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Effect of the Capsular Material of *Cryptococcus neoformans* on the interplay between Microglial cells and Neutrophils

by

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SUPERVISOR'S STATEMENT

As the candidate's supervisor, I agree to the submission of this thesis.

Signed: _____ Date: 2-10-14

Prof A. Willem Sturm

AUTHOR'S DECLARATION

I, Dr P Bhola, hereby declare that:

This thesis contains my own work except where specifically acknowledged, and all experiments were carried out in the department of Infection Prevention and Control, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, under the supervision of Professor A W Sturm.

This research has not been previously submitted to the University of KwaZulu-Natal or any other tertiary institution for the purposes of obtaining any other degree or academic qualification.

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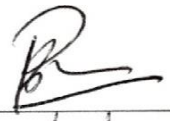
Signed: 

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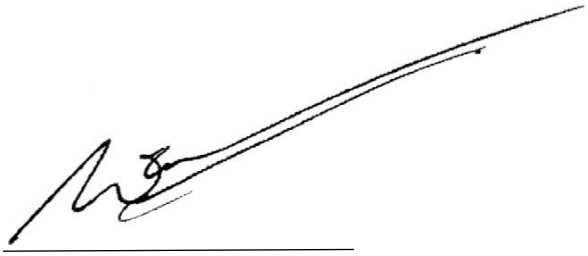
Signed: 

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ETHICS DECLARATION

The Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BE094/12) granted approval for this PhD project.

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LIST OF ABBREVIATIONS AND ACRONYMS

°C	degrees Celsius
AIDS	Acquired Immune Deficiency Syndrome
ART	Anti-Retroviral Therapy
ATCC	American Type Culture Collection
BBB	blood brain barrier
BHI	Brain Heart Infusion
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
CLAT	cryptococcal latex agglutination test
CLR	C-type lectin receptor
CNS	central nervous system
CO_2	carbon dioxide
CR	complement receptors
CSF	cerebrospinal fluid
DC	dendritic cell
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylene-diamine-tetra-acetic acid
GalXM	galactoxylomannan
GXM	glucuronoxylomannan

HIV	human immuno-deficiency virus
HRP	horseradish peroxidase
IFNγ	interferon gamma
IL-1β	Interleukin-1 beta
IL-8	Interleukin-8
LP	lumbar puncture
MCP-1	monocyte chemoattractant protein 1
MIP-1α	macrophage inflammatory protein form 1 alpha
MOI	multiplicity of infection
MP	mannoprotein
MPO	Myeloperoxidase
NET	neutrophil extracellular trap
NK	natural killer
NOD	nucleotide-binding oligomerization domain
OD	optical density
OP	opening pressure
PAMP	pathogen associated molecular pattern
PCR	polymerase chain reaction
PMNL	polymorphonuclear leucocytes
PRR	pattern recognition receptor
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	revolutions per minute

RPMI	Rosewell Park Memorial Institute
RT	reverse transcriptase
SA	South Africa
SOD	superoxide dismutase
Th1	helper T cell type 1
Th2	helper T cell type 2
TLR	toll-like receptor
TMB	tetramethylbenzidine
TNF-α	tumour necrosis factor alpha
µl	microliter
µm	micrometer
× g	g-force

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SUMMARY

Cryptococcal meningitis is an important opportunistic infection in immunocompromised patients. It has been well established that a distinguishing feature of this form of meningitis is a relatively low neutrophil count in the cerebrospinal fluid (CSF) compared to bacterial meningitis. There has been speculation and research undertaken previously to understand this phenomenon, however, little information is available in human studies. Furthermore, there is insufficient information on expression and function of Toll-like receptors (TLR) in the human central nervous system (CNS). The work presented here investigated the effect of the capsular material of a series of clinical isolates of *Cryptococcus neoformans* on neutrophil recruitment at the site of infection and determined whether downregulation occurs at the level of TLR expression. This was done in a multiple component study.

Clinical information was collected from patients with cryptococcal meningitis and baseline blood and CSF investigations were performed, which included the quantification of neutrophils in CSF and blood specimens. The size of the Cryptococcus capsule was measured in each isolate and shed capsular material was quantified in individual CSF specimens. The extent of neutrophil chemotaxis inhibition by individual strains of *C. neoformans* was determined by using a Transwell migration assay. Toll-like receptor (TLR)2 and TLR4 gene expression induced by individual *C. neoformans* isolates in human microglial cells was quantified. The possible associations among these experiments were subsequently evaluated.

As anticipated, a paucity of neutrophils in the CSF was observed. The cryptococcal capsule was larger in isolates of patients with lower CSF neutrophil counts. In addition, patients with lower CSF neutrophil counts shed more capsular material in the CSF. Chemotaxis inhibition occurred in close to 70% of tested isolates. The concentration of shed capsular material in this group was higher compared to the group with no chemotaxis inhibition. Patients presenting with fever had higher CSF neutrophil counts as well as elevated intracranial pressures. The majority of isolates expressed downregulation for TLR2 and TLR4 in microglial cells exposed to *C. neoformans.* CSF neutrophil counts were lower in this group.

These findings imply that the capsular components of *C. neoformans* downregulated recruitment of neutrophils into the CSF. Downregulation of neutrophil recruitment was observed at the level of TLR expression.

CHAPTER 1 – INTRODUCTION

1.1 Background and Research Rationale

Cryptococcus neoformans is a facultative intracellular pathogen. This fungal pathogen is known to cause meningitis in the immunocompromised patient. The capsule of *Cryptococcus neoformans* is an established virulence factor. It has been shown previously that the cryptococcal capsule can change in size in response to environmental conditions [1, 2].

It is well known that a characteristic of cryptococcal meningitis is the relatively low number of leucocytes in the CSF as compared to bacterial meningitis [3-5]. This is associated with a protracted versus an acute clinical presentation. The presence of leucocytes is regulated through chemotaxis. Chemotaxis is dependent on chemokine production at the site of infection and the level of expression of corresponding chemokine receptors on the migrating cells. Chemokines are produced when TLRs on cells at the infection site recognize specific microbes. For *C. neoformans*, TLR2 and TLR4 have been identified as important TLRs [6-8]. TLR2 and TLR4 are membrane protein receptors that are expressed on certain cell surfaces and together with CD14 are able to recognize various microbial products. When these receptors are activated, signaling cascades are triggered which result in a proinflammatory response [8]. While it is evident that TLR expression is significant, there is not much published information on TLR expression and function in human CNS glial cells [7].

High levels of the cryptococcal capsular components glucuronoxylomannan (GXM), galactoxylomannan (GalXM), mannoprotein (MP)-1, and MP-2 in the CSF and blood of affected patients are a characterististic feature of disseminated cryptococcosis [3]. Some of these components have been shown to be involved in chemotaxis inhibition.

Endothelial cells, astrocytes, and mononuclear cells are activated by TNF-α and IL-1β to produce IL-8 in the brain of patients who have meningitis [3]. IL-8 is a strong chemoattractant for polymorphonuclear leucocytes (PMNs). However, in cryptococcal meningitis, there is a paucity of PMNs in the CSF. Previous studies have shown that neutrophil migration does not occur in the presence of GXM because of the absence of a chemotactic gradient across the blood brain barrier [5].

In this study, I hypothesized that the crytpococcal capsular material interferes with neutrophil chemotaxis and that the level of chemotaxis inhibition is related to severity of disease. In order to prevent cryptococcosis, one requires a functional innate immune response followed by an acquired immune response if this is not successful.

1.2 Aims

1.2.1 To investigate the quantitative effect of the cryptococcal capsular material on neutrophil recruitment at the site of infection.

1.2.2 To investigate whether downregulation of neutrophil chemotaxis occurs at the level of TLR expression.

1.3 Objectives

1.3.1 Obtain clinical information from patients with cryptococcal meningitis.

1.3.2 Quantify the number of neutrophils in the CSF and blood.

1.3.3 Measure the thickness of the cryptococcal capsule in each isolate.

1.3.4 Determine the concentration of cryptococcal capsular material in individual CSF and serum samples by using antibodies against capsular components.

1.3.5 Determine the extent of inhibition of neutrophil chemotaxis by individual strains of *C. neoformans*.

1.3.6 Quantify the TLR2 and TLR4 expression induced by individual strains of *C. neoformans* in human microglial cells.

1.3.7 To investigate possible associations between 1.3.1.and 1.3.2 with 1.3.3 to 1.3.6.

CHAPTER 2 – LITERATURE REVIEW

2.1 A Brief History of the Organism *Cryptococcus neoformans*

2.1.1 Nomenclature and Classification

The genus *Cryptococcus* belongs to the basidiomycetous fungi and includes two species that are known to cause human disease, *C. neoformans* and *C. gattii*. In mammals, these pathogens can exist as intracellular yeasts, where they are found freely in tissue and body fluids or they may be enclosed by phagocytic cells, whilst in the environment, they can grow either freely or in soil amoeba [9, 10]. During the mid-twentieth century, scientists used rabbit antisera and defined four capsule serotypes (A – D) [9, 11, 12]. This categorization has been refined over the years by various analyses including DNA sequencing, epidemiology and ecology among a few methods. There are two species in the current classification: *C. neoformans*, var. *grubii* (serotype A) and var. *neoformans* (serotype D) and *C. gattii* (serotypes B and C) [9, 13]. A further eight major molecular types: VNI and VNII (var. *grubii*), VNIV (var. *neoformans*), VNIII (AD hybrids), and VGI – VGIV (*C. gattii*) occur within the two species [9, 14-17]. The genomes of these species have diverged more than 34 million years ago thus revealing species that have distinct ecological and pathological differences [9, 18]. The species names of the genus Cryptococcus still remains controversial. In a recent perspective by Hagen, et al, they discussed the advantages of recognising seven species within the genus rather than two species complexes, which would enhance further research into their possible differences [19]. *C. neoformans* is saprophytic in nature and is distributed worldwide. It has been associated with bird droppings (especially pigeon droppings), decaying vegetables and soil [20]. Although *C. neoformans* changes from yeast to hyphae during its sexual cycle, some do not consider it to be a dimorphic fungus because of its predominant yeast form both outside and within the human host [21]. *C. neoformans* is an important cause of opportunistic infections in the immunocompromised patient whereas *C. gattii* mainly affects immunocompetent individuals [20]. *C. gattii* has predominantly been found in tropical and subtropical areas and is associated with certain species of trees, particularly the eucalyptus tree and is also less responsible for human disease [22].

2.1.2 Cryptococcal Disease: Epidemiology and Infection

Prior to the onset of the Acquired immune deficiency syndrome (AIDS) pandemic, cryptococcal infection was not considered a very common disease entity. There were less than 300 reported cases of cryptococcosis globally during the 1950s [9, 23]. As Human immuno-deficiency virus (HIV) became more prevalent during the following years, a distinct rise in cryptococcosis became evident. During the mid-1980s, the incidence of disease significantly increased with HIV/AIDS accounting for greater than 80% of cases of cryptococcosis worldwide [24, 25]. Subsequent widespread implementation of antiretroviral therapy (ART) during the mid-1990s significantly reduced the incidence of HIV-associated cryptococcosis in most developed countries, although the incidence in other at-risk populations did not change [25, 26]. Furthermore, in settings, where access to health care resources as well as access to ARTs is limited, for example, in sub-Saharan Africa and certain parts of Asia, the prevalence of cryptococcal meningitis remains high [25]. Mortality rate had actually peaked at approximately 600 000 deaths per year at the beginning of the twenty first century [27]. An estimated 957 900 cases of meningoencephalitis was shown to have occurred every year globally, which resulted in more than 600 000 deaths per year [27]. In 2006, sub-Saharan Africa was found to have the most estimated cases (720 000 cases) followed by South and Southeast Asia (120 000 cases) [27]. It has been estimated that at least 222 000 HIV infected patients present with cryptococcal meningitis throughout the world annually [28]. This in turn results in 181 000 deaths every year, of which the majority of cases occur in sub-Saharan Africa [28]. A recent study has implied that cryptococcosis remained the second commonest cause of AIDS-related mortality, and this is only narrowly overtaken by tuberculosis [28].

Cryptococcal meningitis usually occurs in people with impaired cell-mediated immunity and is an important AIDS-related opportunistic infection. A large majority of cases are found in AIDS patients whose CD4 counts drop to less than 100 cells per microliter (cells/μl) [25]. The remaining cases are found in patients with haematological malignancies and transplant patients who are receiving immunosuppressive therapy [29]. Cryptococcal infection in general can be acute, chronic, or even asymptomatic. Generally, a pulmonary infection initially occurs which may spread systemically and ultimately infects the CNS [20]. Most cases of pulmonary infection are asymptomatic. However, infection in the CNS is usually more serious and can be life threatening and usually present as meningitis or meningoencephalitis. Other less common infections include skin, lung, prostate and eye infections. [20, 25].

2.1.3 Virulence Factors of Cryptococcus neoformans

The key virulence factors of *C. neoformans* are its polysaccharide capsule that prevents phagocytosis, melanin production that protects the organism from environmental stresses and ability of the organism to grow at human body temperature of 37ºC [30-32]. There have been various other virulence factors established in *C. neoformans* such as phospholipase B, urease, and many signalling cascades [33-35].

2.1.3.1 Polysaccharide Capsule

The polysaccharide capsule of *C. neoformans* is a significant virulence factor and is situated just outside of the cell wall of the organism. It has been hypothesized that the size of the capsule plays an important role in virulence and that the capability of phagocytes to clear the organism in vitro is related inversely to the size of the capsule [36, 37]. There are two major components of the polysaccharide capsule, which include glucuronoxylomannan (GXM) and galactoxylomannan (GalXM) [38-41]. GXM is the most abundant component and makes up approximately 90% of the capsular mass whilst GalXM constitutes approximately seven percent of the total capsule mass [41]. Mannoprotein (MP) is a nonpolysaccharide component of the capsule, and has been found to represent transient capsular components that are destined for cellular exit. [39, 40, 42, 43].

With regards to environmental growth of the organism, the role of the capsule is not entirely clear however there has been some speculation that the capsule acts as a food source and protects the fungus from dehydration [39, 44]. During infection in mammals, the capsule plays a role in resisting phagocytosis as well as modulating host immune response [39, 45-49]. A possible explanation for resisting phagocytosis is that macrophage receptors that bind most of the antigen determinants are at the cell wall, and the capsule is able to hide them from phagocytic cells [50]. The capsule also functions to protect the fungus after its ingestion, against free radicals, and protects the cell from oxidative bursts [39, 51]. There are various studies that show that the capsular components are secreted and this affects the host immune response in different ways [52, 53]. Much of the data has focused on the capsular component GXM because it is the most abundant. GXM inhibits neutrophil migration in various ways. It prevents leucocytes exiting the blood vessels because of its chemoattracting properties [50, 54]. It also induces shedding of L-selectin and E-cadherin from neutrophils and binds to CD18 thus inhibiting the binding of leucocytes to endothelium [50, 55-58].

The capsule has been shown to produce immunological tolerance in some early studies and this is manifested by an inhibition of antibody production against the capsule components [50, 59, 60]. Further studies of the capsule showed that both GXM and GalXM interfere with production of cytokines and affect dendritic cell maturation and antigen presentation [61-65]. In addition, these components are able to induce apoptosis of leucocytes [66-69].

2.1.3.2 Melanin Production

The accumulation of melanin is protective to *C. neoformans* in that it confers resistance to heat and cold, to free radicals and to ionizing radiation [70-72]. Melanin synthesis is dependent on the enzyme diphenol oxidase, which is encoded by the genes, LAC1 and LAC2 [50, 73]. In *C. neoformans*, production of melanin only occurs when exogenous dihydrophenolic compounds are present, since an endogenous substrate for this organism is not yet known [39].

This pigment displays other important characteristics in that it also binds and decreases the susceptibility to antifungal agents [50, 74, 75]. Melanin seems to be an important factor in the dissemination of disease from lung to brain [76]. It changes host cytokine production and protects against macrophages [50, 77, 78].

2.1.3.3 Survival at Human Body Temperature

Usually, environmental fungi do not tolerate higher temperatures and this includes human body temperature [50, 79]. However, most pathogenic fungi appear to have the ability to replicate at 37ºC. This seems to be an important factor for it becoming a pathogen in the immunocompromised patient [80]. Various processes are involved in enabling replication at higher temperatures. These include antioxidant responses, accumulation of trehalose, and stimulation of certain signalling pathways [50, 81-84].

2.1.3.4 Extracellular Enzymes

C. neoformans, like many other fungi, secrete various degradative enzymes such as proteases, DNases and lipases. During the course of infection, these enzymes enhance tissue destruction promoting survival of the fungus, and interfere with a competent immune response [39].

Urease is considered an important cryptococcal virulence factor [33]. This enzyme contributes to the organism crossing the blood-brain barrier after its escape from the lung, but once inside the brain, it appears not to be required for fungal growth [39, 85]. *C. neoformans* produces extracellular DNase, which degrades host DNA that neutrophils secrete as part of the innate immune response. It could also supply the organism with nucleotides [39, 86]. Two superoxide dismutases (SODs) have been reported in *C. neoformans*, which promotes growth of the organism within macrophages [39, 87]. Interestingly, temperature influences the production of SOD, where expression is increased at 37ºC, and this may also protect the organism against oxidising agents that effector cells of the host produce [39, 88]. The activity of phospholipase supports attachment of the fungal pathogen to host cells and various phospholipases have been identified in *C. neoformans* extracellular supernatants [39, 89, 90]. Phospholipase B has been shown to promote invasion of host tissue by the fungus and is responsible for hydrolysing phospholipids in plasma membrane as well as in lung surfactant [91-93]. It also maintains integrity of the cell wall and provides essential nutrients required by the organism during infection [39]. Another important enzyme that contributes to virulence is protease. This enzyme contributes to colonisation, tissue invasion and alters host defence response [39].

2.2 Cryptococcal meningitis

C. neoformans has the ability to cause infection anywhere in the human body. The lung and CNS however, appear to have a greater predisposition to infection. The lung usually forms the avenue of entry and the symptoms of disease may vary from asymptomatic to severe infection. The most frequent manifestation of cryptococcosis is meningitis [94]. Patients present clinically with a variety of symptoms and signs. These include headache, fever, cranial neuropathies, altered mental state, malaise and loss of memory [25]. Headache appears to be the predominant symptom and is a common finding in various studies [29, 95, 96]. Other symptoms are variable and are dependent on the immune status of the patient. Clinical signs are usually absent, but when present, include meningism, cranial nerve palsies, papilloedema, certain focal neurological deficits and a decreased level of consciousness [94].

Disease severity varies mostly in accordance with degree of immunosuppression and may be acute (a few days to a week), sub-acute (2-4 weeks duration) or chronic (extending beyond 4 weeks) [29]. Disease of the CNS is life threatening and is a major cause of mortality in patients with HIV especially in sub-Saharan Africa with an incidence of 15 to 30% [97]. Of these patients 30 to 60% succumb to their illness within 12 months [20, 97]. In severely immunocompromised patients, there is often a high fungal burden in the CSF and these patients tend to have a more acute clinical presentation, a higher CSF polysaccharide antigen titre and weak CSF inflammatory response determined by low white blood cell count [25, 94].

2.3 Interactions of Cryptococcus with the host immune system

The significant and profoundly more severe course of infection that occurs in the immunocompromised population highlights the significance of an effective immune response to this organism. The innate as well as the adaptive immune responses have an essential role in host defence against cryptococcal infection.

2.3.1 Innate Immune Response to Cryptococci

Various physical barriers are involved in the initial defence against Cryptococcus. Some of these include the skin, nasal mucosa, saliva as well as anti-cryptococcal activity of human serum [98, 99]. However, together with these, the main components of the host's innate immune response to Cryptococcus involve the complement system and the phagocytic effector cells.

2.3.1.1 Complement response

The complement pathway consists of a cascade of serum proteins and plays an important role in mediating phagocytosis of *C. neoformans* by the cells of the innate immune system. Studies have shown that opsonisation by complement improves the ingestion and inevitably, the death of *C. neoformans* by

phagocytes and mediates dendritic cell responses to the organism [100-103]. Complement activation can take place through the classical, lectin or alternative pathway. All of these pathways converge eventually to form C3-convertase and cleaves C3 into C3a and C3b [20]. C3b can either facilitate opsonisation and consequently enhance ingestion of the pathogen by phagocytes or it may facilitate cleavage of C5 into C5a and C5b [20]. C5a and C3a both act as mediators of the inflammatory response and thus attract phagocytic effector cells, while C5b plays a role in initiating membrane attack complex (C5b, C6, C7, C8, C9) formation [20, 104]. During cryptococcal infection, two important functions of the complement system include stimulation of phagocytic effector cell chemotaxis, and secondly, enhancing Cryptococcus cell ingestion by phagocytes [20]. As previously discussed, the polysaccharide capsule of *C. neoformans* is a well-known and important virulence factor especially important in inhibiting phagocytosis. The capsule of *C. neoformans* inhibits complement system activation through the lectin pathway by inhibiting the binding of mannan-binding lectin [20, 105]. Complement component 3 (C3) has been shown to bind to the cryptococcal capsule and is then converted into inactivated C3b (iC3b) [100, 106-108]. This enables phagocytosis to occur via complement receptors (CR). In vitro studies showed that when these complement receptors (CR1, CR3, CR4) are blocked, there is decreased interaction between human macrophages and *C. neoformans* [102]. While complement-mediated phagocytosis has been facilitated by CR3 in murine macrophages, both CR3 and CR4 have the ability to mediate complement-independent phagocytosis [100, 109, 110].

