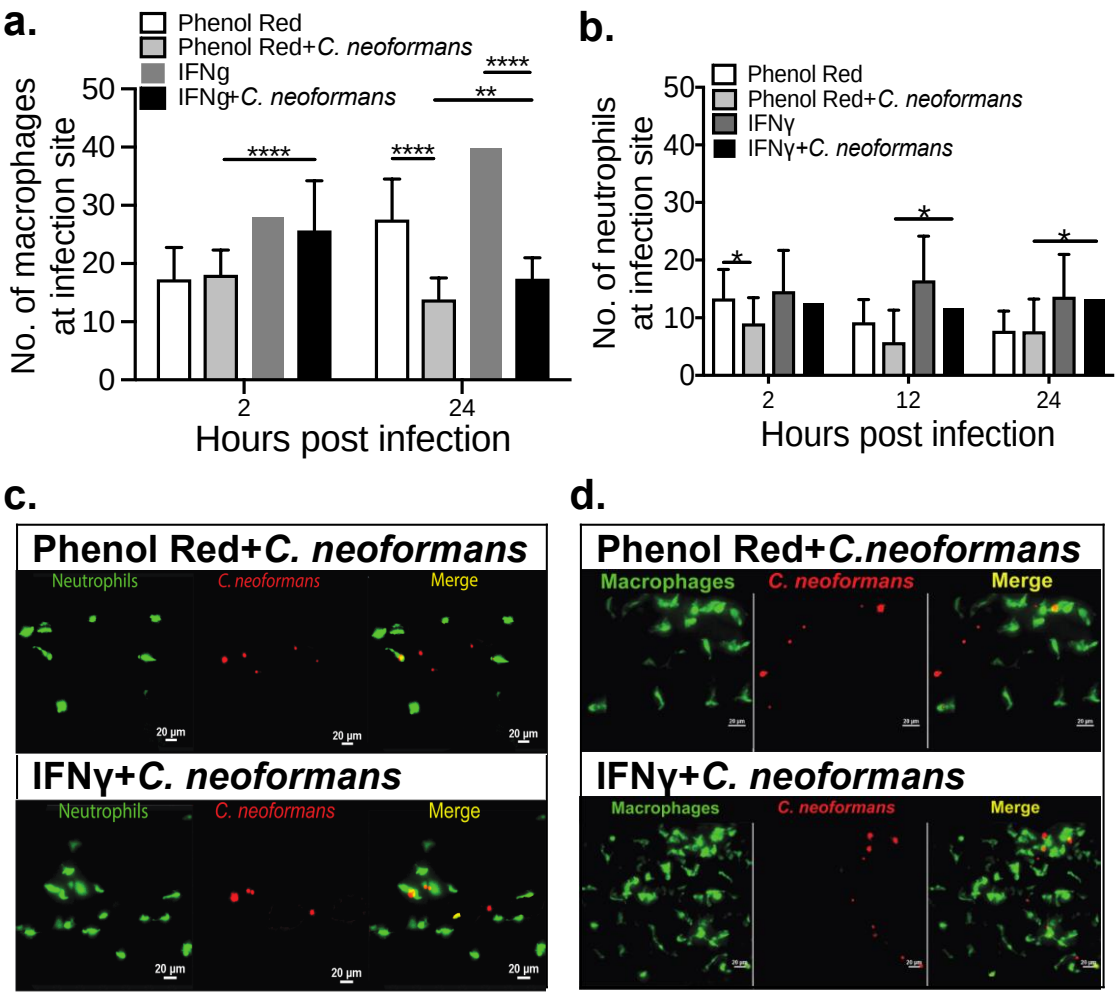

Figure 11: Administration of IFNγ increases influx of phagocytes at the site of infection.

Kinetics of influx of phagocytes quantitated by manual counting at different time-points. Tg(*mpeg1*:Gal4-VP16);Tg(UAS:Kaede) for macrophages and *Tg(mpx:*GFP)i114 for neutrophils larvae were challenged with *C. neoformans* in phenol red alone or supplemented with 5ug/mL IFNγ. Mean number of (a) macrophages, Phenol red+*C. neoformans* vs IFNγ+*C. neoformans*, 2hpi ****P < 0.0001 and 24hpi **P = 0.0022 (b) neutrophils, Phenol red+*C. neoformans* vs IFNγ+*C. neoformans*, 2hpi P=0.18; 12hpi, *P = 0.038 and 24hpi, *P=0.027. In both graphs (a&b), data were obtained from three independent experiments (n=22-24 per group) presented as a mean ±standard deviation and analysed using Ordinary one-way ANOVA with Dunnett's multiple comparison test. Representative fluorescent images of (c) macrophages at 2hpi (d) neutrophils at 12hpi at infection site of larvae inoculated with phenol red+*C. neoformans* alone and larvae inoculated with IFNγ+*C. neoformans*, Scale bar is 20μm.

4.2.3 Interferon-γ is associated with increased fungal uptake by both macrophages and neutrophils during *C. neoformans* infection in zebrafish

Second, to investigate whether IFNγ influences the rate of fungal uptake by phagocytes, phenol red alone or supplemented with IFNγ were co-injected with *C. neoformans* into 3dpf *Tg*(*mpeg1*:Gal4-VP16/UAS:Kaede) and *Tg(mpx:*GFP)i114 zebrafish larvae for macrophage and neutrophils respectively. I found that following two hours of infection in *Tg*(*mpeg1*:Gal4-VP16/UAS:Kaede) larvae, the proportion of internalised *C. neoformans* cells by macrophages was significantly higher in IFNγ-treated larvae compared to phenol red-treated larvae ($P < 0.0001$, Figure 12a). At 12hrs post infection, fungal uptake markedly increased in both groups with IFNγ-treated larvae still having significantly higher uptake than phenol red-treated larvae ($P = 0.0004$). On the other hand, fungal uptake in IFNγ-treated larvae by neutrophils showed a small but significant change compared to phenol red treated larvae at 12 hpi ($P = 0.001$). No significance difference was observed at 2 hpi ($P = 0.505$, Figure 12b). These findings show that by 12 hpi, almost all injected cryptococci are intracellular. To confirm this, *Tg(mpeg1:Gal4-UNM)sh256* macrophages line were crossed with *Tg(mpx:eGFP)i114* neutrophils line to obtain double positive *Tg(mpeg1:Gal4-UNM)sh256;Tg(mpx:eGFP)i114* larvae. Uptake rates of the cryptococci by 12 hpi in 3dpf *Tg(mpeg1:Gal4-UNM);Tg(mpx:eGFP)i114* line were up to 100 % in IFNγ-treated larvae and up to 85 % in phenol red treated larvae ($P < 0.0001$, Figure 12e).

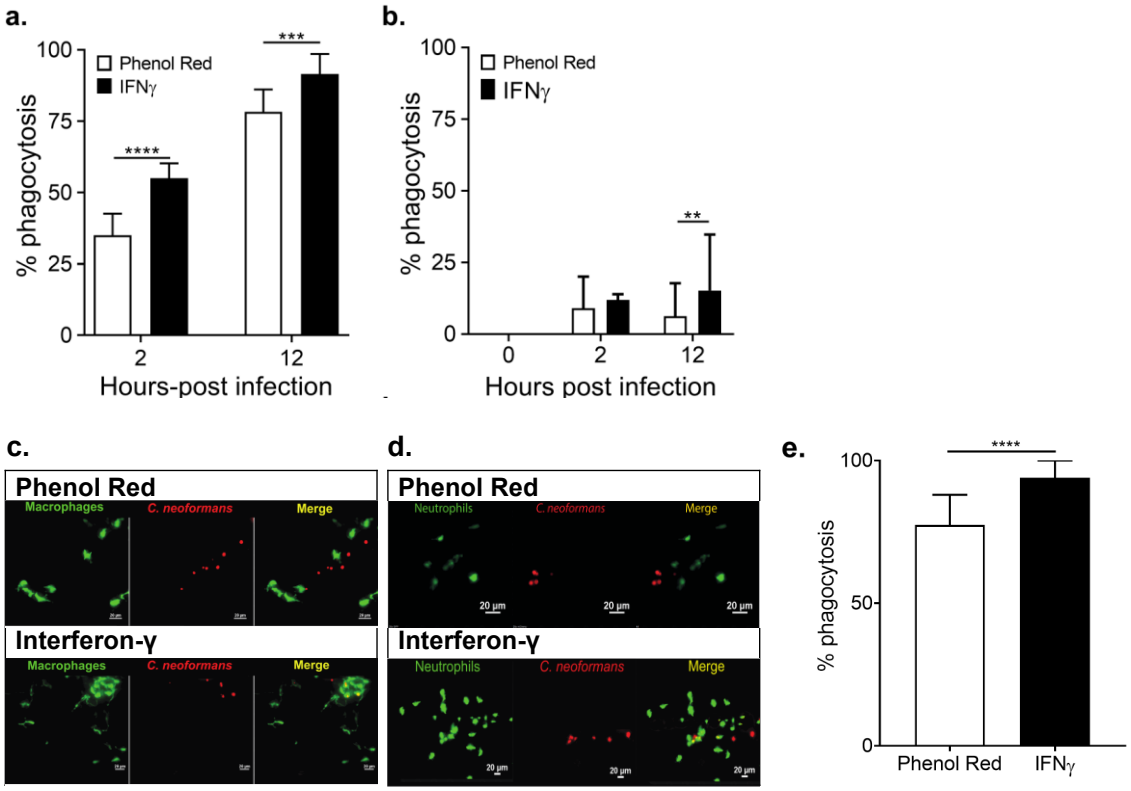

Figure 12: Administration of IFNγ increases phagocytosis of fungal cells by phagocytes.

Phagocytosis of *C. neoformans* in 3dpf larvae following challenge with *C. neoformans* in phenol red alone or supplemented with 5ug/mL IFNγ. Percentage phagocytosis of fungal cells per larva at 2 hpi and 12 hpi by (a) macrophages, Phenol red vs IFNγ, 2hpi ****P < 0.0001 and 24hpi ***P = 0.0004; and (b) neutrophils, Phenol red vs IFNγ, 2hpi P = 0.505, and 12hpi, **P = 0.001. In both graphs (a & b), data representative from three independent experiments (n=13-17 per group) and presented as %, however, statistical analysis was performed on raw data (total number of intracellular cryptococci vs total number of extracellular cryptococci) using Fisher's exact test comparing phenol red- to IFNγ- treated larvae. (c & d) Representative fluorescent images of phagocytosis by (c) macrophages (d) neutrophils at infection site, phenol red vs IFNγ at 2hpi, Scale bar is 20μm. (e) Percentage phagocytosis at 12 hpi in phagocytes (macrophages+neutrophils), Phenol red vs IFNγ, ****P = 0.0001

4.2.4 Relative phagocytic capacity is not significantly different between groups in both macrophages and neutrophils

As shown in the previous section (4.2.3), IFNγ increases phagocytosis of fungal cells by both macrophages and neutrophils. I then investigated whether phagocytes that had taken up fungal cells were primed to take up more cells. Phenol red or IFNγ were co-injected with *C. neoformans* into 3dpf *Tg*(*mpeg1*:Gal4-VP16/UAS:Kaede) and (*Tg(mpx:*GFP)i114 zebrafish larvae as described above. Phagocytic index in both macrophages and neutrophils was determined. The number of fungal count per macrophage (Figure 13a) or neutrophil (Figure 13b) at 2hpi (phagocytic index) were not significantly different between IFNγ and phenol red treated larvae (Macrophages P = 0.112; Neutrophils P = 0.148).

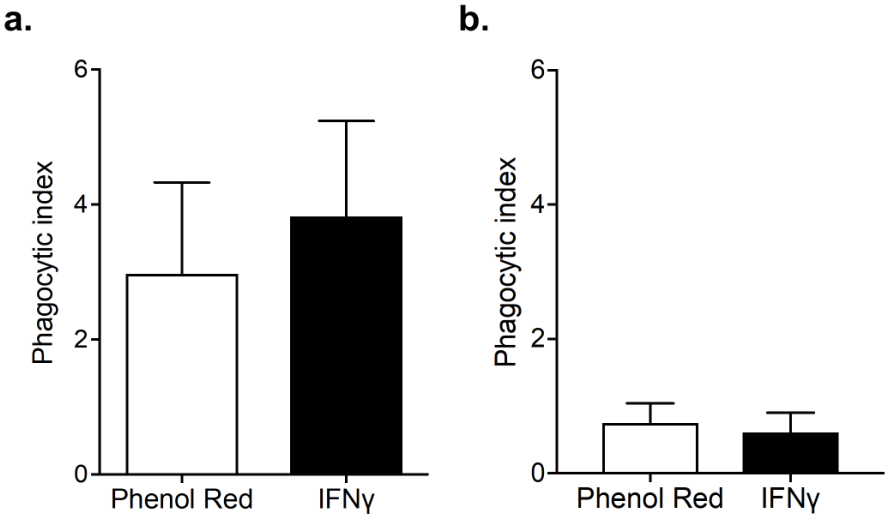

Figure 13: Relative phagocytic capacity is not significantly different between groups in both macrophages and neutrophils.

Average fungal count (phagocytic index) per (a) macrophage, Phenol Red vs INFg, P = 0.112 (b) neutrophil, Phenol Red vs IFNγ, P = 0.148. Data obtained from two independent experiments (n=13-16 per group) and Phagocytic index was analysed using two-tailed, unpaired t-test.

4.2.5 Macrophages are essential for Interferon-γ mediated protection against *C. neoformans*

Previous work in our laboratory demonstrated that deletion of macrophages in an experimental systemic infection with *C. neoformans* in zebrafish, results in increased fungal burden and mortality (Bojarczuk et al, 2016). These have also demonstrated in mammalian models (Monga, 1981; Osterholzer et al, 2009a). As shown above, upon intramuscular injection in zebrafish, *C. neoformans* are phagocytosed mainly by macrophages. To better appreciate the explicit role of macrophages in the presence of IFNγ during cryptococcal infection, *Tg(mpeg1:Gal4-UNM)*sh256 zebrafish were chemically ablated of macrophages. The larvae were then given an intramuscular inoculation with *C. neoformans* and IFNγ as described above. The fungal burden was quantified at 48 hours post inoculation. As expected, the fungal burden was significantly increased in larvae which had been ablated of macrophages (IFNγ Mφ^{-}) compared to control larvae ($P < 0.0001$, Figure 14a). Metronidazole did not inhibit *C. neoformans* growth *in vivo* as demonstrated by no change in fungal burden upon depletion of neutrophils compared to control (Figure 14b – data from Josie Gibson). Larvae depleted of macrophages looked healthy. No significant difference in mortality between groups was observed ($P = 0.368$, Figure 14c). To detect if metronidazole effectively ablated macrophages, a whole-body count of macrophages was performed in *Tg(mpeg1:Gal4-UNM)*sh256 zebrafish larvae at 5dpf. There was significantly reduced number of macrophages in metronidazole-treated larvae compared to control ($P < 0.0001$, Figure 14d&e).

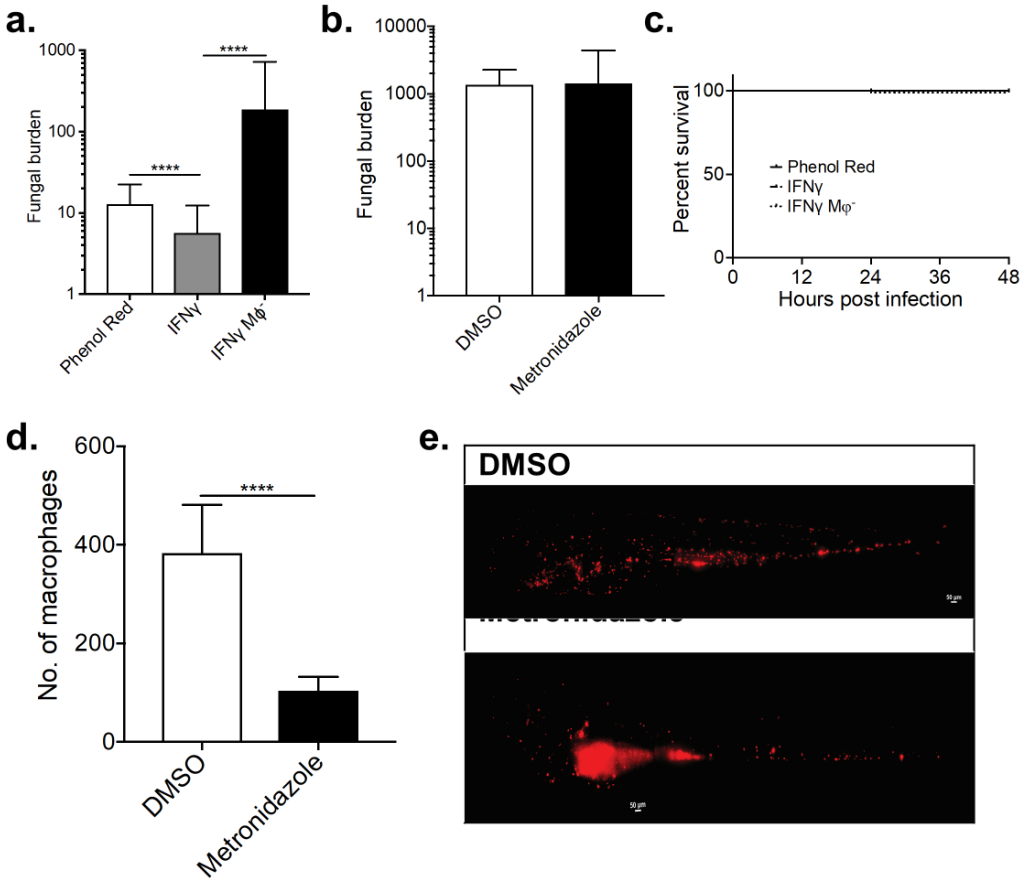

Figure 14: Role of macrophages during *C. neoformans* infection in zebrafish

(a) Fungal burden at 48hpi of *Tg(mpeg1:Gal4-UNM)sh256* larvae either with or depleted of macrophages challenged with *C. neoformans* in phenol red alone or supplemented with 5µg/mL IFNγ: data presented as a mean ±standard deviation and analysed using one-way ANOVA with Dunn's multiple comparison test. IFNγ vs Phenol red ****P < 0.0001; IFNγ vs IFNγ Mφ¯, ****P < 0.0001). n=60-67 per group. (b) Fungal burden at 72hpi of larvae either with (DMSO) or depleted (Metronidazole) of neutrophils challenged with *C. neoformans*. Date was analysed with Mann-Whitney test, DMSO vs Metronidazole, P = 0.13 (c) Survival of *Tg(mpeg1:Gal4-UNM)sh256* larvae with or depleted of macrophages infected with *C. neoformans* with Phenol red alone or supplemented 5ug/mL IFNγ. Data representative of two independent experiments (n=60-67 per group) and analysed using Log-rank (Mantel-Cox) test, Phenol red or IFNγ, P = 0.368. (d) Zebrafish 0dpf were immersed in metronidazole and the number of macrophages counted using *Tg(mpeg1:Gal4-UNM)sh256*, DMSO (control) vs Metronidazole at 5dpi, P < 0.0001. Data obtained from two independent experiments (n= 24 per group) presented as a mean ±standard deviation and analysed using Ordinary one-way ANOVA with Dunnett's multiple comparison test (e) Representative fluorescent images z-stacks of the number of macrophages in DMSO vs metronidazole treated larvae at 5dpf, scale bar is 50µm.

