THE NEUROIMMUNE RESPONSE TO CRYPTOCOCCAL INFECTION

A dissertation submitted to the Faculty of Health Sciences, University of Cape Town in fulfilment of the requirements for the Master of Medical Sciences in Neuroscience degree

by

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Abstract

Cryptococcal meningitis (CM) is a fatal fungal infection of the brain that is responsible for up to 20% of all AIDS-related deaths globally, 75% of which are from Sub-Saharan Africa. CM is characterised by debilitating neurological damage often resulting in death or serious long-term sequelae even after receiving treatment. Despite the brain being the main organ of injury, there is a paucity of data describing the interaction of the fungus with resident immune cells of the brain. The aim of this study was to investigate the neuroimmune response to cryptococcal infection using a novel organotypic brain slice culture system. We treated cultured brain slices with either whole cell *C. neoformans,* its purified capsule (a known major virulent factor) or lipopolysaccharide (LPS), and compared them to untreated control slices. The neuroimmune response was measured by tracking the activation of nuclear factor for interleukin 6 (NF-IL6), and confirmed by measuring the release of IL6 and tumour necrosis factor-α (TNF-α). Our results showed that neither *C. neoformans* nor its purified capsule elicited a neuroinflammatory response as observed in LPS-treated slices. Co-stimulation of LPS-treated slices with *C. neoformans* or its purified capsule did not abolish an LPS-induced inflammatory responses in brain slices. Our findings also show that microglia are the principal cells that phagocytose fungal cells during cryptococcal infection and that even after engulfing fungal cells, microglial cells were not classically activated. Therefore, we concluded that *C. neoformans* recognition by resident immune cells, on its own, may not be responsible for the debilitating inflammatory response observed during CM, and that the purified cryptococcal capsule does not elicit an inflammatory or anti-inflammatory response.

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Abbreviations

Symbol	Description
AIDS	Acquired immunodeficiency syndrome
AmB-D	Amphotericin B deoxycholate
ART	Anti-retroviral treatment
BBB	Blood brain barrier
BSA	Bovine serum albumin
CA3	Cornu ammonis region 3
C-IRIS	Cryptococcosis-induced immune reconstitution inflammatory syndrome
CLR	C-type lectin receptor
CM	Cryptococcal meningitis
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAMP	Damage-associated molecular patterns
ECM	Extracellular matrix
EBSS	Earl's based salt solution
ELISA	Enzyme-linked immunosorbent assays
GalXM	Galactoxylomannan
GFP	Green fluorescent protein
GM	Growth media
GXM	Glucuronoxylomannan
HIV	Human immunodeficiency virus
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-IL6	Nuclear factor for interleukin-6
NF-κβ	Nuclear factor kappa b
NK	Natural killer
NO	Nitric oxide
NTD	Neglected tropical disease
OBSC	Organotypic brain slice cultures
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PRR	Pattern recognition receptors
SSA	Sub-Saharan Africa
TNF-α	Tumor necrosis factor-α
TLR	Toll-like receptor
UCT	University of Cape Town
WHO	World Health Organization
YPD	Yeast peptone dextrose

CHAPTER ONE

1. Introduction

1.1. Background

Cryptococcal meningitis (CM) is an opportunistic fungal infection of the brain that is associated with 15-20% of deaths of people living with HIV/AIDS globally [1]. CM is the major cause of adult acquired meningitis with a global annual incidence of 280 000 cases and a case fatality ratio of 70% at three months [2, 3]. Due to its disproportionately large burden of HIV/AIDS and a substantial population of immunocompromised individuals, Sub-Saharan Africa (SSA) contributes up to 75% of the global cases and CM-related deaths each year [4]. In addition, the top ten countries with the highest annual incidence of CM are all found in SSA, with South Africa contributing the highest number of cases (see Figure 1.1) [2].

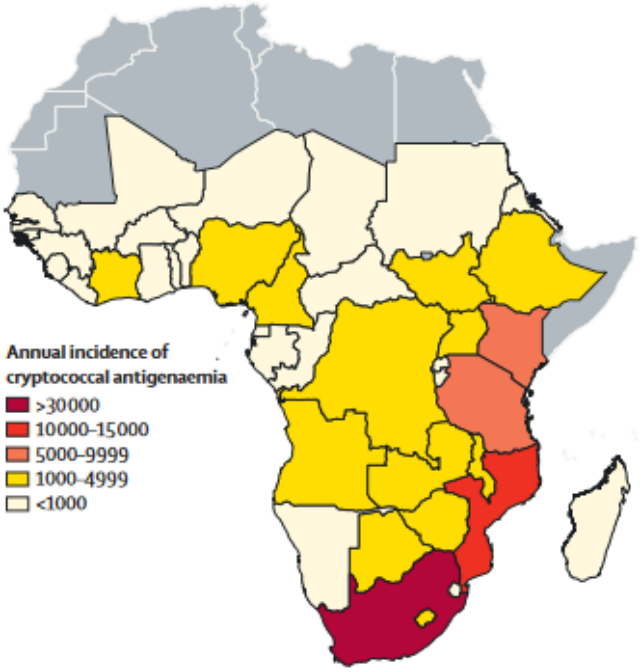

Figure 1.1: Annual incidence of cryptococcal antigenemia in Sub-Saharan Africa

Top ten countries globally based on the incidence of cryptococcal antigenaemia. From highest to lowest: South Africa, Mozambique, Kenya, DR Congo, Tanzania, Zambia, Nigeria, Malawi, Zimbabwe, and Ethiopia [2].

Mortality as a result of cryptococcal infection is between 40-70% with treatment, and up to 100% without treatment [2]. In developing countries, the high mortality rates associated with CM are a result of the unavailability and inaccessibility of affordable and effective treatment strategies [5]. The current World Health Organisation (WHO)-recommended treatment for CM involves induction on Amphotericin B deoxycholate (AmB-D) in combination with flucytosine, followed by consolidation and maintenance on varying doses of fluconazole [5]. However, there have been several challenges in implementation of this recommended treatment regime to CM patients in Africa. First, the antifungal drug flucytosine is not registered in more than 90% of the countries in Africa and therefore unavailable for use as an induction drug with AmB-D. Additionally, AmB-D administration requires hospitalisation and very strict laboratory monitoring because of its toxicity [6, 7]. Lastly, CM patients may require up to 12 months maintenance on fluconazole treatment to clear infection and avoid relapse. In the resource-constrained regions of rural and poor urban communities of SSA, these factors reduce

the overall effectiveness of the WHO-recommended treatment quite significantly and as a result, mortality is extremely high [3].

Despite its high prevalence and the unacceptably high mortalities associated with it, CM remains a neglected tropical disease (NTD) in research, receiving only 0.2% of all available research and development funding [8]. In 2015, CM research received $5.8 million (just over 1% of the total research funding received for tuberculosis research) [9]. As a result of its neglect in research, funding and policy, the pathogenesis of CM (especially in the brain) is poorly described and this has impeded the development of drugs and better management strategies.

1.2. Mycology and serotyping

CM is caused by an encapsulated basidiomycetous fungus (*Cryptococcus*), usually 4-5 μm in diameter, that infects humans by inhalation of basidiospores into the lungs [10, 11]. Of the 30 species found across the globe, two species of the *Cryptococcus* genus are known to be pathogenic to humans namely, *Cryptococcus neoformans* and *Cryptococcus gattii*. Whilst *C. gattii* is associated with disease in immunocompetent individuals, *C. neoformans* is known to primarily affect immunocompromised individuals [10]. These two species can be further classified into different serotypes, with *C. neoformans* var. *grubii* (serotype A) being the species responsible for 95% of all infections in humans and found predominately in Africa [11]. It is found in contaminated soil and pigeon excreta all around the globe. The other 4-5% of infections are caused by *C. neoformans* var. *neoformans* (serotype D) and *C. gattii* (serotypes B/C). Serotype D is primarily observed in European countries and serotypes B/C in tropical and sub-tropical regions like Hawaii, Brazil, Australia and Southeast Asia [11].