The complement system, being the first line of defence in the bloodstream, is significant in preparing for subsequent host responses. The Cryptococcus capsule likely inhibits complement related host responses like phagocytosis by suppressing the classical complement pathway and inhibiting C3-convertase activity by effectively removing C3b which is an important part of the C3-convertase of the alternative pathway [20].

2.3.1.2 Phagocytic effector cells

Various phagocytic cells are involved in phagocytosis of Cryptococcus (Figure 2.2). Phagocytosis occurs either by directly recognising the organism or through receptor mediated recognition, which involves complement or antibodies. Presence of the organism is recognised by cells of the innate immune system using pattern recognition receptors (PRR) which are present on host cells. These PRRs recognise certain conserved molecular structures called pathogen-associated molecular patterns (PAMPs) which can only be produced by pathogens and not host cells. Toll-like receptors (TLRs), non-TLRs, like intracellular nucleotide-binding oligomerization domain (NOD)-like proteins and the C-type

lectin receptors (CLRs) are some of the important PRRs [111-114]. The interaction between PRRs and PAMPs allows for activation of innate immune cells and eventually production and release of mediators that destroy pathogens and control the adaptive immune response [111].

i. Dendritic cells

Dendritic cells (DC) function predominantly in antigen processing and presentation to T-cells and to activate adaptive immunity [100, 115, 116]. They are considered major initiators of protective cell-mediated immunity during cryptococcal infection [20, 117, 118]. These cells are important in connecting the innate and the adaptive immune response [119]. During cryptococcal infection, DCs are responsible for presentation of the major antigens, glycoantigens and mannoproteins which are required for activation of T-cell responses [120, 121]. Apart from antigen presentation to naïve T-cells, DCs regulate the adaptive immune response by producing cytokines [122, 123]. Dendritic cells are able to generate differential helper T-cell responses which depend on the type of co-stimulatory molecules that are expressed on them [122]. There are three main groups of helper T-lymphocytes that play a role in fungal infections: (a) helper T-cell type 1 (Th1) , (b) helper T-cell type 2 (Th2) and (c) helper T-cell type 17 (Th17) [122]. During cryptococcal infection, a Th1 type response is a protective pro-inflammatory response that kills intracellular pathogens whilst a Th2 response is non-protective and promotes an anti-inflammatory immune response [124-126]. Th17 cells are often seen in relation with autoimmune diseases and mucosal immunity and during fungal infections have protective as well as non-protective roles [127-131]. Therefore, in the division of Th-mediated adaptive immune responses, it is important to know the type of antigen-presenting DCs [122].

ii. Macrophages

Macrophages are phagocytic cells that have for decades been considered the first cell of the innate immune system in the host defence against *C. neoformans* [100]. There are two types of macrophages that occur, which include macrophages that remain fixed in tissues, and migrating macrophages which are found mainly in interstitial fluid. Macrophages play an important role in determining disease outcome by either assisting in clearance of the organism or by aiding in its dissemination depending on their activation status [119]. Some researchers have revealed *C. neoformans* to be an intracellular parasite where the organism has found a method to manipulate host macrophages [20, 132]. The fungus survives phagocytosis and proliferates within infected host cells, which subsequently leads to lysis of the cell, and this is an important route for escape of intracellular pathogens [133-135]. Researchers recently have described a non-lytic expulsion

mechanism for the organism to be removed from macrophages without destroying the host cell; a process which depends on living cryptococci and which occurs very rapidly (< 60 seconds) [20, 136, 137]. In addition, cryptococci can be transferred from one macrophage to another laterally and this does not depend on the route of ingestion or strain of Cryptococcus, but this does depend on viability of the cryptococci [20, 133]. A well-known observation is that *C. neoformans* is able to disseminate to different sites in the human body but shows a predilection for the CNS. It seems that the intracellular environment within the macrophage is favourable for the organism in that it protects it from the immune system thus allowing it to proliferate. A "Trojan horse" mechanism of dissemination has been described by some authors, which implies that the replication intracellularly within macrophages, the transfer laterally between them and their exit from macrophages could explain how the organism remains latent and therefore disseminates in the host without activating immediate immune responses [20, 138, 139]. In fact, it is thought that lateral transfer and expulsion particularly, might be responsible for the yeast being able to cross the blood-brain barrier by possibly being transferred by macrophages directly to its endothelial layer and then being released into the CNS [20]. Two phenotypes appear to be important determinants of whether macrophages are beneficial or harmful during cryptococcosis: the classically activated macrophages (M1) or alternatively activated macrophages (M2) [119]. M1 macrophages are involved in eradicating Cryptococcus by their ability to produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) while M2 macrophages allows persistence of infection by supporting intracellular survival and proliferation of cryptococci [119, 140]. The ability to polarize towards either M1 or M2 macrophages depends on the cytokine response during infection [141]. In pulmonary cryptococcal infection for example, the cytokine profile needed to activate M1 macrophages depends on a cytokine environment that is IFN-dominant, whereas for M2 macrophages activation requires a cytokine profile that is IL-4 or IL-3-dominant [119, 142, 143].

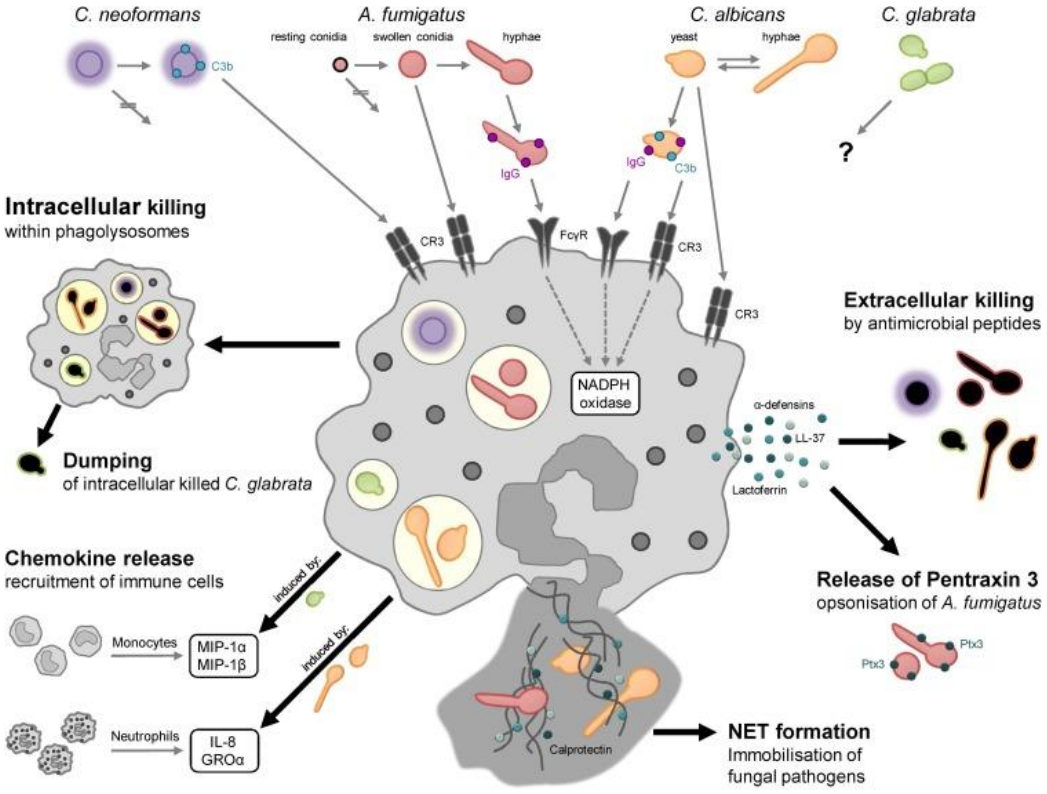
iii. Neutrophils

Neutrophils are phagocytic cells that migrate to the site of infection, release enzymes against microbes and produce neutrophil extracellular traps (NETs) [100, 144]. They are considered the "rapid responders" of innate immunity and are considered the most important immune cells that control the early stages of many fungal infections [145]. These cells are also the most abundant leucocyte type in human blood accounting for 40 – 75% of all leucocytes [145]. Neutrophils are derived from bone marrow precursors, are very motile and importantly, have a relatively short life-span [145]. Although neutrophils have been shown to have clear roles in the innate response to other fungi such as *Aspergillus fumigatus*, their role in defence against *Cryptococcus species* is not clear [100, 146]. Previous in vitro studies have shown human polymorphonuclear leucocytes

(PMNs) to have activity against cryptococci by oxidative and non-oxidative mechanisms like hydrogen peroxide, hypochlorous acid, calprotectin, and defensins [147, 148]. Complement, especially C5b acts as a chemoattractant thus causing neutrophils to migrate towards the C5b coated Cryptococcus [148, 149]. However, despite neutrophils ability to kill this organism, *C. neoformans* capsular component GXM has been shown to inhibit migration of neutrophils, neutrophil extracellular trap (NET) formation, killing and respiratory burst [3, 150-152]. Therefore in view of data that show only low numbers of neutrophils present in infected tissues during early infection it might suggest an immune-regulatory rather than an antimicrobial role for neutrophils [20, 135].

Complement receptor 3 (C3) and Fc-γ R are significant human neutrophil receptors which recognise C3b and IgG opsonised fungal pathogens (Figure 2.1) [145]. Fungal contact with these receptors initiates signalling cascades, which stimulates various mechanisms such as phagocytosis, oxidative burst, NET, and release of antimicrobial peptides from neutrophil granules [145]. All of these contribute to fungal killing. Neutrophils also secrete cytokines and chemokines during fungal contact which leads to recruitment of further immune cells [145].

Figure 2.1 Neutrophil function in antifungal immune response



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iv. Natural killer cells

Natural killer (NK) cells are cytotoxic cells that form part of the innate immune system. Some studies have shown that these cells play a role in defence against cryptococci in murine experiments through direct cytotoxic effect against the organism [100, 153-155]. Other studies in mice suggest that NK cells enhance the ability of macrophages to kill fungal cells by producing interferon gamma (IFNγ) [156, 157]. In human studies, NK cells and T-lymphocytes inhibit growth of Cryptococcus through direct interaction with the organism [158, 159]. NK cells express granulysin and perforin but perforin only is required for anti-cryptococcal activity by NK cells [148, 160].

v. Eosinophils

Eosinophils are granulocytes that are especially known for their role in allergies and parasitic infections [100]. In rats, it was established that *C. neoformans* was phagocytosed by eosinophils and that eosinophils primed B and T cells to generate protective host Th1 responses [100, 161-163]. There is however, no clarity on whether eosinophils have any significant role in innate immunity to *C. neoformans* or whether their recruitment is just a by-product of an inadequate Th2 response [100].

2.3.1.3 Pattern Recognition Receptors

Usually, pathogens are recognised by PRRs on host cells, which recognise PAMPs that are produced by the pathogen. This interaction of PRRs and PAMPs induces signal transduction, which allows for innate immune processes to occur. Components of the cell wall including glucans, mannans and chitin are common fungal PAMPs. *C. neoformans* polysaccharide capsule has the ability to mask these PAMPs and interestingly, PRRs such as C-type lectin receptor (CLR) and Toll-like receptor (TLR) families, that usually detect other fungi, do not play similar roles in recognising *C. neoformans* [100]. The process involved in the recognition of *C. neoformans* by the host therefore, is still not clearly defined [100].

i. Toll-Like Receptors

Of the PRRs , TLRs are the family studied most extensively, with 13 known members coded for in the human genome [119]. These receptors are able to recognise bacteria, viruses and fungal pathogens and they play a role in regulating both the pro-inflammatory as well as the anti-inflammatory immune responses. Most TLRs recognise PAMPs and this initiates signal transduction cascades that are associated with the adaptor molecule myeloid differentiation primary response protein 88 (MyD88) [119]. Evidence that MyD88 is involved in anti-cryptococcal responses in rats supports the possible involvement of TLRs as cryptococcal PRRs [164, 165]. However, there is still insufficient experimental evidence that supports the role of many of the TLRs in cryptococcal infection [100].

TLR2 and TLR4 are able to recognise several pathogen ligands especially cell wall-associated ligands and this has earned them much attention among researchers [119]. TLR2 and TLR4 are cell surface receptors and are expressed on many innate cells such as neutrophils, macrophages, monocytes, and DCs [166]. β-glucans that are expressed in the fungal cell wall are usually recognised by TLR2, however the capsule of *C. neoformans* has the ability to conceal the β-glucan layer [167]. The function of TLR2 during the immune responses to cryptococci appears to be varied among studies [119].

Microglial cells, which are the resident phagocytes within the CNS, as well as meningeal macrophages, make up the first line of defence within the brain during infections of the CNS [168-170]. These cells may be found throughout the brain parenchyma and constitutes between 10 and 20% of the glial cell population in the CNS [171]. Koutsouras et al found that a comparison between microglia and macrophages aided in the understanding of microglial physiology. However, unlike macrophages, microglial cells maintain themselves entirely by self-replication [171]. Perivascular microglial cells, also named perivascular macrophages, are derived from bone marrow and are maintained continuously from the periphery while simultaneously being maintained by the self-replication properties of parenchymal microglia [171, 172]. During infection, these cells release chemokines which increase recruitment of dendritic cells, neutrophils, and lymphocytes from peripheral tissue [171]. Microglial cells express TLRs, which identify PAMPs and are therefore important in regulating the innate immune response [170, 173, 174]. TLR2 as well as TLR4 are known to recognize cryptococcal GXM [170, 175]. It was determined that GXM had the ability to bind to TLR2 and TLR4 with the co-receptor CD14 but it was unable to activate the MAPK (mitogen-activated protein kinase) pathway and produce TNF-α [170, 175]. TLRs also have the ability to form heterodimers like TLR1/2 and TLR2/6 that recognize GXM in the cryptococcal capsule [176]. In vitro studies show that O-linked mannans activate TLR4 in *C. neoformans* [119]. Furthermore, pro-inflammatory responses were increased in *C. neoformans* infection when

microglial cells were stimulated with TLR1/2, TLR3, TLR4, and TLR9 (TLR agonists), however, the significance is not clear [170].

While TLR function in the lymphatic system has stimulated much interest in recent years, not much information is available on the expression and role of TLRs in CNS microglial cells [7]. It is however, well established that these cells do participate in the innate immune response [177]. Emerging data is becoming available on TLR expression in the rodent CNS, however minimum information is available on expression of TLRs in human microglial cells [7].

ii. C-type Lectin Receptors

The C-type Lectin Receptors (CLRs) are a group of receptors that recognise fungal carbohydrate ligands such as β-glucans and mannans [100]. CLRs usually initiate signalling pathways through their own intracellular signalling domain or via signalling adapters that have an immunoreceptor tyrosine-based motif (ITAM) [100]. It has been established that CLRs play a role in host immune responses to other fungi, however their role in *C. neoformans* is not significant [100, 178]. Evidence exists that β-glucans are accessible on capsules of *C. neoformans*, however, in vivo, it seems evident that the capsule interferes with many of these interactions [179, 180].

iii. Nucleotide-Binding Oligomerization Domain (NOD)-Like Receptors

NOD-like receptors (NLRs) are cytoplasmic PRRs that share a role in immunity and are able to recognise PAMPs and damage-associated molecular patterns (DAMPs) [119]. Some studies recently, have shown that NLRs were involved in sensing the presence of fungi [119]. *C. neoformans* cells stimulated formation of the NLRP3 inflammasome, and studies with mice that are deficient in the NLRP3 inflammasome components showed that they were more susceptible to infection [181-183]. However, sufficient information on the role of NLRs in response to *C. neoformans* is not available [100].

2.3.2 Adaptive Immune Response

2.3.2.1 Cell-mediated Immunity: T-cells

The patient group predominantly at risk for developing cryptococcal disease usually have severe T-cell function defects [148]. This clearly indicates that T-cell mediated immunity plays a vital role in the control of cryptococcal infections (Figure 2.2). Both CD4 and CD8 cells inhibit *C. neoformans*, either directly or by producing pro-inflammatory cytokines that are required for recruiting and activating other phagocytic cells to kill the organism [122, 184-186]. A review of in vitro studies indicate that CD4 and CD8 T cells both produce Th1 cytokines, however the sole source of Th2 cytokines are the CD4 T cells [122]. Various T cell subsets exist that have a function in cryptococcal infection. Regulatory T-cells are protective against cryptococcal infection in that they suppress the damaging Th2 immune response [122]. Other examples of T-cell subsets include NK cells, Natural killer T (NKT) cells and gamma delta T (γδT) cells which all play a role in in protective immunity against cryptococcal infection [122, 187, 188]. T-cells however, are also able to downregulate the protective Th1 response [189].

The proliferation of naïve T-cells may be induced by complete cryptococcal cells or by cell extracts such as cell wall and cell membrane protein extracts [190, 191]. To ensure presentation of *C. neoformans* antigen to T-lymphocytes, it is necessary for phagocytosis and protein processing to occur [192]. Cryptococcal cells are then either inhibited or killed by CD4 and CD8 T cells.

2.3.2.2 Antibody-Mediated Immunity

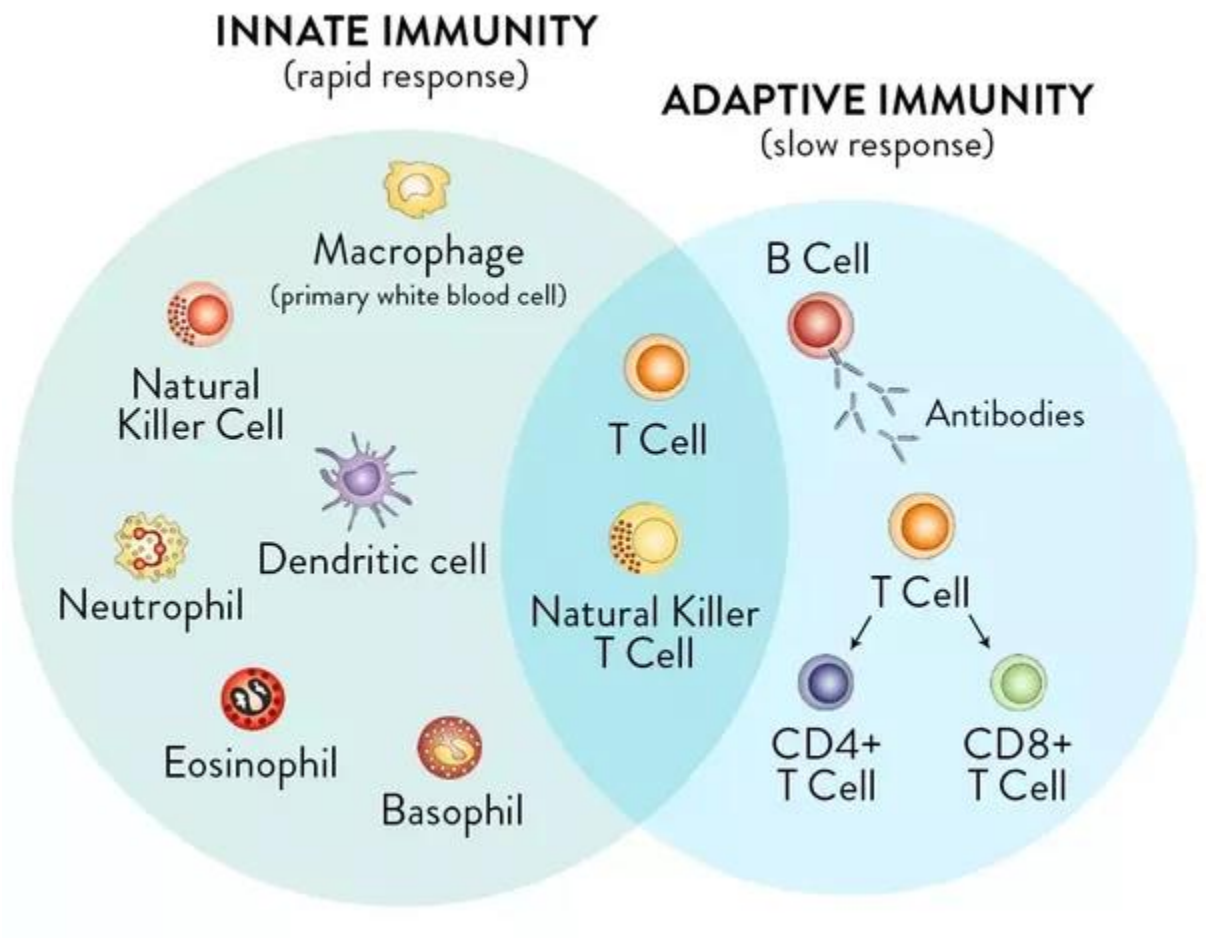
There has been conflict regarding the role of antibody-mediated immunity in cryptococcal infections. There are however, many reports of cryptococcal infections in patients who have B-cell deficiencies, as well as antibody or even lymphoproliferative deficiencies [193]. In addition, antibodies against cryptococcal proteins and capsular components have been found in people without infection [20, 194, 195]. In HIV-patients, there is an association with cryptococcal infections when there is a decrease in B-cells secreting IgM [196]. Anti-cryptococcal antibodies opsonise the pathogen resulting in Fc receptor-dependant phagocytosis and activation of the classical complement pathway [20]. Studies in mice indicate that the protective effect of antibodies may be to an extent, due to interactions with cell-mediated immunity [20]. Some studies show that antibody responses may amplify cryptococcal disease

in humans [122]. In non-HIV patients with normal CD4 counts autoantibodies have been associated with infection [197].