4.2.6 Interferon-γ promotes macrophage fungistatic activity in zebrafish

Cryptococcus neoformans is known to survive and replicate within macrophages (Levitz et al, 1999). I developed an *in vivo* proliferation assay to determine whether IFNγ influences cryptococcal proliferation inside macrophages. Phenol red or IFNγ was co-inoculated with *C. neoformans* and the fungal proliferation was monitored within macrophages over 12 hours. Treatment with IFNγ significantly reduced fungal replication within macrophages than phenol red treatment alone (P = 0.001, Figure 15).

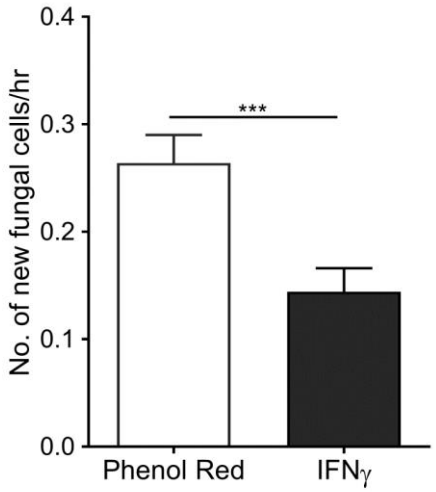

Figure 15: Interferon-γ promotes macrophage fungistatic activity in zebrafish.
The number of new fungal cells per hr within macrophages; 3dpf transgenic zebrafish, *Tg*(*mpeg1*:Gal4-VP16/UAS:Kaede) were challenged with *C. neoformans* in Phenol red alone or supplemented with 5µg/mL IFNγ fungal cells were monitored for proliferation within macrophages over 12 hours. Data obtained from three independent experiments (n=23-26 per group) presented as a mean ±standard deviation and analysed using unpaired t-test, Phenol red vs IFNγ, ***P = 0.001

4.2.7 Pro-inflammatory cytokine involvement

Inflammatory cytokines are a group of cytokines that enhance inflammation. Inflammatory cytokines including IL-1β, IL-6, IL-8 and TNFα have been shown to be secreted in response to *C. neoformans* (Retini et al, 1996).

4.2.7.1 Interferon-γ does not alter expression of *TNFα GFP* in cryptococci infected macrophages

Previous studies by Kawakami et al. (1999) showed that TNFα-producing macrophages induced by IFNγ display anti-cryptococcal activity *in vitro* (Kawakami et al, 1999b). In a murine model of cryptococcosis, resistant mice strains exhibit elevated levels of TNFα than susceptible mice strains (Guillot et al, 2008b). Levels of TNFα in the CSF of patients with HIV-associated cryptococcal meningoencephalitis are higher in survivors following anti-fungal therapy (Lortholary et al, 2001). In addition, Siddiqui et al. (2005) showed a correlation between TNFα levels and clearance of infection in HIV infected patients with cryptococcal meningitis (Siddiqui et al, 2005a). Patients with increased production of TNFα had no mortality reported following IFNγ treatment (Jarvis et al, 2013). Therefore, I examined whether IFNγ-induced fungistatic activity of macrophages involves the production of TNFα. To specifically track TNFα-expressing macrophages, the TNF promoter BAC driving GFP transgenic zebrafish line *TgBAC(tnfa:GFP)pd1028* (Marjoram et al., 2015) was crossed to the macrophage reporter line *Tg(mpeg1:mCherryCAAX)sh378* (Bojarczuk et al, 2016) to produce a double transgenic line *TgBAC(tnfa:GFP); Tg(mpeg1:mCherryCAAX)*. Three dpf *TgBAC(tnfa:GFP); Tg(mpeg1:mCherryCAAX)* were co-inoculated intramuscularly with *C. neoformans* in phenol red alone or supplemented with IFNγ. Expression of TNFα GFP in infected macrophages was measured at different time-points during infection. I found that *C. neoformans* induced the expression of TNFα but addition of IFNγ did not significantly alter the expression compared to phenol red at 12 ($P = 0.422$), 24 ($P = 0.918$) or 36 ($P = 0.211$) hpi as measured by relative fluorescent intensity (Figure 16).

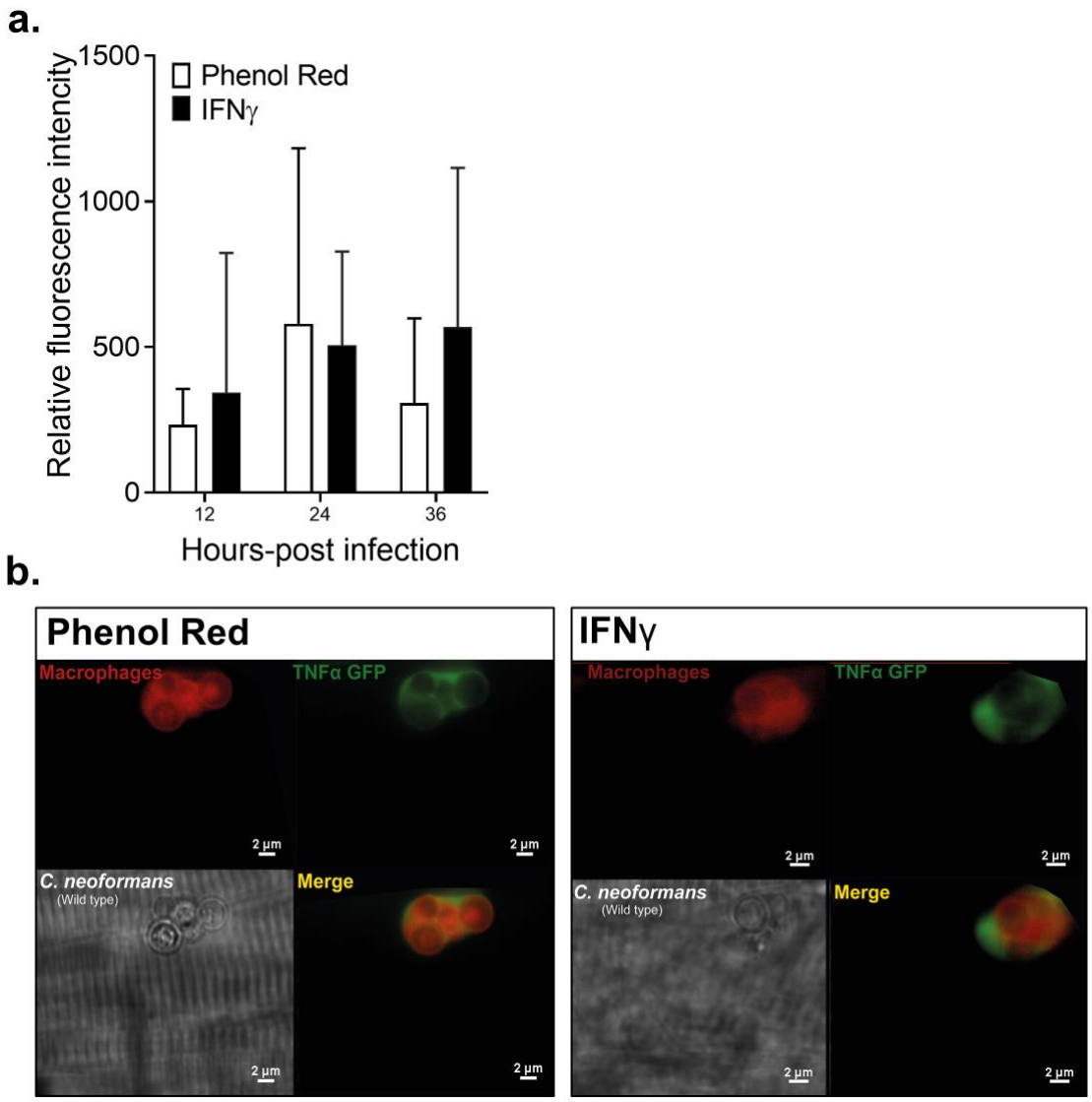

Figure 16: Expression of TNFα GFP in macrophages infected with cryptococci in zebrafish treated with IFNγ

(a) Cumulative relative fluorescent intensity of macrophages per fish. 3dpf double transgenic zebrafish larvae *Tg*(*tnfa:GFP/mpeg1:mCherryCAAX)* were challenged with *C. neoformans* in Phenol red alone or supplemented with 5µg/mL IFNγ and imaged macrophages for TNFα expression. The relative fluorescent intensity of TNFα GFP; data is representative of three independent experiments (n=10-11 fish per group) and expressed as a mean ± standard deviation and analysed by unpaired t-test. Phenol red vs IFNγ at 12hpi, P = 0.422; 24hpi, P = 0.918 and 36hpi P = 0.211. (b) Representative fluorescent images z-stacks of the TNFα GFP expression in Phenol red vs IFNγ at 12hpi treated larvae, scale bar is 2µm.

4.2.7.2 Interferon-γ treated larvae have higher expression of pro-inflammatory cytokine IL-1 GFP at the site of infection

The *Cryptococcus* cells and purified capsular polysaccharide has been shown to induce the production of pro-inflammatory cytokines such as IL-1β by human neutrophils although the levels are significantly lower than LPS stimulation (Retini et al, 1996). *In vivo*, brains of mice with meningeal cryptococcosis have increased expression of IL-1β (Uicker et al, 2005). Marroni et al. (2007) report described an apparently immunocompetent patient with pulmonary cryptococcosis. This patient had functional defects of IL-1β, TNFα and nitric acid production by phagocytes (Marroni et al, 2007) suggesting that IL-1β may be potentially important in host immune response against *C. neoformans*. Therefore, I investigated whether IL-1β played a role in IFNγ-mediated fungistatic activity. Three dpf *Tg(il1:GFP)sh445* zebrafish larvae were inoculated intramuscularly with *C. neoformans* in phenol red alone or with IFNγ and imaged at different time-points for expression of IL-1β GFP. I found that there was significantly higher expression of IL-1β GFP in IFNγ-treated larvae at 12hpi ($P = 0.02$) but not at 2 ($P = 0.91$) and 6 ($P = 0.995$) hpi (Figure 17).

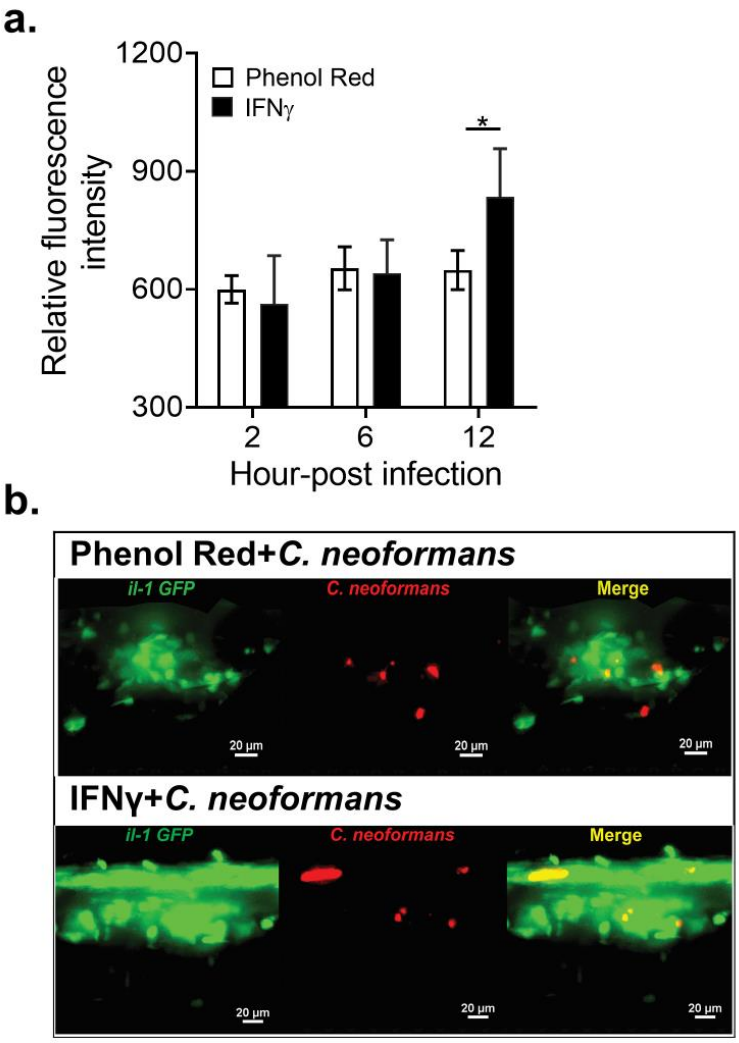

Figure 17: Interferon-γ treated larvae have higher expression of pro-inflammatory cytokine IL-1 GFP at the site of infection

(a) Relative fluorescent of IL-1 GFP at the site of infection. 3dpf transgenic zebrafish line *Tg(il1:GFP)sh445* were challenged with *C. neoformans* in Phenol red alone or supplemented with 5ug/mL IFNγ and imaged to determine IL-1 GFP expression. Data is representative of three independent experiments (8-10 fish per group.) presented as a mean ± standard deviation and analysed by Two-way ANOVA with Bonferroni's multiple comparison test. Phenol red vs IFNγ at 2hpi, P = 0.91; 6hpi, P = 0.995 and 12hpi, *P = 0.02. (b) Example of fluorescence z-stacks of the infection site of 12hpi in the presence or absence of IFNγ, Scale bar is 20µM

4.2.8 Nitrosylation is not detectable in zebrafish macrophages but in neutrophils following *C. neoformans* challenge

Once taken up by phagocytes, intracellular pathogens such as *C. neoformans* are exposed to several antimicrobial mediators, including reactive nitrogen species (Shoham & Levitz, 2005). *C. neoformans* is susceptible to nitric oxide produced by M1 macrophages (Aguirre & Gibson, 2000; Alspaugh & Granger, 1991; Hardison & Brown, 2012a; Müller et al, 2007; Osterholzer et al, 2009b). iNOS knockout mice have a high fungal burden in the brain and fail to clear the infection from the lungs than wild-type mice (Aguirre & Gibson, 2000). Macrophages isolated from iNOS deficient mice fail to control intracellular proliferation of *C. neoformans* (Leopold Wager et al, 2015) suggesting that iNOS is essential for immune resistance to cryptococcal infection. Thus, I hypothesised that IFNγ induces fungistatic activity of macrophages via nitric oxide production. However, the direct measurement of the reactive nitrogen species production has proven difficult due to their high reactivity nature (Elks et al, 2013). To get around this, an anti-nitrotyrosine antibody test has been previously employed in zebrafish and in other fish models of infection (Elks et al, 2013; Forlenza et al, 2008; van der Vaart et al, 2012). Nitrotyrosine is a downstream product of nitric oxide and is formed when nitric oxide reacts with superoxide to produce peroxynitrite (Ischiropoulos et al, 1992). Peroxynitrite reacts with tyrosine residues of proteins in an irreversible process to form nitrotyrosine (Beckman et al, 1990). The major advantage of tyrosine nitrosylation is that it forms a stable protein, which enables the detection of the historical nitric oxide production (Elks et al, 2013). To accurately locate anti-nitrotyrosine staining in macrophages, three dpf macrophage reporter line *Tg(cfms:GFP)sh377* were infected with *C. neoformans* in phenol red alone or supplemented with IFNγ and imaged at different time-points. Surprisingly, anti-nitrotyrosine staining was not detected in macrophages in either group at 2, 6, 12 and 24 hpi (Figure 18a). However, staining was found in other cells, likely neutrophils in both groups (Figure 18a). The nitrosylation levels in neutrophils were not significantly different in the larvae

treated with IFNγ compared to the phenol red treated larvae (P = 0.128, Figure 18b).