1.3. The pathogenesis and clinical manifestation of cryptococcal meningitis

C. neoformans infection occurs through inhalation of yeast cells or cryptococcal spores resulting in a primary lung infection. In immunocompetent individuals, the infection is often cleared by the peripheral immune system or may remain in granulomata in a latent state for years before symptoms develop (Figure 1.2). In immunocompromised patients, clearance or containment of the fungus often fails, allowing fungal cells to establish an active pulmonary infection followed by subsequent hematogenous dissemination to other organs.

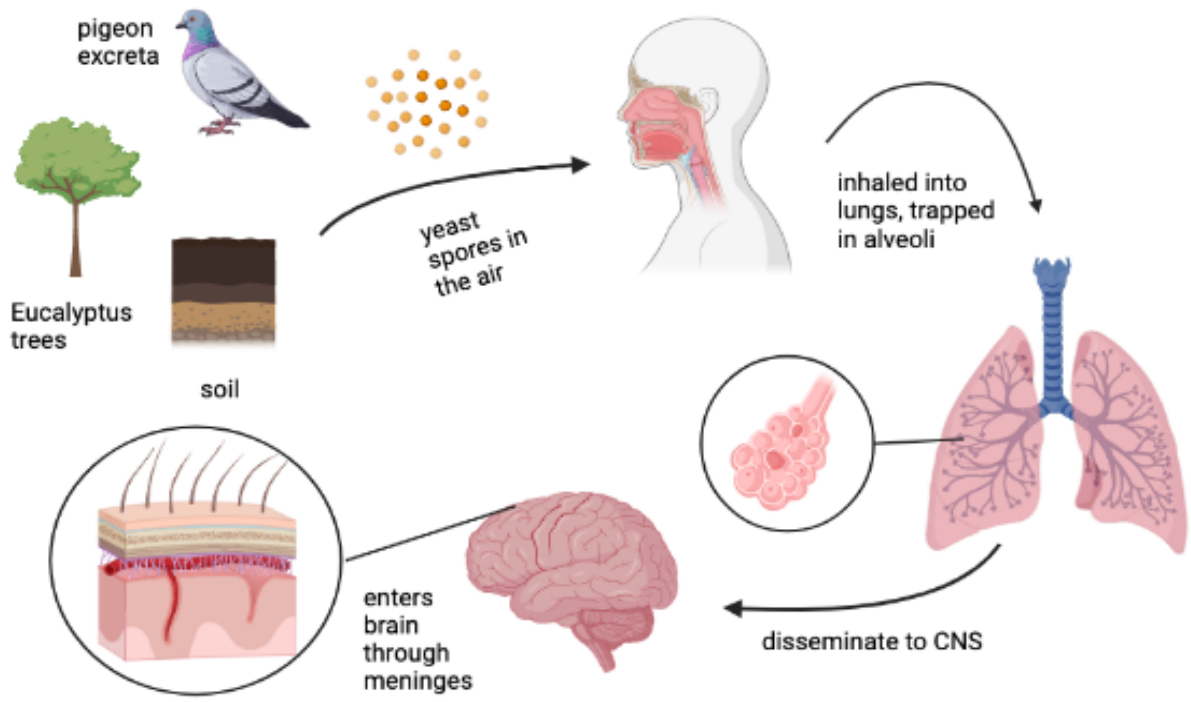

Figure 1.2: The pathogenesis of cryptococcal infection in humans
A schematic diagram describing the pathogenesis of cryptococcal meningitis. The host inhales yeast spores resulting in a lung infection and subsequent dissemination to the brain. Schematic made using BioRender.

C. neoformans is known to have a predilection for the central nervous system (CNS) and several studies have shown that it has the ability to cross the blood-brain barrier (BBB) [10,

12, 13]. Current research shows that *C. neoformans* uses three main mechanisms (Figure 1.3) to traverse the blood brain barrier; (1) a transcellular, transmigratory Trojan horse method whereby phagocytosed fungal cells are transported through the endothelium via infected monocytes, (2) transcytosis whereby the fungus crosses the BBB into the brain through the microvascular endothelial cells by exploiting the endocytic pathways without disrupting the integrity of the tight junctions, and (3) a paracellular transport method by which the fungus compromises the integrity of the BBB by releasing urease and other proteases to degrade the extracellular matrix (ECM) and basement membrane. Additionally, *C. neoformans* can bind to host plasminogen and promote its conversion to plasmin, a protein that digests BBB components [13-18]. However, how the fungus interacts with the brain's innate immune system after having crossed the BBB is not yet fully understood.