2.3.2.3 Cytokine response

Knowledge regarding cytokine response has been obtained predominantly in mouse models and therefore information and evidence regarding their response in cryptococcal infection relates to mouse models [198-200]. Cytokines consist of proteins, peptides or glycoproteins that are secreted by immune cells and have a role in mediating and regulating immunity. Various cytokines and chemokines are involved in cryptococcal infection. These cytokines and chemokines either induce the Th1 response and/or suppress Th2 immune responses [122]. Both protective and non-protective cytokines are produced during infections by *C. neoformans*. Protective cytokines include IFN-γ, IL-12 and IL-2 which have been shown in humans as well as mouse models [122]. Additionally, other protective cytokines have been identified which include TNF-α, IL-6, IL-8, IL-18, IL-23, and IP-10 [122]. Non-protective cytokines include IL-5 and IL-13, which are the Th2 cytokines, and these cytokines stimulate cryptococcal disease. There are certain cytokines/chemokines that may play a dual role in protection as well as disease aggravation during cryptococcal infection, and these include IL-4, IL-8, IL-10, IL-1β, MCP-1 (monocyte chemoattractant protein 1), MIP-1α (macrophage inflammatory protein form 1 alpha) and RANTES (CCL5) [122].

Figure 2.2 Innate vs Adaptive Immunity



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2.4 Paucity of neutrophils in cryptococcal meningitis

Cryptococcal meningitis is known to be characterized by a relative paucity of leucocytes in the CSF compared to that in bacterial meningitis. [3-5, 56, 201]. The presence of leucocytes is regulated through chemotaxis. Chemotaxis is dependent on chemokine production at the infection site and the level of expression of corresponding chemokine receptors on the migrating cells. Chemokines are important in mediating recruitment of leucocytes into infection sites, including infections with *C. neoformans* [202]. Microglial cells in the brain are important sources of IL-8, IP-10, MIP-1α, MIP-1β, RANTES, KC, and MCP-1[203]. Chemokines are produced when TLRs on cells at the site of infection recognize specific microbes. *For C. neoformans*, TLR2 has been identified as a significant TLR [6, 7, 175].

TLR2 and TLR4, as described above, are receptors found on the cell surface that enable phagocytic inflammatory responses to various microbial products. When these receptors are activated, signaling cascades are triggered and this results in a pro-inflammatory response including production of TNFα. [175]. Whilst it is clear that TLR expression is significant, there is not much published information on expression and function of TLRs in human CNS microglial cells [7].

High levels of the capsular components GXM, GalXM, MP-1, and MP-2 is characteristic of disseminated cryptococcal infection, in the CSF and serum of patients [3]. These components induce peripheral blood monocytes and polymorphonuclear neutrophils (PMN) to produce the cytokines TNF-α and IL-1β [3]. TNF-α and IL-1β stimulates endothelial cells, astrocytes and mononuclear cells to produce IL-8 in the brain of patients with meningitis.[3]. IL-8 is a potent chemo-attractant for PMNs [3]. However, in cryptococcal meningitis, there is a paucity of PMNs [4, 5, 56, 201].

A study by Lipovsky et al [5] confirmed what others have observed that GXM has chemoattractant activity. They have suggested that IL-8 production occurs in the brain but neutrophils do not cross the blood brain barrier (BBB) in response to IL-8. Their explanation is that migration of PMNLs does not occur because a chemotactic gradient does not exist across the blood brain barrier. Furthermore, a phenomenon called cross-desensitization of the IL-8 receptor could be another possible explanation for chemotaxis inhibition by GXM [5, 204].

Glucuronoxylomannan has been observed to inhibit adhesion of PMNs to activated endothelium in a concentration-dependent manner [56]. Furthermore, another component of the capsule, MP-4, was studied as a new capsular antigen which activates neutrophils and desensitizes them toward a chemotactic challenge [3].
Another possibility for the paucity of leucocytes is that even though IL-8 was produced in the CSF of patients with cryptococcal meningitis, the circulating GXM was downregulating recruitment of leucocytes, perhaps through shedding of L-selectin, thus impairing migration of leucocytes into the CSF [205, 206].

A series of steps are involved during the inflammatory response with regards to leucocytes and endothelium interactions [57]. These progress from rolling to adhesion and ultimately migration into tissues. Margination and rolling of leucocytes on the cytokine-activated endothelium are the first steps in adhesion [57, 207]. It was shown that GXM impedes the early rolling phase of neutrophil adhesion to endothelium and to Chinese Hamster Ovary (CHO) cells that were E-selectin-transfected [57].

Various observations as described above suggest the presence of neutrophil chemokines in the brain of infected patients and that microglial cells are a potent source, yet a paucity of neutrophils still occurs in cryptococcal infection. Toll-like receptors have been described as receptors involved in initiating phagocytic inflammatory responses, however little evidence is available on its expression and function in human microglial cells. This study therefore aims to describe this.

CHAPTER 3 – METHODS

3.1 Patient Recruitment and Clinical Data

A prospective study was conducted at a regional hospital in KwaZulu-Natal, South Africa, from 16 July 2013 to 03 February 2016. HIV infected adult patients with a diagnosis of meningitis with *Cryptococcus neoformans* as causative agent were invited to participate in the study. The criteria for exclusion were age <18 years, HIV uninfected, and concomitant infections other than HIV. The diagnosis was made on clinical grounds and confirmed by laboratory examination of CSF. Only patients with isolates identified as *C. neoformans* were included (3.3). Patients who provided written informed consent were enrolled in the study within 24 hours of presentation. Clinical details were recorded and a second lumbar puncture was performed 48 hours following presentation as part of the routine patient work-up and CSF opening pressure was recorded. Two tubes with CSF as well as two with venous blood collected at this time point were transported to the laboratory. A total of 71 patients who fulfilled all the criteria were enrolled in the study. None of the patients had received prior antifungal treatment. Antifungal therapy was commenced after the first CSF specimen was collected.

3.2 Quantification of Immune Cells in CSF and Blood

3.2.1 Quantification of Immune Cells in CSF

Cells in the CSF were quantified using a haemocytometer (Thermo Fischer Scientific, Massachusetts, USA). One drop of trypan blue was added to a clean tube using a sterile glass pipette. Nine drops of CSF was then added to the same tube using an equivalent glass pipette. This made a 1:10 dilution. This was left to stand for five minutes to allow the cells to absorb the stain. The solution was mixed by gently tapping the bottom of the tube. A coverslip was placed over the counting chamber and using a capillary tube the counting chamber was filled with the diluted specimen. It must be noted that the counting chamber can only accommodate a predetermined fixed volume of 20 µl. The slide was then viewed under a light microscope using a 10× objective and the number of cells were counted using a cell counter. A standard formula was used to calculate number of cells (Appendix A1).

3.2.2 Quantification of Immune Cells in Blood

A full blood count was performed using an automated system (Advia 2120i, Siemens, Munich, Germany). A differential count was performed using the same instrument. Enumeration of neutrophils was done by establishing the percentage of these cells from the total white blood cell count using the same automated instrument.

3.3 Microscopy and Culture of CSF Specimens

The first CSF received from the patient was centrifuged at 1109 × g for 10 minutes. The supernatant was aseptically decanted into a sterile capped tube labelled with the study number. The deposit was resuspended in the remaining supernatant by gently tapping the bottom of the tube. A smear was prepared by placing one drop of the resuspended deposit onto a sterile glass slide using a sterile pipette. The slide was allowed to air dry followed by Gram staining (Appendix A2). Fifty microliters of the deposit were inoculated onto sheep blood agar (Appendix B1), and incubated at 37°C for 48 hours in a regular incubator. Each isolate was identified to species level using the automated Vitek® 2 system (bioMérieux, France). The CSF supernatant was used to perform a Cryptococcus latex agglutination test (3.4).

3.4 Concentration of Capsule Material Components in CSF and Serum

3.4.1 Principle of the Cryptococcal Antigen Latex Agglutination Test

The Cryptococcal Antigen Latex Agglutination System (CALAS® - Cryptococcal Antigen Latex Agglutination System, Meridian Bioscience Inc., Ohio, USA) was used to measure the titre of cryptococcal antigen in serum and in CSF. This was used as a proxy for the amount of shed capsular material. The procedural instructions by the manufacturer were followed to complete the tests. CALAS utilizes latex particles coated with rabbit anti-cryptococcal IgG in glycine buffered saline (pH 8.4 ± 0.1) that contains a preservative thimerosal (< 0.01%). This is referred to as detection latex. Visible agglutination is observed when the antibodies on the latex particles and the cryptococcal polysaccharide antigen bind together. Latex particles that were coated with immune-globulins not containing

cryptococcal binding sites were used as negative control. The positive control consisted of purified *C. neoformans* polysaccharide antigen, which contained the preservative thimerosal (0.01%). Goat anti-rabbit serum with thimerosal (0.01%) was the antibody control. Macroglobulins such as rheumatoid factor may be present that could cause nonspecific agglutination. If this does occur specimens are treated with pronase (Appendix B2) to remove these macroglobulins.

3.4.2 Procedure of the Cryptococcal Antigen Latex Agglutination Test

One free falling drop of positive control was added to the two designated rings on the reaction card provided with the kit. Twenty-five microliters each of the antibody and negative controls were pipetted to the relevant rings. Twenty-five microliters of patient CSF supernatant was pipetted into each of the two rings on the test-card. Thereafter, one free falling drop of the detection latex was added into each ring. Similarly, one drop of negative control latex (3.4.1) was added into the additional rings. The contents of the different rings were then mixed using separate applicator sticks. Subsequently, the card was placed on a horizontal rotator and rotated at 0.4 × g for 5 minutes. Results were read immediately.

3.4.2.1 Titrations

Two-fold serial dilutions of each specimen were done until the agglutination end point was reached. Two hundred and fifty microliters of sample diluent (Appendix B3) was added into five labelled test tubes and placed in a rack. Using a clean pipette, 250 µl of CSF or serum was added into tube labelled 1 and mixed well. Two hundred and fifty microliters from tube 1 was then transferred to tube 2 and mixed well. Following this pattern, the dilution series was continued through to tube five. Two hundred and fifty microliters from tube 5 was transferred into a "holding" tube to allow for further dilutions that may be necessary. Thereafter, the same antigen detection procedure as described in 3.4.2 was followed.

3.5 Storage of CSF and Blood specimens

The second tube with CSF from each patient was centrifuged at 1109 × g for five minutes. The supernatant was aseptically decanted into at least two sterile cryovials labelled with the study number and stored at -80°C. The deposit was resuspended by gently tapping the bottom of the tube and inoculated onto sheep blood agar (Appendix B1). After 48 hours of incubation at 37°C, the growth was harvested using a sterile plastic loop and suspended in 1 mL of sterile distilled water. These suspensions were stored at room temperature for further work (Appendix A3).

Blood specimens were centrifuged at 1109 × g for 5 minutes. The serum was then aseptically decanted into at least two sterile cryovials with labelled with the study number and stored at -20°C.

3.6 Retrieval of Stored Cryptococcus Cultures for Determination of Capsule Thickness

Stored Cryptococcus suspensions were plated out on Brain Heart Infusion Agar plates (Oxoid Ltd, Cheshire, England) supplemented with 10% Horse blood (Appendix B4). These were incubated for 48 hours at 37°C aerobically.

3.6.1 Determination of capsule thickness

A microscopy slide was made on the first subculture of organisms grown from the 48-hour specimen obtained by lumbar puncture (3.6). Anthony's capsule stain was applied to visualize the polysaccharide capsule [208].

3.6.1.1 Principle of Anthony's Stain

This procedure for staining capsules contains crystal violet as the primary stain, which is taken up by all parts of the cell. A 20% copper sulphate solution is used as a decolorizing agent as well as a counter stain. When the copper sulphate washes out the crystal violet, the capsule becomes decolorized but not

the cell. As the copper sulphate decolorizes the capsule, it simultaneously counter stains the capsule. The capsule therefore can be visualised as a faint blue halo surrounding a purple cell.

3.6.1.2 Preparation of the inoculum

i. Determining Yeast cell concentration of 1-McFarland Standard for Cryptococcus

A suspension of the culture was made in phosphate buffered saline, pH = 7.2 (Appendix B6) and adjusted to a 1-McFarland suspension (Appendix B5) which is equivalent to a suspension of 3.0×10^8 CFU/mL for bacteria of average size (0.5 – 2.0 μm). Considering that *C. neoformans* is larger than an average bacterial cell (5 – 10 μm vs 0.5 – 2.0 μm), the concentration of the yeast cell equivalent to a 1-McFarland standard was determined. A series of 1:10 dilutions was made in RPMI with L-Glutamine (Lonza Group Ltd, Basel, Switzerland) starting with a density of 3.0×10^8 CFU/mL and ending at 1.0×10^2 CFU/mL. Ten microliters of each suspension was inoculated on individual sheep blood agar plates and incubated aerobically for 48 hours at 37°C. This was performed in duplicate. Thereafter, colonies were counted on the plates that contained on viewing 20 – 200 colonies. The average number of colonies was then adjusted for volume and inoculum size and thus it was determined that the bacterial 1-McFarland standard was equivalent to 1.0×10^6 CFU/mL for *C. neoformans*.

3.6.1.3 Anthony's staining procedure

Fifty microliters of the cryptococcal suspension was placed on a glass slide and a thin film was made using a second slide under an approximately 45^o angle. After air drying, the slide was flooded with 1% crystal violet for two minutes [208]. It was then rinsed gently with a 20% copper sulphate solution and air-dried. It is not recommended to blot the slide because blotting removes the un-fixed microbes from the slide and cause disruption of the capsule [208]. The slide was viewed under an oil immersion lens (Leica DM 3000; Leica microsystems, Wetzlar, Germany). Bacterial cells and the proteinaceous background appeared purplish while the capsules appeared transparent. Photographs were taken of the organisms on the slide and viewed at a magnification of 100×. A camera (Image Pro-Plus of Media Cybernetics, Inc., Maryland, USA), was used to capture these images. The number of images taken depended on the density of cryptococcal cells on the slide. The images were used to measure the capsule thickness. This was done by first measuring the diameter of the entire cell including the capsule

followed by measuring the smaller diameter of the cell excluding the capsule. The difference between the two diameters was recorded as the capsule thickness in micrometers. The average capsule size of each isolate was determined using three different cryptococcus capsule measurements per isolate. Three cells per isolate were randomly selected on three microscopic fields and capsule measurements were performed as described above.

3.7 Neutrophil Chemotaxis Inhibition

Neutrophil chemotaxis inhibition experiments were performed to compare the number of migrating neutrophils toward the chemoattractant (*E. coli*), with the number of neutrophils that migrate toward the chemoattractant mixed with the cryptococcal isolates. The amount of inhibition was determined by calculating the difference between these two values (no. of migrated neutrophils towards *E. coli* – no. of migrated neutrophils towards *E. coli/Cryptococcus* combination = Chemotaxis inhibition).
A qualitative approach using the Boyden Chamber method was used. A gradient separation method was used to separate neutrophils from the rest of the human blood cells (*3.7.2.2*).

3.7.1 Boyden Chamber assay

The Boyden Chamber method [209, 210] was used to determine the rate of neutrophil chemotaxis in the presence and absence of *C. neoformans* yeast cells. *Escherichia coli* ATCC 25922 was used as the chemoattractant. This assay is based on a chamber that consists of two compartments that are separated by a microporous membrane. The assay was developed originally by Boyden to analyse leucocyte chemotaxis [211]. Cells added into the upper compartment migrate through the membrane pores into the lower compartment, which potentially contains the chemotactic agents. The chamber is incubated for an appropriate/predetermined time, following which the membrane between the two compartments is fixed and stained and the number of migrated cells in the lower compartment is determined using a cell counting method. This assay has therefore also been called “filter membrane migration assay”, “trans-well migration assay”, or “chemotaxis assay” [211]. Various different Boyden chamber devices are available commercially. Here, Transwell® inserts (Corning Life Sciences, New York, USA) in 24-well tissue culture plates were used.

3.7.2 Preparation for the Transwell Migration Assay

3.7.2.1 Acquisition of neutrophils from volunteer donors

Healthy adult volunteers were recruited with informed consent to donate blood on the day of an experiment. Approximately 25 mL of blood was drawn from one donor. Blood samples were collected in EDTA tubes (BD Life Sciences, New Jersey, USA) and transported immediately to the laboratory for neutrophil separation.

3.7.2.2 Neutrophil separation

A gradient separation method using Histopaque®-1119 (Sigma-Aldrich, Missouri, USA) and Histopaque®-1077 (Sigma-Aldrich, Missouri, USA) was used in the laboratory to separate neutrophils from the rest of the human blood cells. The procedure was followed according to specifications by the manufacturer. Before commencing on the separation procedure, cells and plasma were mixed evenly by gentle tilting of vials. Twelve millilitres Histopaque®-1119 was added to a 50 mL conical centrifuge tube using a sterile needle and syringe. Twelve milliliters of Histopaque®-1077 was then carefully layered onto the Histopaque®-1119 using a sterile needle and syringe. This was followed by carefully layering 24 mL of whole blood onto the upper gradient of the tube using a sterile needle and syringe. Without disturbing the gradients, the tube was centrifuged at 700 × g (centrifugal force) for thirty minutes at room temperature (25°C). Two distinct opaque layers were visible, one containing mononuclear cells and platelets and the other containing granulocytes including the neutrophils. The granulocyte layer was gently aspirated using a sterile Pasteur pipette (Sigma-Aldrich, Missouri, USA) and cells were transferred to a sterile 15 mL conical centrifuge tube. The cells were washed by adding 10 mL of isotonic phosphate buffered saline (PBS), pH = 7.2 (Appendix B6). This was centrifuged at 200 × g for 10 minutes at room temperature (~25°C). The supernatant was removed using a sterile Pasteur pipette and discarded. The cells were resuspended by gentle aspiration three times with a sterile Pasteur pipette. The wash step was repeated for a further two times. Cells were then resuspended in isotonic PBS, pH = 7.2 to achieve a cell count of 1×10^6 cells/mL [212]. To determine the dilution factor to obtain this neutrophil concentration, a cell count was done using a haemocytometer as described previously (Appendix A1). Further dilutions were made as required. The purity of the neutrophil suspension was tested by means of the Giemsa staining method (Appendix A4)

3.7.2.3 Preparation of the inoculum of C. neoformans isolates and E. coli ATCC 25922

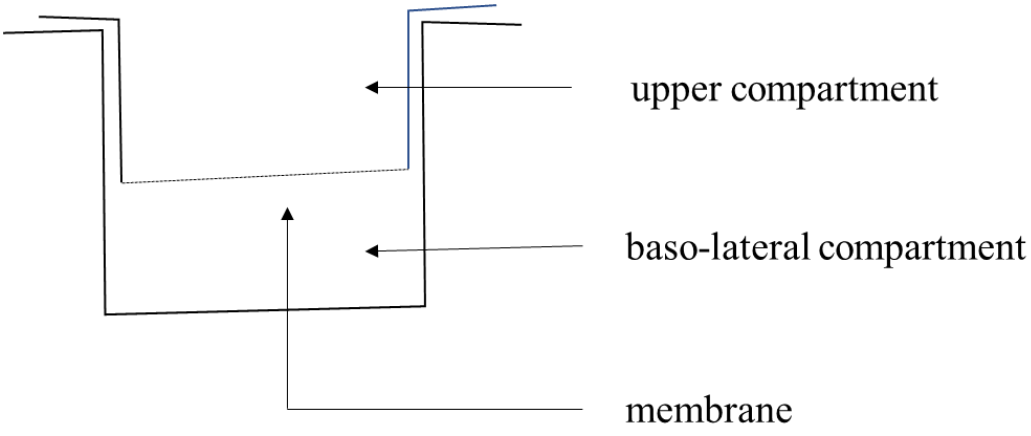
For *C. neoformans*, in a series of preliminary experiments (3.6.1.2), the appropriate density was determined to be 1.0×10^6 CFU/mL and for *E. coli*, it was determined to be 3.0×10^8 CFU/mL. Therefore, a 1-McFarland suspension of each organism suspension was used in the chemotaxis assay. For the *E. coli*/*Cryptococcus* mixture, equal volumes of the *E. coli* ATCC 25922 (3.0×10^8 CFU/mL) and *C. neoformans* (1.0×10^6 CFU/mL) suspensions were mixed in a sterile tube at a 1:2 dilution for each organism. Thus, a suspension of each culture was made in PBS, pH = 7.2 containing 0.5×10^6 CFU/mL Cryptococcus and 1.5×10^8 CFU/mL *E. coli*. The mixed suspension contained the same amount of microbes of each species as above. For the Transwell assay the cryptococcal suspension, the *E. coli* suspension as well as the mixed suspension were used.