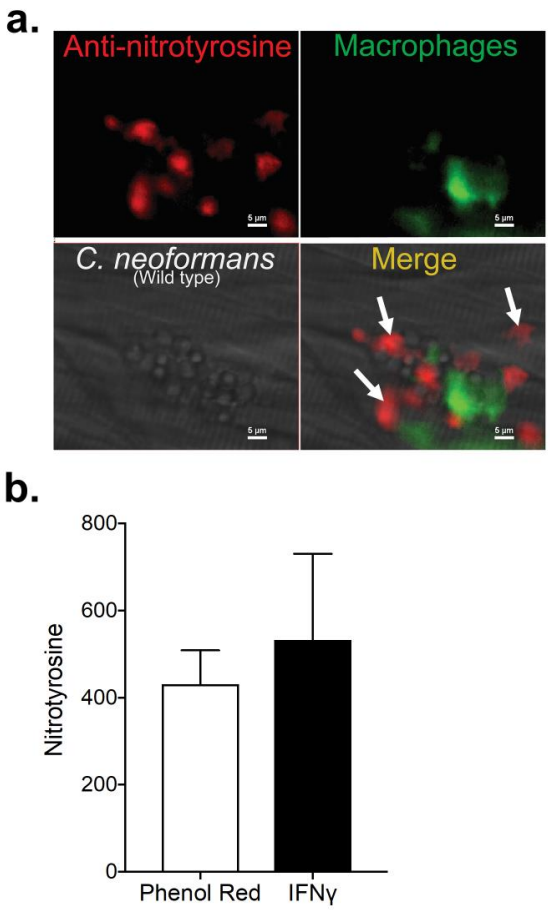

Figure 18: Nitrosylation is not detectable in zebrafish macrophages following *C. neoformans* challenge

Three-dpf transgenic zebrafish line *Tg(cfms:GFP)sh377* were challenged with *C. neoformans* in Phenol red alone or supplemented with 5ug/mL IFNγ and imaged to determine anti-nitrotyrosine staining. (a). Example of fluorescence z-stacks of the infection site of larvae stained with Alexa-657 labelled anti-nitrotyrosine antibody (red), imaged at 6hpi in presence and absence of IFNγ. Anti-nitrotyrosine is not co-localised with macrophages (labelled with *Tg(cfms:GFP)sh377* - green), however, some cells non-green labelled cells, possibly neutrophils, had anti-nitrotyrosine staining (white arrowhead). (b) Relative fluorescence intensity levels of anti-nitrotyrosine antibody z-stacks in Phenol red vs IFNγ (P = 0.128) treated larvae. Data is a representative of three independent experiments (n=25-30 fish per group) presented as mean ± standard deviation and analysed by unpaired t-test.

4.2.9 Low lysosomal pH in macrophages of larvae treated with IFNγ during *C. neoformans* infection

The uptake of pathogenic organisms begins with the attachment of the pathogen to the cell followed by formation of an endocytic vesicle known as phagosome. The phagosome then matures into the phagolysosome which eventually digests the pathogen (reviewed in Tjelle et al., 2000). The maturation of the phagosome results from the fusion of the phagosome to the lysosome to form phagolysosome. Once the internalised material reaches the lysosome, it is bombarded with microbicides including reactive oxygen and nitrogen species, acidic pH and cathepsin hydrolases (Bogdan, 2015; Cross & Segal, 2004; Delamarre et al, 2003; Torres et al, 2006; Trombetta et al, 2003). Similarly, following uptake by macrophage and other phagocytes, *C. neoformans* enters the lysosomes (Artavanis-Tsakonas et al., 2011, Artavanis-Tsakonas et al., 2006, Levitz et al., 1999, Weber and Levitz, 2001, Wozniak and Levitz, 2008). Lysosomes are membrane-bound organelles with a low pH of 4.75 compared to neutral pH of the surrounding cytosol. Lysosomal pH is maintained by alveolar ATPase (Christensen et al, 2002; Kinchen & Ravichandran, 2008). Despite the low lysosomal pH, macrophages fail to control the intracellular growth of *C. neoformans* (Levitz et al., 1999). Therefore, I examined whether administration of IFNγ altered pH levels in infected macrophages. To specifically locate lysosomal pH in macrophages, three dpf macrophage reporter line *Tg(cfms:GFP)sh377* were infected with *C. neoformans* in phenol red alone or supplemented with IFNγ and imaged at different time-points. Low lysosomal pH was observed in infected macrophages of larvae treated with IFNγ compared to controls (P = 0.0036, Figure 19).

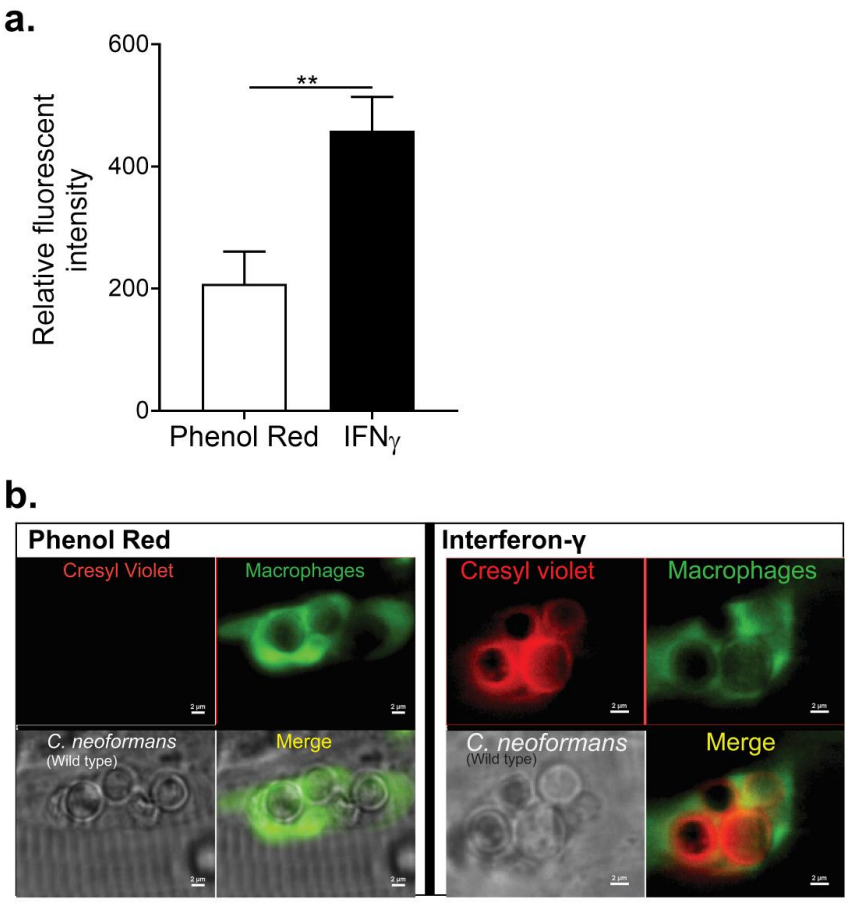

Figure 19: Low lysosomal pH in macrophages of larvae treated with IFNγ during *C. neoformans* infection

(a) Relative fluorescence intensity levels of lysosomal acidity z-stacks in Phenol red vs IFNγ, **P = 0.0036 treated larvae. Data shown is a representative of three independent experiments and presented as mean ± standard deviation from and analysed by unpaired t-test. n=9-10 larvae per group (b) example of fluorescence z-stacks of macrophages at infection site of larvae injected with cresyl violet (red), imaged at 24hpi in presence and absence of IFNγ. pH co-localised with *C. neoformans* (wild-type) in macrophages (labelled with *Tg(cfms:GFP)sh377* - green), Phenol red vs IFNγ treatment. Scale bar is 2μm.

4.3 Discussion

Evidence from clinical and experimental studies consistently suggest that a Th1-type immune response is predominantly required for protection against cryptococcosis (Blasi et al, 1994; Buchanan & Doyle, 2000; Hill & Harmsen, 1991; Huffnagle et al, 1991; Shoham & Levitz, 2005) and is mediated by Th1-type cytokines such as IFNγ and IL-12 (Bauman et al, 2003; Herring et al, 2002; Kawakami et al, 1996a). IFNγ is specifically essential for anti-cryptococcal host defences induced by most pro-inflammatory cytokines (Zhou et al, 2006; Kawakami et al, 1996c). IFNγ is a prototype Th1-type cytokine that induces Th1 differentiation and directs the host immune response towards a protective Th1 phenotype (Schroder et al, 2004).

Using the zebrafish *Cryptococcus* infection model, I have shown that administration of a single dose of IFNγ alone augments host immunity against *C. neoformans,* resulting in reduced fungal burden and increased fungal clearance. These findings corroborate and extend previous observations in experimental murine models and clinical cryptococcosis (Jarvis et al, 2012; Lutz et al, 2000; Pappas et al, 2004). Although in my study IFNγ was given concurrently with *C. neoformans*, only a single dose was enough to yield lower fungal counts. Similarly, in a phase-2 clinical trial by Jarvis et al. (2012), a short course of adjunctive IFNγ with anti-fungals were as effective as a full two-week course of IFNγ (Jarvis et al, 2012) suggesting that IFNγ treatment can have long-lasting effects.

There are several possible mechanisms accounting for enhanced *C. neoformans* clearance. I found that IFNγ induces an influx of macrophages and neutrophils to the infected site early (2hpi) and later (12hpi) during infection respectively. Previous murine data evaluating local cell-mediated immunity at day 3, 7 and 14 days post inoculation showed increased leukocyte recruitment to the lungs in response to IFNγ-producing *C. neoformans* H99γ strain at day 7. However, no significant increase in macrophage influx was observed (Wormley et al, 2007). These discrepancies may result from differences in time-points in which

recruitment was investigated. This is more likely because macrophage numbers at the site of infection were diminished by 24hpi in my experiments. Another possible explanation for this inconsistency may be related to zebrafish larvae immune defences that rely entirely on innate immunity. Thus, it is possible that innate immune cells such as macrophages will respond more readily to the exogenous IFNγ than cells in adult immunocompetent mice would. Interestingly, significantly increased infiltration of leukocytes, including macrophages but not neutrophils, has also been observed at week 3 post inoculation in IFNγ knockout (KO) mice with experimental pulmonary *C. neoformans* infection (Arora et al, 2005). The IFNγ knockout mice display Th2-type responses, alternatively activated macrophages and loss of fungistatic effect resulting in high fungal burden (Arora et al, 2005). In addition, IFNγ-R KO mice display elevated neutrophil infiltration at week 5 post inoculation and a higher fungal burden than wild-type mice (Chen et al, 2005). These findings suggest that early recruitment of immune cells is required for protection against cryptococcal infection. Another intriguing observation arising from phagocyte recruitment assays was that administration of IFNγ alone showed a markedly increased recruitment of both neutrophils and macrophages than when administered with *C. neoformans*. Monari et al. (2002) showed that the encapsulated *C. neoformans* or the addition of GXM suppresses the expression of C5aR (Monari et al, 2002) which is required for migration of neutrophils towards *C. neoformans* (Sun et al, 2015). The possibility that *C. neoformans* may be inhibiting recruitment of macrophages also requires further exploration in the future.

An induction of phagocytosis accompanied the influx of both neutrophils and macrophages. Almost all fungal cells were intracellular by 12 hpi in IFNγ-treated larvae. IFNγ has recently been shown to promote phagocytosis of *C. neoformans* by macrophages *in vitro* (Ikeda-Dantsuji et al, 2015). Phagocytosis plays a vital role in the outcomes of infection by *C. neoformans*. Histopathological examinations of the lungs of mice infected with low-mortality *C. neoformans* strain showed significantly higher numbers of internalised yeasts than high-mortality strains (Okubo et al, 2013). It is interesting to note that *C. neoformans* is more

readily phagocytosed by leukocytes *in vivo* compared to *in vitro* assays which require opsonisation. It has been suggested that since humans begin to develop antibodies to *C. neoformans* as early as 2 years of age (Goldman et al, 2001); perhaps these antibodies may act as opsonins for phagocytosis of *C. neoformans* in the human lung. However, the mechanism of phagocytosis or how IFNγ enhances uptake of the cryptococci by phagocytic effector cells in the zebrafish model remains to be elucidated. I also observed that the numbers of intracellular cryptococci per neutrophil or macrophage were not different between groups. This suggests that phagocytes that had already taken up fungal cells were not primed to take up more fungal cells.

Macrophages have been shown to play a significant role in *C. neoformans* infection. Indeed, I found that more macrophages are recruited and take up more cryptococci than neutrophils following infection, which is consistent with recent findings (Davis et al., 2016, Tenor et al., 2015). The administration of IFNγ in larvae that were depleted of macrophages prior to intramuscular injection of *C. neoformans* could not arrest fungal proliferation suggesting that IFNγ mediates protection predominantly through macrophages. Similar findings have been demonstrated in murine and zebrafish models of cryptococcosis (Bojarczuk et al, 2016; Osterholzer et al, 2009a) highlighting the critical role of macrophages across vertebrates. Besides, for the first time, I have shown that the proliferation of intracellular cryptococci is markedly lower in macrophages of IFNγ-treated larvae (*in vivo*). It has been observed that mice immunised with IFNγ-producing cryptococci display classically activated macrophages (Hardison et al, 2010), and macrophages isolated during secondary infection suppress cryptococcal growth *in vitro* (Hardison et al, 2012b). However, *in vitro* experiments with J774 mouse cell lines or human macrophages treated with IFNγ demonstrate either no or lower fungistatic activity compared to untreated controls (Levitz & Farrell, 1990; Reardon et al, 1996; Voelz et al, 2009). It has been hypothesised that macrophages *in vitro* may already be activated by the addition of cryptococci and/or experimental conditions such as tissue culture environments (Voelz et al, 2009), hence the lower fingistatic activity.

Data from Josie Gibson confirms previous findings that neutrophils are not essential in host immunity against *C. neoformans* infection. Cryptococcosis is found primarily in patients with impaired adaptive immunity and not in neutropenic patients. Murine studies have shown improved survival following concurrent transient depletion of neutrophils and pulmonary infection with *Cryptococcus* (Mednick et al, 2003). This finding appears to be attributable to the absence of neutrophil-mediated inflammation (Wozniak et al, 2012). Interestingly, neutrophils have been shown to kill *Cryptococcus in vitro* (Chaturvedi et al, 1996; Mambula et al, 2000; Qu & Wang, 1991). Neutrophils can eliminate *Cryptococcus* directly from the brain vasculature in mice, an activity that is associated with efficient recruitment of neutrophils (Sun et al, 2016; Sun et al, 2015; Zhang et al, 2016). Thus, further investigation of the role of neutrophils and how they can be exploited for developing new therapies for cryptococcal infections would be valuable.

Interestingly, I did not observe a significant increase in the expression of the pro-inflammatory cytokine TNFα in macrophages of IFNγ-treated larvae at any time-point examined. TNFα has previously been shown to be associated with protective immune responses against *C. neoformans* in experimental murine models (Uicker et al., 2005, Kawakami et al., 1996a, Herring et al., 2002) and human disease. TNFα levels in the CSF of patients with cryptococcal meningitis are significantly higher in survivors compared with non-survivors (Siddiqui et al, 2005a). Transient TNFα depletion in mice results in high fungal burden and inhibition of fungal clearance (Herring et al, 2005; Huffnagle et al, 1996; Xu et al, 2016). In fact, anti-TNFα therapies are associated with an increased predisposition to invasive fungal infections, including cryptococcosis (Ellerin et al, 2003; Tsiodras et al, 2008). Mice infected with IFNγ-producing *C. neoformans* H99γ strain had significantly higher levels of pro-inflammatory cytokines, including TNFα, than wild-type strain-infected mice (Wormley et al, 2007). Similarly, survivors of cryptococcal meningitis at two weeks following IFNγ therapy had higher proportions of IFNγ– and TNFα-producing $CD4^+$ T cells and a trend towards more rapid clearance of infection from the CSF (Jarvis et al, 2013). However, it should be noted that in the previous studies in mice by Wormly

et al, (2007), TNFα production was increased at day 7 post inoculation (Wormley et al, 2007). Thus, while my results may not completely correlate with those observed in mice and humans, they suggest a more significant role of other cytokines such as IL-1β (see below), in the elicitation of protective responses against *C. neoformans* infections.

Testing of another pro-inflammatory cytokine, IL-1β showed that its expression is increased in IFNγ-treated zebrafish larvae at 12 hpi. The *Tg(il1:GFP)sh445* expression in this line is not cell lineage specific. IL-1β has been demonstrated to be expressed in many cells (reviewed Sims & Smith, 2010). As with TNFα, IL-1β elevation has been shown to be increased in *C. neoformans* H99γ strain infection in mice (Wormley et al, 2007). The role of IL-1β in *C. neoformans* infection has been highlighted in a clinical case report of a 21-year-old Italian female smoker with impaired IL-1β, TNFα and nitric oxide production who developed pulmonary cryptococcal disease (Marroni et al, 2007). No further studies have been reported regarding IL-1β in human cryptococcal disease, thus more studies would be needed to determine whether IL-1β is involved in host defence against *C. neoformans* infection.

Macrophages can be induced towards phenotypically and functionally distinct effector cells, mostly influenced by the cytokine environment (reviewed in Martinez et al, 2009; Mosser & Edwards, 2008). The macrophage polarisation status is a well characterised phenomenon in mouse and human macrophages. The most used markers of M1 macrophages are inducible nitric oxide synthase (iNOS) or iNOS generated nitric oxide (NO). NO production by M1 macrophages in mice is required for control of the intracellular replication of *C. neoformans* (Leopold Wager et al, 2015). IFNγ cytokine environments induce M1 macrophages. Although M1 subsets have been identified in zebrafish with TNFα as a marker (Nguyen-Chi et al, 2015), I could not find the nitric oxide-derived inflammatory oxidant, nitrotyrosine, in macrophages of infected zebrafish treated with IFNγ at any of the time-points examined. However, I detected nitrotyrosine staining exclusively in the neutrophils, both infected and uninfected. Elks et al.

(2013) showed that nitrosylation in zebrafish was predominantly observed in neutrophils of larvae injected with *Mycobacterium marinum* or vehicle control (phenol red) (Elks et al, 2013). The nitrosylation observed in zebrafish neutrophils in the absence of infection is thought to be due to myeloperoxidase activity, a zebrafish-specific neutrophil enzyme (Eiserich et al, 1998; Forlenza et al, 2008; Lieschke et al, 2001). Recently, anti-nitrotyrosine staining was detected in macrophages of larvae infected with *Mycobacterium laprae* (Madigan et al, 2017). Elks et al. (2013) have also reported staining of other cells thought to be macrophages in uninfected larvae. However, these findings could not be replicated in my hands using the same method. Further experimentation of the role of iNOS/NO in cryptococcal immunity is required.