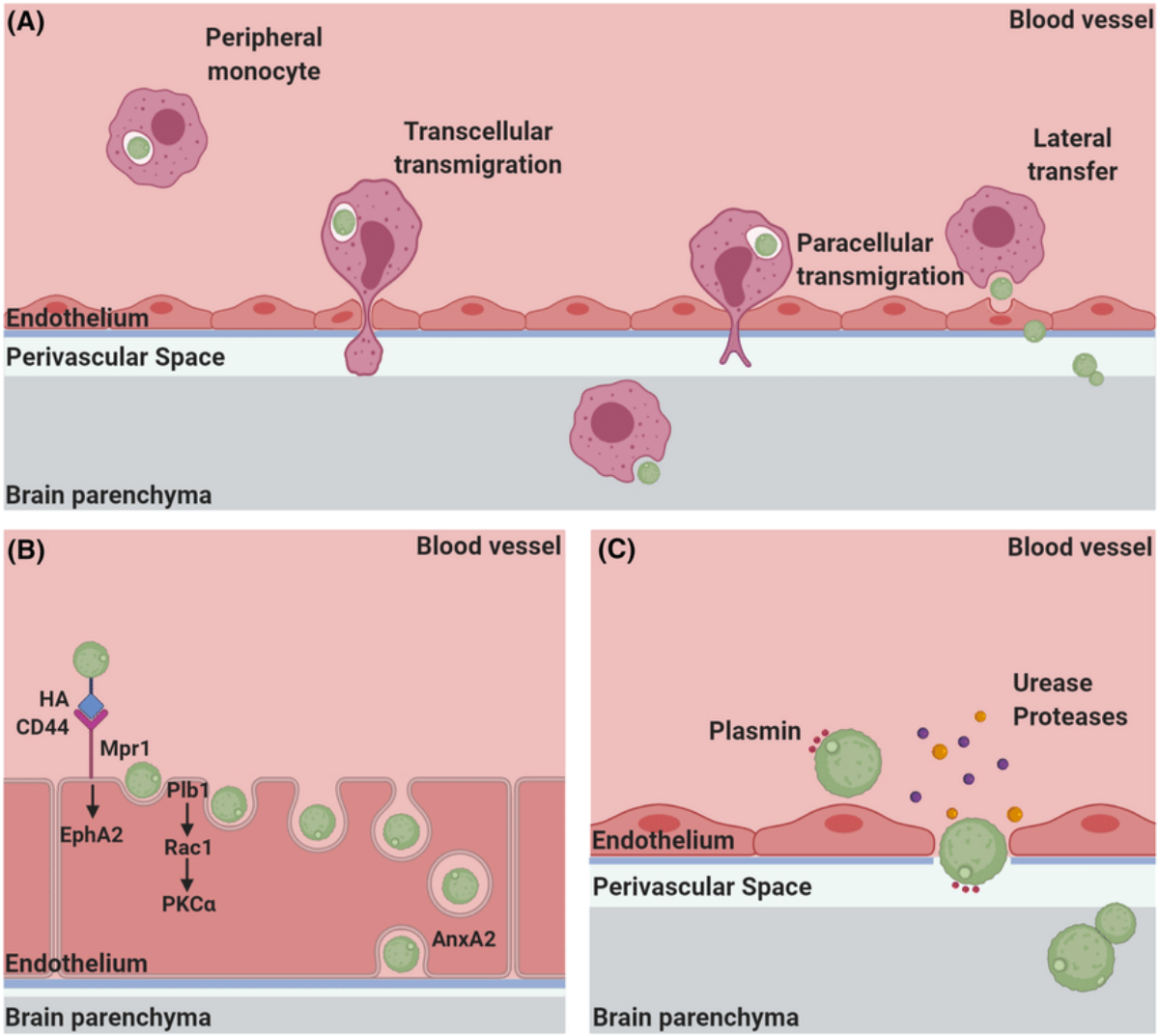

Figure 1.3 Mechanisms by which *Cryptococcus neoformans* crosses the blood brain barrier

A) The Trojan horse method: monocytes in the periphery (dark pink) phagocytose *C. neoformans* (green) and shuttle fungal cells to the brain's blood vessels. Monocytes are able to move fungal cells across the BBB via transcellular or paracellular crossing. Additionally, monocytes can transfer fungal cells directly to endothelial cells (red) resulting in lateral transfer. **B)** Transcytosis: *C. neoformans* (green) can exploit host endocytic pathways to enter endothelial cells (red) and traverse the BBB. **C)** Paracellular crossing due to barrier integrity loss: *C. neoformans* disrupts the BBB by means of converting host plasminogen to plasmin that digests BBB components (dark pink). The fungus also secretes urease (purple) and other proteases (yellow) that degrade the ECM, in order to migrate into the parenchyma [18].

CNS invasion by *C. neoformans* is often characterised by meningoencephalitis; inflammation of the meninges and the parenchyma, which is the most fatal form of CM [13]. This life-threatening form of the disease brings on a milieu of symptoms and complications for patients,

including increased intracranial pressure, hearing and vision loss, fever, headaches, stiff neck, photosensitivity, cranial neuropathy, impaired executive functions, and possibly cognitive impairment and gait ataxia due to obstructive hydrocephalus [10, 19]. Severe CM is reported in between 10 - 50% of HIV-positive patients upon recruitment of anti-retroviral treatment (ART) [20]. Paradoxically, CM symptoms worsen upon receiving ART, as the immune reconstitution afforded by ART can result in a hyperinflammatory response. This phenomenon is described as Cryptococcosis-induced immune reconstitution inflammatory syndrome (C-IRIS) [3, 20].

1.4. Cryptococcal virulence

C. neoformans has three known major virulent factors that aid in the pathogenesis of the disease, namely its ability to proliferate at 37 ºC, the production of melanin, and most importantly the significant thickening of the fungal cell's outer capsule upon entering the host (Figure 1.4) [21-23]. Whilst the ability to proliferate at 37 ºC is what allows the fungus to successfully spread in the human body, the fungus' ability to convert diphenolic compounds to form melanin may partially explain its predilection for the brain, an area with high concentrations of diphenolic catecholamines [11]. The cryptococcal capsule is considered the main virulent factor that allows the fungus to evade host detection and destruction. On its own, the cryptococcal capsule is believed to contribute to 25% of the total virulence of *C. neoformans* to the point where non-encapsulated mutants are not virulent [24]. The capsule helps protect the fungus exposed to the external environment by preventing desiccation and protecting from oxidative stress, moreover increased capsule size results in stronger protection from phagocytosis as has been seen in interactions between amoeba and *C. neoformans* [24]. In the event that fungal cells are phagocytosed by host immune cells, the capsule allows

cryptococcal cells to replicate and proliferate within phagocytes [25]. Through the capsule, the fungus thus evades destruction in the periphery and can successfully disseminate to other organs.

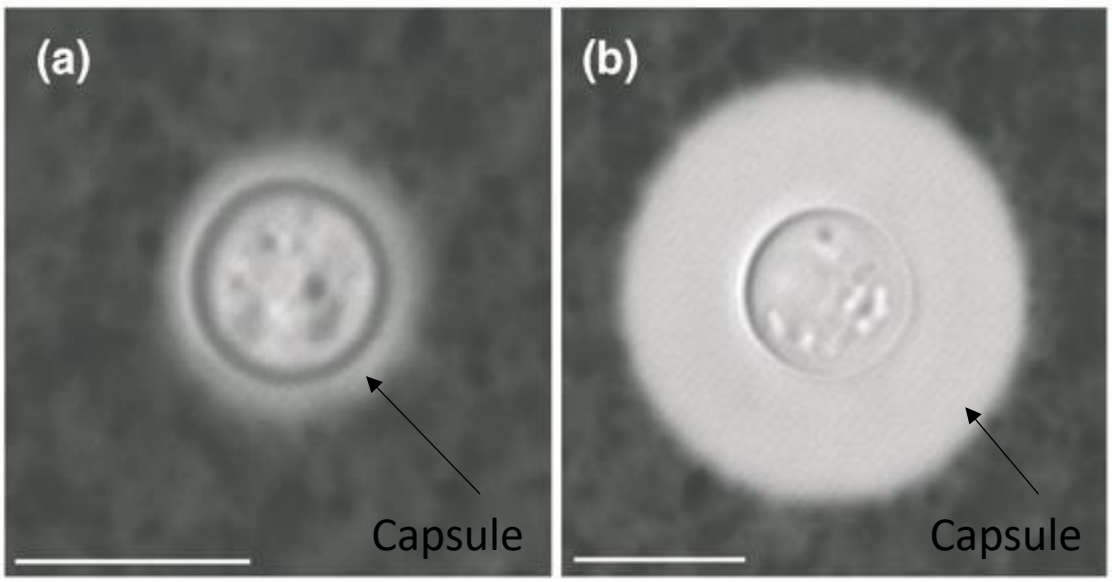

Figure 1.4: *Cryptococcus neoformans* yeast cells stained with India Ink under phase contrast microscopy

A is a yeast cell with a thin capsule, with **B** showing a yeast cell with a thickened capsule, due to exposure to the host internal environment, which appears as a halo around the yeast cell (Adapted from [26])

The cryptococcal capsule is made of one major and two minor polysaccharide molecules. The major molecule is glucuronoxylomannan (GXM) which makes up approximately 90% of the capsule [24]. GXM prevents leucocyte migration, recruitment of inflammatory cells and inhibits phagocytosis by alveolar macrophages (Kumar V, 2021). The two minor components are galactoxylomannan (GalXM) and mannoproteins. Mannoproteins have been identified as highly diverse and immunogenic in the periphery, activating T-cells and inducing Tumor necrosis factor-α (TNF-α) release from monocytes [24, 27]. Some of the pathogenic effects of GalXM are well known, e.g., it induces apoptosis in different immune cells, stimulates the

release of TNF-α and promotes the activation of dendritic cells in the periphery. The full extent to which capsule interacts with the brain's innate immune cells, is yet to be fully described.