3.7.3 Transwell Assay

Twenty-four well tissue culture plates that have inserts in the plate with a collagen-coated membrane of 3-μm pore size were used (Figure 3.1). Transwell® inserts (Corning Life Sciences, New York, USA) were used. A membrane with a 3 μm pore size was used to ensure that neutrophils actively migrate through them to the lower chamber (baso-lateral compartment) and move through freely [213]. Six hundred microliters of organism suspension was added to the bottom of a well of a 24-well plate. The Transwell membrane filter was then placed carefully in the same well. Typically 1.0×10^6 cells/mL is used for nearly all types of cells when using the 24-well Transwell insert [212]. One hundred microliters of neutrophil cell suspension was added onto the top (upper compartment) of the filter membrane. These volumes were determined to ensure that the suspension medium in the bottom of the well makes contact with the membrane to form a chemotactic gradient [212]. The assay was incubated at 37°C for 30 minutes. The number of migrated cells was determined using the Myeloperoxidase (MPO) Human SimpleStep ELISA® Kit (Abcam®, Cambridge, UK)). As described in 3.7, the chemotaxis assay was performed with each cryptococcal isolate alone and a combination of the same cryptococcus plus the chemoattractant (*E. coli* ATCC 25922). *E. coli* (ATCC 25922) alone was used as the control. The amount of inhibition was determined by calculating the difference between two values (no. of migrated neutrophils towards *E. coli* – no. of migrated neutrophils towards *E. coli/Cryptococcus* combination = Chemotaxis inhibition). All experiments were performed in duplicate.

The following preliminary experiments were performed to determine i. the most appropriate chemoattractant (control organism) for the experiment, ii. the most appropriate incubation time to allow for chemotaxis, iii. the most appropriate density or concentration of test organism (*C. neoformans*), and iv. the most appropriate means for quantification of migrated neutrophils across the transwell membrane.

Figure 3.1 Transwell Migration Assay Diagram



3.7.3.1 Preliminary Experiments

i. Determination of chemoattractant

Four organisms were tested as possible chemoattractants in the Transwell migration inhibition assay as described above. These were *C. albicans* ATCC 14053, *E. coli* ATCC 25922, *H. influenzae* ATCC 49427, and *K. pneumoniae* BAA 1705. Six 1:10 serial dilutions of a 1-McFarland standard for each organism were made using RPMI with L-glutamine (Lonza Group Ltd, Basel, Switzerland) as the diluent. The Transwell Migration procedure as described above (3.7.3) was performed for each of the four organisms with each of the dilutions. Migrated neutrophils were counted using a haemocytometer and enumeration of neutrophils were performed manually according to predetermined calculations (Appendix A1). The highest yield of migrated neutrophils was obtained with the highest dilution

microbe concentration for three of the four isolates tested. *E coli* yielded a total count of migrated neutrophils of 1.0×10^5 cells/mL, *H. Influenzae*, 0.2×10^5 cells/mL, and *K. pneumonia*, 0.2×10^5 cells/mL. *C. albicans* ATCC 14053 was determined to be an inappropriate chemoattractant control to be used in this assay, because it was difficult to distinguish between the organism and the migrated neutrophils in the haemocytometer. Therefore, it was determined that a McFarland-1 suspension of *E. coli* ATCC 25922 would be used.

ii. Determination of incubation time for chemotaxis

E. coli ATCC 25922, *H. influenzae* ATCC 49427, and *K. pneumoniae* BAA 1705, at densities of 3.0×10^8 CFU/mL were used in this experiment. The Transwell Migration procedure as described above (3.7.2) was performed for each of the three control organisms for the following incubation periods: 30 minutes, 60 minutes and 90 minutes. The results obtained are illustrated in Table 3.1.

TABLE 3.1: Neutrophil count after 30, 60, and 90 minutes incubation for control organisms

		incubation time (minutes)		
	control organism	30	60	90
no. of neutrophils (cells/mL)	*E. coli* ATCC 25922	1.0×10^5	0.3×10^5	0.0
	H. influenzae ATCC 49427	0.2×10^5	0.2×10^5	0.0
	K. pneumoniae BAA 1705	0.2×10^5	0.0	0.0

From this experiment, *E. coli* ATCC 25922 displayed the highest cell count at an incubation period of 30 minutes. The other two organisms had lower cell counts than *E. coli* after 30 minutes of incubation. After 60 minutes of incubation, all three organisms yielded lower counts compared to 30 minutes of incubation. After 90 minutes, the cell count for all three organisms was zero. Therefore, an incubation time of 30 minutes was adopted.

iii. Inoculum size for C. neoformans

Inoculum size for *E. coli* ATCC 25922 was determined to be 3.0×10^8 CFU/mL following preliminary experiments to determine chemoattractant (3.7.3.1.i). For *C. neoformans*, a series of 1:10 dilutions was made in RPMI with L-Glutamine (Lonza Group Ltd, Basel, Switzerland) starting with a density of 3.0×10^8 CFU/mL and ending at 1.0×10^2 CFU/mL (3.6.1.2.i). The Transwell Migration procedure as described above (3.7.3) was performed on each of the prepared dilutions. Migrated neutrophils were counted using a haemocytometer and enumeration of neutrophils was performed manually according to predetermined calculations (Appendix A1). At this point, due to similar size, it was difficult to distinguish microscopically between the organism and the migrated neutrophil in the lower chamber of the assay using the haemocytometer. Therefore, an alternative method for quantification of migrated neutrophils was determined. Using this method (described below), it was determined that the lowest concentration at which Cryptococcus caused neutrophil migration was at a density of 1.0×10^6 CFU/mL.

iv. Determination of quantification method for migrated neutrophils – The Myeloperoxidase (MPO) Human Simple Step ELISA® (Abcam)

Myeloperoxidase (MPO) is an enzyme belonging to the subfamily of peroxidases. A variety of cells including neutrophils, lymphocytes [214-216] monocytes, and macrophages [214, 217] are able to express this enzyme. The azurophilic granules of neutrophils contain stored myeloperoxidase. When the neutrophil is stimulated, exocytosis or degranulation occurs, and the content of the granules is released out to the extracellular space [214, 218, 219]. Although various other antimicrobial enzymes and proteins are present in these granules, MPO is most abundant and accounts for 5% of neutrophils' dry weight and 25% of azurophilic granular proteins [214, 220]. A modification to the Boyden Chamber method was made by Kristina Somersalo, et al, where the response to chemotaxis was determined by measuring myeloperoxidase (MPO) specific to neutrophils and monocytes [213]. They found that the MPO Boyden chamber method had the advantages of being faster, was easier to carry out and was more objective than the traditional cell count method [213].

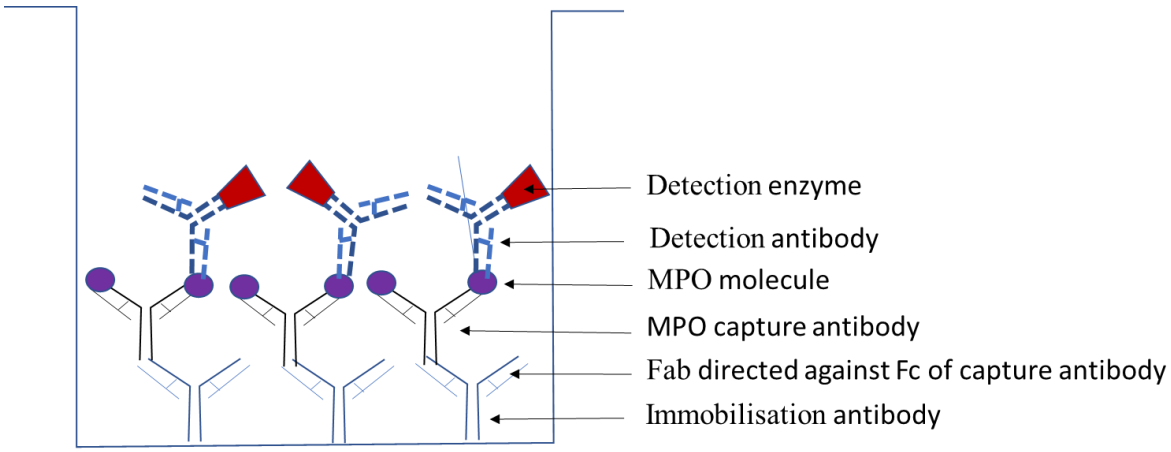
Considering the difficulty encountered when counting cells using the conventional method described above (haemocytometer) especially when using Cryptococcus in the preliminary experiments, it was decided to measure chemotactic response in this study, by measuring myeloperoxidase in the cell extracts after the 30 minute incubation step in the Transwell assay procedure (3.7.3).

3.7.3.2 Myeloperoxidase ELISA

i. Principle of the test

In this study, a commercially available Myeloperoxidase (MPO) *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit (Abcam®, Cambridge, UK) was used. Abcam's Myeloperoxidase (MPO) *in vitro* SimpleStep ELISA® kit is designed to quantitatively measure human myeloperoxidase in various cell culture supernatants, plasma, human serum, urine, milk, saliva, as well as cell and tissue extracts. Two antibodies are used in this ELISA, which capture the analyte (MPO) in the solution, and these include an affinity tag labelled capture antibody and a reporter conjugated detector antibody (Figure 3.2). Immobilization of the capture antibody/analyte/detector antibody-complex occurs next through immunoaffinity of an anti-tag antibody that coats the well. When performing the assay, standards and samples are added first to the designated wells. The antibody mix is added next. Following incubation, the wells are washed to ensure removal of any unbound material. TMB substrate (3.3′,5.5′-tetramethylbenzidine) is then added and the reaction is catalysed by horseradish peroxidase (HRP) during an incubation step. A blue colour change is observed. The reaction is stopped by adding Stop Solution, which causes a change in colour from blue to yellow. The colour intensity is proportional to the quantity of bound MPO and is measured on a suitable spectrophotometer at 450 nm. This is a sensitive assay (the lowest detection limit is 0.026 ng/mL) [221].

Figure 3.2 Myeloperoxidase Assay



The reaction follows the steps described in 3.7.3.2. The detection enzyme changes the colour of the substrate

ii. Preparation of reagents, standards and samples

A 96 well plate was used that was provided in the SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit (Abcam, Cambridge, UK). All reagents were equilibrated to room temperature (18-25°C) prior to use. The kit contained sufficient reagents for 96 wells (Appendix A5). Sample volumes were adjusted as required for the number of wells in each experiment. Serially diluted standards were prepared immediately before use. This was constituted in accordance with manufacturer's instructions (Appendix A6).

The samples on which the assay was performed were obtained for each cryptococcal isolate following the Transwell procedure described above (3.7.3) i.e. the solution containing migrated neutrophils retrieved in individual wells for each cryptococcal isolate. This was centrifuged at 18 000 × g at 4°C for 20 minutes. Supernatants were transferred to clean tubes and pellets were discarded. A standard 1:200 dilution was made for all samples that were assayed.

iii. Assay procedure

Fifty microliters each of sample or standard was added to appropriately labelled wells. Fifty microliters of prepared antibody cocktail consisting of capture and detector antibodies in antibody diluent (Appendix A5) was then added to each well. A plate sealer was used to seal the plate and this was incubated for one hour on a plate shaker set at 3.6 × g, at room temperature. Each well was then washed with 3 × 350 µl of 1 × Wash buffer PT (Appendix A5). Care was taken to ensure that all excess liquid was removed following the last wash step. One hundred microliters of TMB substrate was added to individual wells and incubated for 10 minutes in the dark on a plate shaker set at 3.6 × g. Following this, 100 µl of Stop Solution was immediately added to each well and the plate was again placed for 1 minute on a plate shaker to mix the solution. The OD was recorded at 450 nm using a microplate reader software (Thermo Fischer Scientific, Massachusetts, USA).

3.8 Gene Expression – Quantitation of TLR2 and TLR4 in Human Microglial Cells

A human microglial cell line (M059K, ATCC® - CRL-2365™) was purchased from American Type Culture Collection (ATCC), Virginia (USA). Cells were exposed to cryptococcal isolates for a period of two hours.

3.8.1 Cell Culture

3.8.1.1 Cell propogation

M059K cells (ATCC® CRL-2365™) as described above (3.8), were regrown from frozen stock as advised by ATCC. Using strict aseptic conditions, the cell suspension was rapidly thawed (not more than two minutes) by agitating gently in a water bath at 37°C. The outside of the vial was then disinfected with 70% ethanol. Within a laminar flow cabinet the contents of the vial was transferred to a 15 mL centrifuge tube, which contained nine millilitres of complete culture medium (Appendix A7). This was centrifuged (Heraeus multifuge 3S-R, 2006, Thermo Electron Corporation) at 125 × g for five minutes and the supernatant was discarded. This process is recommended to remove cytotoxic preservatives such as dimethylsulphoxide (DMSO) that may be present in the freezing fluid. The cell pellet was then resuspended in 10 mL of pre-warmed complete culture medium (Appendix A7) and transferred into 75 cm^3 tissue culture flasks. Pre-warming of culture medium for 15 minutes at 37°C was done to ensure that the medium reached the normal pH (7.0 – 7.6) and to prevent cold-shock of the cells. Cells were then incubated at 37°C in 5% CO_2 until approximately 90% confluent monolayer was obtained. Duration of incubation was determined to be between four to six days to achieve 90% confluence. Cells were maintained by refreshing the culture medium every 48 hours. Cell growth was observed at 40 × magnification using an inverted light microscope (Nikon Diaphot, Carl Zeis (PTY) Limited, Germany).

3.8.1.2 Cell counts

Cells were enumerated using the "trypan blue exclusion method". This staining method is one of the traditional methods used to do cell viability analyses. Various academic research laboratories and industrial biotechnology units use this technique as their standard procedure for this purpose [222]. The purpose of this technique is to determine and quantify viable cells in a cell suspension. The test is based on the concept that live cells possess cell membranes that are intact and exclude certain dyes, such as trypan blue and eosin while this is not the case in dead cells [223]. In this test, 10 µl of cell suspension was diluted in 10 µl of phosphate buffered saline (PBS). An equal volume (20 µl) of 0.4 % trypan blue was added to this suspension. Ten microliters of the cell-dye suspension was then filled into a Neubauer Haemocytometer slide (Appendix A1) with a cover slip and cells were counted using a Coulter counter within 3 minutes. Viable cells appeared clear microscopically whilst nonviable cells appeared blue.

The number of cells per millilitre was calculated using the following formula:

$$\text{Number of Viable Cells/mL} = \frac{\text{total number of viable cells counted} \times \text{dilution factor } (= 4) \times 10^4}{\text{number of squares counted } (4)}$$

The factor 4 corrects for the dilution with PBS and trypan blue and 10^4 for the volume.

3.8.1.3 Passaging of cells

Once a sufficiently confluent monolayer was attained, the monolayer was trypsinised with 0.25% Trypsin-0.53 mM EDTA solution (Lonza Group Ltd, Basel, Switzerland). Trypsin is a proteolytic enzyme that degrades the extracellular tissue protein matrix, which causes cells to adhere to each other. The EDTA is a chelating agent, and removes calcium and magnesium ions, which allow attachment of the cells to the flask wall. To achieve this, old medium was removed from the flask and discarded. Cells remaining were washed three times with PBS, pH = 7.2 (Appendix B6). One millilitre of trypsin-EDTA was added and spread evenly by gently rocking the flask. The trypsin-covered monolayer was then incubated for 30 seconds at 37°C to facilitate dispersal. Cells were observed at 40 × magnification under an inverted microscope (Nikon Diaphot, Carl Zeis (PTY) Limited, Germany) until the cell layer was completely dispersed. Care was taken to expose cells to trypsin for the shortest possible duration (not more than five minutes) because trypsin may damage living cells. One millilitre of foetal bovine serum (FBS) (Appendix B7) was added to the flask and gently dispersed to counteract the effect of trypsin. Cells were gently aspirated and collected into sterile tubes. The cell concentration was adjusted according to a predetermined ratio of 1:6. Complete growth medium was added to this suspension and seeded to new culture flasks, which were incubated at 37°C in 5% CO_2 until a confluent monolayer was obtained.

3.8.1.4 Cryopreservation of cells

Cryopreservation medium containing 95% complete culture medium and 5% DMSO was added to the cell suspension, slowly to prevent cell destruction. A stepwise process was followed to ensure slow freezing of cells. This entailed first placing cells at 4°C for one hour followed by a 24-hour storage at -20°C. Thereafter, cells were stored at -70°C until required for use.

3.8.2 Exposure of cells to Cryptococcus

Prepared M059K cells were exposed to cryptococcal isolates for a period of two hours at a ratio of 1000 cryptococcal cells to 1.0×10^5 M059K cells per well. The Cryptococcus suspension was prepared in a series of 1:10 dilutions as previously described (3.7.2.2) to achieve this concentration.

3.8.2.1 Confirmation of attachment of C. neoformans to M059K cells

M059K cells were regrown in a $75cm^3$ flask using complete culture medium as described previously (3.8.1.1) until confluent. The cells were trypsinized (3.8.1.3) and 100 µl of a 1.0×10^5 cells/mL was seeded into the wells of a 96 well flat bottom tissue culture plate and incubated at 37°C in 5% CO_2 for 24 hours. Thereafter, the media was carefully removed from the wells by aspiration and the adherent cells were gently washed with PBS, pH = 7.2 three times. Cells were next exposed to 1.0×10^3 CFU/mL of *C. neoformans* and incubated at 37°C in 5% CO_2 for 2 hours. The wells with organism-exposed cells were subsequently washed with PBS, pH = 7.2 without disturbing the attached infected cells. These cells were fixed with 70% ethanol for 10 minutes in the wells and stained thereafter with 1% crystal violet for 5 minutes. The stained cells were visualized under the inverted microscope (Nikon Diaphot, Carl Zeis (PTY) Limited) at 100 x magnification. Cells infected with *C. neoformans* were visualized to confirm infection before gene expression assay was carried out.

3.8.3 Gene Expression

3.8.3.1.RNA extraction

The TaqMan® Gene Expression Cells-to-CT™ Kit (Thermo Fischer Scientific, Massachusetts, USA Part No. 4399002) was used to produce DNase I digested cell lysates in accordance with manufacturer's instructions. Reagents were provided in the kit. Cells were exposed to *C. neoformans* and incubated at 37°C in 5% CO_2 for 2 hours as described previously (3.8.2.1). The culture medium was then aspirated and discarded from the wells. One wash of each well with 50 µl PBS, pH = 7.2 was done. Without

disturbing the cells, the PBS was aspirated and removed from each well. To remove genomic DNA that may be present, DNase 1 was diluted into Lysis Solution at a ratio of 1:100. Fifty microliters of Lysis Solution with DNase 1 was added to each well and mixed well by gentle pipetting. The lysis reactions were incubated for 5 minutes at room temperature following which 5 µl of Stop Solution was added to each well. This was incubated at room temperature for 2 minutes. RNA lysates were stored at -20ºC until cDNA synthesis was performed. Six RNA extractions were performed for each sample and these were tested separately.

3.8.3.2 cDNA synthesis

We used the TaqMan® Gene Expression Cells-to-CT™ Kit (Thermo Fischer Scientific, Massachusetts, USA, Part No. 4399002) to generate cDNA from RNA lysates. The cDNA synthesis was carried out as per manufactures instruction. Under aseptic conditions, 22.5 µl of RNA lysate was added to 27.5 µl master mix consisting of 2X RT Buffer, 20X RT Enzyme Mix in nuclease free water and mixed well. All steps were performed on ice. The cDNA synthesis was performed on a SimpliAmp™ Thermal Cycler (Thermo Fischer Scientific, Massachusetts, USA, Cat no. A24811) which was programmed as shown in Table 3.2.

TABLE 3.2: Thermal cycle programme for reverse transcription

sequence	no. of cycles	temperature	time (minutes)
reverse transcription (RT)	1	37°C	120
RT inactivation	1	95°C	5
hold	1	4°C	indefinite

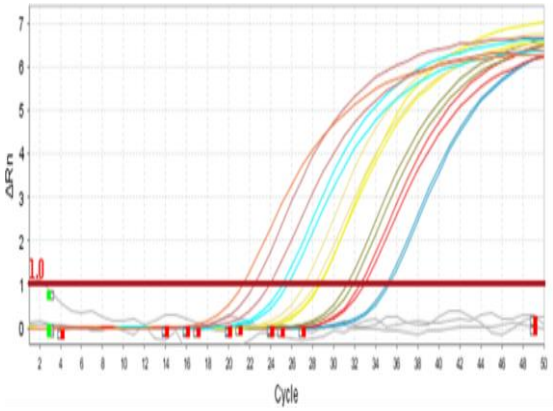
The total cDNA concentration was quantified using a Nanodrop 1000 spectrophotometer (Thermo Fischer Scientific, Massachusetts, USA) and samples were only used if the optical density at 260 nm (OD260)/OD280 ratio was 1.8 or higher.

3.8.3.3 Real Time Polymerase Chain Reaction

Quantitative real time Polymerase chain reaction (PCR) was performed on the following genes, 18S (Thermo Fischer Scientific, Massachusetts, USA, Cat no: Hs99999901_s1 18S), TLR2 (Thermo Fischer Scientific, Massachusetts, USA, Cat no: Hs02621280_ s1 TLR2) and TLR4 (Thermo Fischer Scientific, Massachusetts, USA, Cat no: Hs00152939_ m1 TLR4). TaqMan probes were specifically designed from highly conserved regions of the genes. Each cDNA sample was diluted 1:10 for real time PCR analysis. Each PCR reaction comprised of 0.5 µl FAM™ labelled probe primer mix for individual targets, 5.0 µl TaqMan® Gene Expression Master Mix (2X) (Thermo Fischer Scientific, Massachusetts, USA, Part No. 4399002), 1.0 µl cDNA and 3.5 µl water resulting in an end volume of 10 µl. Reactions were run on a Quant Studio 5 real time PCR machine (Thermo Fischer Scientific, Massachusetts, USA, Cat no: A28140) with one cycle at 95 ˚C for 10 minutes, followed by 45 cycles consisting of denaturation at 95 ˚C for 15 seconds, and annealing for one minute at 60 ˚C. Detection of the fluorescent products was carried out at the end of the 60 ˚C annealing period. Standard curves were made for each gene from a pool of cDNA from every sample (Figures 3.3, 3.4, 3.5). Serial dilutions of cDNA from RNA lysates were made for each target gene. Standard curves were made from these and used for quantitative analysis. All samples where run in duplicate. Data is represented as normalized gene expression to 18S.