Finally, I report that phagolysosomal acidification is enhanced in the presence of IFNγ. The effect of pH, independent of host, on the *C. neoformans* growth has been studied since the 1950s. An acidic environment enhances *C. neoformans* growth whereas an alkaline milieu inhibits growth of the fungus (Howard, 1961; Mosberg & McAlpine, 1951). More recent studies support those findings. Levitz et al, (1997) demonstrated that pH5 is optimal for *C. neoformans* growth, but the fungus is significantly inhibited at pH values that are slightly above neutrality (Levitz et al, 1997). Since during the pathogenesis process of *C. neoformans*, the fungus goes through different pH phases from neutral to acidic conditions in the mature phagolysosome. It is not known whether lower pH (i.e. < 5) inhibits *C. neoformans* growth. In contrast to previous findings (Levitz et al., 1999), recent results by Smith et al. (2015) showed that live but not heat or UV killed cryptococci are capable of manipulating host pH in macrophage phagolysosome (Smith et al, 2015). However, how *C. neoformans* can alter phagosome maturation is currently unclear. The phagolysosome containing cryptococci has been demonstrated to permeabilise immediately following phagocytosis (Chayakulkeeree et al., 2011, Tucker and Casadevall, 2002, Johnston and May, 2010) allowing dilution of phagosomal content and increase in the luminal pH from the phagolysosome. Interestingly, failure of acidification of the phagosome has been observed in microglia cells, in which only one-third of phagolysosomes reached full

acidification (Orsi et al, 2009). Recently it has been shown that treatment of macrophages with IFNγ protects the phagolysosomal membrane from damage and enhances the fungicidal activity of macrophages (Davis et al, 2015). Taken together, low pH of the phagolysosome compartments may not be the only important anti-cryptococcal mechanism by macrophages. However, maintenance of the phagolysosome membrane integrity may prevent dilution of the lysosomal content, thus, enabling other antimicrobial effects, including reactive nitrogen species, to kill intracellular fungi. In addition, these observations suggest that mediations that protect the integrity of the phagosome membrane to maintain low pH, could have potential treatment applications.

Collectively, my data show that intramuscular infection with *C. neoformans* and a single dose of IFNγ activated rapid influx of macrophages, increased uptake of the cryptococci and caused highly acidic phagolysosomes. This resulted in reduced replication of yeast within macrophages, low fungal burden and increased fungal clearance. These findings provide a critical framework from which to develop our understanding in formulating strategies for inducing local protective immune responses against invasive fungal infections. The administration of a host cytokine to a local infection site in a 'see-through' animal model is a novel way to investigate immune responses to *C. neoformans* infection *in vivo*. Using this approach, we can examine the pathogen-phagocyte interactions directly during infection. Although larger clinical studies are needed to explore the benefits of IFNγ treatment in cryptcoccosis, its underlying mechanism needs to be understood. My study and other studies committed to unravelling the mechanism of IFNγ will be helpful in developing potential immune-based treatments for human mycoses. In addition, although caution should be taken in deducing the results of animal studies as translatable to humans; my findings are consistent with clinical outcomes. My findings collaborate with previous findings that administration of IFNγ in patients with cryptococcal meningitis augments anti-cryptococcal chemotherapy resulting in increased rates of cryptococcal clearance from the CSF (Jarvis et al, 2012). In contrast, treatment of human macrophages *in vitro* fail to show increased

fungistatic activity (Levitz & Farrell, 1990; Reardon et al, 1996; Voelz et al, 2009) highlighting the usefulness of using a range of models to study human disease.

The necessity for new treatments remains a prominent matter considering an increasing patient population with impaired immunity. These include individuals with HIV/AIDS, those receiving immunosuppressant therapy and organ transplant recipients. Thus, the viability of immunotherapy in immunocompromised individuals may not be a cause of concern. However, considering the diversity of the patient population at risk of cryptococcosis, identifying individuals who can benefit from immunotherapy cannot be overemphasized. In addition, reflecting on an ongoing outbreak of *C. gattii* in the immunocompetent hosts (Byrnes and Heitman, 2009b), indicates that there is a need to develop therapeutic strategies to protect both healthy and immunocompromised individuals from fungal infections. Since cryptococcal infection in immunocompetent persons is more often confined to the lungs, any strategies like those presented here, in which therapies are administered in such a way as to enhance local anti-cryptococcal host immune responses, can provide protection against future infections or existing latent infections. Added together, the findings presented here, promote the concept that strategies that exploit Th1-type cytokines against life-threatening fungal infections including cryptococcosis can result in the clearance of infections.

CHAPTER 5

INTRAMUSCULAR ADMINISTRATION OF *S. AUREUS* CELL WALL PREPARATIONS PROTECTS ZEBRAFISH AGAINST *C. NEOFORMANS* INFECTION

5.1 Introduction to immunomodulation

Immunomodulation involves exploitation by either suppression or enhancement of the natural immune defences depending upon the circumstances required to treat specific disease. The ability to modulate the immune response has proved to be a valuable therapeutic strategy in a wide range of ailments including prevention and treatment of infections, dampening of autoimmune and inflammatory responses, and induction of antitumor immune responses in patients with cancer (Ulevitch, 2004, Hamill et al., 2008). The objective of immunomodulation for the treatment of infections is to initiate or potentiate protective antimicrobial immunity without causing detrimental effects such as inflammatory tissue damage. One advantage of immunomodulation is the targeting of the host rather than the pathogen thereby avoiding selective pressure that may lead to the development of microbial resistance. Immunomodulatory molecules for the stimulation of protective antimicrobial immunity include agonists of pattern recognition receptors (PRRs), immunomodulatory host defence peptides (also referred to as antimicrobial peptides), and natural bacterial signalling molecules.

The toll-like receptor (TLR) family is one of the major classes of PRRs. The TLR family in humans includes ten transmembrane proteins. Each member is specialised for the recognition of specific classes of ligands (agonists) which are typically signature components of microbes. Such microbial components include lipopolysaccharides (LPS), flagellin, DNA, lipoteichoic acid, lipoproteins, dsRNA, SS-RNA etc. (for further details see Chapter 1). The interaction of microbial signature components with TLRs results in the induction of signal transductions pathways, such as MAPK and NF-κB through TIR domain-containing adaptor proteins (including MYD88, TIRAP, TRIF) leading to activation of innate immunity. This in turn, activates the production of, for example, pro-inflammatory cytokines and induces antimicrobial effector functions such as phagocytosis, oxidative burst and production of antimicrobial peptides (Reviewed in Hancock et al., 2012). This stimulation of innate immunity that is referred to as stimulated innate resistance appears to provide a broad-spectrum protection. The stimulation of innate

immune resistance by TLR agonists has been demonstrated in mice models of infection. For example, LPS (a TLR4 agonist) protects mice from *Bordetella pertussis* infection when administered concurrently (Evans et al., 2010). The administration of flagellin (a TLR5 agonist) gives protection against *Streptococcus pneumonia* and *Pseudomonas aeruginosa* infections in mice when given concurrently (Munoz et al., 2010, Yu et al., 2010). Besides, flagellin also reduces colonisation of vancomycin resistant *Pseudomonas aeruginosa* in the guts of mice undergoing a broad-spectrum antibiotic therapy (Kinnebrew et al., 2010). The TLR agonists have also been shown to treat fungal infection in a mouse model. The exogenous administration of LPS or imiquimod (a TLR7 agonist) helped to clear *Fonsecaea pedrosoi* infection in mice. *F. pedrosoi* is a fungus that causes a chronic skin infection known as chromoblastomycosis (Sousa et al., 2011).

5.2 Rationale of immune modulation

The innate receptors' agonists for inducing resistance to infection have been used in research for some time now (Hancock et al., 2012). The rationale is to initiate or enhance an innate response that significantly exceeds in quantity and quality the innate response induced by the pathogen itself while minimising inflammation-associated tissue damage. The rationale to explore immunomodulation of innate immunity as a strategy to infer protection against *C. neoformans* are two-fold:

Firstly, *C. neoformans* do not appear to induce a robust pro-inflammatory response both *in vitro* and *in vivo*. Without the use of opsonins in *in vitro* assays, phagocytosis of *C. neoformans* by macrophages is minimal (Levitz, 2002). In *in vitro* assays, *C. neoformans* fails to stimulate monocytes and macrophages to produce pro-inflammatory cytokines such as TNFα (Levitz et al., 1994; Naslund et al., 1995, Vecchiarelli et al., 1995), IL-1β (Vecchiarelli et al., 1995), IL-6 (Delfino et al., 1997) or IL-12 (Retini et al., 1999) and induces low levels of IFNγ in T cells (Vecchiarelli et al., 1994). Instead, as shown in experimental mouse models, *C. neoformans* activates the Th2-type immune responses resulting in the generation of M2 macrophages (Osterholzer et al., 2009, Arora et al., 2005).

Recently Davis et al. (2013) showed that following intratracheal infection, *C. neoformans* proliferates rapidly to high titre in the first week and mice display Th2-type response characterised by M2 macrophages (Davis et al., 2013). *C. neoformans* laccase, urease and the polysaccharide capsule have been implicated in cryptococcal-mediated immune modulation of macrophages towards M2 (Osterholzer et al., 2009, Qiu et al., 2012, Almeida et al., 2001).

Secondly, TLR signalling is critically required for the generation of protective innate immune defences against *C. neoformans* infection. Toll-like receptor (TLR)$9^{-/-}$ mice have impaired pulmonary clearance of *C. neoformans* and are associated with diminished $CD4^{+}$, $CD8^{+}$ T cell, $CD19^{+}$ B cells, macrophage recruitment, increased induction of arg1 and FIZZI (found in an inflammatory zone) but reduced nitric oxide synthase (Nakamura et al., 2008, Zhang et al., 2010). TLR2 and TLR4 recognise *C. neoformans* GXM (Shoham et al., 2001, Yauch et al., 2004, Yauch et al., 2005) but GXM fail to induce MAPK pathways and TNFα secretion (Shoham et al., 2001). $TLR2^{-/-}$ mice are more susceptible to cryptococcal infection than wild-type (Yauch et al., 2004, Biondo et al., 2005, Nakamura et al., 2008). TLR4 and C-type lectin receptors including mannose receptor and DC-SIGN recognise *C. neoformans* but play a minor role, if any, in resistance to cryptococcosis (Yauch et al., 2004, Nakamura et al., 2006, Mansour et al., 2006, Dan et al., 2008b). Dectin-1, Dectin-2, Dectin-3 or Mincle do not appear to have any role in anti-cryptococcal immunity (Campuzano et al., 2017, Nakamura et al., 2007, Walsh et al., 2017, Nakamura et al., 2015, Heung, 2017).

The contribution of TLR or IL-1R signalling in host defence against cryptococcal disease is highlighted in MyD88 deficient mice model. Mice deficient in MyD88, an adaptor molecule required for signalling by all TLR except TLR3 and IL-1R (reviewed in Warner and Nunez, 2013), succumb to *C. neoformans* early during infection. TLR2 and myD88 deficient mice display decreased expression of pro-inflammatory cytokines, IL-12p40, TNFα, IFNγ in various organs and reduced production of IFNγ in peritoneal macrophages (Biondo et al., 2005, Yauch et al., 2004).

Despite these observations, little work has been done on the exploitation of TLR-driven responses as potential therapy for cryptococcal infections. TLRs are already being investigated as drug targets for many infectious diseases in ongoing experimental studies and clinical trials. Considering the weak immunogenic nature of *C. neoformans* and the vital role of TLRs in cryptococcal disease, I hypothesised that; activation of TLR signalling may enhance appropriate protective immune responses and suppression of cryptococcal infections (Figure 20).

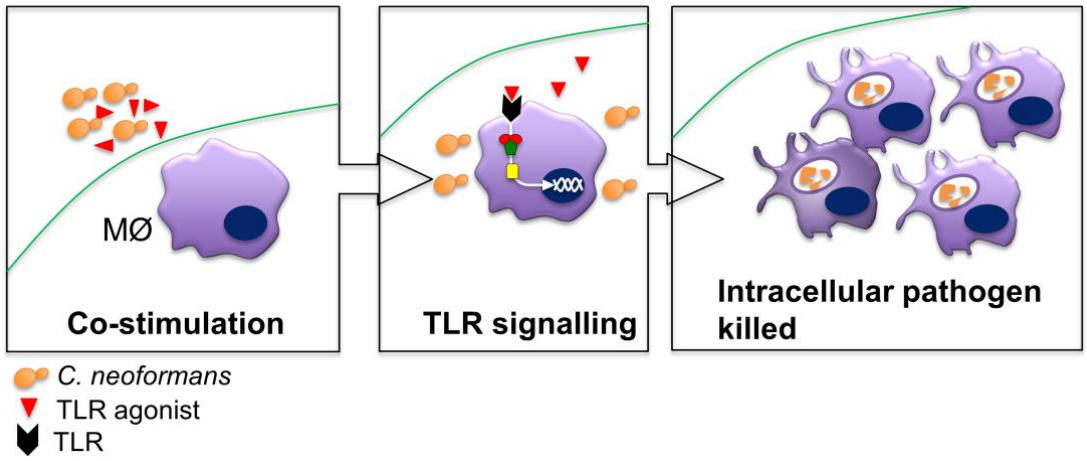

Figure 20: Hypothesis

Activation of TLR signalling activates new and appropriate protective innate defences against *C. neoformans* infection.

5.3 TLR agonists tested

5.3.1 Lipopolysaccharides (LPS)

Lipopolysaccharide is a bacterial endotoxin and a major structural component of the outer membrane of Gram-negative bacteria. LPS is a large molecule comprised of three units, a lipid A, an inner and outer core oligosaccharide and a highly variable O-antigen consisting of repeating oligosaccharide units (Reviewed in Erridge et al., 2002). LPS has been shown to be recognised by both human and murine TLR4 (Hoshino et al., 1999, Schwartz, 2001) leading to induction of overwhelming immune response that can cause life-threatening septic shock in humans.

5.3.2 Imiquimod

Imiquimod, which is also known as R837, is a small molecule imidazoquinoline amine compound belonging to a group of immune response modifiers that possess antitumor and antiviral effects (Beutner et al., 1998, Craft et al., 2005). Imiquimod is used for the treatment of genital warts, caused by human papillomavirus infection, and malignant tumours of the skin (Smits et al., 2008). Imiquimod is an agonist that preferentially binds to TLR7 (Lee et al., 2003) leading to activation of MyD88 and induction of NF-κB (Hemmi et al., 2002). Recently imiquimod has been shown to have beneficial effects in the treatment of a chronic subcutaneous mycosis, chromoblastomycosis (de Sousa et al., 2014).

5.3.3 *Vibrio anguillarum* DNA

Vibrio anguillarum DNA (*Va*DNA) is phenol-extracted genomic DNA from *Vibrio anguillarum* ATCC19264 cells (Sepulcre et al., 2009). This was a generous gift from Victoriano Mulero at Department of Cell Biology and Histology, Faculty of Biology, University of Murcia, Spain. Most dsDNAs are recognised by mammalian TLR9. *Va*DNA has been shown to stimulate expression of TLR21 in zebrafish (Gao et al., 2013).

5.3.4 Pam2CSK4

Pam2CSK4 is a synthetic diacylated lipopeptide that induces immune signalling through TLR2 (Buwitt-Beckmann et al., 2005).

5.3.5 CpG ODN 2359

CpG ODN 2359 is a synthetic class C CpG oligonucleotide that comprises unmethylated CpG dinucleotides. CpG ODN is recognised by human, mouse and zebrafish TLR9, and chicken and zebrafish TLR21 leading to immunostimulatory effects (Vollmer et al., 2004, Brownlie et al., 2009, Keestra et al., 2010, Hemmi et al., 2000, Bauer et al., 2001, Chuang et al., 2002).

5.3.6 *Staphylococcus aureus* cell wall preparations

Staphylococcus aureus is as a gram-positive bacterium characterised by a thick peptidoglycan layer in the cell envelope that retains gentian violet following staining with Gram stain. *S. aureus* cell wall is composed of an inner (plasma) membrane and the outer peptidoglycan with different glycopolymers, teichoic acids and lipoprotein, that are attached to either the peptidoglycan or lipid part of the plasma membrane (reviewed in Weidenmaier and Peschel, 2008) (Figure 21). The cell wall glycopolymers stretch through the peptidoglycan layers to the bacterial cell surface. Here they mediate interactions with the host by binding with host receptors and triggering an innate immune response such as inflammation, complement activation or opsonisation.

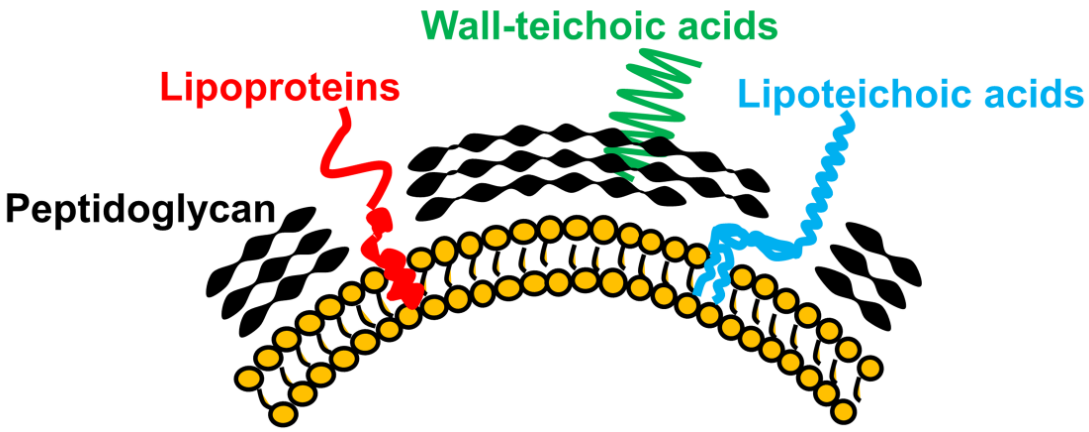

Figure 21: Schematic structure of *S. aureus* cell wall

Peptidoglycan – Peptidoglycan (PGN) is made up of linear glycan strands of alternating *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac) residues cross-linked by short peptides, β-1→4 bonds (Schleifer and Kandler, 1972). Peptidoglycan is located on the outside of the plasma membrane (Guan and Mariuzza, 2007). The core functions of peptidoglycan are to preserve the integrity of the cell, maintain cell shape, and anchor other cell envelops including teichoic acid and proteins (Dramsi et al., 2008, Neuhaus and Baddiley, 2003). Peptidoglycan has been shown to be detected by the pattern recognition receptors (PRRs) present on monocytes, macrophages and dendritic cells. The PRRs that recognise peptidoglycan include TLR2 (though not conclusively) (Travassos et al., 2004, Dziarski and Gupta, 2005), NOD1 (Chamaillard et al. 2003) and membrane-bound adaptor molecule CD14 (Dziarski et al., 1998).