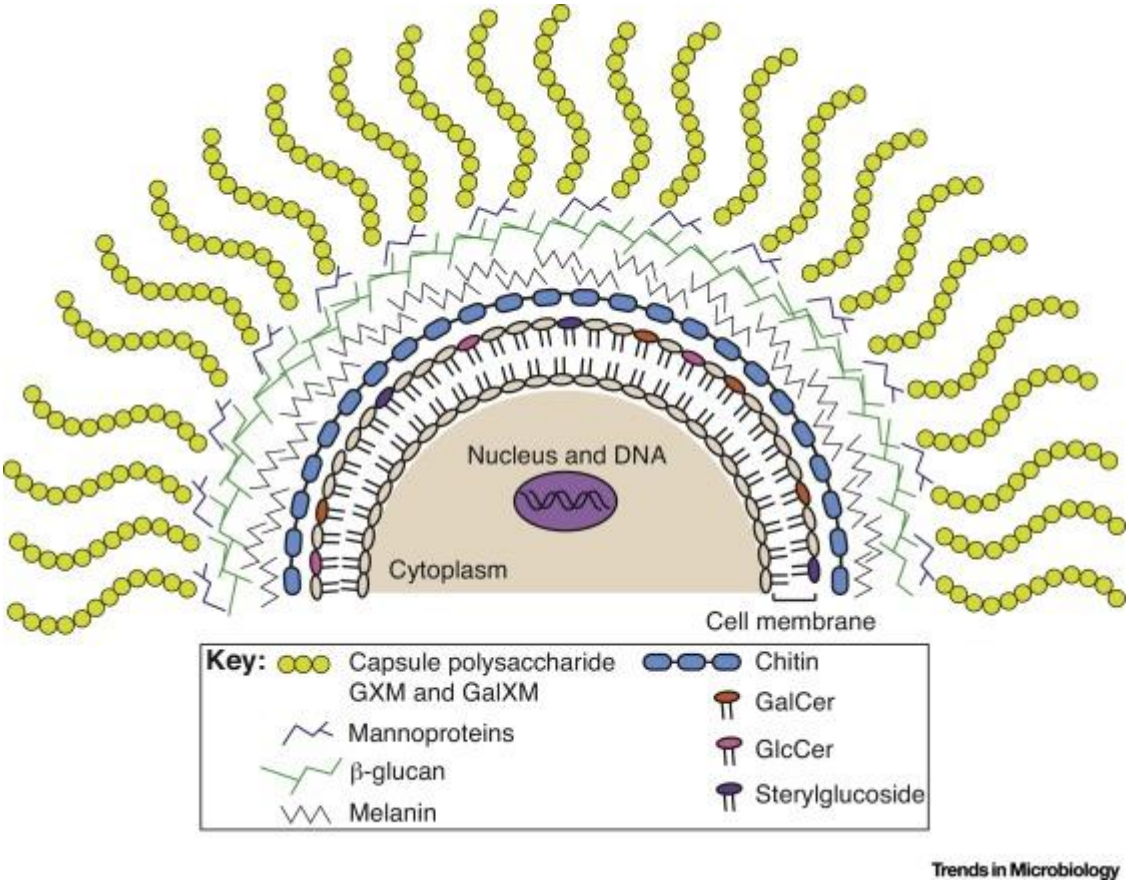

Figure 1.5: The composition of the cryptococcal cell wall and capsule

Schematic of the cell wall (which includes mannoproteins, melanin, chitin and β-glucan) and capsule (which includes capsular polysaccharides GXM and GalXM) [28].

C. neoformans modifies its capsule during titanisation, a process by which the fungal cell grows to a much larger diameter, increases its DNA, thickens its cell wall, and forms a dense cross-linked capsule in response to the host's internal environment [21]. The changes to fungal cell walls during this process is an important survival step for *C. neoformans,* as changes in chitin content in titan cell walls result in host immune responses that promote disease progression in murine models [29]. Capsule composition differs between titan and normal fungal cells, with titan cells possessing more mannose and xylose and increased capsule GXM, with similar GalXM levels compared to normal cells [21].

1.5. The peripheral immune response to *C. neoformans*

In immunocompetent individuals, the peripheral immune response to cryptococcal infection involves recognition of the fungus by the innate immune cells such as dendritic cells, neutrophils, and alveolar macrophages which ingest the yeast cells, digest them and present them to cells of the adaptive immune response (see [30] for a comprehensive review). Recent studies on the interaction between macrophages and *C. neoformans* have described the fungus as an intracellular parasite which can grow and proliferate inside macrophages, eventually leading to macrophage lysis [24, 31]. Neutrophils have also been shown to contribute strongly to the innate immune response to cryptococcal infection by killing fungal cells by means of respiratory bursts [32, 33].

Cell-mediated immunity in CM involves $CD4^+$, $CD8^+$ and natural killer (NK) cells which exhibit anti-cryptococcal activity by secreting perforin and granulsyin to induce cryptococcal lysis [34].The presentation of cryptococcal antigens by dendritic cells and macrophages is associated with proliferation and differentiation of naïve T-cells into three subtypes of helper T-cells (Th1, Th2 and Th17) [31]. Th1 and Th17 cells are associated with the release of pro-inflammatory cytokines e.g., interferon (IFN)-γ and interleukin (IL)-17 for fungal clearance, whereas Th2 cells release anti-inflammatory cytokines IL4, IL5 and IL13, which are permissive to fungal multiplication and dissemination. GXM has been shown to promote a more permissive Th2-type response during *C. neoformans* infection [35].

Despite the extensive research that has been done to describe the interactions of *C. neoformans* and its virulent factors with the peripheral immune system, very little has been done on how

the fungus or its virulent factors interact with the resident immune cells of the CNS and the contribution of these interactions to CM pathology in the brain remain poorly described.

1.6. The neuroimmune response to cryptococcal infection

1.6.1. The CNS response to infection

The CNS was for a long time believed to be an immunologically inert zone that was completely separated from the peripheral immune system, but recent research has demonstrated significant immune-related activity in the CNS following infection [36]. The CNS has resident immune cells and may also recruit peripheral immune cells to mediate responses to viral, bacterial, fungal and parasitic pathogens [37]. The term neuroinflammation is often used to describe the broad range of protective immune responses taking place within the CNS, usually involving the BBB, innate immune system and in some cases the adaptive immune system [38-40].

Previously, the BBB was thought to completely separate the CNS from the body's periphery. However, recent studies have shown that certain neurotropic pathogens are able to traverse the BBB [37]. The BBB can also be stimulated to facilitate leukocyte migration into the brain and has also been shown to be permeable to numerous pro-inflammatory cytokines [41, 42]. Most clinically relevant CNS infections involve fungi, bacteria, viruses or protozoans, where the adaptive immune response to these pathogens is meditated by $CD4^+$ and $CD8^+$ T-cells that release IFN-γ [43]. IFN-γ production induces a wide variety of anti-microbial effects [44], with the most critical role in driving local innate immune cells to produce toxic oxygen species like nitric oxide (NO) to control intracellular parasites [45-47].