Figure 3.3 Amplification Plot and Standard Curve of the 18S Gene

Amplification Plot of 18S



Standard Curve for 18S

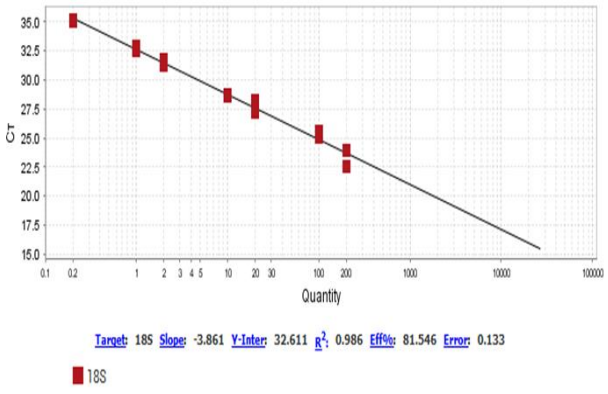
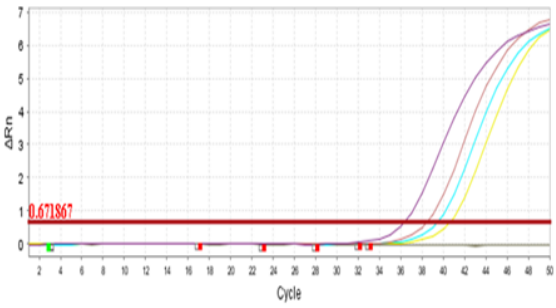
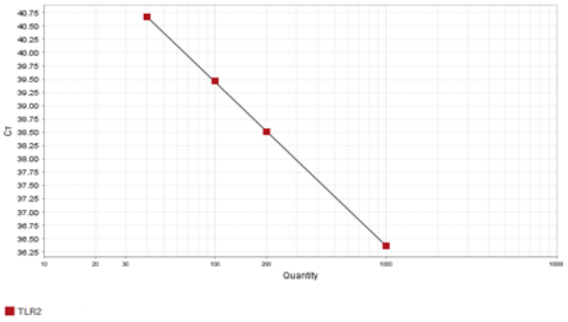


Figure 3.4 Amplification Plot and Standard Curve for the TLR2 Gene

Amplification Plot of TLR2



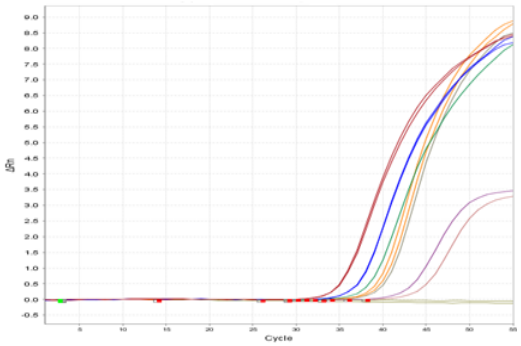
Standard Curve for TLR2



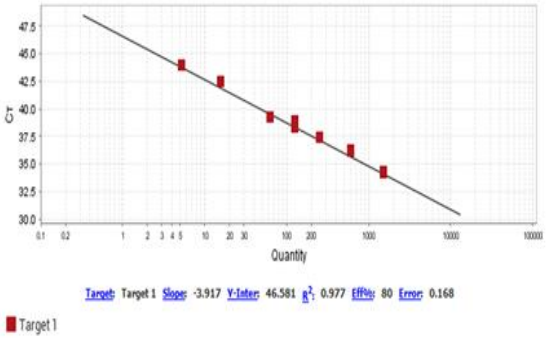
Target: TLR2 Slope: -3.076 Y-Inter: 44.856 R^2: 1 Eff%: 111.396 Error: 0.016

Figure 3.5 Amplification Plot and Standard Curve for the TLR4 Gene

Amplification Plot of TLR4



Standard Curve for TLR4



Target: Target 1 Slope: -3.917 Y-Inter: 46.581 R^2: 0.977 Eff%: 80 Error: 0.168

3.9 Statistical Analysis

All experiments were done in triplicate. The distribution of cell count and neutrophil data were summarized using medians and interquartile ranges. Mann-Whitney non-parametric tests were used for two group comparisons and Kruskal-Wallis for more than two groups. Numeric variables such as CSF, capsule size and opening pressure were also categorized and chi square tests or Fisher's exact were used to compare sub-groups. The association between two number variables was examined using scatter plots and Spearman's correlation coefficient. Stata v13 statistical software was used in the analysis.

CHAPTER 4 - RESULTS

4.1 Description of Clinical Data

Seventy-one participants were enrolled in the study. *Cryptococcus neoformans* was identified in the CSF of all 71 patients. The general clinical parameters indicative of meningitis (fever, headache and neck stiffness) were recorded (Table 4.1.1). The commonest clinical finding was headache (71 patients), followed by neck stiffness (48 patients). Only 15 patients presented with fever. The mean age was 35 years with a range of 18 – 62 years. There were 11 more male than female patients (41 vs 30). Fifty-nine patients had positive Gram stain microscopy for Cryptococcus whilst all patients had an initial Cryptococcal Latex Agglutination Test (CLAT) positive.

TABLE 4.1.1: Clinical and laboratory parameters of 71 patients presenting with meningitis at the time of admission

	no. (%)
Glasgow coma scale = 15	60 (85)
headache	71 (100)
fever	15 (21)
neck stiffness	48 (68)
papilloedema	0 (0)
cryptococcus seen on Microscopy	59 (83)
qualitative CLAT titre	71 (100)

At the baseline diagnostic lumbar puncture (LP), 65 patients had CSF opening pressure (OP) measured (Table 4.1.2). Thirty-nine of these patients had an opening pressure >20 cm H_2O. CSF chemistry was slightly abnormal with a median protein of 0.83 g/L (IQR = 0.43 – 1.35) and glucose median of 2.1 mmol/L (IQR = 1.2 – 2.6). The CSF neutrophil count was within the normal range (IQR = 0 – 4 cells/mL). CSF lymphocytes however were elevated at baseline with a median of 21 cells/mL (IQR = 8 – 84). Notably, the blood neutrophil counts of all patients were within the normal range (median = 4.29; IQR = 2.58 – 6.10). As a means to determine the amount of shed capsular material in CSF and serum, two-fold serial dilutions of each specimen was done until the endpoint of CLAT antigen positivity was reached. The median CSF and serum CLAT titres were both 2048, however the IQRs

were different (CSF IQR = 9256 – 8192; serum IQR = 256 – 4096). Raw data for CLAT titre investigations are displayed in Appendix C1. The average capsule size was measured as described earlier (3.6.1). Raw data for determination of capsule size is available in Appendix C2. Measurement of capsule thickness was performed on Cryptococcus cells isolated from the 65 culture positive CSF specimens. These organisms were all passaged once after initial isolation (3.6.1). The median capsule thickness was 1.2 μm (IQR = 1.04 – 1.39).

TABLE 4.1.2: Baseline blood and cerebrospinal fluid (CSF) investigations of 71 patients presenting with meningitis

	no. tested	median (IQR)
blood CD4	70	39.5 (14 - 96)
blood neutrophils *	62	4.29 (2.58 - 6.10)
serum CLAT titre	71	2048 (256 - 4096)
CSF opening pressure	65	22 (16 - 29)
CSF protein	71	0.83 (0.43 - 1.35)
CSF chloride	70	117 (113 - 121)
CSF glucose	71	2.1 (1.2 - 2.6)
CSF neutrophils **	70	0 (0-4)
CSF lymphocytes **	70	21 (8 - 84)
CSF RBC **	70	0 (0 - 30)
CSF CLAT titre	71	2048 (9256 - 8192)
average capsule size ***	65	1.2 (1.04 - 1.39)

CLAT: cryptococcal latex agglutination test; * $\times 10^9$/L; ** cells/μl; *** μm

4.2 Association between CSF neutrophil and blood neutrophil count

It is known that cryptococcal meningitis is characterized by a relative paucity of neutrophils in the CSF as compared to that in bacterial meningitis [3-5, 56, 201]. Blood neutrophil and CSF neutrophil counts of patients were obtained at time of admission and comparisons were made (Table 4.2.1). Complete data (which included blood neutrophil and CSF neutrophil counts) were available for 62 patients. The majority (47) had a CSF neutrophil count below five cells per microliter (cells/μl) of who 39 patients had no neutrophils in the CSF at all. Only 15 patients had a CSF neutrophil count of > 5 cells/μl. Of the 47 patients with CSF counts below five cells/μl, 40 (85.11%) had blood-neutrophil counts at the higher

end of the normal range (2 - 7 $\times 10^9$ cells/L). Nine patients had an elevated blood neutrophil count. Eight of these had CSF neutrophil counts of < 5 cells/µl. An association was noted between CSF neutrophil and blood neutrophil count where a lower CSF neutrophil count appeared to be associated with higher blood neutrophil count. However, this was not statistically significant (p = 0.37).

Table 4.2.1: Comparison between blood neutrophil and CSF neutrophil count

CSF neutrophils (cells/µl)	no. (%) of patients with blood neutrophils *:			Total
	<2	2 to 7	>7	
<5	7 (15)	32 (68)	8 (17)	47
>5	1 (7)	13 (87)	1 (7)	15
Total	8 (13)	45 (73)	9 (62)	62

* x 10^9 cells/L

4.3 Association between clinical parameters and neutrophil count, cryptococcal capsule size and shed cryptococcal capsular material

In keeping with meningitis, all of the study patients presented with headache as the predominant clinical symptom. There were however only 15 patients who presented with fever. The association between fever and total CSF and blood neutrophil counts, cryptococcal capsule size and cryptococcal shed antigen (CSF CLAT titre) were further investigated (Table 4.3.1). Nine of the 15 patients with fever had a CSF neutrophil count of < 5 cells/µl (p=0.05). Forty-six patients presented without fever. Of statistical significance was that patients with fever had a higher CSF neutrophil count (p = 0.003). In contrast with the CSF neutrophil-count, those patients who presented with fever had a lower blood-neutrophil count. This displayed a borderline significance (p = 0.08). However, the blood neutrophil count remained within the normal range in all patients with or without fever. The capsule size of cryptococcal cells isolated from patients with fever tended to be smaller than in patients without fever, however, there was no statistical significance (p = 0.18). Similarly, the comparison between shed capsular material in the CSF of patients with fever and those without fever showed no significant difference (p = 0.86).

Table 4.3.1: Association between fever and neutrophil count, cryptococcal capsule size and shed cryptococcal capsular material in 71 patients presenting with meningitis

	no. of patients	no. of patients with:		p-value
		fever (n=15)	no fever (n=55)	
CSF neutrophils > 5 cells/µl	15	6	9	0.05
median (cells/µl) CSF neutrophils (IQR)	70	4 (0 - 16)	0 (0 - 2)	0.003
median (×10⁹ cells/L) blood neutrophils (IQR)	62	3.47 (2.37 - 4.46)	4.36 (2.94 - 6.25)	0.08
median (µm) capsule size (IQR)	65	1.06 (1.02 - 1.33)	1.23 (1.06 - 1.46)	0.18
median CSF CLAT titre (IQR)	71	2048 (512 - 8192)	2048 (256 - 8192)	0.859

CLAT: cryptococcal latex agglutination test

All 71 patients presented with headache as a common clinical symptom. Fifteen patients with headache had fever and 48 had neck stiffness (Table 4.3.2). Of all the patients with headache, 55 had CSF neutrophil counts of ≤ 5 cells/µl of which 46 had no neutrophils at all in the CSF. (There was missing data (CSF neutrophil count) for one patient).

Table 4.3.2: Headache in association with six other parameters in 71 patients presenting with meningitis

	no. of patients	no. (%/IQR) of patients with:
fever	71	15 (21)
neck stiffness	71	48 (68)
CSF neutrophils:	70	
≤5 (cells/µl) median CSF neutrophils	55	0.0 (0 -0)
>5 (cells/µl) median CSF neutrophils	15	16 (8- 60)
median CSF neutrophils	70	0.0 (0 - 4)
median capsule size(µm)	65	1.198 (1.034 - 1.418)
median CSF CLAT titre	71	2048 (256 - 8192)
median blood neutrophils (×10⁹cells/L)	62	4.29 (2.58 - 6.13)

4.4 Shedding of cryptococcal capsular material: Effect on opening pressure, CSF neutrophil count and capsule size

Shed capsular material was quantified as described previously (3.4). Twenty-five CSF specimens had a CLAT titre below 512; 25 had a titre between 1024 and 4096 and 21 samples had a titre ≥ 8192. Raw data for CSF CLAT titre is shown in Appendix C1. The CSF opening pressure appeared to decrease with increasing CLAT titre (Fig 4.1). There was a tendency for the average capsule size to decrease with increased amount of shed capsular material. Spearman's correlation co-efficient determined that this was not statistically significant, however (Spearman's rho = − 0.2) (Fig 4.2).

Figure 4.1: Shed Crytpococcal Capsular Material: Association with Opening Pressure

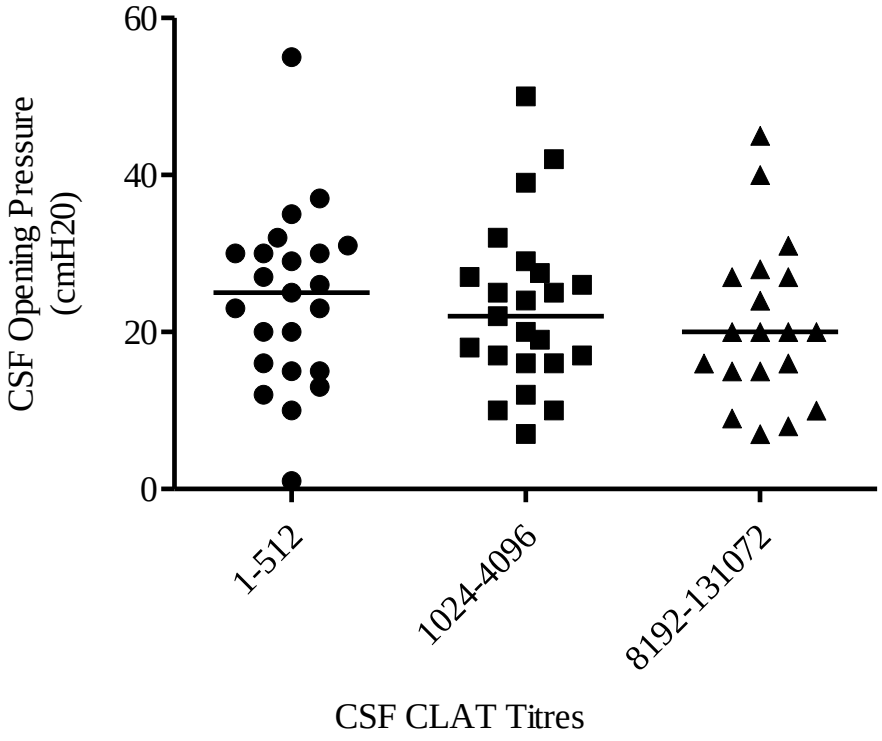
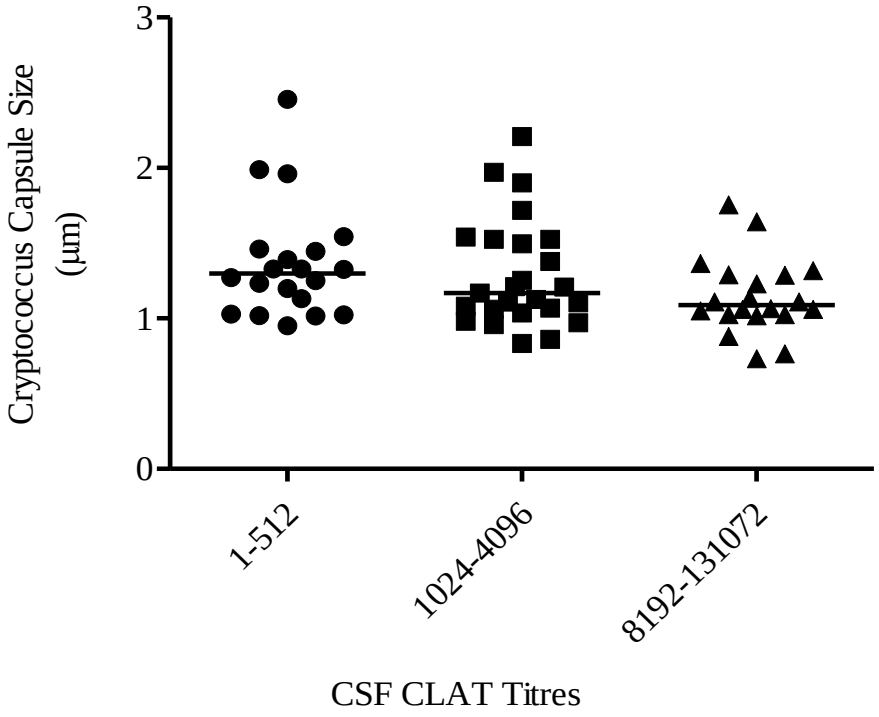


Figure 4.2: Shed Crytpococcal Capsular Material: Association with Capsule size



There was a significant association with shed capsular material (CSF CLAT titre) and number of neutrophils in CSF (Table 4.4.1). With a higher CSF CLAT titre there were significantly more patients with lower CSF neutrophils (p = 0.03, Spearman's rho = − 0.3).

TABLE 4.4.1: Shed capsular material in relation with CSF neutrophils, opening pressure and capsule size

		CSF CLAT titre		p-value
		<1000	>1000	
CSF neutrophils (cells/μl)	≤5	16	39	0.03
	>5	9	6	
opening pressure (cmH_20)	≤20	8	18	0.15
	>20	15	24	
median capsule size (IQR)		1.30 (1.08 - 1.45)	1.11 (1.04 - 1.37)	0.14

CLAT: cryptococcal latex agglutination test

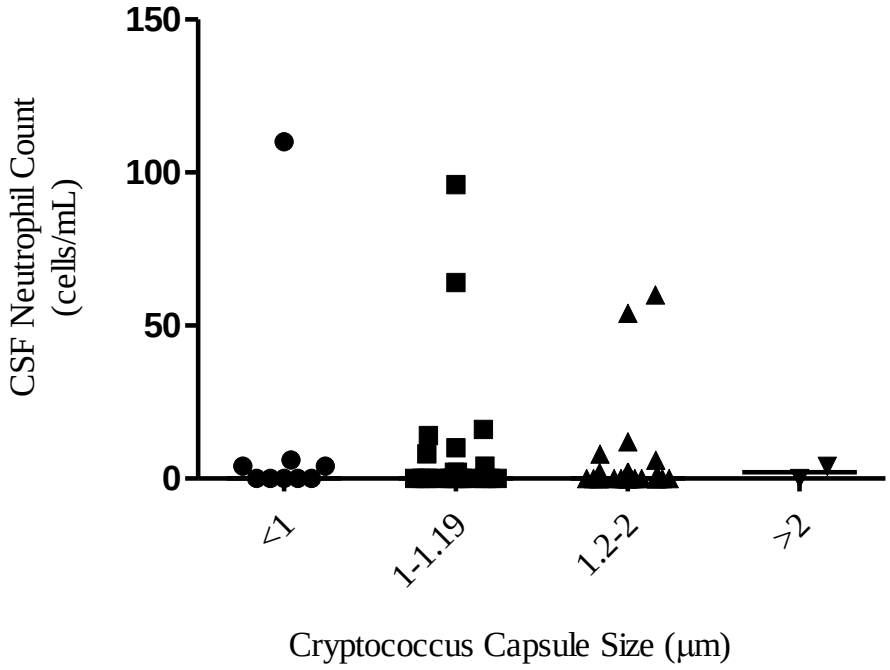
4.5 Capsule thickness and its association with opening pressure, blood and CSF neutrophils and CSF CLAT titre

The capsule of *C. neoformans* is a known virulence factor. Previous studies have shown that the capsule can change in size in response to environmental conditions. In this study, capsule size was measured as described previously (3.6.1). Raw data for determination of capsule size is shown in Appendix C2. Average capsule size ranged between 0.73 μm – 2.46 μm and based on the size of the capsule patients were divided into four groups for further analysis (Table 4.5.1). The majority of cryptococcal cells (n = 54) had capsule sizes that ranged between one and two micrometers respectively. The opening pressure was elevated in all four groups with the highest opening pressures recorded in those patients with cryptococci with an average capsule size > 2 μm. CSF neutrophil counts were lowest in this last group with cells that had capsule size > 2 μm and highest in patients with cryptococcal cells with capsule size < 1 μm. Blood neutrophil counts were similar in all four groups. However, like CSF neutrophil counts, blood neutrophil counts were also lowest in the patients whose cryptococci had a capsule size of > 2 μm. Those CSF samples with *C. neoformans* cells that had a capsule size > 2 μm also displayed the lowest shed cryptococcal capsular material (average CSF CLAT titre = 768) compared to the other three groups.