Teichoic acids – Teichoic acids (TAs) are cell-wall glycopolymers that are constitutively produced. TAs are either attached to peptidoglycan (wall-teichoic acids, WTAs) or lipids of the plasma membrane (lipoteichoic acid, LTA). WTA is composed of 40 ribitol phosphate units that are substituted with D-alanine ester and *N*-acetylglucosaminyl residues. The chain is covalently linked to peptidoglycan via a phosphodiester linkage to the C6 hydroxyl of the *N*-acetyl muramic acid sugars (Neuhaus and Baddiley, 2003). However, LTA is composed of approximately 25 poly (1-3)-glycerol phosphate linked to a diacylglycerolipid anchor which attaches to the surface of cytoplasmic membrane (Fournier and Philpott, 2005). The actual function of TAs is not well understood, but WTA is not required for viability of the bacteria under laboratory conditions but plays a vital role during infection *in vivo* (Weidenmaier et al., 2004, D'Elia et al., 2006, D'Elia et al., 2009). On the other hand, LTA is required in growth condition below 37° C and deletion of both WTA and LTA is lethal (Oku et al., 2009, Schirner et al., 2009), suggesting that TAs are essential for viability of the bacteria. WTA and LTA are exposed on the bacterial surface and interacts with several host receptors. Soluble C-type lectin receptor, Mannose-binding lectin (MBL) has been shown to recognise both WTA and LTA. WTA binds to MBL and function as an opsonin but only in the serum of infants who have not developed anti-WTA

IgG antibodies (Park et al., 2010). MBL-deficiency individuals are more susceptible to bacterial infection including *S. aureus* especially during infancy (Kurokawa et al., 2016). The WTA deficient *S. aureus* mutants exhibit defects in adherence to epithelial and endothelial cells, nasal colonisation and dissemination of the bacteria from the blood stream to sub-endothelial tissues in endovascular infections in animal models (Weidenmaier et al., 2004, Weidenmaier et al., 2005, Weidenmaier et al., 2008). Recently the first receptor of WTA on nasal epithelial cells has been reported. F scavenger receptor, SREC-1 was shown to bind to WTA and facilitated adhesion to nasal epithelial cells *in vitro* (Baur et al., 2014). LTA also binds MBL and L-ficolin leading to activation of lectin-initiated complement pathway and subsequently bacterial opsonisation (Polotsky et al., 1996, Lynch et al., 2004, Nahid and Sugii, 2006). LTA has also been shown to elicit innate immune system through TLR2 (Morath et al., 2001, Hermann et al., 2002, Draing et al., 2008). However, this has been controversial because the commonly used LTA preparations are contaminated with bacterial lipopeptides, which are major inducers of TLR2 mediated pro-inflammatory responses (Hashimoto et al., 2006, Zahringer et al., 2008, Hashimoto et al., 2007).

Lipoproteins – Lipoproteins (LPP) are a distinctive class of surface proteins comprised of N-terminal lipid modification of proteins with *N*-acyl-*S*-diacyl-glyceryl-cysteine (Hantke and Braun, 1973). Lipoproteins in the Gram-positive bacteria are anchored by a lipid moiety at the N terminus in the outer leaflet of the plasma membrane and extends into the cell wall. During *S. aureus* interactions with the host, LPP are required for virulence and TLR2 signalling (reviewed in Nguyen and Gotz, 2016). *S. aureus* mutants lacking matured LPP (Δ*lgt*) have decreased induction of pro-inflammatory cytokines via the TLR2-MyD88 signalling pathway in murine peritoneal macrophages. Also, Δ*lgt* display severely impaired pathogenicity in the C57BL/6 mouse sepsis model (Stoll et al., 2005, Schmaler et al., 2009).

5.4 Results

5.4.1 *S. aureus* CWP and *Va*DNA reduce fungal burden in zebrafish

The following TLR agonists were used to activate innate immunity in cryptococcal infection: lipopolysaccharide, imiquimod, *Vibrio anguillarum* genomic DNA (*Va*DNA) and *Staphylococcus aureus* SH1000 strain cell wall preparations (CWP), denoted as *S. aureus* CWP. These agonists were selected based on the following previously published and unpublished work. LPS and imiquimod induced robust inflammatory responses to the fungus *Fonsecaea pedrosoi* resulting in clearance of infection in mice (Sousa et al., 2011). *Vibrio anguillarum* DNA had been shown to induce inflammatory genes in zebrafish (Sepulcre et al., 2009) whereas *S. aureus* CWP elicit severe inflammation in zebrafish infected with non-lethal inoculum of *S. aureus* (Boldock, 2016). To test the capacity of TLR agonists to control *C. neoformans* infection, 3dpf *nacre* zebrafish larvae were injected intramuscularly with *C. neoformans* in phenol red alone (vehicle control) or supplemented with individual TLR agonists. Fungal burden was determined at 48-hours post challenge. The mean cryptococcal burden in larvae treated with LPS or imiquimod were not significantly different from phenol red treated larvae (LPS P = 0.31; Imiquimod P=0.89; Figure 22a). However, cryptococcal burden was significantly lower in larvae that were injected with *S. aureus* CWP or *Va*DNA compared to phenol red treated larvae (*S. aureus* CWP, P = 0.03; *Va*DNA, P < 0.0001; Figure 22a). It is worth mentioning that IFNγ was slightly more effective at reducing fungal burden than *S. aureus* CWP (P = 0.04).

TLR2 recognises *Staphylococcus aureus* CWP while TLR9 or TLR21 recognises *Va*DNA. Thus, I supposed that administration of synthetic TLR2 or TLR9/21 agonists, Pam2CSK4 and CpG oligodeoxynucleotides (CpG ODN) respectively, would yield similar results of *S. aureus* CWP and *Va*DNA. CpG ODN is a ligand to zebrafish TLR9 and TLR21 (Yeh et al., 2013) and both *Vibrio anguillarum* and CpG ODN have been shown to induce the expression of TLR21 in zebrafish (Gao et al., 2013). Surprisingly, there was no significant reduction of the fungal burden in larvae injected with Pam2CSK4 or CpG ODN than larvae which were treated

with vehicle control (Figure 22b&c; Pam2CSK4 P = 0.29; CpG ODN P = 0.29). As a control, the agonists were tested for their effect on the fungal growth *in vitro* at the concentration injected. None of the agonists showed any effect on the growth of *Cryptococcus* (Figure 22d).

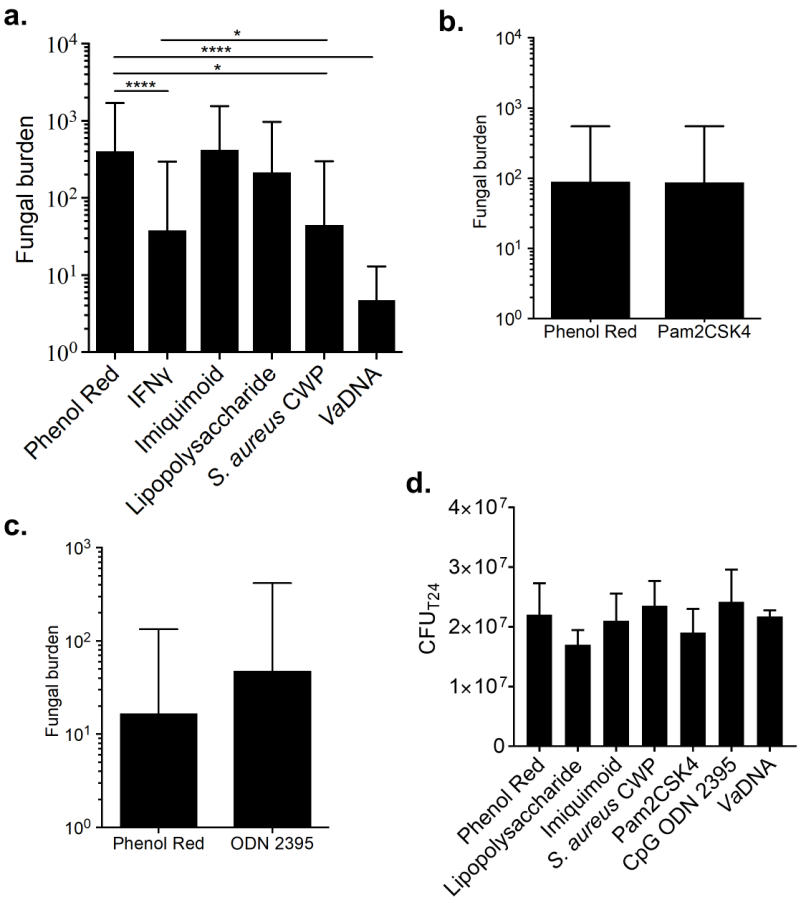

Figure 22: *S. aureus* CWP and *VaDNA* reduce fungal burden in zebrafish.

Fungal burden at 48hpi of 3dpf *nacre* larvae challenged with (a) *C. neoformans* in phenol red alone or supplemented either with 10 µg/mL lipopolysaccharide, or 10 µg/mL imiquimod, or 5 mg/mL *S. aureus* CWP or 5µg/mL *Va*DNA. Data from three independent experiments (n=130-140 per group) presented as a mean ±standard deviation analysed by one-way ANOVA with Dunn's Multiple comparison test for each treatment group versus phenol red. Phenol red vs IFNγ, ****P < 0.0001, *S. aureus* CWP, *P = 0.03, *Va*DNA, ****P < 0.0001 (b) *C. neoformans* in phenol red alone or with 10 µg/mL PAM2CSK4. Data from three independent experiments (n=78-83 per group) presented as a mean ± standard deviation analysed by Mann-Whitney test; Phenol red vs PamCSK4, P = 0.29. (c) *C. neoformans* in phenol red alone or with 50 µg/mL ODN2395, data from three independent experiments (n=105-106 per group) presented as a mean ± standard deviation analysed by Mann-Whitney test, Phenol red vs CpG ODN 2395, P = 0.29. (d) Colony forming units of *C. neoformans* at 24h following *in vitro* exposure to TLR agonists at concentration used above. Data from three repeats presented as a mean ± standard deviation analysed by Ordinary one-way ANOVA with Dunnett's multiple comparisons test of Phenol red vs LPS (P = 0.33); imiquimod (P = 0.99); Peptidoglycan (P = 0.97); Pam2CSK4 (P = 0.71); CpG ODN 2395 (P = 0.89); *Va*DNA (P = 0.99).

5.4.2 *S. aureus* Lipoproteins are not required for CWP-mediated protection against *C. neoformans* infection in zebrafish

As described above, the *S. aureus* cell consists of the inner plasma membrane, outer peptidoglycan, lipoproteins, and glycopolymers that are wall teichoic acid and lipoteichoic acid (Figure 21). Therefore, I tried to determine which of these cell wall components are required for *S. aureus* CWP mediated protection against *C. neoformans*. I first tested whether lipoproteins were required for protection. Three dpf *nacre* zebrafish larvae were challenged intramuscularly with *C. neoformans* in phenol red alone or supplemented with CWP made from lipoprotein-null *S. aureus* Δ*lgt* mutant or CWP from *S. aureus*. Fungal burden was determined at 48 hpi as described before. The fungal burden in the larvae that were treated with Δ*lgt* CWP or *S. aureus* was significantly lower compared to the larvae injected with phenol red alone (Figure 23; Δ*lgt* CWP, $P < 0.0001$; *S. aureus* CWP, $P < 0.0001$). The fungal burden between Δ*lgt* CWP and *S. aureus* CWP-treated larvae were not significantly different ($P = 0.35$; Figure 23). These results suggest that lipoproteins are not essential for *S. aureus* CWP-mediated protection against *C. neoformans.*

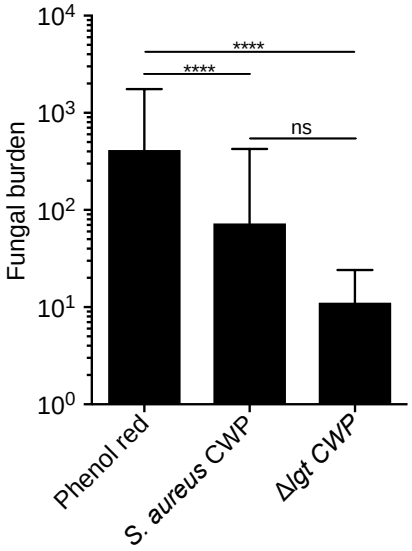

Figure 23: *S. aureus* lipoproteins are not required for CWP mediated protection against *C. neoformans* infection in zebrafish.

Fungal burden at 48hpi of 3dpf *nacre* larvae challenged with *C. neoformans* in phenol red alone or supplemented either with 5mg/mL *S. aureus* CWP or with 5 mg/mL Δ*lgt* CWP. Results are a combination of three independent experiments (n=54-58 per group) and data presented as a mean ± standard deviation and tested for statistical significance using one-way ANOVA with Dunn's multiple comparison test for each treatment group versus phenol red. *S. aureus* CWP vs Phenol red, ****P < 0.0001; *S. aureus* CWP vs Δ*lgt* CWP, P = 0.35.

5.4.3 *S. aureus* wall-teichoic acid is required for CWP-mediated protection against *C. neoformans* infection

I then evaluated whether *S. aureus* cell wall glycopolymer, wall-teichoic acid (WTA) may be essential in *S. aureus* CWP driven protection against *C. neoformans* infection in zebrafish. I used the CWP made from *S. aureus* that have been treated with hydrofluoric acid (denoted as *S. aureus* CWP-HF) to remove wall teichoic acid and the CWP made from WTA-null *S. aureus* strain, Δ*tarO*. The *tarO* is the first gene in the WTA biosynthetic pathway (Weidenmaier et al., 2004). Interestingly, there was no significant difference in the mean fungal burden in larvae injected with the *S. aureus* CWP-HF or the Δ*tarO* CWP compared to larvae treated with phenol red (Figure 24; *S. aureus* CWP-HF, P = 0.67; Δ*tarO* CWP, P = 0.12). These results strongly suggest that WTA is essential for protection. However, efforts to test wall teichoic acid alone for protection proved impossible because we were unable to obtain pure wall teichoic acid.

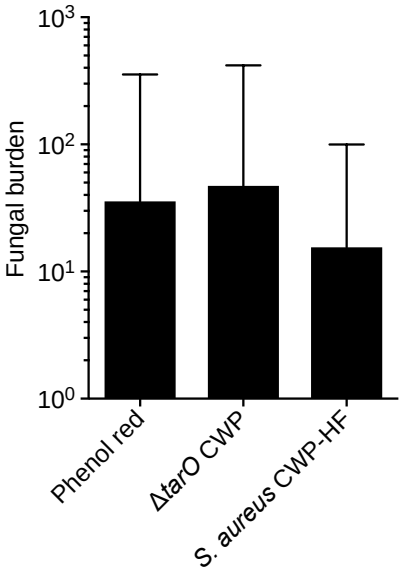

Figure 24: *S. aureus* wall-teichoic acid is required for CWP-mediated protection against *C. neoformans* infection.

Fungal burden at 48hpi of 3dpf *nacre* larvae challenged with *C. neoformans* in phenol red alone or supplemented either 5mg/mL Δ*tarO* CWP or 5mg/mL *S. aureus* CWP-HF. Results are a combination of three independent experiments (n=99-124 per group) and data presented as a mean ± standard deviation. Tested for statistical significance using one-way ANOVA with Dunn's multiple comparison test for each treatment group versus phenol red. Phenol red vs Δ*tarO* CWP, P = 0.12; Phenol red vs *S. aureus* CWP-HF, P = 0.67.

5.4.4 *S. aureus* CWP must be particulate to induce protection against *C. neoformans*

Unlike soluble PRR agonists such as Pam2CSK4 and lipoteichoic acid, *S. aureus* CWP is an insoluble particulate antigen. Particulate antigens bind and are likely internalised by macrophages through phagocytosis. To determine whether the particulate form of *S. aureus* CWP was needed to induce protection, *S. aureus* CWP were treated with mutanolysin to solubilise the preparation. Mutanolysin cleaves β-*N*-acetylmuramyl-(1→4)-N-acetylglucosamine linkage of PGN without degrading WTA (Shimada et al., 2010). All types of mutanolysin-digested CWP including Δ*lgt* CWP or *S. aureus* CWP that showed efficacy in particulate form failed to induce innate immunity for protection against *C. neoformans* (Figure 25). These results profoundly suggest that a particulate form of *S. aureus* CWP is needed to induce protection.

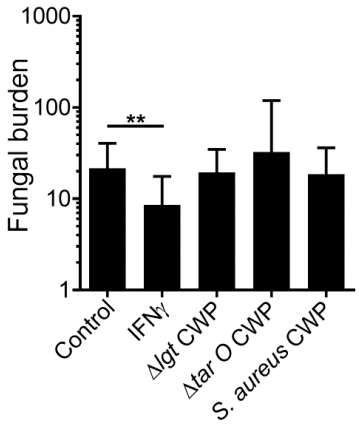

Figure 25: Mutanolysin-digested *S. aureus* CWP fails to induce protection against *C. neoformans* in zebrafish.