The innate immune response of the CNS is mediated by a number of immunocompetent cells, namely macro- and microglia. The most abundant cell group in the CNS are macroglia and they consist of cell types such as astrocytes and oligodendrocytes [12]. Macroglia have numerous functions, but most importantly are essential in providing support for neurons and endothelial cells structurally, and in terms of nutrition. Whilst oligodendrocytes have been shown to be targeted during viral infections, no notable involvement during infections with other pathogens has been reported [12]. The most abundant cell type in the CNS is astrocytes and they are an important component of the BBB. By either blocking movement, through tight junctions, or promoting movement, using chemokines, astrocytes are able to regulate the movement of leukocytes across the BBB [48, 49]. These cells have also been shown to be involved in immune responses within the CNS where they undergo a process called astrogliosis. This process results in astrocytes that are morphologically and functionally different and is driven by the activation of microglial cells [50, 51]. These phenotypically different astrocytes promote infection resistance by expressing complement proteins or mediate tissue repair by secreting neurotrophic factors [50, 51]. A few studies have used *in vitro* models to show that astrocytes may be involved in the CNS response to *C. neoformans* infection and may potentially activate protective T-cell responses [12, 52, 53]. However, other researchers argue that neuroinflammation from astrogliosis may not necessarily be always protective in the context of infection and that in bacterial meningitis, and that these reactive astrocytes are linked to immunopathology in the brain [12]. Despite playing a major role in preventing pathogens from entering the CNS, once infiltrating the parenchyma, these microbes are primarily removed or destroyed by microglial cells.

1.6.2. Microglia: sentinels of the CNS against infection

The main phagocytes in the CNS are microglial cells, the macrophage analogue [12]. They are found throughout the parenchyma and have long dendritic projections that survey the surrounding environment [54]. Microglia possess pattern recognition receptors (PRRs) such as, toll-like receptors (TLRs) and C-type lectin (CLRs) receptors that are responsible for the identification of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [55]. Studies have shown that CLRs have no role in pathogen detection in *C. neoformans* infection in the CNS due to the protective capsule that masks the fungal PAMPs from CLRs. Therefore, one of the main methods of identification of cryptococcal PAMPs is via the TLRs [56, 57].

Microglial cells exist in two broad states, a resting state or an activated state [58]. In a healthy brain and under normal physiological conditions, resting microglia appear as ramified cells, with a rod-shaped soma and long dendritic projections [59]. Upon detecting homeostatic changes in the brain, microglia becoming activated and undergo functional and morphological changes. Activated microglial cells rapidly retract their processes and enlarge their soma [59, 60]. These activated microglial cells can be characterised into two opposite types: M1 (classic) and M2 (alternative). M1 microglia, typically induced by lipopolysaccharide (LPS) or IFN-γ and produce pro-inflammatory cytokines and chemokines (IL6, IL1β, IL12 CC chemokine ligand 2) [61]. The release of these pro-inflammatory cytokines follows a cascade of intracellular inflammatory signalling events. Upon contact with PAMPs, PRRs is activated, resulting in activation of intracellular pathways that eventually upregulate the production of transcription factors such as nuclear factor for IL6 (NF-IL6) and nuclear factor kappa B (NF-κB) [62, 63]. They express nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which produce reactive oxygen species, inducible nitric oxide synthase (iNOS), major histocompatibility complex-II (MHC-II) as well as integrins, costimulatory molecules and Fc

receptors [64]. The M2 phenotype is induced by anti-inflammatory cytokines IL4 and IL13. M2 microglia release the anti-inflammatory cytokine IL10, growth factors, fibroblast growth factor, colony stimulating factor-1 and various neurotrophic factors [64]. These microglial cells promote phagocytosis of cell debris, and promote tissue repair and extracellular matrix reconstruction [65].

1.6.3. Cryptococcal interactions with resident immune cells of the CNS

During cryptococcal infection, astrocytes have been shown to play a vital role in stimulating anti-cryptococcal activation in microglia via S100β production [66]. In murine models of cryptococcal infection astrocytes that have undergone astrogliosis have been found around cryptococcomas [52]. Astrocytes also upregulate the expression of MHC-II proving that these cells respond to cryptococcal infection [53]. Activated astrocytes also release NO, inhibiting *C. neoformans* growth [67]. Woo and Martinez suggest that the capsule of *C. neoformans* may contribute to astrocyte dysregulation, however further study is required to validate this hypothesis [68].

The exact response of microglial cells to cryptococcal infection is poorly described and the few studies that have described the response of microglia to *C. neoformans* have yielded conflicting conclusions. Some argue that *C. neoformans* activates microglial cells and subsequently the release of proinflammatory cytokines [12, 69]. In support of this argument, another study showed that heat-killed *C. neoformans* that had been injected intracranially increased the expression of proinflammatory cytokines by microglia [70]. However, others have shown that live *C. neoformans*, both encapsulated and acapsular, did not upregulate the expression of proinflammatory cytokines in microglial cells lines [71]. Additionally, further findings of this study suggest that both whole cell *C. neoformans* and its purified capsule may possess anti-

inflammatory properties as seen in a decreased LPS-induced TNF-α response in *C. neoformans* treated BV2 microglia [71]. Other research exploring the possible anti-inflammatory properties of the cryptococcal capsule have identified that GalXM supresses T-cell activation and induces apoptosis of these cells [72-74]. The conflicting evidence with regards to microglial response to *C. neoformans* infection warrants further research into the effects of both live whole cell *C. neoformans* and its purified capsule on microglial activation.

1.7. Rationale

CM is a serious threat to brain health globally but more so in Africa. Despite the high burden and unacceptably high mortality rates associated with the disease, CM has been neglected in research and as a result the pathogenesis of this disease is grossly understudied. From what we know, *C. neoformans* utilises potent virulent factors such as the fungal capsule to evade destruction by the peripheral immune system but our knowledge of how the fungus behaves within the CNS is limited. A good knowledge of how the fungus interacts with resident immune cells of the brain is critical to inform the development of much needed safer and more effective treatment strategies for CM. Therefore, there is an urgent need to investigate how the fungus and its major virulent factor, the capsule, interacts with the resident immune cells of the brain. This study aims to develop a working model of cryptococcal infection in mouse hippocampal organotypic brain slices that will allow us to investigate the innate neuroimmune response to *C. neoformans* infection, and to utilise this model to examine the effect of both live whole cell *C. neoformans* and its purified capsule on microglial activation.

1.8. Aims and objectives

1.8.1. Aim

The overarching aim of this study is to determine the microglial response to whole cell *C. neoformans* and its cell capsule in mouse hippocampal organotypic brain slices.

1.8.2. Objectives

1. To develop an *ex-vivo* model of cryptococcal brain infection.
2. To investigate the innate neuroimmune response to whole cell *C. neoformans* and its purified capsule by using:
    a. dual immunofluorescent staining to track inflammatory activation
    b. enzyme-linked immunosorbent assays (ELISA) to measure the release of IL6 and TNF-α from *C. neoformans*-infected brain slices.
3. To determine the role of microglia in mediating the host response to whole cell *C. neoformans* or its purified capsule by using dual immunofluorescent staining to track inflammatory activation.

CHAPTER TWO

2. Materials and methods

2.1 Animal care and ethics

Seven-day-old, male and female C57BL/6 mice pups were used for this study and were housed in the University of Cape Town (UCT) Research Animal Facility. Mice were kept in acrylic cages lined with wood shavings, in a temperature-controlled room (22 ± 2°C) with 12-hour light/dark cycles. Dams were given uncontrolled access to food and water, and all mouse cages were checked daily to monitor for stress and any litters born. Any mice found in distress were humanely euthanized. All experiments were approved by the UCT Research Animals Ethics Committee (AEC 018/010).