TABLE 4.5.1 : Capsule size in relation to opening pressure, blood and CSF neutrophils and CSF CLAT titre

average capsule size (μm)	no. of patients	average (SD) opening pressure (cmH_20)	average (SD) CSF neutrophils (cells/μl)	average (SD) blood neutrophils ($\times 10^9$ cells/L)	median (IQR) CSF CLAT titre
<1	9	23.44 (10.41)	13.78 (36.16)	5.23 (3.45)	2048 (1024 - 8192)
1 - 1.19	24	22.86 (12.90)	9.0 (22.85)	4.34 (1.92)	4096 (768 - 12288)
1.2 - 2.0	30	22.24 (10.12)	4.87 (14.46)	4.35 (2.53)	1024 (256 - 4096)
>2	2	25.50 (0.71)	2.0 (2.83)	3.73 (2.03)	768 (512 - 1024)

Figure 4.3: Cryptococcus Capsule size in Relation to CSF Neutrophil Count



In this analysis, the capsule size is larger in those patients with lower CSF neutrophil counts (Table 4.5.2). However, this is not statistically significant (p = 0.28). There was no relationship between capsule size and blood neutrophils. Blood neutrophils were essentially within the normal range in the study group.

TABLE 4.5.2: Capsule size in relation to neutrophil count

		no. of patients	median (IQR) capsule size (μm)	p-value
CSF neutrophils *:	≤5	52	1.21 (1.06 - 1.41)	0.28
	>5	13	1.06 (1.02 - 1.39)	
Blood neutrophils **:	<2	8	1.51 (1.12 - 1.81)	0.22
	2 to 7	41	1.10 (1.03 -1.33)	
	>7	8	1.26 (1.03 - 1.45)	

* cells/μl ; ** x 10^9 cells/L

4.6 Neutrophil chemotaxis inhibition

Various explanations are presented in the literature regarding the possibility of inhibition of neutrophil chemotaxis in the CSF. In this study, chemotaxis experiments were conducted as described earlier (3.7) and chemotaxis inhibition was calculated. Raw data is displayed in Appendix C3. There was no inhibition by 21 cryptococcal isolates while 44 displayed chemotaxis inhibition (Table 4.6). The amount of shed cryptococcal capsular material (CLAT titre) was higher in the group that displayed chemotaxis inhibition (but not statistically significant). Capsule size was similar in both groups. The number of neutrophils in the CSF was significantly higher in the group that displayed chemotaxis inhibition (10.82 vs 0.67 cells/mL respectively; p = 0.04).

TABLE 4.6: Chemotaxis inhibition in relation to CSF CLAT titre, capsule size and CSF neutrophils

chemotaxis	no. of patients:	median (IQR) CSF CLAT titre	average (SD) of patients with: capsule size (μm)	average (SD) of patients with: CSF neutrophils (cells/mL)
activation	21	1024 (512 - 4096)	1.27 (0.30)	0.67 (1.59)
inhibition	44	4096 (384 - 8192)	1.26 (0.37)	10.82 (25.38)

CLAT: cryptococcal latex agglutination test

4.7 Expression of TLR2 and TLR4 genes in human microglial cells

In this study, we attempted to show TLR2 and TLR4 gene expression induced by the individual strains of Cryptococcus in human microglial cells. This was performed on 64 *C. neoformans* isolates. Gene expression was performed as described earlier (3.8.3). Forty-five (70 %) of these isolates displayed downregulation for TLR2 and 19 showed upregulation (Table 4.7.1). Capsule size did not vary remarkably in both these groups. Neutrophil counts were higher in those CSF specimens that grew cryptococci that upregulated TLR2 expression more than 10 times, and was lower in those specimens

that showed downregulation, but this finding is not statistically significant. The amount of shed capsular material was also similar in all the groups.

TABLE 4.7.1: Expression of TLR2 genes in relation to average capsule size, CSF neutrophils and CSF CLAT titre

TLR2/18S	no.of patients	average (SD) capsule size (μm)	average (SD) CSF neutrophils (cells/μl)	median (IQR) CSF CLAT titre
<1	45	1.30 (0.38)	7.06 (19.82)	4096 (512 - 8192)
1.0 - 10	12	1.27 (0.30)	5.67 (18.39)	1024 (288 - 6144)
>10	7	1.15 (0.18)	13.71 (36.28)	4096 (256 - 32768)

TLR4 gene expression was similar to TLR2 regarding upregulated (n = 21) and downregulated (n = 43) gene expression (Table 4.7.2). There was a strong correlation (Spearman's rho = 0.9) between expression of the two genes. The average capsule size did not display much variation among the different groups. The CSF neutrophil counts however were lower in the isolates that showed upregulation above 10x and there were in fact zero cells per milliliter in this small group. This was not statistically significant. Shed capsular material was similar in the different groups.

Raw data for expression of TLR2 and TLR4 is shown in Appendix C4.

TABLE 4.7.2: Expression of TLR4 genes in Relation to Average Capsule size, CSF neutrophils and CSF CLAT titre

TLR4/18S	no.of patients	average (SD) capsule size (μm)	average (SD) CSF neutrophils (cells/μl)	median (IQR) CSF CLAT titre
<1	43	1.31 (0.38)	6.70 (20.27)	4096 (512 - 8192)
1.0 - 10	15	1.18 (0.32)	12.93 (28.21)	1024 (512 - 8192)
>10	6*	1.21 (0.12)	0.0 (0.0)	1453 (64 - 8192)

*5 of these had the same value for TLR2 expression

CHAPTER 5 – DISCUSSION AND CONCLUSION

Cryptococcal meningitis is an important opportunistic infection especially in the immunocompromised patient. It is well known that cryptococcal meningitis characteristically has a relative paucity of polymorphonuclear neutrophils (PMN) in the CSF when compared to bacterial meningitis [3-5]. This is despite evidence that the capsular material induces production of the early proinflammatory cytokines IL-1β and TNF-α by peripheral blood PMNs and monocytes [3, 36, 224]. Mouse model studies previously showed that the inhibition of PMN infiltration was mainly due to major capsule component GXM [225]. Several studies have attempted to determine the mechanisms involved in regulating passage of leucocytes through endothelial cells yet the mechanism by which signaling in brain inflammation occur, is still unclear [3]. In this study, we hypothesized that the capsular material interfered with neutrophil chemotaxis and that this had an inverse impact on severity of disease. Few studies have been done on human infection and therefore this study focused on human infection and effect on host inflammatory response. Similar to previous studies, our study shows typically few mononuclear cells and virtually no PMNs in patients' CSF and interestingly, all patients' blood PMNs were within the normal range that is, neither elevated nor decreased. Furthermore, we observed that a low CSF neutrophil count was associated with a higher blood neutrophil count although this was not statistically significant.

It is important to note that the sample size in this study had to be considerably reduced (by 70%) due to a general decrease in patients presenting with crytpococcal meningitis to hospital. The recruitment of patients for the study was therefore significantly compromised. A possible explanation for this drastic decrease could be the fact that highly active antiretroviral therapy (HAART) was introduced to patients infected with HIV, and studies show that there is a positive impact of HAART to patients with crytpococcal infection especially in Western countries [226, 227].

Although there is no severity of disease score for patients with cryptococcal meningitis, a trend in clinical parameters was evident in these patients in keeping with the literature. Of the clinical parameters measured, headache was the predominant clinical symptom in all patients followed by neck stiffness. Fever occurred in only a fraction of the patients. These findings are consistent with other studies that describe the clinical course of cryptococcal meningitis as subacute or chronic with headache being a predominant finding [228, 229]. In fact, it is known that patients with AIDS may even remain asymptomatic early in the disease process and that the classic symptoms of meningitis (headache, photophobia and meningism) may be absent [230, 231]. Furthermore, it has been observed that cryptococcal encephalitis may remain undiagnosed due to poor inflammation at the site of infection [139, 232]. Vaidhya et al identified fever, headache and neck stiffness respectively as the predominant symptoms in their cohort, which differs from the findings in this study where fever occurred in only a

small fraction (21%) of patients [96]. Clinical signs such as papilloedema, hydrocephalus and focal seizures during cryptococcal meningitis is a common occurrence in immunocompetent patients [228, 233]. This study population included only immunocompromised patients and consistent with this observation, none of the patients showed signs of papilloedema. More than 50% of patients also showed evidence of mildly raised intracranial pressure (ICP) with a median opening pressure of 22 cmH_20. This finding is noteworthy considering that in this study the opening pressure was recorded only at the second lumbar puncture where it is performed routinely to reduce intracranial pressure. Raised intracranial pressure is a common complication of cryptococcal meningitis and contributes to morbidity and mortality [234, 235]. It is not clearly understood what the causes of raised intracranial pressure in cryptococcal meningitis are, however, some hypotheses are that it may be related to impaired CSF reabsorption caused by the organism or perhaps its shed polysaccharide capsule [234, 236]. It was also noted in this study that the CSF chemistry (protein and glucose) was only slightly abnormal. This relates to previous studies that describe clear differences in CSF chemistry between cryptococcal meningitis (moderate elevation in protein and moderate decrease to normal glucose) and bacterial meningitis (high protein and low glucose) [228, 237].

Significantly, patients who presented with fever had a higher CSF PMN count compared to patients with no fever. Patients with fever also had a lower blood PMN count. Cellular components of the innate immune system (including neutrophils) form part of the first line of defense in cryptococcosis [119]. These cells recognize and phagocytose the yeast resulting ultimately in their degradation. Fever is often a manifestation of an aggressive inflammatory response [238]. This was confirmed in our finding that patients who mounted a neutrophil response presented clinically with fever. The concept of the association of fever with leucocyte infiltration dates as far back as the 19th century [239]. The hypothalamus in the brain regulates and maintains normal body temperature. The febrile response is a prominent component of various inflammatory and immunologically mediated diseases [239]. These infections, including bacterial, viral and fungal infections, may result in the production of pyrogens that alter the "thermostat setting" of the hypothalamus to raise body temperature leading to fever. Pyrogens may be both exogenous as well as endogenous. Cytokines that are known to be intrinsically pyrogenic are IL-1α, IL-1β, TNF-α, TNF-β, IFN-γ, and IL-6 [239]. Following CNS invasion by *C. neoformans*, the microglial response is critical. Microglial cells together with astrocytes recognize fungal PAMPS. During fungal antigen exposure, cytokines, which include the pyrogenic cytokines (IL-1β, TNF-α, IFN-γ, and IL-6) recruit peripheral CD4 and CD8 T-cells, peripheral macrophages, and neutrophils, which are then able to seed into the CNS [171]. In addition, the literature demonstrates that neutrophils also produce and express various cytokines either constitutively or upon activation by micro-environmental stimuli [240]. The pyrogenic cytokines are known examples of cytokines that neutrophils can potentially express and/or produce [240]. The relationship between fever and PMN counts in CSF or blood in cryptococcal meningitis has not been explored previously. Fernandes et al noted that there was

a positive correlation between fever and increased capsule thickness [238]. We found no significant correlation between fever and phenotypic characteristics of Cryptococcus such as the capsule thickness or the amount of shed capsule.

The polysaccharide capsule of *C. neoformans* is one of its most important virulence factors and plays a role in evading phagocytosis as well as regulating the immune response [39, 45-49]. Some studies suggest that the ability of phagocytes to clear the organism inversely relates to the capsule size [36, 37]. The phenomenon of capsular enlargement during infection is important as this has an effect on the size of the cell thus creating a problem for phagocytosis [101, 109, 241]. Various studies suggest an association between increased capsule size and resistance to phagocytosis [241-243]. In particular, one compartment in which induction of capsule increase took place, although less efficiently than in the lung, was found to be the brain [241, 244]. Littman demonstrated that the capsule size was quite variable and depended on the organ affected [241, 245]. Charlier et al reported that tissue invasion was associated with changes in size and composition of the organism's capsule in the brain of AIDS patients with both acute and chronic meningoencephalitis [1]. In our study, the capsule size ranged between 0.7 μm and 2.5 μm. The normal size of the cryptococcal capsule ranges between 2.0 – 4.0 μm, which indicates that the capsule size was not enlarged in the study group. There were however, a large number (97%) that expressed a capsule size below the normal range. A possible explanation for this is that the organisms were isolated from CSF on sheep blood agar (3.3) and passaged once in vitro on Brain Heart Infusion Agar plates supplemented with 10% horse blood (3.6) before the capsule measurements took place. In an in vitro murine study, Dykstra et al observed that cryptococcal cells in diseased tissue had large capsules, but when grown on ordinary media such as Sabouraud glucose agar, there was notable variation in capsule size [246]. They postulated that capsule size decreased in high osmolarity media and enlarged in media of low osmolarity. Furthermore, they suggested that although cells may produce the same amount of capsular material despite osmolarity, the volume occupied in high osmolarity media could possibly be less due to shrinkage [246]. In our study, the capsule size was larger in patients with lower CSF neutrophil counts, although this was not statistically significant. This is in keeping with previous findings that the capsule suppresses immune cell infiltration into the CNS [247, 248]. Our study also suggests that patients' whose isolates had larger capsules also had higher CSF opening pressures. Robertson et al demonstrated that patients who had *C. neoformans* infection had a greater chance of having raised intracranial pressures and diminished CSF inflammatory response when infecting strains produced larger capsules [248]. This finding corroborates an earlier study in a rat model that showed that capsular phenotype influences raised intracranial pressure [248, 249].

A major polysaccharide component of the Cryptococcus capsule is glucuronoxylomannan (GXM), which is shed freely from the capsule and accumulates in body fluids and tissue of patients during infection [2, 151]. In disseminated cryptococcal infection, there are high serum and CSF titres of GXM in affected patients and this has been associated with disease progression as well as a diminished

inflammatory response [3, 151, 250]. In this study, we quantified shed capsular material in individual patient CSF samples and analyzed the effect of shed capsular material on CSF neutrophil count as well as on CSF opening pressure and capsule size. There was a significant association with shed capsular material and number of neutrophils in CSF. With a higher CSF CLAT titre there were significantly more patients with lower CSF neutrophils ($p = 0.03$). Various explanations have been described in the literature regarding the possibility of inhibition of chemotaxis in the CSF. One such explanation is a phenomenon called cross desensitization of the IL-8 receptor which describes the relationship of FMLP and C5a (also neutrophil chemoattractants) to IL-8 [5, 204]. In that study, rapid desensitization for IL-8 was shown to have occurred when PMNLs were previously exposed to the chemoattractants IL-8 itself, C5a or FMLP. Receptor phosphorylation and IL-8 receptor internalization was believed to be the mechanism involved during this process [204]. In another study, GXM was observed to inhibit polymorphonuclear leucocyte (PMNL) adhesion to activated endothelial cells in a concentration dependent manner [56]. They used a static adhesion assay and found that GXM exerts this action by affecting both PMNs and endothelial cells [56]. Another possibility for the paucity of leucocytes described in the literature was that although production of interleukin-8 (IL-8) occurred in the CSF of patients with cryptococcal meningitis, the circulating GXM was downregulating recruitment of leucocytes, thus leading to impaired migration of leucocytes into the CSF [205, 206].

In this study, chemotaxis experiments were performed on individual isolates of Cryptococcus obtained from patients CSF to determine the extent of inhibition of neutrophil chemotaxis. Close to 70% of isolates displayed chemotaxis inhibition. Although this was not statistically significant, the amount of shed cryptococcal capsular material was higher in the group that displayed chemotaxis inhibition. This is in keeping with several previous studies that showed evidence of capsular material especially GXM inhibiting leucocyte migration [3-5, 56, 57]. Lipovsky et al, further determined that the different GXM serotypes varied in their ability to induce production of IL-8 [5]. They also found that most clinically important isolates of *C. neoformans* were of the serotype A. They concluded in their study that the cryptococcal polysaccharide capsule inhibited neutrophil migration toward IL-8 in both GXM serotypes A and D [5]. The number of neutrophils in the CSF was significantly higher in the group that displayed chemotaxis inhibition ($p=0.04$). This finding is interesting because it was expected that with chemotaxis inhibition, there would be a lower number of neutrophils in the CSF. This is also contrary to the finding that fewer neutrophils were found in the CSF of patients whose cryptococcal isolates shed more capsular material in the CSF. A possible explanation for this could be that there was a small percentage of patients (7%) in this group that had very high neutrophil counts (above 60 cells/mL) in their CSF, which may have influenced the average neutrophil count in this group. When this group (7%) was excluded and the average neutrophil count in the CSF was recalculated, this observation turned out not to be true. The presence of leucocytes is regulated through chemotaxis. Chemotaxis depends on chemokine production at the site of infection and the level of expression of corresponding chemokine receptors on

the migrating cells. Chemokines are major mediators of leucocyte recruitment into infection sites, including infections with *C. neoformans* [202]. In the brain, microglial cells are known sources of not only IL-8, but also other chemokines such as IP-10, MIP-1α, MIP-1β, RANTES, KC, and MCP-1 [203]. Therefore, a further explanation for this discrepant finding could be the varying effects of chemokines at the site of infection. Chemokines were not measured in this study and this finding stimulates further research.

Capsule size was similar in both groups (chemotaxis inhibition and no inhibition) although interestingly, there was a tendency for the average capsule size to decrease with an increased amount of shed capsular material. This is in accordance with studies that showed how the capsule of *C. neoformans* responded to various environmental factors [251-253]. Larger capsules were related to greater production of polysaccharides, a greater and more extensive assembly of polysaccharide, and also less shedding of capsule [251]. This could imply that with greater shedding of capsular material, the capsule size would also be smaller, as was observed in our study. In a recent study by Fernandes et al, cryptococcal capsule size was found to differ between individual isolates, genotypes, as well as species [238]. They have also stated that it is not clear whether a large capsular phenotype enhances the overall virulence of Cryptococcus. However, a finding in their study was that capsule size correlated strongly with yeast cell size, and as such, the possibility existed that capsule alone has less significance than overall size of the cell [238].

Microglial cells, which are phagocytic cells within the central nervous system, along with meningeal and perivascular macrophages, make up the first line of defence in the brain during CNS infections [168-170]. These cells are major regulators of the innate immune response because they express Toll-like receptors (TLRs) which can identify pathogen associated molecular patterns (PAMPs) [170, 173, 174]. Chemokines are produced when TLRs recognize specific microbes. For *C. neoformans*, TLR2 has been identified as the most important TLR [6, 7, 175]. It has been shown that TLR2 and TLR4 recognize cryptococcal capsular component GXM [170, 175]. GXM bound to both TLR2 and TLR4 with the co-receptor CD14 but it was unable to activate the MAPK (mitogen-activated protein kinase) pathway and produce TNF-α [170, 175]. Although increasing interest in expression of TLRs is becoming evident, little information is available on expression and function of TLRs in the CNS microglial cells [7]. Some data is becoming available on TLR expression in rodent CNS, however little information exists on TLR expression in human microglial cells [7].

In this study, we measured TLR2 and TLR4 gene expression induced by the individual strains of Cryptococcus in human microglial cells. Both TLR2 as well as TLR4 were expressed similarly in microglial cells by individual cryptococcal isolates. This concurs with earlier studies that associated both TLRs with cryptococcal infection [170, 175]. Biondo et al, however, concluded in their study, that TLR2 but not TLR4 contributed to anti-cryptococcal defenses [164]. We showed that exposure to the

majority (nearly 70%) of cryptococcal isolates induced downregulation for both TLRs in the glial cells and only a small proportion resulted in upregulation. This supports one of our research questions that downregulation of neutrophil chemotaxis occurs at the level of TLR expression.

In keeping with this finding was that neutrophils were lower in the CSF specimens where there was downregulation (TLR4/18s <1) of the TLR4 genes in human microglial cells. This was however not true for TLR2 gene expression. Netea et al reported the effect of TLRs in another fungal pathogen (*Candida albicans*) [254, 255]. Their experiments in mice which express defective TLR4 genes showed impaired neutrophil recruitment to the site of infection [254]. Further studies on the fungal pathogen *C. albicans* showed conflicting results with TLR2, which displayed either protective or harmful effects on the pathogen [167, 255, 256]. Furthermore, various research on *Aspergillus fumigatus* described the function of TLR2, TLR4 and MyD88 in neutrophil activation and cytokine secretion [255, 257-259]. Redlich et al stimulated microglial cells with various TLR agonists and found that this enhanced phagocytosis of fungi as well as bacteria [170, 260, 261]. They have also shown that a role of TLRs in the phagocytosis of *C. neoformans* requires the MyD88 signaling cascade, which is used by all TLRs except TLR3. Although there is some evidence of the role of TLRs in the early immune response to fungal infection, none of these studies shows clarity on the role of TLRs and their effect on neutrophil activation in cryptococcal infection in humans. In our study there is a possible association between neutrophil recruitment and TLR expression in human microglial cells, although further studies are needed to confirm this.

There was no significant association between the amount of shed capsular material and expression of TLRs in our study. Several studies have reported the ability of capsular GXM to activate a TLR-mediated immune response [165, 175, 176, 262]. GXM has been shown to interact with both TLR2 and TLR4, which is believed to influence response to cryptococcal infection [164, 165, 175, 263]. In our study, a possible explanation for the lack of an association could be the small sample size. Further studies would be beneficial to confirm this observation.