Fungal burden at 48hpi of 3dpf *nacre* larvae challenged with *C. neoformans* in phenol red alone or supplemented with either solubilised 5 mg/mL *S. aureus* CWP or 5 mg/ml Δ*tarO* CWP or 5 mg/mL Δ*lgt* CWP. Results are a representative of three independent experiments (n=36-47 per group) and data presented as a mean ± standard deviation and tested for statistical significance using one-way ANOVA with Dunn's multiple comparison test for each treatment group versus phenol red. Phenol red vs IFNγ, **P = 0.0019; *S. aureus* CWP, P = 0.25; vs Δ*tarO* CWP, P = 0.49; vs Δ*lgt* CWP, P = 0.6.

5.4.5 Immune correlates associated with *S. aureus* CWP protective effect

So far, I have shown that *S. aureus* CWP induces zebrafish innate defence against *C. neoformans*. I have also demonstrated that the glycopolymer wall teichoic acid, but not lipoproteins are required for this induction. In the sections below, I will try to decipher how *S. aureus* CWP promotes protective anti-cryptococcal defences. My work in Chapter 4 had shown that IFNγ-mediated protection was associated with increased recruitment of macrophages, phagocytosis and expression of IL-1 GFP. Therefore, I investigated whether *S. aureus* CWP effected innate resistance through the same.

5.4.5.1 *S. aureus* CWP is associated with increased macrophage recruitment

As I have shown in the previous chapter, a potential mechanism by which *S. aureus* CWP reduced fungal burden in zebrafish larvae is through the alteration of macrophage recruitment. To address this, I quantified the number of macrophages at the site of infection in phenol red-, Δ*tarO* CWP- and *S. aureus* CWP-treated larvae infected with *C. neoformans*. The Δ*tarO* CWP was used as a negative control to test for *S. aureus* CWP because the Δ*tarO* CWP did not induce protection. Macrophage recruitment in the Δ*lgt* CWP-treated larvae were also tested. There was a significant increase in the infiltration of macrophages at 2hpi but not at 24 hpi in the larvae injected with *S. aureus* CWP compared to Δ*tarO* CWP-treated larvae (2hpi $P = 0.001$; 24hpi $P = 0.051$; Figure 26a). Interestingly, larvae that received Δ*tarO* CWP showed intermediate recruitment of macrophages but significantly higher than phenol red treated larvae at 2 ($P = 0.002$) and 24 ($P = 0.02$) hpi (Figure 26a). Moreover, the number of macrophages remained unchanged between 2 and 24hpi in both the Δ*tarO* CWP and *S. aureus* CWP-treated larvae (Δ*tarO* CWP $P = 0.54$; *S. aureus* CWP $P = 0.58$; Figure 26a). The Δ*lgt* CWP also induced significantly higher macrophage influx than phenol red treatment at 2 and 24hpi (Figure 26b; 2hpi $P < 0.0001$; 24hpi $P = 0.01$) but the number of recruited macrophages was lower at 24hpi than at 2hpi in Δ*lgt* CWP-treated larvae ($P < 0.0001$; Figure 26b).

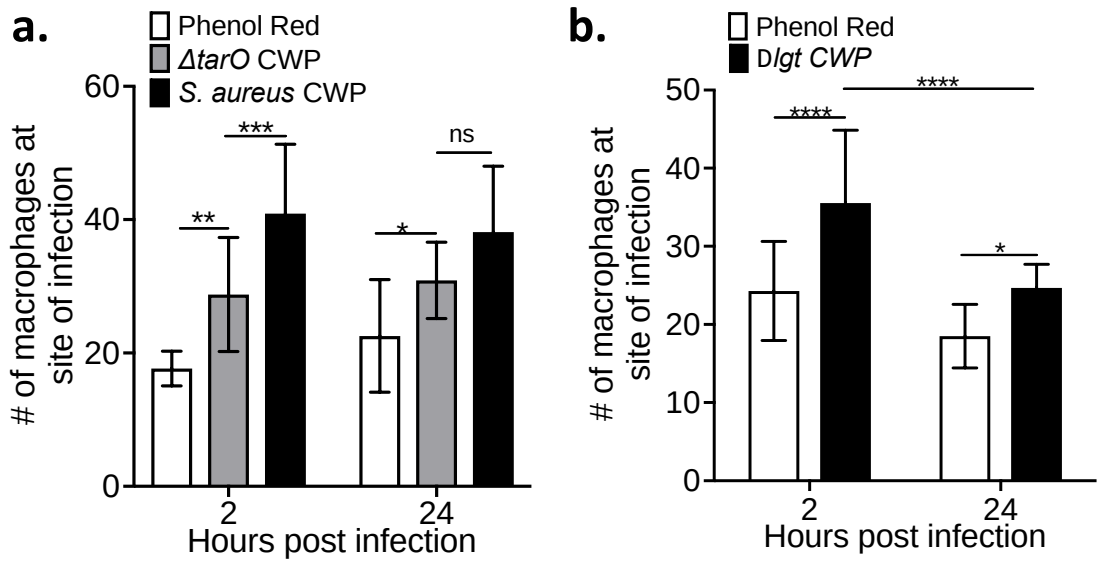

Figure 26: *S. aureus* CWP is associated with increased macrophage recruitment.

Number of macrophages at the site of infection at 2 and 24 hpi of 3dpf *Tg(mpeg1*:Gal4-VP16/UAS:Kaede) larvae challenged with *C. neoformans* in phenol red alone or supplemented with either (a) 5 mg/mL *S. aureus* CWP or 5 mg/ml Δ*tarO* CWP. Results are a representative of three independent experiments (n=9 per group) and data presented as a mean ± standard deviation and tested for statistical significance using two-way ANOVA and Dunnett's multiple comparison test for each treatment group versus Δ*tarO* CWP. At 2 hpi, Δ*tarO* CWP vs *S. aureus* CWP, ***P = 0.001; vs Phenol red, **P = 0.002. At 24hpi, Δ*tarO* CWP vs *S. aureus* CWP P = 0.051; vs Phenol red, *P = 0.02. Comparison between time-points, in Δ*tarO* CWP-treated larvae, 2hpi vs 24h P = 0.54; *S. aureus* CWP, 2hi vs 24hpi, P = 0.58). Data analysed using un-paired t-test (b) Δ*lgt* CWP data is a combination of three independent experiments (n=23-29 per group/exp.) and data presented as a mean ± standard deviation and tested for statistical significance using two-way ANOVA with Dunnett's multiple comparison tests, Δ*lgt* CWP vs Phenol red, ****P < 0.0001 at 2hpi, and *P = 0.01 at 24hpi. Comparison between time-points, in Δ*lgt* CWP -treated larvae, 2hpi vs 24 ****P < 0.0001, data analysed using unpaired t-test.

5.4.5.2 *S. aureus* CWP does not enhance phagocytosis of *C. neoformans* by macrophages

The ability of microglial cells to take up *C. neoformans* is increased *in vitro* following stimulation by TLR agonists, (Redlich et al., 2013). I hypothesised that induction of innate immunity with *S. aureus* CWP will enhance phagocytosis of *C. neoformans* by macrophages *in vivo*. Unlike IFNγ (see Chapter 4), macrophages in larvae treated with the *S. aureus* CWP did not have increased phagocytosis than Δ*tarO* CWP-treated larvae at 2hpi (P = 0.75; Figure 27).

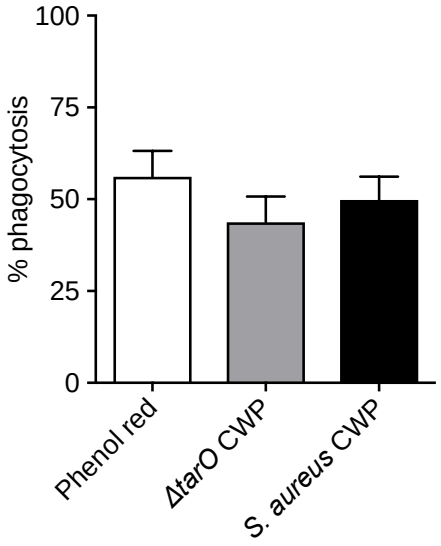

Figure 27: *S. aureus* CWP does not enhance phagocytosis of *C. neoformans* by macrophages.

Proportion of internalised fungal cells per larva at 2 hpi macrophages. Data is combination of two independent experiments (n=16-17 per group) and presented as %, however, statistical analysis was performed on raw data (total number of intracellular cryptococci vs total number of extracellular cryptococci) using Fisher's exact test Δ*tarO* CWP vs *S. aureus* CWP, P = 0.75; vs Phenol red, P = 0.34.

5.4.5.3 *S. aureus* CWP does not increase TNFα GFP expression in infected macrophages

I further evaluated whether *S. aureus* CWP modulated the behaviour of macrophages. I analysed the expression of the pro-inflammatory cytokine TNFα GFP, in zebrafish macrophages. To specifically follow TNFα expressing macrophages, a TNFα promoter BAC driving GFP transgenic zebrafish line *TgBAC(tnfa:GFP)pd1028* was crossed to a macrophage reporter line *Tg(mpeg1:mCherryCAAX)sh378,* to produce double transgenic line *TgBAC*(*tnfa:GFP);Tg(mpeg1:mCherryCAAX)*. Three dpf double transgenic larvae *TgBAC*(*tnfa:GFP);Tg(mpeg1:mCherryCAAX),* were injected with *C. neoformans* with phenol red or with Δ*tarO* CWP or *S. aureus* CWP. The expression of TNFα GFP was examined in macrophages with internalised cryptococci at 12, 24 and 36hpi. The cumulative relative fluorescent intensity of TNFα GFP was significantly higher in both *S. aureus* CWP (P = 0.004) and Δ*tarO* CWP (P = 0.003) at 12hpi compared to phenol red treated larvae. However, there was no significant difference in the cumulative relative fluorescent intensity of TNFα GFP in larvae that were treated with *S. aureus* CWP compared to Δ*tarO* CWP-treated larvae at 12 (P = 0.9), 24 (P = 0.951) and 36 (P = 0.602) hpi (Figure 28).

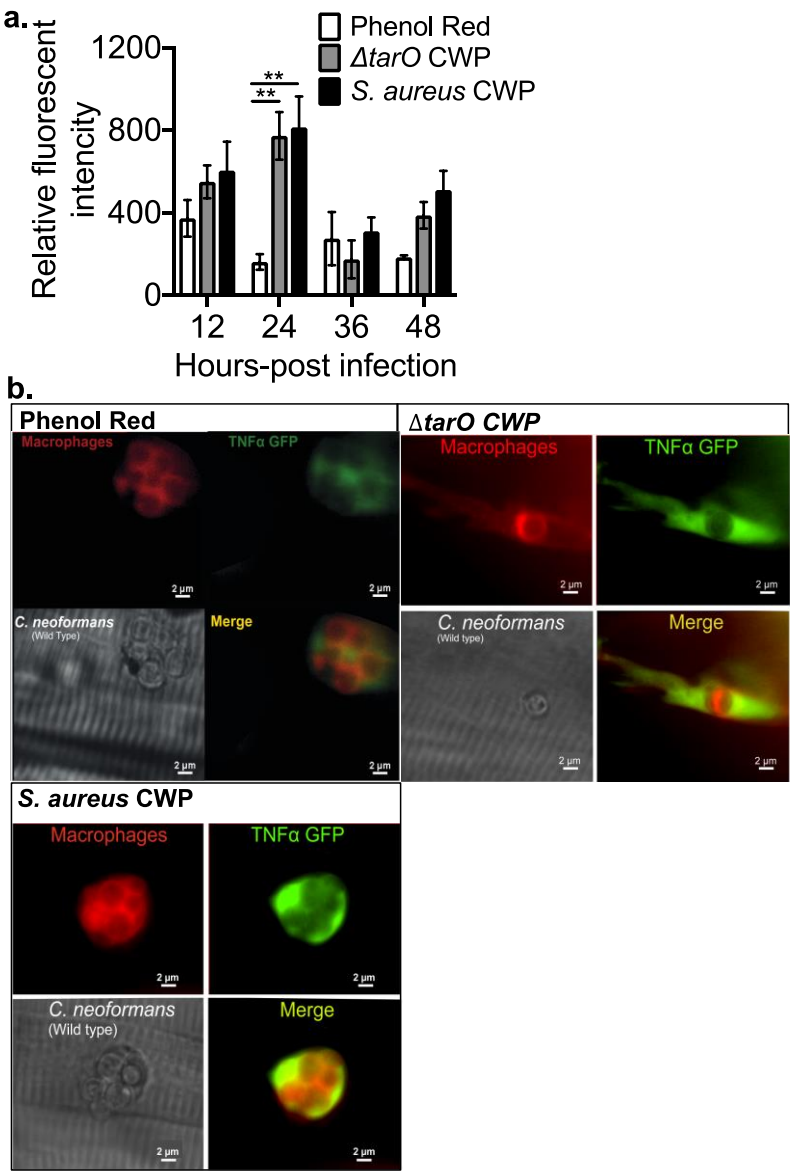

Figure 28: *S. aureus* CWP does not increase TNFα GFP expression in infected macrophages.

(a) Relative fluorescent intensity of TNFα GFP in 3dpf double transgenic larvae *TgBAC(tnfa:GFP);Tg(mpeg1:mCherryCAAX)* challenged with *C. neoformans* in phenol red alone or supplemented either with 5 mg/mL *S. aureus* CWP or with 5 mg/ml Δ*tarO* CWP. Results are a representation of three independent experiments (n=7-10 per group) and data presented as a mean ± standard deviation and tested for statistical significance using Ordinary one-way ANOVA with Dunnett's multiple comparison test for each treatment group versus Δ*tarO* CWP. At 24 hpi, Phenol red vs Δ*tarO* CWP, **P = 0.004; vs *S. aureus* CWP, **P = 0.003. (b) Representative fluorescent images z-stacks of the TNFα GFP expression in Phenol red, Δ*tarO* CWP and *S. aureus* CWP at 24hpi treated larvae, scale bar is 2µm

5.5 Discussion

Here I describe a new approach of stimulated innate immune resistance to *C. neoformans* that effectively reduced fungal burden by use of PRR agonists in a zebrafish infection model. Firstly, I show that TLR agonists including *Va*DNA and *S. aureus* CWP but not LPS and imiquimod reduced cryptococcal burden *in vivo*. Although LPS and imiquimod have been shown to exert innate immune resistance to *Fonsecaea pedrosoi* infection in mice (Sousa et al., 2011), they failed to display the same effect on *C. neoformans* infection in the zebrafish model. Besides, *Va*DNA and *S. aureus* CWP are TLR9 and TLR2 agonists respectively and induced protection. However, agonists CpG ODN and Pam2CSK4 of the same TLRs, failed to induce resistance. CpG ODN is recognised by zebrafish TLR9 and TLR21 (Yeh et al., 2013, Meijer et al., 2004) and mammalian TLR9 (Vollmer et al., 2004). Interestingly, immunisation with CpG ODN three days before cryptococcal infection has been shown to reduce cryptococcal burden in mice with pulmonary cryptococcosis via induction of IFNγ production by T cells (Edwards et al., 2005) perhaps that is why it takes longer to induce anti-cryptococcal innate resistance. This may explain why CpG ODN failed to induce protection in zebrafish larvae because they lack functional T cells (Edwards et al., 2005). In addition, the sequence of the CpG ODN motif used in the previous study is different from my study. It has been shown that different sequences of the CpG ODN motifs produce varied responses. For example, CpG ODN with GACGTT or AACGTT has a better activity to TLR9 while motifs that are recognised by both TLR9 and TLR21 trigger more potent cytokine production than those that bind to TLR9 or TLR21 alone (Yeh et al., 2013).

The mechanism of action of *Va*DNA reduction of cryptococcal burden requires further investigation because the corresponding TLR9 agonist, CpG ODN could not produce similar findings. Administration of *Va*DNA has been shown to increase expression of inflammatory genes in spotted green pufferfish but fail to induce NF-κB in MyD88 zebrafish morphants (Sepulcre et al., 2009). *Vibrio anguillarum* causes vibriosis in aquatic animals. Interestingly, *Debaryomyces hansenii* and *Yarrowia lypolitica* yeasts protect zebrafish from *Vibrio anguillarum*

infection by reducing bacterial burden and pro-inflammatory responses (Caruffo et al., 2016). Perhaps a synergistic or antagonistic effect exists between the yeast *C. neoformans* and *Va*DNA which leads to a required host response capable of suppressing cryptococcal growth.

Secondly, I identified that wall-teichoic acid but not lipoproteins are required for *S. aureus* CWP-mediated innate immune resistance to *C. neoformans*. WTA is widely known as a binding site of Gram-positive bacteria for uptake by host immune cells. WTA-deficient *S. aureus* does not adhere to polymorphonuclear leukocytes, dendritic cells and is not phagocytosed as efficiently as the wild-type strain (Kurokawa et al., 2013, Hong et al., 2017). WTA has been implicated in *S. aureus* attachment to nasal epithelial cells during nasal colonisation (Weidenmaier et al., 2008, Weidenmaier et al., 2004). WTA is also required for maturation and activation of DCs (Hong et al., 2017). Thus, WTA appears to facilitate bacterial adherence and internalisation by phagocytes leading to activation of host immune cells. Although I was unable to test wall teichoic acid alone, the presence of intact WTA in solubilised *S. aureus* CWP failed to induce innate immune resistance to *C. neoformans*. This suggests that free WTA alone may not induce protection and perhaps WTA attached to intact peptidoglycan is required to elicit protection against *C. neoformans* infection.