2.2 Mouse hippocampal organotypic brain slice cultures

Organotypic brain slice cultures (OBSCs) are an *ex-vivo* technique whereby brain slices are cultured in media and preserved in an incubator under physiologic-like conditions. OBSCs are a well-established technique that allow the use of living, three-dimensional tissue that has all major brain cell types with their connections preserved [75]. To prepare OBSCs, mouse pups were cervically dislocated and decapitated and their brains were carefully recovered and placed in ice-cold dissection media (Earls balanced salt solution (EBSS) with 6.1 g/l HEPES, 6.6 g/l D-glucose and 5 µM saturated sodium hydroxide). Recovered brains were moved to a cooled stage where the cerebellum and a portion of the frontal lobe were removed. The brains were cut along the great longitudinal fissure to separate hemispheres. The hippocampus was then located in each hemisphere and recovered with care to preserve its structure. After removal,

both hippocampi were placed in parallel on cutting disks to ensure each hippocampus was cut at the correct angle. The disks were placed on an Mcllwain tissue chopper (Brinkmann, Mickle) where the hippocampus was cut into 350 µm thick slices. Chopped slices were washed off the disks into a falcon tube and separated by light swirling. The slices were transferred to a mini petri dish, where slice quality was checked using a light microscope. Hippocampal slices with good structural architecture were plated onto semi-permeable Millicell-CM cell culture inserts (Merck) within 6-well culture plates with exposure to growth media (GM). The GM consisted of 50 % Minimum essential medium with Glutamax, 23 % EBSS, 25 % heat-inactivated horse serum (Biochrom), 6.5 g/l glucose and 2 % B27 (recipe obtained from [76]). Six hippocampal slices were plated per well. Plated slices were cultured in an incubator at 37°C and 5% CO_2 for 7 days to allow them to recover from slicing injury before they could be used for experiments. GM was changed once every two days to ensure proper nutrients were provided to the slices. A summary of the organotypic slice culture preparation is given in Figure 2.1

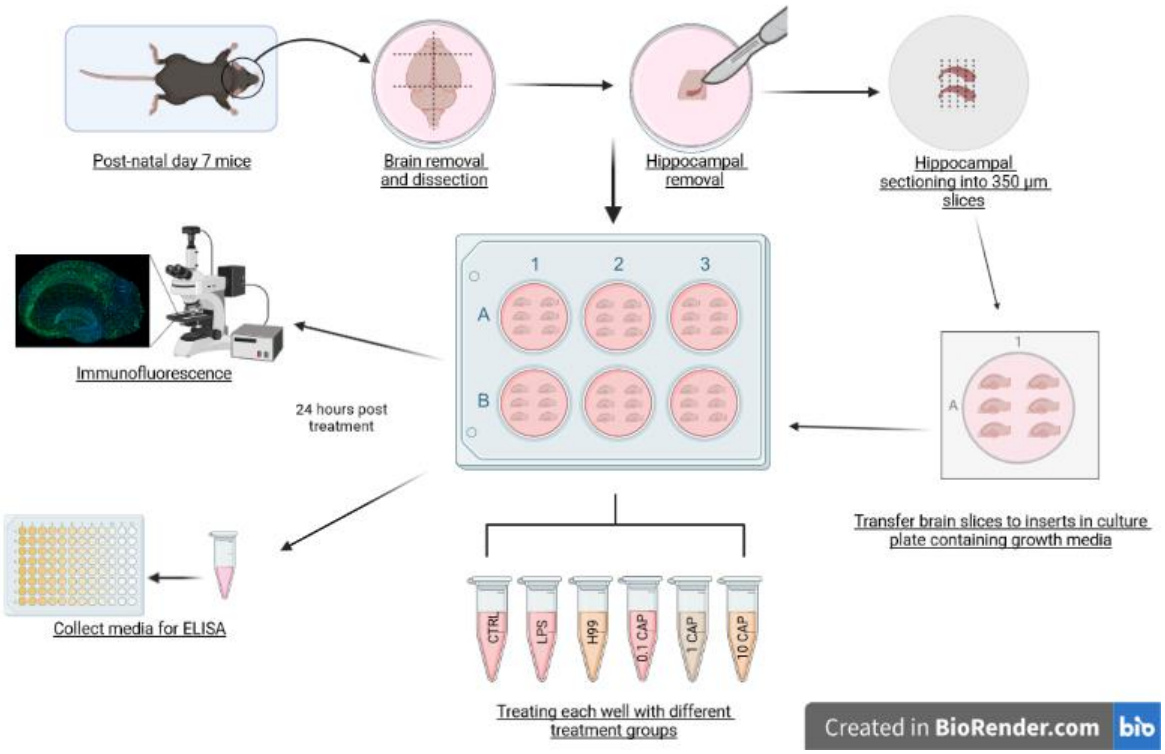

Figure 2.1: The organotypic brain slice culture system set up for studying the neuroimmune response to cryptococcal infection

Organotypic brain slices were made from healthy, living hippocampal tissue obtained from 7-day old mouse pups. Sectioned hippocampal slices were cultured on membrane inserts with access to growth media (GM) for 6 days before infection with *C. neoformans* or its purified capsule for 24 hours. Slices and cultured media were recovered post-infection for determination of inflammatory activation using dual immunofluorescence staining and ELISAs. Created in BioRender.

2.3 *Cryptococcus neoformans* culture

A green fluorescent protein (GFP)/mCherry expressing strain of *C. neoformans* (donated by Associate Professor Rebecca Drummond from the University of Birmingham) was used for this study. The *C. neoformans* strain was grown from a frozen glycerol stock. A loopful of fungal stock was streaked on a yeast peptone dextrose (YPD) agar plate and inoculated plates were left to stand for 48 hours at room temperature in the presence of light to let the fungus grow. Thereafter, a loopful of the *C. neoformans* colony grown on the YPD agar plate was used to inoculate 10 ml of YPD broth in a T75 flask. The inoculated broth was incubated for 22

hours at 30 °C in the presence of light on the shaker (at 150 rpm). Thereafter, the liquid *C. neoformans* culture was centrifuged (2465 rcf, at 27 °C for 5 minutes). The supernatant was removed, and the pellet was washed with 10 ml of 1X phosphate-buffered saline (PBS). The washed pellet was centrifuged (2465 rcf, at 27 °C for 5 minutes) and the supernatant was removed. This washing step was repeated twice before the number of cells were counted using a Neubauer haemocytometer. Cells were diluted as necessary to achieve a final concentration of 10^6 cell/ml. The living fungus was then resuspended in growth media and it was used for treating hippocampal tissue.

2.4 Preparation of capsular extracts

Purified cryptococcal cell capsular extracts were used for stimulating slices. Capsular extracts were prepared from the H99 strain of *C. neoformans* that was grown on Sabouraud dextrose agar plates (Remel, Lenexa, KS)[77]. A colony that was grown was inoculated into 1 liter of yeast nitrogen base (with amino acids) culture medium and left to shake overnight at 30ºC. The inoculum was centrifuged to form a pellet of fungi. The supernatant containing the required soluble polysaccharide was recovered while the fungal pellet was discarded. The polysaccharide was precipitated by slowly adding sodium acetate in powder form to make the solution 10% (weight/volume) followed by pH adjustment to 7.0 using acetic acid. Two and a half volumes of ethanol were added that formed a precipitate and the solution was left on the countertop overnight until the polysaccharide formed a glaze at the bottom of the flask. The supernatant was then decanted and the flask was inverted to remove any ethanol, whilst left to air dry. The polysaccharide was then dissolved in 2-3 ml of distilled water. The extracted polysaccharide capsular pellet was recovered and freeze dried and thereafter was ready for use. Purified capsular extracts were reconstituted in GM to an initial stock solution of 10 mg/ml followed by ultrasonication at 18 Hz for 30 seconds (Soniprep 150, MSE France) to evenly disperse the contents.