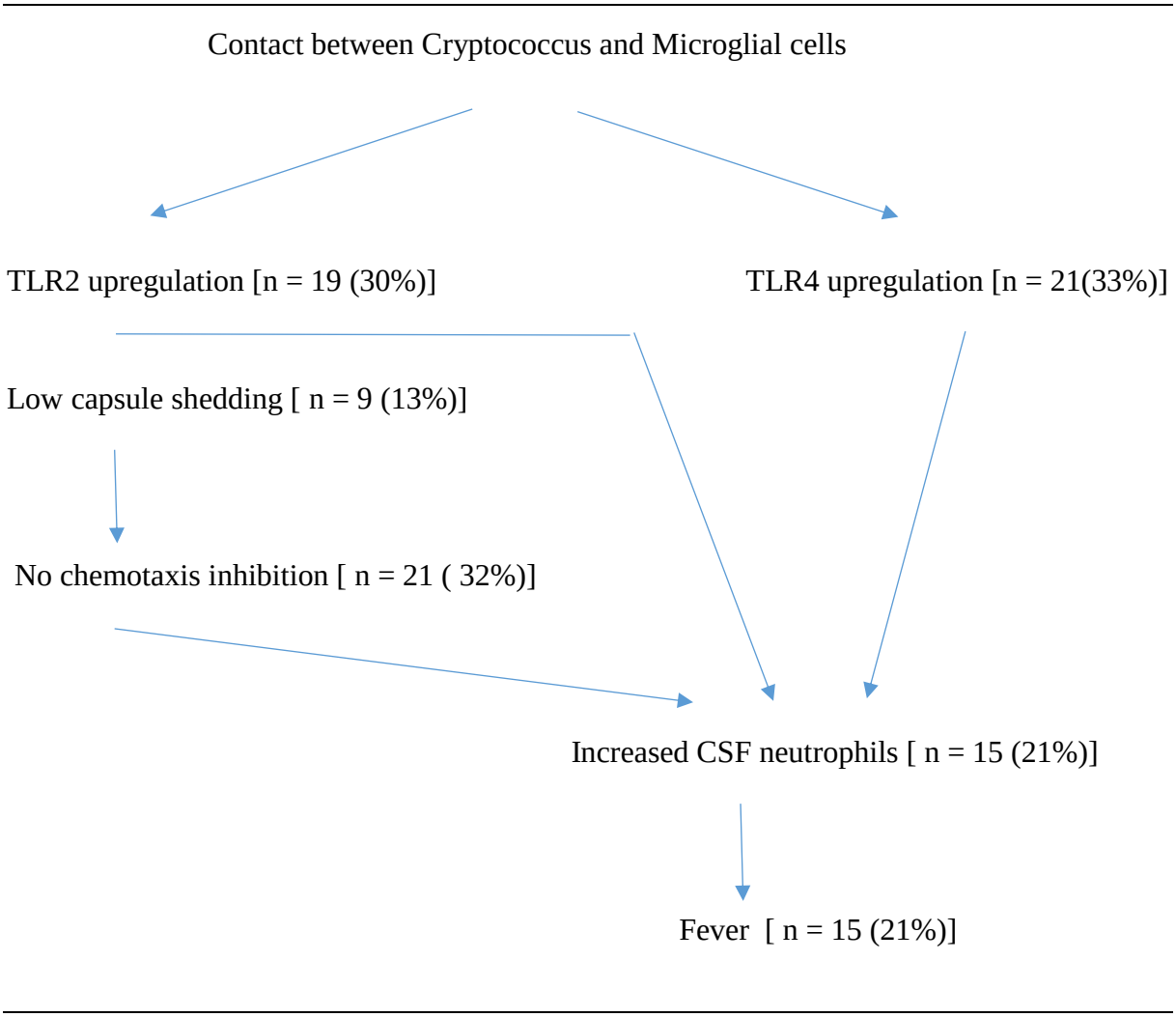
In conclusion, there is increasing interest and research focusing on understanding the pathogenesis of cryptococcosis. Most of this research has thus far, focused on animal models. This study is among the first to examine the host-pathogen relationship in human microglial cells. It is also the first study to show a possible association between neutrophil recruitment and TLR expression in human microglial cells. Our hypothesis that the cryptococcal capsular material interfered with neutrophil chemotaxis and that the level of chemotaxis inhibition was related to severity of disease was pursued in a multicomponent approach.

A relative paucity of CSF neutrophils in the study cohort was observed, which is consistent with the literature. Although the capsule of *C. neoformans* was not enlarged, we noted an association between larger capsule size and lower CSF neutrophil counts in these patients. In addition, a significant

association occurred between shed capsular material and CSF neutrophil counts. Lower CSF neutrophil counts occurred in patients whose CSF had higher amounts of shed capsular material. These findings imply that the capsular components (mainly GXM) do in fact downregulate recruitment of neutrophils into the CSF. Chemotaxis inhibition occurred in almost 70% of the isolates tested. This group also had a higher amount of shed capsular material in the CSF, which further supports the hypothesis that the capsular material interfered with neutrophil chemotaxis.

There is no known severity of disease score for cryptococcal meningitis, however this study shows that chemotaxis inhibition influences clinical parameters. Headache was a predominant finding in all patients. Significantly, patients who presented with fever had higher CSF neutrophil counts, which suggests that these patients are able to mount an immune response, a finding that has not been explored previously. The association between fever and raised CSF neutrophil counts is demonstrated in Figure 5. These patients also had raised intracranial pressures.

Figure 5: Summary of Association between Fever and CSF Neutrophils



In support of one of our research questions, we showed that downregulation for both TLR2 and TLR4 occurred in microglial cells exposed to *C. neoformans* in the majority of isolates. A strong correlation between TLR2 and TLR4 gene expression was demonstrated. To further support this, CSF neutrophil counts were lower in the group where downregulation of TLR4 occurred.

This study contributes to understanding the host-immune response to cryptococcal meningitis in humans. Our findings address the possible reasons for the paucity of neutrophils in the CSF of individuals with cryptococcal meningitis. Further research on a larger study population would be valuable to support these findings.

SUMMARY OF THE STUDY LIMITATIONS

The study sample size was smaller than planned initially. During the course of the study, there was a general decrease in the number of patients presenting with cryptococcal meningitis, which significantly affected patient recruitment. As discussed above, this could possibly be explained by the roll-out of HAART in the HIV-infected population.

A second limitation was that cryptococci were isolated from CSF on sheep blood agar and passaged once on BHI plates supplemented with 10% horse blood before capsule measurements were performed. Some studies have suggested variations in capsule size when grown in different media with rich media resulting in smaller capsules. In this study, all cryptococci were passaged only once on the same media, therefore results obtained for each isolate could be compared.

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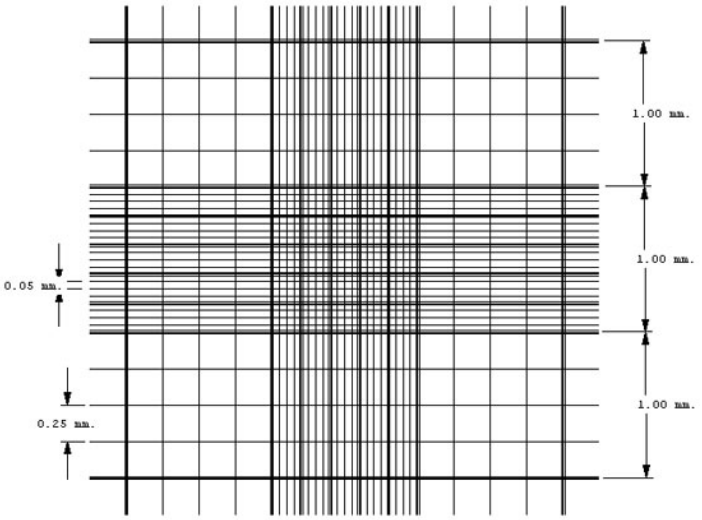
APPENDIX A: PROCEDURES

A1. Cell Count: Neubauer Chamber

Neubauer's chamber is a thick glass plate with the size of a glass slide (30 x 70 x 4 mm). The counting region consists of two square shaped ruled areas. There are depressions on either side on which the squares are marked, giving it an "H" shape. The ruled area is 3 mm² and it is divided into nine large squares each with an area of 1 mm^2. The large central square is divided into 25 medium squares with double or triple lines. Each of these 25 squares is divided into 16 small squares with single lines, so that each of the smallest squares has an area of 1/400 mm^2. The coverslip is a square glass 22 mm in width . The glass cover is placed on the top of the Neubauer chamber and must cover the central area. The ruled area is 0.1 mm lower than the rest of the chamber to allow for a gap of 0.1 mm (1/10 mm) between the cover slip and the ruled area.



RBCs are counted in the central large square. Polymorphs and lymphocytes are each counted in the four corner squares.

Formula:

$$\text{Total number of Cells} = \frac{\text{Number of Cells}}{\text{Depth} \times \text{Dilution Factor} \times \text{Area counted}}$$

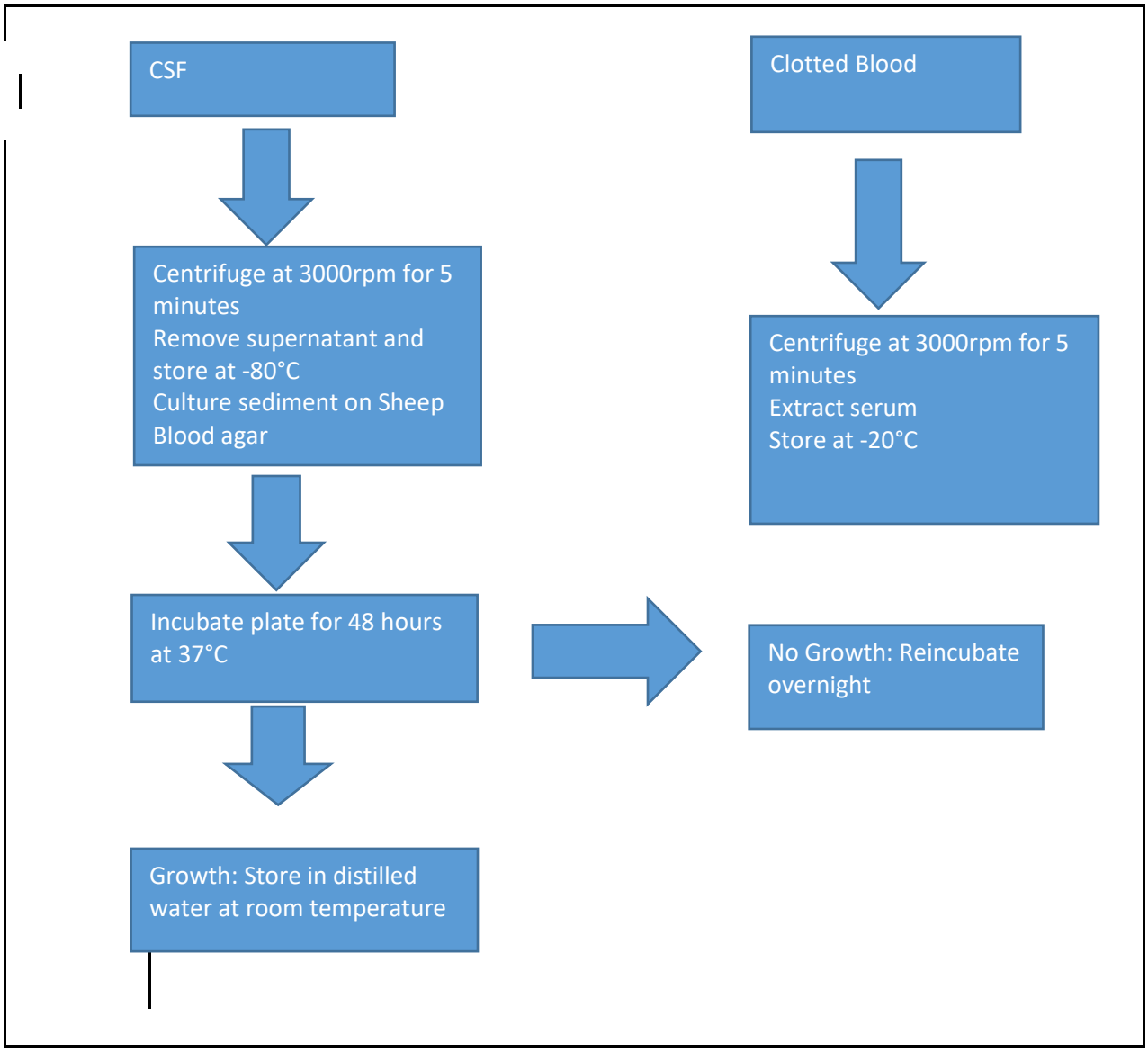
A2. Gram Staining Principle and Procedure

The Gram stain is a staining method used to differentiate bacteria into two groups (gram positive and gram negative). Bacteria stain differentially on the basis of their cell wall composition and structure. Gram positive organisms have a thick peptidoglycan layer and are not affected by decolourisation, therefore retain the initial stain, appearing deep violet. Gram negative organisms have a thinner peptidoglycan layer overlaid by an outer membrane protein layer. The outer membrane is damaged by the alcohol decolouriser, allowing the crystal violet-iodine complex to leak out and be replaced by the counter stain.

Staining Procedure:

1. Flood the heat fixed smear (slide) with crystal violet for 30 seconds.
2. Rinse with running tap water.
3. Add iodine (which binds to crystal violet and traps it in the cell) and leave for 30 seconds .
4. Rapidly decolourise with ethanol or acetone.
5. Rinse immediately with water.
6. Counterstain with carbol fuchsin/safranin for 30 seconds
7. Rinse with water
8. Drain excess water (blot dry with blotting paper)
9. Examine smears microscopically (Light microscope)

A3. Flow Diagram for Storage of Specimens



A4. Giemsa Staining Procedure (Electron Microscopy Sciences, USA)

1. Place 1.0 mL of the Wright-Giemsa Stain upon the smear, in sufficient quantity to cover the entire surface, for 3-4 minutes.
2. Add 2.0 mL distilled water or Phosphate Buffer, and let stand twice as long as in step 1.
3. Rinse stained smear with water or the Phosphate Buffer, until the edges show faintly pinkish red.
4. The film is allowed to air dry. The preparation may be blotted gently to hasten drying.
5. Permanent mounts can be made with mounting medium

A5. Reagent Preparation for MPO Human SimpleStep ELISA® Kit (Abcam, Cambridge, UK)

1. 1× Cell Extraction Buffer PTR

Prepare 1× Cell Extraction Buffer PTR by diluting 5× Cell Extraction Buffer PTR and 50× Cell Extraction Enhancer Solution to 1× with deionized water. To make 10 mL 1× Cell Extraction Buffer PTR combine 7.8 mL deionized water, 2 mL 5× Cell Extraction Buffer PTR and 200 µl 50× Cell Extraction Enhancer Solution. Mix thoroughly and gently. If required protease inhibitors can be added.

2. 1× Wash Buffer PT

Prepare 1× Wash Buffer PT by diluting 10× Wash Buffer PT with deionized water. To make 50 mL 1× Wash Buffer PT combine 5 mL 10× Wash Buffer PT with 45 mL deionized water. Mix thoroughly and gently.

3. Antibody Cocktail

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 4BI. To make 3 mL of the Antibody Cocktail combine 300 µl 10× Capture Antibody and 300 µl 10× Detector Antibody with 2.4 mL antibody Diluent 4BI. Mix thoroughly and gently.

A6. Standard Preparation for MPO Human SimpleStep ELISA® Kit (Abcam, Cambridge, UK)

The Myeloperoxidase lyophilized standard sample is reconstituted by adding 1mL 1× Cell Extraction Buffer PTR by pipette. Mix thoroughly and gently. Keep this at room temperature for 10 minutes and mix gently. This is the 5,000 pg/mL Stock Standard Solution. Label eight tubes, Standards 1– 8. Add 180 µl 1× Cell Extraction Buffer PTR into tube number 1 and 150 µl of 1× Cell Extraction Buffer PTR into numbers 2-8. Use the Stock Standard to prepare the dilution series. Standard #8 contains no protein and is the Blank control.

A7. Complete Culture Medium (for M059K cells)

Cells are grown in a medium containing a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 medium with 2.5 mM L-glutamine adjusted to contain 15 mM HEPES, 0.5 mM sodium pyruvate, and 1.2 g/L sodium bicarbonate (Lonza Group Ltd, Basel, Switzerland) supplemented with 0.05 mM non-essential amino acids and 10% foetal bovine serum.

APPENDIX B: MEDIA AND REAGENTS

B1. Sheep Blood Agar

Requirements for 1 litre media:

1. 40 grams blood agar base (Oxoid Ltd, Cheshire, England)
2. 1 litre distilled water
3. 70 mL horse blood (7%)
4. Petri dishes

Method:

1. Suspend 40 g blood agar base (Oxoid Ltd, Cheshire, England) in 1 litre distilled water.
2. Bring to boil to dissolve completely.
3. Sterilise by autoclaving at 121°C for 15 minutes.
4. Remove from autoclave. Place in waterbath at 56.5°C to cool.
5. Once cooled, remove from waterbath, allow to cool to at least 50°C.
6. Add 70 mL to 930 mL agar solution and mix gently, without creating bubbles.
7. Pour immediately into petri dishes at a volume of approximately 18 – 20 mL per petri dish.
8. Keep at room temperature overnight in sterile environment.
9. Store prepared plates at 2-8°C until use.

B2. Pronase (Meridian Bioscience, Inc, Ohio, USA)

Non-specific protease. It's activity extends to both denatured and native proteins leading to complete or nearly complete digestion into individual amino acids.

B3. Sample Diluent (Meridian Bioscience, Inc, Ohio, USA)

Glycine buffered saline (pH 8.4 ± 0.1) containing bovine serum albumin and 0.01% thimerosal as preservative

B4. Brain Heart Infusion Agar with Horse Blood

Requirements for 1 litre media (Approximately 50 plates):

1. 47 grams BHI agar powder (Oxoid Ltd, Cheshire, England)
2. 1 litre distilled water
3. 100 mL horse blood (10%)
4. Petri dishes

Method:

1. Add 47 grams BHI Agar powder (Oxoid, England) in 1 litre distilled water
2. Autoclave at 121°C for 15 minutes
3. Remove from autoclave. Place in waterbath (56.5°C) to cool.
4. Once cooled, remove from water bath, allow to cool to at least 50°C.
5. Add 100 mL blood to 900 mL agar solution and mix gently, without creating bubbles.
6. Pour immediately into petri dishes at a volume of approximately 18 – 20 mL per petri dish.
7. Keep at room temperature overnight in sterile environment.
8. Store prepared plates at 2-8°C until use.

B5. McFarland Turbidity Standard 1

1. Add 0.5 mL of concentrated H_2SO_4 (Merck, Kenilworth, USA) to 49.5 mL of sterilized distilled water to make a 1% H_2SO_4 solution

2. Add 0.175 gram of $BaCl_2.2H_2O$ (Sigma-Aldrich, Missouri, USA) to 10 mL of sterilized distilled water to make a 1% $BaCl_2.2H_2O$ solution

    - For a McFarland Standard of 1.0, mix 0.1 mL of the 1% $BaCl_2.2H_2O$ solution with 9.9 mL of the 1% H_2SO_4 solution

3. Cover tube with tin foil and store away from direct sunlight

B6. Phosphate Buffered Saline

1. Add 10 PBS tablets (Oxoid, England) to 1000 mL distilled water in a 3-litre Erlenmeyer flask

2. If it is required that bacterial clumping be minimized, add (1%) 10 mL Tween-80 (Merck, SA)

3. Slowly bring to boil and swirl flask and to mix contents

4. Sterilize by autoclaving at 121°C for 15 minutes

5. Cool down at room temperature

6. Dispense into the required volumes

7. Store at 4 to 8°C until use

B7. Foetal Bovine Serum (FBS)

1. Heat inactivate by de-complementing at 56°C for 1 hour.
2. Filter sterilize, aliquote into 10 mL tubes and store at -20°C.
3. Quality control by incubating 5 mL serum at 37°C for one week. Observe for no growth.