Interestingly, *S. aureus* has been shown to kill *C. neoformans in vitro* and the addition of soluble capsular polysaccharide prevents the killing (Saito and Ikeda, 2005). Killing occurs when the glycolytic enzyme of *S. aureus* recognises mannose residues in the backbone of GXM (Ikeda et al., 2007). However, the possibility of *S. aureus* CWP killing *C. neoformans* was ruled out because the fungi did not show any growth defects following co-incubation with *S. aureus* CWP. This suggests that stimulated innate resistance by *S. aureus* CWP is the only possibility that lead to reduced fungal burden in zebrafish. Recently at the 10th international conference of *Cryptococcus* and cryptococcosis, a poster by Tseng et al. (2017) presented the case of a 25-year-old patient who had both *S. aureus* and *C. neoformans* infection. The patient only developed signs of cryptococcal meningitis and showed positive CSF culture for *C. neoformans*

following commencement of antibiotics (Tseng, 2017). Perhaps these findings not only reveal that *S. aureus* may kill *C. neoformans in vivo* but also highlight the induction of anti-cryptococcal immunity by *S. aureus*.

Further work is required to identify receptors of WTA and PGN in zebrafish. Wall teichoic acid binds to MBL (Park et al., 2010). Other *S. aureus* cell wall components that bind to MBL include PGN (Ma et al., 2004) and LTA (Polotsky et al., 1996, Ip et al., 2008). MBL is a soluble CLR and is a member of the collectin family which is found in human serum and other animals (Holmskov et al., 2003). Human MBL binds to mannose and GlcNAc residues present on the glycopolymers of bacteria (Weis and Drickamer, 1996) and functions as an opsonin, activating the lectin complement pathway (Fujita, 2002). The activation of the lectin pathway induces opsonophagocytosis of *S. aureus* by PMNs in the serum of infants lacking Anti-WTA (Park et al., 2010). However, adult serum containing Anti-WTA immunoglobulin elicits opsonophagocytosis of *S. aureus* following binding of the antibody-WTA complex with C1q, leading to activation of the classical pathway (Park et al., 2010). Thus, I hoped to see increased phagocytosis in *S. aureus* CWP-treated larvae. However, uptake rates were not significantly higher at 2hpi and there is need to investigate the rates at later time points. The activation of the lectin pathway results in opsonisation of the pathogen by complement fragments including C4b and C2a. This leads to the formation of anaphylatoxins C3a and C5b that stimulate inflammation and act as chemoattractants of macrophages and neutrophils (Ricklin et al., 2010). Since I have shown that *S. aureus* CWP-treated larvae have higher influx of macrophages, thus it would be interesting to investigate whether this recruitment is mediated by MBL-WTA complex.

I further demonstrated that the particulate nature of CWP from *S. aureus* is essential for the activation of innate immune resistance. The particulate *S. aureus* PGN is required for activation of NLRP3 inflammasomes and secretion of IL-1β in mouse macrophages. This effect requires that the *S. aureus* PGN particles must be internalised via phagocytosis (Shimada et al., 2010). Nod-like receptor (NLR) family, including NOD1 and NOD2 recognise peptidoglycan in the

cytoplasm. NOD1 recognises peptidoglycan fragments of Gram-negative and some Gram-positive rods while NOD2 senses peptidoglycan fragments from all bacteria (Girardin et al., 2003, Chamaillard et al., 2003, Inohara et al., 2003). Although the functions of the NLR family members are not well characterised in zebrafish, the canonical members of their mammalian counterparts, NOD1, NOD2, and NOD3 are present (Stein et al., 2007). Morpholino-mediated depletion of Nod1 or Nod2 results in higher bacterial counts and reduced survival of embryos infected with *Salmonella enterica* (Oehlers et al., 2011) suggesting that NLRs play a vital role in innate antibacterial immunity in zebrafish. Whether zebrafish NLRs recognise bacterial peptidoglycan fragments remains to be elucidated. It is worth to note that ligand recognition by some but not all innate receptors in zebrafish correspond to their mammalian counterparts. For example, zebrafish TLR2 recognises lipopeptides and Pam3CSK4 (Yang et al., 2015, Meijer et al., 2004) which are both recognised by murine and human TLR2 (Kang et al., 2009, Jin et al., 2007, Zahringer et al., 2008, Omueti et al., 2005). However, zebrafish TLR4 is not responsive to LPS stimulation (Li et al., 2017), highlighting the similarities and differences between zebrafish and mammalian models.

On testing the expression of inflammatory cytokine TNFα, I found higher expression in both *S. aureus* CWP and Δ*tarO* CWP treatment compared to controls. However, the TNFα expression in *S. aureus* CWP treatment was not significantly higher than Δ*tarO* CWP treatment. WTA is not very inflammatory. Previous studies have shown that PGN but not WTA is required for *S. aureus* cell wall-mediated induction of TNFα (Leemans et al., 2003, Majcherczyk et al., 2003). This may explain why the two CWP induced similar levels of TNFα expression. WTA interfere with certain cellular immune responses. For example, *S. aureus* inhibits the secretion of superoxide in macrophages, to escape killing following phagocytosis (Watanabe et al., 2007). However, a WTA-deficient *S. aureus* strain induces increased superoxide concentration and is readily killed compared to wild-type *S. aureus* (Shiratsuchi et al., 2010). Thus, WTA acts to protect both the bacteria from killing and the host from inflammatory damage. It has also been shown that WTA interferes with the recognition of peptidoglycan

by PGRP-SA in *Drosophila* resulting in reduced production of AMP-like drosomycin (Tabuchi et al., 2010) suggesting that WTA act as a regulator of inflammation and less as a pro-inflammatory activator.

In this age of emerging pathogens, the development of antibiotic resistance and an increasing immunosuppressed population, infections are becoming hard to treat. The management of cryptococcal disease remains a challenge, particularly in the immunocompromised. Despite anti-fungal treatment in HIV-associated cryptococcal disease mortality remains excessively high (reviewed in Williamson et al., 2017), demonstrating a pressing need to develop new therapeutic strategies to successfully treat this disease. Well-characterised interactions are required to identify treatments that target PRRs, particularly the TLRs. The TLRs fulfil most of the requirements as potential treatment targets. These include diminished disease resistance in knockout animal models, overexpression in disease and genetic alteration in TLRs or signalling molecules are associated with a risk of disease in humans. Despite few examples of effective interventions that target TLRs currently in use in the clinic, one profound example, imiquimod, stands out. Imiquimod is a TLR7 and TLR8 agonist that was approved over a decade ago for treatment of genital warts and keratosis (Lebwohl et al., 2004, Stockfleth et al., 2004). Recently imiquimod showed efficacy in four cases of chromoblastomycosis, a chronic skin infection caused by the fungus *Fonsecaea pedrosoi* (de Sousa et al., 2014). This is also a prime example of local induction of host immunity and resonates with my findings. *S. aureus* CWP is potent at enhancing local innate immunity to suppress *C. neoformans* infection in a somite zebrafish model.

Several studies have examined the administration of bacterial or fungal lysates or whole-heat killed microbes to induce protective defences against infection (Liu et al., 2011, Clemons et al., 2014, Braido et al., 2007). However, the undefined nature of these preparations becomes problematic when designing therapies for human use. In light of this, I identified that wall-teichoic acids but not lipoproteins are required for *S. aureus* CWP augmentation of anti-cryptococcal innate immune defences. Although there is need for further characterisation, it highlights the

potential of using well-defined PAMPs as the basis for developing immunomodulatory anti-infective treatments.

There are still questions as to whether severely immunocompromised patients, such as those at risk of *C. neoformans* infection can mount a robust immune response following treatment with PRR agonists. A good example is the use of imiquimod in HIV-positive patients for the treatment of neoplastic human papillomavirus-associated conditions produce positive outcomes (Conley et al., 2002, De Panfilis et al., 2002, Silverberg et al., 2002, Richel et al., 2013, Fox et al., 2010). This indicates that immunomodulatory treatment is relevant in the severely immunocompromised patients. However, prolonged treatment is required and recurrences usually occur (Richel et al., 2013, Fox et al., 2010).

Considering the diversity of patients at risk of cryptococcosis, the target population for such treatment strategies will have to be identified. Besides, the use of zebrafish, which mainly rely on innate immunity, can provide insights into immune responses to immunomodulation therapy in the immunocompromised host. Overall, my findings have clearly identified a new compound to stimulate local innate immunity that may be exploited therapeutically to treat *C. neoformans* infections.

CHAPTER 6

DISCUSSION AND FUTURE WORK

6.1 Anticryptococcal Immunomodulation

The ability to modify specifically the immune system has long shown success in the treatment of many diseases. Immunomodulation involves the direct elicitation of host innate defences and enabling natural host directed killing mechanisms to control pathogens. Most current immunomodulatory treatments typically work by correcting an acquired or congenital immune system defect or by enhancing the immune responses to a pathogen that poorly provokes an immune response. Immunomodulation approaches share a number of advantages. They include, being intrinsically incapable of endangering the development of resistance of the targeted pathogen, broad-spectrum activity and minimal side effects. Current clinical use and ongoing clinical trials are based on a mass of evidence from animal models. The evidence demonstrates that immunomodulation by the administration of cytokines or other immune modulatory agents such as PRR agonists can act as potent treatments to stimulate the clearance of an existing infection.

Despite the treatment with anti-fungal regimens, patients with HIV/AIDS-associated cryptococcal meningitis show reduced response. Mortality levels are prohibitively high among these patients and survivors often require lifelong anti-fungal therapy for prevention of relapses. The pre-requisite of a successful treatment is the clearance of fungal cells from the infected site. Macrophages are highly specialised for host defence against *C. neoformans.* However, their effector functions are defective in the presence of very low $CD4^+$ T cells. The current treatments mainly target the pathogen and are unable to resolve macrophage inactivity. Thus, there is a demand to identify novel therapeutic strategies that specifically target macrophage activation mechanisms to clear *C. neoformans* infection in the absence of $CD4^+$ T cells.

The zebrafish model is ideal for screening compounds as it takes a short period to obtain results. Zebrafish can be placed in a niche between *in vitro* cell-based assays and *in vivo* mammalian models. Testing several compounds in murine models can be cumbersome because infection establishment and resolution take time. It is comparatively straightforward to perform infection burden and host-pathogen interaction assays on a zebrafish larva, enabling the identification

and interrogation of potential immune modulatory compounds that elicit the desired activity.

6.2 The zebrafish model of *C. neoformans* infection

I have developed a zebrafish model of *C. neoformans* infection and designed an infection outcome assay. The aim was to identify potential immunomodulatory compounds that could drive suppression or clearance of infection and examine their mechanisms of action. In this model, *C. neoformans* infection can be cleared, controlled or fail to be controlled leading to exponentially high fungal counts. By taking advantage of the zebrafish transparency, fungal cells can be enumerated in live fish larva during infection. Because of the localised nature of the infection, most detailed *in vivo* analysis of the interaction of *C. neoformans* and immune cells was carried out. Thus, the immune response to *C. neoformans* can indeed be interrogated and manipulated therapeutically using this zebrafish model.

6.3 Immune correlates of IFNγ-mediated protective immunity against cryptococcal infection

6.3.1 What do we know so far? – *In vitro*, murine and human studies

The use of IFNγ in preventing or treating infectious disease is not a new phenomenon. In clinical practice for example, IFNγ is given as a prophylaxis to prevent infection in patients with chronic granulomatous disease (Marciano et al., 2004). The investigations of IFNγ as a potential agent to elicit anti-cryptococcal activity in effector cells *in vitro* or *in vivo* date back nearly 30 years. The therapeutic potential of IFNγ has been studied predominantly in macrophages *in vitro*, mice experimental cryptococcosis and human cryptococcal disease. There appears to be a difference in IFNγ signalling in mice and human macrophages in *in vitro* assays. IFNγ has been clearly demonstrated to increase the fungicidal effect of mouse macrophages (Flesch et al., 1989). However, in human macrophages, IFNγ treatment results in no change in intracellular proliferation rates (Voelz et al., 2009). In fact, another study showed increased intracellular replication in human macrophages upon addition of IFNγ *in vitro* (Reardon et al., 1996). In mice, treatment with IFNγ using a *C. neoformans* strain engineered to produce IFNγ, leads to reduced fungal burden. This infection is

accompanied by increased leukocyte recruitment, the classically activated macrophages that is shown by iNOS expression mediated via STAT1, and the production of Th1-type cytokines such as IFNγ, TNFα, IL-1α, IL-2, IL-12 p40, p70, IL-17, G-CSF, MIP-1α and RANTES (Wormley et al., 2007, Hardison et al., 2010, Hardison et al., 2012b, Leopold Wager et al., 2014). The Th2-type response appears to be detrimental in mice. It is characterised by the production of IL-10, IL-4 and IL-13, and abrogated fungicidal activity by innate effector cells (Arora et al., 2011, Hardison et al., 2012b, Wozniak et al., 2012). The administration of IFNγ in patients with HIV-associated cryptococcal meningitis significantly increased the rate of clearance of cryptococcal infection from the CSF (Jarvis et al., 2012). Survivors have a higher proportion of IFNγ-, TNFα-producing $CD4^+$ T cells (Jarvis et al., 2013) and higher levels of pro-inflammatory cytokines in their CSF, including IL-6, IL-8, TNFα and IFNγ (Siddiqui et al., 2005). Non-survivors have higher levels of MCP-1 and MIP-α than survivors (Jarvis et al., 2013). In contrast to findings in mice, non-survivors of human cryptococcal disease do not display increased Th2-type cytokine production. In fact, survivors have higher levels of IL-10 (Jarvis et al., 2013; Figure 29).

6.3.2 What has this research revealed? – Zebrafish

In zebrafish, my findings from Chapter 4 reveal that treatment of IFNγ results in increased clearance of *C. neoformans* infection. The immune correlates for IFNγ-mediated protective immunity include increased recruitment of phagocytes to the site of infection and increased fungal uptake by macrophages. Besides, most fungal cells are taken up by macrophages, and macrophage-deficient larvae fail to clear or suppress cryptococcal infection despite treatment with IFNγ. In addition, there is low lysosomal pH and elevated expression of IL-1 in infected macrophages of IFNγ-treated larvae (Figure 29). Furthermore, my results reveal that neutrophils do not play an essential role in *C. neoformans* infection. Although neutrophils take up the fungus, their depletion does not alter the *C. neoformans* burden.

6.3.3 Where do we go from here?

6.3.3.1 Recruitment of macrophages

Following infection of mice by *C. neoformans*, there is a mass recruitment of leukocytes to the lungs (Wormley et al., 2007). The alveolar macrophages are one of the first immune host cells to interact with *C. neoformans* as evidenced by the frequent presence of the fungi in lung macrophages (Feldmesser et al., 2000). External signals, particularly cytokines such as IFNγ, induce the recruitment of phagocytes (Gordon, 2003). However, the mechanism of how IFNγ mediates macrophage recruitment is poorly understood. CCL2 (MCP-1) is a chemokine that mediates monocyte chemotaxis and binds to its receptor CCR2 (Deshmane et al., 2009). Previous studies have shown that CCR2 deficient mice have impaired accumulation of macrophages and diminished clearance of *C. neoformans* (Traynor et al., 2002, Traynor et al., 2000). Macrophages primed with IFNγ display increased expression of CCR2 and exhibit enhanced migration in response to CCL2 (Hu et al., 2005). Recently, a functional zebrafish CCR2 orthologue has been identified. Zebrafish larvae that are deficient in CCR2 have reduced macrophage migration in response to human CCL2 (Cambier et al., 2014). Thus, it would be interesting to investigate whether IFNγ enhances expression of zebrafish CCR2.

In addition, further work is required to determine more immune correlates of IFNγ effects. For example, molecular factors involved in chemotaxis of phagocytes, uptake, killing of fungal cells, and signalling pathways in zebrafish have not been identified. An interesting way to investigate this would be to use RNAseq to define transcriptome features associated with IFNγ treatment. To do this, cells can be obtained from zebrafish larvae from infected sites as described previously (Nguyen-Chi et al., 2015). Macrophages can be isolated using flow cytometry for RNAseq. Expression profiles from RNAseq data of cleared infections can then be compared with unresolved infections. Blocking with antibodies, using morpholinos or generation of transgenic lines can be used to confirm which hits obtained from the RNAseq may be essential for clearance. Imaging can also provide valuable information regarding phenotypic behaviours of macrophages and their interactions with *C. neoformans* in the transgenic zebrafish lines.

6.3.3.2 Phagocytosis

Phagocytosis is mediated via the attachment of antibodies or complement factors to pathogens. IFNγ has been shown to activate FcγRI, a receptor for IgG-type antibodies (Pan et al., 1998, Mendoza-Coronel and Ortega, 2017), increase expression of early components of complement cascade, including C1q, C3 and C4a and induce antibody/complement mediated opsonophagocytosis (Chakrabarty et al., 2010, Barnum et al., 1992, Mitchell et al., 1996). Both FcγR (Yoder et al., 2007) and some complement components have been identified in zebrafish and display similar functions demonstrated in humans (reviewed in Zhang and Cui, 2014). Considering that zebrafish larvae used in my study may not have developed antibodies, perhaps IFNγ mediates phagocytosis via activation of the complement cascade. Further experimentation is required to determine whether complement components are essential in fungal uptake by zebrafish immune effector cells.

6.3.3.3 IL-1

One other finding of the immune mechanism of IFNγ was that of IL-1β induction. Unlike TNFα production in human monocytes, IL-1β is not inhibited by purified capsular polysaccharide nor by inhibition of TNFα (Vecchiarelli et al., 1995). IL-1β is associated with protective immune responses in the *C. neoformans* infected brains of mice (Uicker et al., 2005). In fact, transcriptome analysis of monocytes following exposure to *C. neoformans* revealed increased expression of IL-1β (Chen et al., 2015). However, there are conflicting reports of the effect of IFNγ on IL-1β production. Several studies have shown that IFNγ can inhibit the production of IL-1β in LPS stimulated macrophages (Ghezzi and Dinarello, 1988, Ruschen et al., 1989, Schindler et al., 1989, De Boer et al., 2001) via the STAT1 dependent pathway (Ghezzi and Dinarello, 1988). In contrast, other studies have reported increased production of IL-1β by IFNγ following stimulation by certain TLR agonists such as *S. aureus* (Boraschi et al., 1984, Arenzana-Seisdedos et al., 1985, Gerrard et al., 1987, Burchett et al., 1988, Schindler et al., 1990) which in my study may be facilitated by *C. neoformans*. Although the induction of IL-1β has only been shown in a single graph and time point, it would be particularly useful to investigate further to ascertain if IL-1β is particularly required in host immunity against *C. neoformans*. An interesting strategy to test this would be to

adapt the infection assay but use IL-1$^{-/-}$ zebrafish larvae with or without IFNγ to determine whether IL-1 is essential for protection or whether IFNγ protective activity is mediated via IL-1. Interestingly, IFNγ inhibits IL-1β in mice cells but promotes IL-1β production in human cells (Masters et al., 2010). This suggests why IFNγ treatment sometimes may not be beneficial in a human disease, though success is demonstrated in mouse models of the disease. Thus, diseases outcomes may differ among vertebrates.