2.5 Stimulation of cultured slices

2.5.1 Stimulation of cultured slices with whole cell *C. neoformans*

To test the neuroimmune effects of whole cell *C. neoformans*, hippocampal slices were co-cultured with the GFP/mCherry-expressing strain (H99, Serotype A) of *C. neoformans*. Preliminary experiments were done to determine the dose of cryptococcal cells that could sufficiently penetrate the brain slice and the optimum dose for stimulation that was used for all

experiments was 10 µl at 10^6 cells/ml. Cultured brain slices were allocated to receive one of three treatments:

1. *Group 1* – 1.2 ml of normal GM: these slices served as untreated controls.
2. *Group 2* - 10 µl at 10^6 cells/ml of whole cells of GFP- expressing cryptococcal cells dropped directly onto slices resting in normal 1.2 ml GM
3. *Group 3* - 100 ng/ml of LPS in 1.2 ml GM, and these slices served a positive control for neuroinflammation.

LPS is a cell wall component of Gram-negative bacteria and a powerful TLR 4 agonist therefore it is a good choice to use as positive control for comparison with *C. neoformans*--induced neuroinflammation which is mediated by the same TLR [56, 78].

2.5.2 Stimulation of cultured slices with cryptococcal cell capsule extracts

In order to determine the neuroimmune response to the cryptococcal capsule, slices were treated with three tenfold dilutions of purified capsular extracts. The cultured slices were allocated the following treatments:

1. *Group 1* – 1.2 ml of normal GM, these slices served as an untreated control.
2. *Group 2* – 0.1 mg/ml of purified capsular extracts in 1.2 ml of GM
3. *Group 3* – 1.0 mg/ml of purified capsular extracts in 1.2 ml of GM
4. *Group 4* – 10.0 mg/ml of purified capsular extracts in 1.2 ml of GM
5. *Group 5* - 100 ng/ml of LPS in 1.2 ml of GM, and these slices served a positive control for neuroinflammation.

2.5.3 Co-stimulation of cultured slices with LPS and whole cell *C. neoformans*

To test for a possible anti-inflammatory response to whole cell cryptococcal infection, cultured slices were treated with both LPS and whole cell GFP/mCherry expressing *C. neoformans*. Cultured slices were allocated to receive one of the following treatments:

1. *Group 1* – 1.2 ml of normal GM, these slices served as an untreated control.
2. *Group 2* - 10 µl at 10^6 cells/ml of whole cells of GFP- expressing cryptococcal cells dropped directly onto slices resting in 1.2 ml GM containing 10 ng/ml of LPS
3. *Group 3* - 100 ng/ml of LPS in 1.2 ml GM and these slices served a positive control for neuroinflammation

2.5.4 Co-stimulation of cultured slices with LPS and cryptococcal cell capsule extracts

To test whether the cryptococcal capsule has a possible anti-inflammatory effect, slices were co-cultured with a combination of LPS and cryptococcal cell capsule at three ten-fold concentrations. Cultured slices were allocated the following treatment groups:

1. *Group 1* – 1.2 ml of normal GM, these slices served as an untreated control.
2. *Group 2* – 0.1 mg/ml of purified capsular extracts and 100 ng/ml of LPS in 1.2 ml of GM
3. *Group 3* – 1.0 mg/ml of purified capsular extracts and 100 ng/ml of LPS in 1.2 ml of GM
4. *Group 4* – 10.0 mg/ml of purified capsular extracts and 100 ng/ml of LPS in 1.2 ml of GM
5. *Group 5* - 100 ng/ml of LPS in 1.2 ml of GM and these slices served a positive control

2.6 Immunofluorescence staining

Neuroinflammatory activation was characterised using double immunofluorescence staining. Treated brain slices were collected from culture 24 hours post-treatment and fixed in ice-cold 4% paraformaldehyde (PFA) for 20 minutes. Fixed slices were rinsed three times for 10 minutes in a wash solution (0.3% of Triton X in PBS) to remove any excess PFA. Thereafter, slices were incubated in a blocking solution (5% bovine-serum albumin (BSA) and 1% Triton-X in PBS) for 5 hours at room temperature. After blocking, slices were incubated with an appropriate dilution of primary antibody (Table 2.1) at 4°C for 16 hours, rinsed three times with wash solution before incubation in an appropriate dilution of secondary antibodies (Table 2.1) for 5 hours at room temperature. Following secondary antibody incubation, slices were rinsed three times before a final incubation with a 1:5000 dilution of a nuclear marker (Table 2.1) for 15 mins at room temperature. After one final rinse in 1x PBS for 10 minutes, stained slices were mounted onto glass slides using an aqueous mounting medium (Mowiol, Merck (81381)), cover slipped, and left to dry overnight before imaging.

Table 2.1: List of antibodies used for immunofluorescence staining

Antibody name	**Dilution**	**Catalogue number**	**Supplier**
Primary antibodies			
C/EBP β Antibody (mouse anti-NF-IL6)	1:5000	sc-7962	Santa Cruz
Recombinant anti-IBA1 rabbit monoclonal antibody	1:500	ab178846	Abcam
Secondary antibodies			
Donkey anti-mouse cy3 (red)	1:500	705-166-147	Jackson immunoresearch
Donkey anti-rabbit Alexa 488 (green)	1:500	ab150073	Abcam
Donkey anti-rabbit Alexa 647 (far red)	1:500	ab150107	Abcam
Nuclear marker			
Hoescht (blue)	1:5000	H1399	ThermoFisher

Key: NF-IL6 = nuclear factor for interleukin 6; IBA1 = ionised calcium binding adapter molecule 1

2.7 Confocal Microscopy

Stained brain slices were imaged using a confocal microscope (ZEISS confocal LSM 880 with Airyscan). Two areas in the cornu ammonis 3 (CA3) region were selected at 10X magnification. At each area, z-stacks were taken from the microglial sheath to 20 layers (≈ 0.7 microns for each Z) above at 40x magnification. The raw data was exported to ImageJ and processed through an analysis pipeline developed by A/Prof JV Raimondo (UCT). Data was analysed using GraphPad Prism (Version 9).

2.8 Enzyme-linked immunosorbent assay

The levels of pro-inflammatory cytokines IL6 and TNF-α released by cultured slices were measured using ELISA kits from R&D systems (Table 2.2). ELISA plates (Thermofischer 96-well Maxisorp) were coated with 50 µl of capture antibodies at the recommended concentration (Table 2.2) and incubated overnight at room temperature. The next day, each plate was washed three times using wash solution (1X PBS and 0.05% Tween) and 200 µl of blocking buffer (1% BSA in 1X PBS) was added before incubation at 4 ºC overnight. After blocking, plates were washed three times before 50 µl of experimental samples and prepared cytokine standards (Table 2.2) were added and plates incubated overnight at 4 ºC. Plates were then washed three times and 50 µl of detection antibody at the recommended concentration (Table 2.2) was added to each well followed by incubation for 1 hour at 37 ºC. After another three washes, 50 µl of streptavidin alkaline phosphatase diluted to the recommended concentration in diluent solution (5 g of BSA and 0.1 g of NaN_3 in 300 ml of 1X PBS) was added to each well, and incubated for 1 hour at 37 ºC. Finally, after three washes, 50 µl of phosphatase substrate diluted to the recommended concentration in substrate solution (0.03 g NaN_3, 14.6 ml di-ethanolamine and 0.12g $MgCl_2.6H_2O$ in 100 ml of diH_2O) was added to each well. The plates were covered and allowed to develop for 30-45 minutes. Absorbance was measured for each well at 405 nm with a reference wavelength of 492 nm using a microplate reader (GloMax Discover Promega). To estimate the cytokine concentration, we first subtracted the absorbance of the blanks from all the samples, then we plotted a 4-parameter curve using our duplicate recombinant standards and finally we used the standard curve to estimate the concentrations of the unknown samples. Cytokine data was exported to GraphPad Prism (Version 9) for analysis.