APPENDIX C: RAW DATA FOR CSF CLAT TITRES, CAPSULE SIZE, CHEMOTAXIS INHIBITION AND GENE EXPRESSION

C1. CSF CLAT Titre with Opening pressure, CSF Neutrophils and Capsule size

i. CSF CLAT Titre 1 - 512

Study No	Opening pressure (cmH_20)	CSF neutrophils (cells/mL)	CSF CLAT Titre	Capsule size range (μm)	Average capsule size (μm)
4		0	256	1.015-1.036	1.025
6	31	60	128		
9	55	8	256	0.770-1.163	1.017
10	27	0	2		
15	26	4	512	1.850-3.739	2.457
18	23	12	4	0.955-1.454	1.961
19	29	24	2		
20	23	4	512	0.600-1.507	0.952
26	15	0	256	1.035-1.574	1.325
27	30	0	256	0.895-1.780	1.328
28	15	4	16	1.032-1.229	1.132
33	1	0	32	0.960-1.434	1.233
40	13	0	512	1.100-1.744	1.445
44	35	60	256	1.154-1.916	1.391
45	37	0	16	1.304-2.114	1.989
46		0	64	1.092-1.515	1.272
47	32	2	16	0.891-1.430	1.198
48	30	64	512	0.779-1.232	1.020
56	<20	10	1	0.828-1.236	1.030
59	16	0	64		
62	10	0	512		
65	25	54	16	0.894-2.067	1.543
67	30	0	256	1.059-1.575	1.252
69	20	2	32	1.306-1.700	1.461
70	12	8	256	0.841-2.090	1.329

ii. CSF CLAT Titre 1024 - 4096

Study No	Opening pressure (cmH_20)	CSF neutrophils (cells/mL)	CSF CLAT Titre	Capsule size range (μm)	Average capsule size (μm)
1	16	6	4096	1.052-2.127	1.495
2	<10	0	1024	1.368-2.396	1.902
3	42	0	1024	1.282-1.848	1.526
5	32	0	1024	0.959-1.613	1.208
11	26	110	1024	0.567-1.122	0.835
13	17	0	2048	0.823-1.098	0.958
14		0	4096	1.313-2.014	1.719
16	19	0	4096	0.960-2.048	1.526
17	27,5	2	4096	1.027-1.610	1.210
21		0	4096	1.464-2.808	1.970
22	25	0	1024	1.635-3.011	2.209
23	39	0	2048	1.021-1.225	1.108
24	22	0	2048	0.684-1.232	0.971
25	12	0	4096	1.012-1.848	1.379
29	27	14	1024	0.680-1.764	1.068
34	24	0	1024	0.764-1.162	1.061
35	29	2	1024	1.070-1.413	1.254
41	18	0	4096	0.819-1.572	1.082
51	7	0	1024	0.846-1.171	0.982
54	20	0	4096	0.680-1.024	0.860
55	16	0	4096	0.835-1.426	1.127
57	17	0	4096	0.714-1.575	1.103
58	>50	0	4096	0.894-1.307	1.169
64	25	0	>2650	0.951-1.193	1.037
71	10	0	2048	1.027-2.051	1.541

iii. CSF CLAT Titre 8192 - >131072

Study No	Opening pressure (cmH_20)	CSF neutrophils (cells/mL)	CSF CLAT Titre	Capsule size range (μm)	Average capsule size (μm)
7	31	0	8192	0.410-1.237	0.733
8		0	8192	0.960-1.308	1.110
12	45	4	8192	0.708-0.892	0.767
30		16	32768	0.525-1.654	1.018
31	24	0	8192	0.814-1.575	1.231
32	20	6	16384	0.682-1.132	0.882
36	9	0	8192	1.092-1.501	1.110
37	8	2	16384	0.818-1.369	1.027
38	15	0	16384	0.818-1.229	1.027
39	16	0	16384	0.819-1.780	1.365
42	27	0	>131072	0.980-1.902	1.319
43	10	0	8192	0.754-1.358	1.051
49	28		>131072		
50	16	96	>131072	0.753-1.438	1.061
52	7	0	32768	0.876-1.368	1.136
53	>20	0	>131072	0.886-1.213	1.062
60	20	0	131072	0.910-1.642	1.292
61	15	0	8192	0.972-1.645	1.289
63	27	0	8192	0.821-1.309	1.065
66	20	0	8192	0.985-2.482	1.756
68	40	0	8192	1.367-2.102	1.643

C2. Capsule size with Opening Pressure, CSF Neutrophils, Blood Neutrophils and CSF CLAT Titre

i. Capsule size 0.5 – 1.198 μm

Study No	Opening pressure (cmH_2O)	CSF neutrophils (cells/mL)	Blood neutrophils (n $\times 10^9$cells/L))	CSF CLAT Titre	Capsule size range (μm)	Average capsule size (μm)
7	31	0	13.25	8192	0.410-1.237	0.733
12	45	4	1.93	8192	0.708-0.892	0.767
11	26	110	6.61	1024	0.567-1.122	0.835
54	20	0	7.16	4096	0.680-1.024	0.860
32	20	6	3.8	16384	0.682-1.132	0.882
20	23	4	4.15	512	0.600-1.507	0.952
13	17	0	4.46	2048	0.823-1.098	0.958
24	22	0	2.25	2048	0.684-1.232	0.971
51	7	0	3.97	1024	0.846-1.171	0.982
9	55	8	6.1	256	0.770-1.163	1.017
30		16	3.25	32768	0.525-1.654	1.018
48	30	64	4.58	512	0.779-1.232	1.020
4		0	2.31	256	1.015-1.036	1.025
37	8	2	2.58	16384	0.818-1.369	1.027
38	15	0	5.39	16384	0.818-1.229	1.027
56	<20	10	4.11	1	0.828-1.236	1.030
64	25	0	6.25	>2650	0.951-1.193	1.037
43	10	0	5.53	8192	0.754-1.358	1.051
34	24	0	5.29	1024	0.764-1.162	1.061
50	16	96	2.57	>131072	0.753-1.438	1.061
53	>20	0		>131072	0.886-1.213	1.062
63	27	0	4.26	8192	0.821-1.309	1.065
29	27	14	3.69	1024	0.680-1.764	1.068
41	18	0	4.33	4096	0.819-1.572	1.082
57	17	0	4.29	4096	0.714-1.575	1.103
23	39	0		2048	1.021-1.225	1.108
8		0		8192	0.960-1.308	1.110
36	9	0	1.89	8192	1.092-1.501	1.110
55	16	0		4096	0.835-1.426	1.127
28	15	4	1.18	16	1.032-1.229	1.132
52	7	0	3.46	32768	0.876-1.368	1.136
58	>50	0	6.2	4096	0.894-1.307	1.169
47	32	2	9.57	16	0.891-1.430	1.198

ii. Capsule size >1.198μm

Study No	Opening pressure (cmH_2O)	CSF neutrophils (cells/mL)	Blood neutrophils ($n \times 10^9$cells/L)	CSF CLAT Titre	Casule size range (μm)	Average capsule size (μm)
5	32	0	4.02	1024	0.959-1.613	1.208
17	27,5	2	2.37	4096	1.027-1.610	1.210
31	24	0	6.72	8192	0.814-1.575	1.231
33	1	0	7.88	32	0.960-1.434	1.233
67	30	0	2.75	256	1.059-1.575	1.252
35	29	2	4.4	1024	1.070-1.413	1.254
46		0		64	1.092-1.515	1.272
61	15	0	7.71	8192	0.972-1.645	1.289
60	20	0	4.34	131072	0.910-1.642	1.292
42	27	0	2.15	>131072	0.980-1.902	1.319
26	15	0	3.54	256	1.035-1.574	1.325
27	30	0		256	0.895-1.780	1.328
70	12	8	6.34	256	0.841-2.090	1.329
39	16	0	7.03	16384	0.819-1.780	1.365
25	12	0	4.14	4096	1.012-1.848	1.379
44	35	60	2.46	256	1.154-1.916	1.391
40	13	0	4.37	512	1.100-1.744	1.445
69	20	2		32	1.306-1.700	1.461
1	16	6	0.82	4096	1.052-2.127	1.495
3	42	0	5.08	1024	1.282-1.848	1.526
16	19	0	1.31	4096	0.960-2.048	1.526
71	10	0	4.28	2048	1.027-2.051	1.541
65	25	54	8.97	16	0.894-2.067	1.543
68	40	0	1.43	8192	1.367-2.102	1.643
14		0		4096	1.313-2.014	1.719
66	20	0	9.95	8192	0.985-2.482	1.756
2	<10	0	5.05	1024	1.368-2.396	1.902
18	23	12	3.12	4	0.955-1.454	1.961
21		0	0.84	4096	1.464-2.808	1.970
45	37	0	1.97	16	1.304-2.114	1.989
22	25	0	2.29	1024	1.635-3.011	2.209
15	26	4	5.16	512	1.850-3.739	2.457

C3. Neutrophil Chemotaxis Inhibition data with CSF Neutrophil counts, CSF CLAT Titre and Capsule size

i. No Chemotaxis Inhibition

Study No	CSF neutrophils (cells/mL)	CSF CLAT Titre	Average capsule size (μm)	MPO ELISA Data (OD = 450nm)			
				Cryptococcus/E coli combination	*Cryptococcus* only	*E coli* only	Chemotaxis inhibition
21	0	4096	1.970	0.1305	0.053	0.109	-0.00215
5	0	1024	1.208	0.247	0.070	0.243	-0.004
25	0	4096	1.379	0.113	0.053	0.109	-0.004
20	4	512	0.952	0.117	0.068	0.109	-0.008
23	0	2048	1.108	0.1195	0.056	0.109	-0.0105
26	0	256	1.325	0.1405	0.072	0.109	-0.0315
27	0	256	1.328	0.1475	0.062	0.109	-0.0385
3	0	1024	1.526	0.360	0.065	0.243	-0.117
4	0	256	1.025	0.386	0.091	0.243	-0.143
2	0	1024	1.902	0.393	0.063	0.243	-0.15
24	0	2048	0.971	0.284	0.062	0.109	-0.175
7	0	8192	0.733	0.464	0.064	0.243	-0.221
69	2	32	1.461	0.4745	0.103	0.243	-0.2315
33	0	32	1.233	5.469	5.971	5.237	-0.232
1	6	4096	1.495	0.5485	0.070	0.243	-0.3055
40	0	512	1.445	5.561	5.780	5.237	-0.324
35	2	1024	1.254	5.701	5.389	5.237	-0.464
31	0	8192	1.231	5.715	5.042	5.237	-0.478
34	0	1024	1.061	5.7395	5.665	5.237	-0.5025
39	0	16384	1.365	5.8485	5.910	5.237	-0.6115
38	0	16384	1.027	5.912	5.671	5.237	-0.675

ii. Positive Chemotaxis Inhibition

Study No	CSF neutrophils (cells/mL)	CSF CLAT Titre	Average capsule size (μm)	MPO ELISA Data (OD = 450nm)			
				Cryptococcus/E coli combination	*Cryptococcus* only	*E coli* only	Chemotaxis inhibition
22	0	1024	2.209	0.091	0.055	0.109	0.018
30	16	32768	1.018	0.0715	0.056	0.109	0.0375
28	4	16	1.132	0.068	0.059	0.109	0.041
29	14	1024	1.068	0.0645	0.054	0.109	0.0445
68	0	8192	1.643	1.627	0.122	1.710	0.083
36	0	8192	1.110	5.1275	6.000	5.237	0.1095
71	0	2048	1.541	0.0755	0.053	0.243	0.1675
37	2	16384	1.027	5.061	6.000	5.237	0.176
48	64	512	1.020	0.066	0.065	0.243	0.177
58	0	4096	1.169	0.066	0.056	0.243	0.177
70	8	256	1.329	0.0635	0.053	0.243	0.1795
9	8	256	1.017	0.0625	0.053	0.243	0.1805
18	12	4	1.961	0.062	0.058	0.243	0.181
47	2	16	1.198	0.0605	0.057	0.243	0.1825
50	96	>131072	1.061	0.0605	0.053	0.243	0.1825
51	0	1024	0.982	0.0565	0.054	0.243	0.1865
32	6	16384	0.882	5.0395	5.141	5.237	0.1975
53	0	>131072	1.062	0.0401	0.072	0.243	0.2029
46	0	64	1.272	5.7375	5.477	6.000	0.2625
44	60	256	1.391	5.668	5.031	6.000	0.332
8	0	8192	1.110	5.6485	4.252	6.000	0.3515
41	0	4096	1.082	5.5325	4.845	6.000	0.4675
17	2	4096	1.210	5.4475	4.819	6.000	0.5525
66	0	8192	1.756	1.0875	0.077	1.710	0.6225
43	0	8192	1.051	5.3305	5.360	6.000	0.6695
45	0	16	1.989	5.249	6.000	6.000	0.751
11	110	1024	0.835	5.1795	4.800	6.000	0.8205
42	0	>131072	1.319	5.1385	5.132	6.000	0.8615
14	0	4096	1.719	4.942	4.695	6.000	1.058
67	0	256	1.252	0.645	0.075	1.710	1.065
61	0	8192	1.289	0.5755	0.081	1.710	1.1345
63	0	8192	1.065	0.539	0.092	1.710	1.171
15	4	512	2.457	4.8085	4.716	6.000	1.1915
12	4	8192	0.767	4.7935	4.785	6.000	1.2065
65	54	16	1.543	0.4915	0.099	1.710	1.2185
54	0	4096	0.860	0.434	0.062	1.710	1.276
64	0	>2650	1.037	0.4165	0.102	1.710	1.2935
60	0	131072	1.292	0.352	0.102	1.710	1.358
55	0	4096	1.127	0.3505	0.083	1.710	1.3595
13	0	2048	0.958	4.5875	5.091	6.000	1.4125
57	0	4096	1.103	0.2485	0.072	1.710	1.4615
56	10	1	1.030	0.190	0.071	1.710	1.52
52	0	32768	1.136	0.1185	0.065	1.710	1.5915
16	0	4096	1.526	4.4035	5.059	6.000	1.5965

C4. TLR2 and TLR4 Gene Expression with Capsule size, CSF Neutrophils and CSF CLAT Titre

i. TLR2 Expression with Capsule size, CSF Neutrophils and CSF CLAT Titre

Study No	18S	TLR2	TLR2/18S	Average Capsule size (μm)	CSF neutrophils (cells/mL)	CSF CLAT Titre
17	2 294,964	60,661	0,026432272	1.210	2	4096
18	2 265,975	93,697	0,041349735	1.961	12	4
14	1 981,369	92,927	0,046900188	1.719	0	4096
9	6 154,244	312,673	0,050806008	1.017	8	256
22	2 100,092	124,734	0,059394704	2.209	0	1024
25	455,093	27,135	0,059625389	1.379	0	4096
15	650,646	39,298	0,060397783	2.457	4	512
7	16 713,160	1 016,068	0,060794479	0.733	0	8192
36	420,346	27,712	0,065925527	1.110	0	8192
24	2 626,968	175,981	0,066990088	0.971	0	2048
37	685,151	48,447	0,070710671	1.027	2	16384
8	2 818,096	205,638	0,072970383	1.110	0	8192
4	19 745,727	1 509,424	0,076443082	1.025	0	256
16	536,981	47,055	0,087629236	1.526	0	4096
28	1 948,331	202,018	0,103687608	1.132	4	16
1	3 690,052	388,610	0,105312901	1.495	6	4096
11	1 382,593	151,777	0,10977693	0.835	110	1024
71	546,041	65,906	0,120698172	1.541	0	2048
27	537,477	71,055	0,132200652	1.328	0	256
20	1 637,611	219,187	0,133845604	0.952	4	512
3	946,743	131,188	0,138567397	1.526	0	1024
12	24 245,568	3 371,908	0,139073186	0.767	4	8192
38	218,612	36,861	0,168614471	1.027	0	16384
34	124,800	23,025	0,184497434	1.061	0	1024
2	652,840	127,875	0,195874664	1.902	0	1024
60	4 316,575	858,120	0,198796512	1.292	0	131072
21	1 815,927	368,559	0,202958964	1.970	0	4096
58	99,963	26,062	0,260712545	1.169	0	4096
39	238,221	62,182	0,261026419	1.365	0	16384
57	1 710,332	450,786	0,263566623	1.103	0	4096
42	5 777,510	1 555,493	0,269232438	1.319	0	>131072
13	2 252,085	642,026	0,285080576	0.958	0	2048
40	322,352	99,226	0,307819171	1.445	0	512
30	2 831,050	1 048,441	0,370336553	1.018	16	32768
32	159,672	65,942	0,412982795	0.882	6	16384
63	498,928	222,402	0,445760166	1.065	0	8192
69	146,038	74,622	0,510978429	1.461	2	32
65	380,572	211,396	0,555467891	1.543	54	16
41	4 804,269	2 728,569	0,567946778	1.082	0	4096
68	191,610	150,010	0,782889092	1.643	0	8192

44	2 368,476	1 874,138	0,791284363	1.391	60	256
29	30,195	25,219	0,835215402	1.068	14	1024
53	272,440	252,851	0,928099114	1.062	0	>131072
56	41,524	40,743	0,981180815	1.030	10	1
67	587,515	577,637	0,983185793	1.252	0	256
51	52,161	68,345	1,310271498	0.982	0	1024
55	5 059,433	6 946,552	1,372990271	1.127	0	4096
31	43,820	68,113	1,554383304	1.231	0	8192
48	52,265	119,380	2,28414244	1.020	64	512
43	501,151	1 154,218	2,303133241	1.051	0	8192
66	280,022	702,605	2,509107253	1.756	0	8192
23	236,879	879,060	3,711001352	1.108	0	2048
45	40,914	153,671	3,755951138	1.989	0	16
5	5,841	27,565	4,719394713	1.208	0	1024
35	983,777	4 929,847	5,01114318	1.254	2	1024
47	88,278	492,042	5,573766105	1.198	2	16
46	3,315	32,414	9,778819152	1.272	0	64
50	36,034	474,061	13,15577383	1.061	96	>131072
52	3,965	52,571	13,25874626	1.136	0	32768
54	26,767	397,587	14,85380324	0.860	0	4096
61	77,637	2 661,790	34,28529245	1.289	0	8192
33	473,438	21 647,914	45,72492868	1.233	0	32
64	45,667	2 136,321	46,7801742	1.037	0	>2650
26	251,322	39 695,664	157,9473017	1.325	0	256

ii. TLR4 Expression with Capsule size, CSF Neutrophils and CSF CLAT Titre

Study No	18S	TLR4	TLR4/18S	Average Capsule size (μm)	CSF neutrophils (cells/mL)	CSF CLAT Titre
17	2 294,964	106,345	0,046338	1.210	2	4096
14	1 981,369	122,941	0,062049	1.719	0	4096
9	6 154,244	437,227	0,071045	1.017	8	256
4	19 745,727	1 499,273	0,075929	1.025	0	256
13	2 252,085	178,796	0,079391	0.958	0	2048
12	24 245,568	2 214,537	0,091338	0.767	4	8192
7	16 713,160	1 555,925	0,093096	0.733	0	8192
22	2 100,092	206,800	0,098472	2.209	0	1024
8	2 818,096	283,355	0,100549	1.110	0	8192
24	2 626,968	277,665	0,105698	0.971	0	2048
1	3 690,052	395,961	0,107305	1.495	6	4096
15	650,646	70,163	0,107836	2.457	4	512
28	1 948,331	212,416	0,109025	1.132	4	16
16	536,981	66,460	0,123767	1.526	0	4096
3	946,743	122,351	0,129234	1.526	0	1024
37	685,151	96,414	0,14072	1.027	2	16384
20	1 637,611	273,631	0,167091	0.952	4	512
60	4 316,575	770,819	0,178572	1.292	0	131072
25	455,093	92,539	0,20334	1.379	0	4096
11	1 382,593	284,593	0,20584	0.835	110	1024
57	1 710,332	369,721	0,216169	1.103	0	4096
21	1 815,927	454,140	0,250087	1.970	0	4096
36	420,346	109,359	0,260164	1.110	0	8192
18	2 265,975	615,022	0,271416	1.961	12	4
38	218,612	59,645	0,272834	1.027	0	16384
27	537,477	149,452	0,278063	1.328	0	256
30	2 831,050	800,647	0,282809	1.018	16	32768
42	5 777,510	1 779,079	0,307932	1.319	0	>131072
65	380,572	118,138	0,310423	1.543	54	16
41	4 804,269	1 661,625	0,345864	1.082	0	4096
2	652,840	272,093	0,416784	1.902	0	1024
40	322,352	142,002	0,44052	1.445	0	512
39	238,221	110,497	0,463843	1.365	0	16384
67	587,515	277,476	0,472287	1.252	0	256
34	124,800	68,884	0,551955	1.061	0	1024
63	498,928	283,300	0,567817	1.065	0	8192
44	2 368,476	1 486,484	0,627612	1.391	60	256
69	146,038	95,009	0,650582	1.461	2	32
71	546,041	355,259	0,650608	1.541	0	2048
55	5 059,433	3 793,110	0,749711	1.127	0	4096
53	272,440	233,399	0,856697	1.062	0	>131072
68	191,610	177,153	0,924549	1.643	0	8192
58	99,963	98,001	0,980367	1.169	0	4096

31	43,820	50,523	1,152969	1.231	0	8192
32	159,672	206,113	1,290857	0.882	6	16384
56	41,524	56,142	1,352031	1.030	10	1
48	52,265	80,358	1,537522	1.020	64	512
43	501,151	804,667	1,605639	1.051	0	8192
51	52,161	129,441	2,481561	0.982	0	1024
23	236,879	656,257	2,770427	1.108	0	2048
66	280,022	798,036	2,849905	1.756	0	8192
29	30,195	92,281	3,056175	1.068	14	1024
45	40,914	134,885	3,296804	1.989	0	16
35	983,777	3 457,118	3,514128	1.254	2	1024
47	88,278	424,330	4,806735	1.198	2	16
50	36,034	194,437	5,395878	1.061	96	>131072
5	5,841	34,795	5,957274	1.208	0	1024
54	26,767	183,914	6,87099	0.860	0	4096
33	473,438	7 274,291	15,36483	1.233	0	32
52	3,965	84,838	21,3965	1.136	0	32768
61	77,637	1 987,636	25,60182	1.289	0	8192
64	45,667	1 242,781	27,21384	1.037	0	>2650
46	3,315	127,953	38,60136	1.272	0	64
26	251,322	12 193,188	48,51616	1.325	0	256