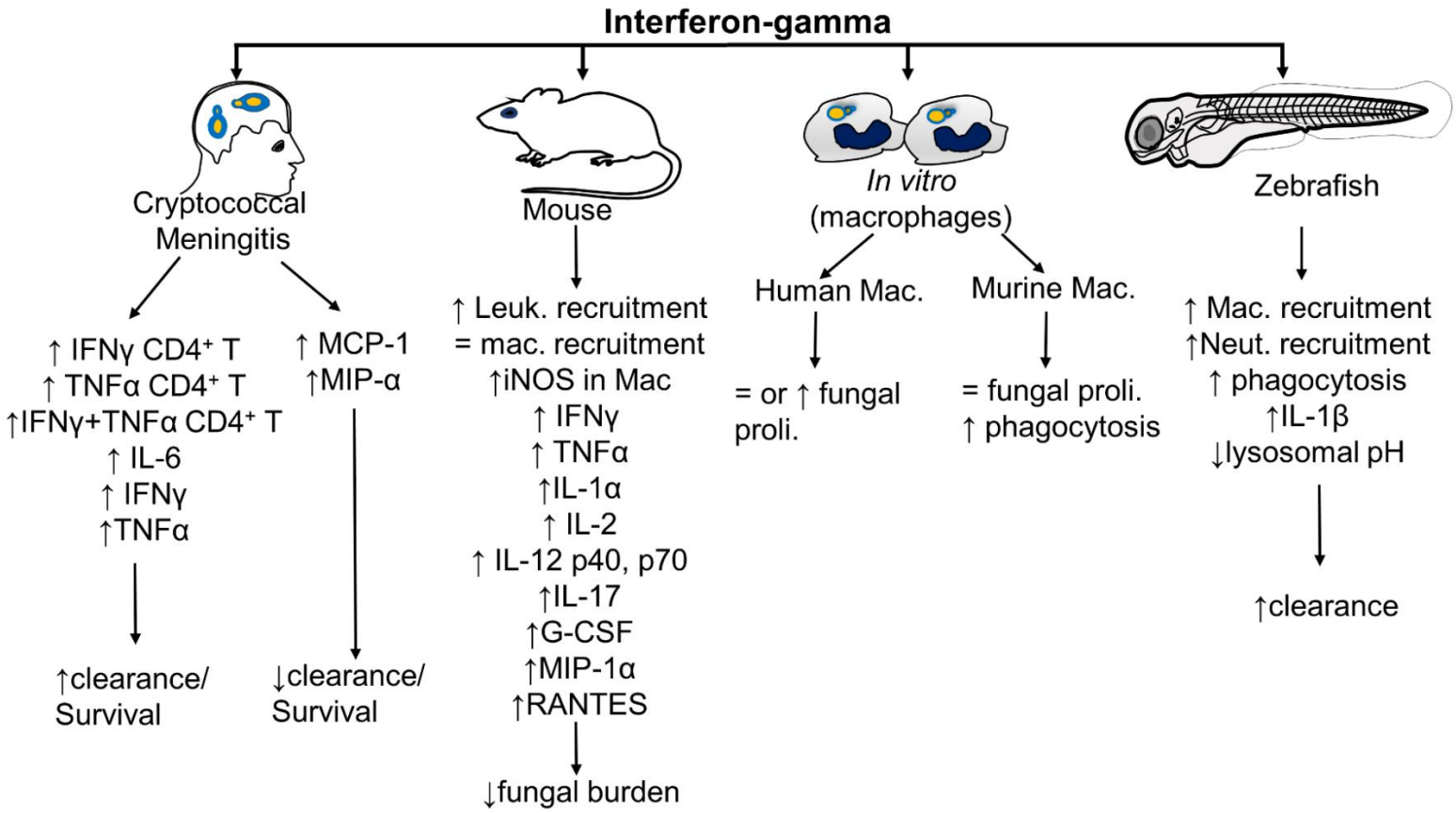

Figure 29: Summary of immune correlates of IFNγ-mediated protective immunity against cryptococcal infection

↑- increased; ↓- reduced; = - no change; Leuk. – Leukocytes; mac. – Macrophages; proli. – Proliferation.

6.4 Immunomodulation via activation of TLR signalling to treat *C. neoformans* infections

To date, there is only one published study that has shown the exploitation of the TLR signalling for treatment of *C. neoformans*. In this study, immunisation with CpG ODN three-days prior to cryptococcal inoculation resulted in reduced fungal burden in mice with pulmonary cryptococcosis (Edwards et al., 2005). Recently, it was shown that treatment of microglia cells with PAM3CSK4 and CpG ODN resulted in activation. The activated microglial cells showed increased phagocytosis activity, enhanced intracellular killing of *C. neoformans* and increased secretion of TNFα, CXCL1, IL-6, IL-10 and MIP-2 (Redlich et al., 2013). These studies highlight the potential use of agonists for the treatment of cryptococcal infections.

6.4.1 What has this research shown?

6.4.1.1 *S. aureus* CWP is a potent inducer of innate defences against *C. neoformans*

Perhaps the most exciting outcome of this work is the identification of a compound that can induce innate immune resistance to *C. neoformans in vivo*. I demonstrate that *S. aureus* CWP is a potent inducer of innate immune resistance to *C. neoformans*. *S. aureus* cell wall components are recognised by TLR2. However, a synthetic TLR2 agonist, Pam2CSK4, failed to elicit innate immune resistance to *C. neoformans*. An involvement of more than one TLR in *S. aureus* CWP efficacy could be a possible reason for the discrepancy. I have also shown that wall teichoic acids, but not lipoproteins, are required for *S. aureus* CWP-mediated protection against cryptococcal infection in zebrafish.

6.4.1.2 What are the potential receptors and signalling pathways involved?

At this stage, the actual receptors of wall teichoic acid and/or peptidoglycan in zebrafish have not been identified. Therefore, further work is required to determine the receptor(s) and the signalling pathway(s) that are involved. The potential targets might be TLR2 and NOD1/2, as peptidoglycan (PGN) has been

shown to bind to these receptors (Dziarski and Gupta, 2005, Chamaillard et al., 2003, Dziarski et al., 1998). However, the role of TLR2 as the major PGN receptor has been challenged (Travassos et al., 2004). The NOD1/2, which are located in the cytosol are recruited to the plasma membrane. After recognition of peptidoglycan during bacterial invasion, NOD1 or NOD2 binds to Rip2. The ubiquitination of K63 of Rip2 leads to initiation of NF-κB and MAPK signalling and subsequent chemokine and cytokine production (Reviewed in Fujimoto et al., 2012) (Figure 30).

On the other hand, the potential targets of wall teichoic acid receptors would be MBL (Baur et al., 2014) (See chapter 5 for further details) and a recently reported class F scavenger receptor termed Scavenger receptor expressed by endothelial cell-I (SREC-I). SREC-I is present on nasal human epithelial cells and binds to WTA. Addition of SREC-I antibody results in reduced nasal colonisation in a murine model (Baur et al., 2014). Alias SCARF1, SREC-1 is known to bind to LDL, LPS, apoptotic bodies, Hsp70, Hsp90, calreticulin, gp96, and zymogen granule protein 2 (Reviewed in Murshid et al., 2016). SREC-1/SCARF1 has been shown to trigger an inflammatory immune response in collaboration with plasma membrane TLRs including TLR2, TLR3 and TLR4. For instance, SREC-I/SCARF1 in association with TLR2 recognise β-glucans from *C. neoformans* and induces the production of IL-1β, CXCL2 and CXCL1 (Means et al., 2009). The downstream signalling of SREC-1/SCARF1 have been shown with the ligand Poly I:C in collaboration with TLR3. The complex leads to activation of NF-κB, MAPK and IRF3 signalling and production of the pro-inflammatory cytokines such as IL-8 and IL-6 (Murshid et al., 2015a). Interestingly, IL-1α, IL-1β, and TNFα were demonstrated to hinder SREC-I/SCARF1 promoter activity, possibly denoting a negative feedback loop to limit inflammatory signalling in circumstances where levels of pro-inflammatory cytokines are high (Adachi and Tsujimoto, 2002). A zebrafish homology of the human scarf-1 has been shown to be expressed in endothelial cells (Sumanas et al., 2005).

Thus, further experimentation would be to test whether similar receptors are involved in zebrafish. An interesting way to identify receptors and signalling

pathways involved would be to use the same approach described above (Section 6.3.3.1) in which macrophages in treated and untreated larvae can be isolated and their transcription profile can be characterised using RNAseq. To identify receptors of *S. aureus* CWP, expression levels of potential receptors could be compared to untreated larvae. Zebrafish knockouts of the potential receptors identified by RNAseq can be generated using CRISPR

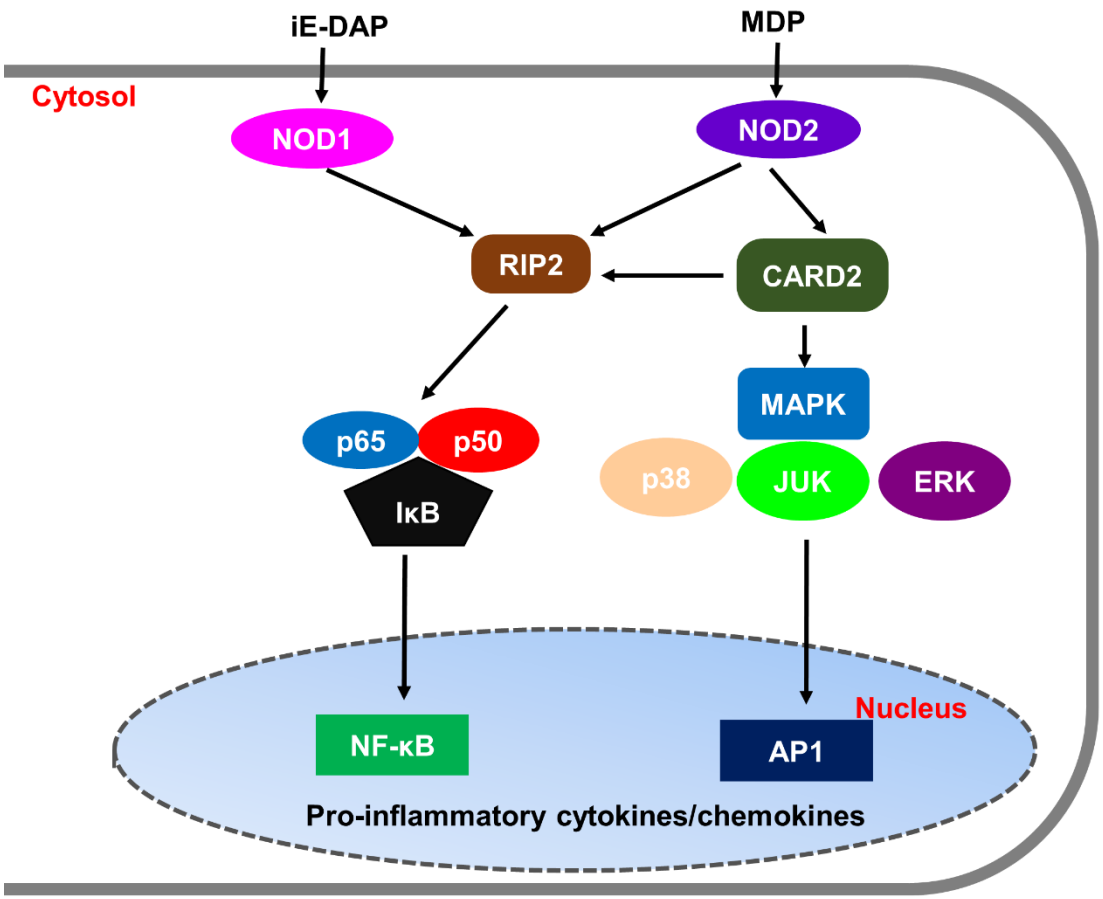

Figure 30: Schematic diagram of NOD1 and NOD2 signalling pathways

NOD1 and NOD2 recognise peptidoglycan fragments iE-DAP (γ-D-Glu-mDAP) and MDP (Muramyl dipeptide) respectively. NOD induced pro-inflammatory signalling involving NF-κB and MAPK transcription factors through recruitment of common adaptor RIP2 by homophilic CARD-CARD interactions. This follows phosphorylation of IκBα and freeing of NF-κB subunits, p50 and p65 and at the same time, TAK1 recruits TAB1 and TAB2 leading to activation of MAPK (ERK, p38 and JNK) pathways. Induction of both pathways culminates in the production of pro-inflammatory cytokine.

6.4.1.3 Cellular response of other phagocytes

Further work is required to test whether *S. aureus* CWP affects other effector cells such as neutrophils in exerting its influence. Neutrophils are required for immune defences against *S. aureus* infections. Individuals with neutrophil deficiencies are highly at high risk of severe *S. aureus* infections (Lekstrom-Himes and Gallin, 2000). Therefore, performing experiments to assess neutrophil infiltration and phagocytosis in the presence of *S. aureus* CWP treatments might be informative along with whether the effect on fungal burden is maintained following depletion of macrophages. Investigating phenotypic changes of immune cells such as shape, movement and speed could also give valuable information.

6.4.1.3 *Va*DNA efficacy, what's next?

Although I also demonstrated that *Va*DNA elicited innate immune resistance to *C. neoformans*, its corresponding TLR9/21 agonist CpG ODN 2395 did not. The administration of *Va*DNA and CpG ODN 1668 in zebrafish cause increased TLR21 expression (Gao et al., 2013). *Va*DNA also causes increased expressions of *Il-1b*, *Il-6* and *tnfα* in zebrafish (Sepulcre et al., 2009). *Vibrio anguillarum* (*Va*) is one of the main fish pathogens that causes septicaemia. This may explain the strong effect of *Va*DNA on the outcome of cryptococcal infection due to its ability to activate zebrafish TLR21. It would be worth mentioning that the preparation of *Va*DNA is not clean and may be contaminated with LPS or any other protein which may have caused this effect. To investigate whether a protein may be involved, *Va*DNA can be treated with DNAse and then tested for efficacy. On the other hand, to test whether the DNA of *Vibrio anguillarum* is solely responsible for this effect, the *Va*DNA prep could be boiled to denature any proteins present and then tested for efficacy. In addition, it has been shown that activation of the TLR21 depends on the sequences of the CpG ODN motifs. Perhaps testing for CpG ODN 1668 as opposed to CpG ODN 2395 would trigger the same immune signalling as *Va*DNA and provide protection. However, whether these findings are translatable to mammalian models considering they lack TLR21 requires further exploration.

6.5 Benefits versus potential side effects, how to strike the balance?

The strategies of immune modulation employed in this project have the advantage of being intrinsically unable to engender resistance of *C. neoformans*. A potential caution against their use in the clinic is the necessity to establish a proper balance where an immunomodulatory compound induces an effective host immune response and controls infection but also limits host damage through inflammation. Since most immunomodulatory agents are typically non-specific in their induction of immune responses, it would be ideal to measure the consequences of such stimulation using zebrafish inflammation model. Inflammation resolution assays are well established in zebrafish. This can include determining the inflammation resolution by the numbers of neutrophils or macrophages at the site of infection following treatment with immunomodulatory agents. Clearance of immune cells particularly neutrophils from the site of infection is essential for successful inflammation resolution (Savill et al, 1989). Thus, it would be particularly useful to carry out resolution assays in addition to immune enhancement experiments.

6.6 Overall strategy

Concomitant administration of *C. neoformans* with immune modulatory compounds markedly enhanced anti-cryptococcal immunity. However, immunomodulatory compounds can be administered as prophylaxis in high-risk groups to boost immunity against subsequent infections. For example, cryptococcal meningitis usually occurs in individuals that are severely immunocompromised i.e. those that have less than 100 $CD4^+$ T cells/mm^3. Such patients can benefit from prophylactic treatments. To explore the prophylactic potential of these compounds to cryptococcal infection using this model, immunomodulatory agents could be injected at least 24 hours before inoculation of *C. neoformans*.

6.7 Thesis Summary

To summarise, in this project I have described the intramuscular zebrafish-*C. neoformans* infection and immunomodulatory model. This has been possible by exploiting the special advantages of transparency and ability to create

localised infection. I aimed to test IFNγ and several TLR agonists' ability to stimulate host innate immunity and their subsequent mechanism to control *C. neoformans* infection in zebrafish. Using this model, I have successfully identified the induction of innate immunity that can suppress *C. neoformans* infection by IFNγ and two other TLR agonists namely *Va*DNA and *S. aureus* CWP. Using transgenic zebrafish lines, I established that macrophages are required for IFNγ-mediated control of *C. neoformans*. The most interesting of these was the discovery of *S. aureus* CWP to induce the host innate immunity to arrest *C. neoformans* infection and that WTA but not lipoproteins are essential for this mechanism.

More work will be required to determine the mechanism of immunomodulation and its mediation of infection clearance. This will involve identifying receptors, signalling pathways in zebrafish and characterisation of effector cells involved. This work demonstrates that the zebrafish larva is an exciting new animal model to study immunomodulatory therapy for *C. neoformans* infection.

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