Table 2.2: List of antibodies used for ELISA

Antibody name	**Working concentration**	**Catalogue number**	**Supplier**
R&D systems mouse TNF-α kit (DY410)			
Capture antibody	800 ng/mL	840143	R&D systems
Detection antibody	37.5 ng/mL	840144	R&D systems
Standard	31.2-4000 pg/mL	840145	R&D systems
R&D systems mouse TNF-α kit (DY410)			
Capture	2 µg/mL	840171	R&D systems
Detection	75 ng/mL	840172	R&D systems
Standard	15.6-4000 pg/mL	840173	R&D systems
Substrates			
Streptavidin alkaline phosphatase	1000-fold dilution	551008	BD biosciences
Phosphatase substrate	0.01 g/mL	P4744	Sigma-Aldrich

2.9 Data analysis

Raw data from confocal microscopy was processed through an analysis pipeline in ImageJ where the total number of nuclei, activated cells and microglia were counted. The raw cytokine data was processed in excel, where readings that were lower than detection limit were approximated to the detection limit. All processed data was exported to GraphPad Prism (Version 9) where data was tested for normality. All data was presented as a mean ± standard deviation. After performing the Shapiro-Wilk normality test, Kruskal-Wallis tests were employed for datasets that had more than two treatment groups and Mann-Whitney tests where only two treatment groups were compared.

CHAPTER THREE

3. Results

3.1. Mouse organotypic brain slice cultures: a good model for studying the pathogenesis of cryptococcal meningitis

We set up the mouse hippocampal organotypic slice culture system for studying neuroimmune responses to cryptococcal infection. Figure 3.1.A shows a two-dimensional low-magnification (10X) confocal microscope tile scan of a mouse hippocampal organotypic brain slice with a distinctive, well-preserved cellular architecture at seven days post-culture (nuclei in blue and microglial cell bodies in green). Figure 3.1.B-C shows the presence of the major immune cell types of the brain within the slice namely microglia (immunofluorescent staining with Iba1) (B) and astrocytes (immunofluorescent staining with GFAP) (C) with good cell morphology and numbers.

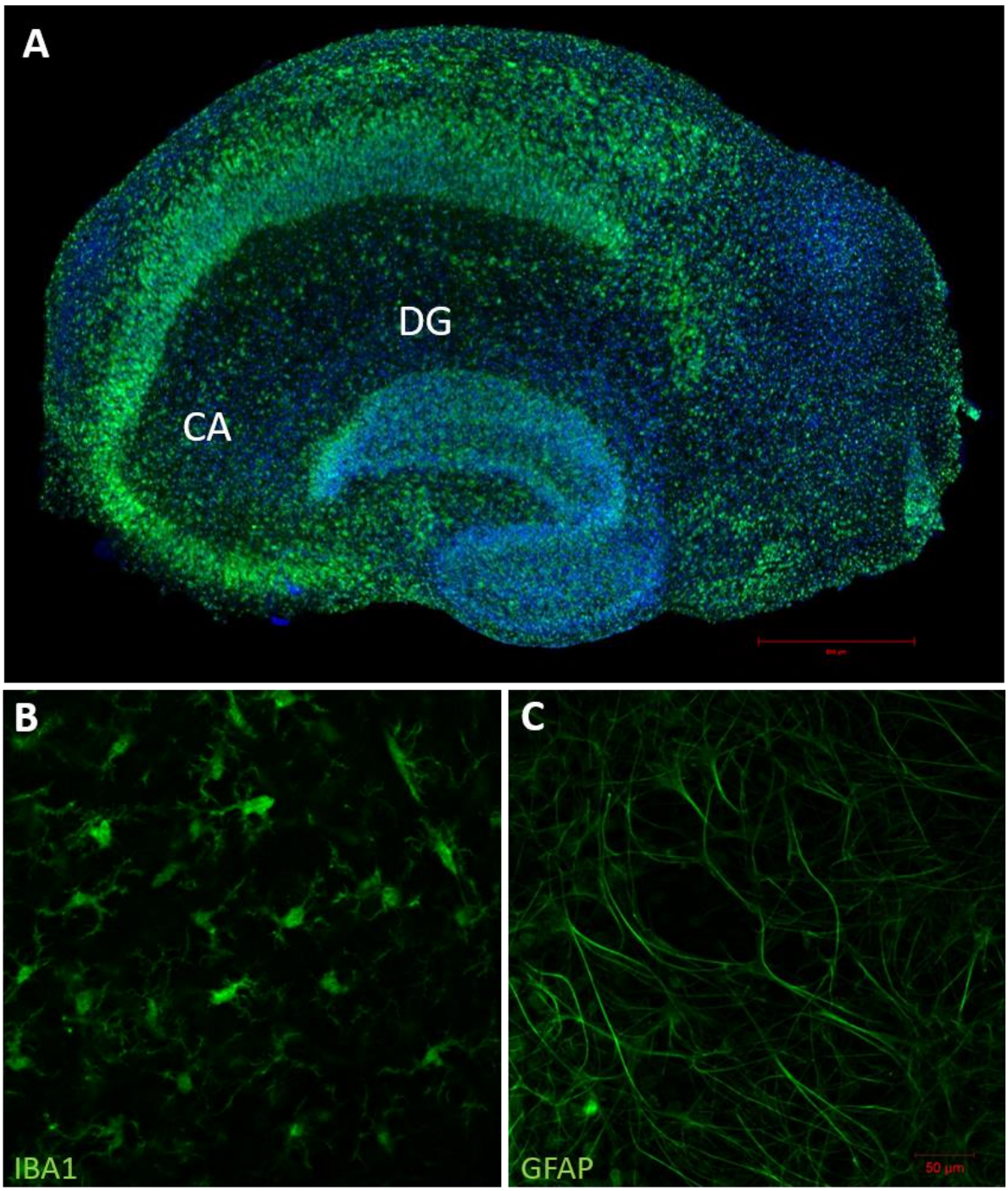

Figure 3.1: The architecture and cell types present in organotypic brain slices
A is a wide tile-scan image (10X) of a whole hippocampal slice with distinct architecture intact (nuclei in blue (Hoechst stain) and cell bodies of microglial cells in green (Iba1 stain)). DG is the dentate gyrus and CA is the cornu ammonis region (scale bar 500 µm). **B** is a representative image of microglia at 40x magnification (green - Iba1 stain) and **C** is a representative image of astrocytes at 40x magnification (green - GFAP). Scale bar in **B** and **C** is 50 µm

Having confirmed the presence of the main resident immune cells of the brain within our cultured hippocampal slices, we developed a protocol to infect the cultured slices with *C. neoformans*. We used a fluorescent experimental strain of *C. neoformans* expressing GFP and mCherry reporters (Figure 3.2A) which allowed us to visualise the fungal cells under a fluorescent microscope. The fluorescent strain's mCherry reporter is expressed in galactose-enriched media and the GFP is expressed in glucose-enriched (low-galactose) media which mimics normal physiological conditions of the human body.

The in-built fluorescent reporters helped us to track *C. neoformans* in the brain slices and in brain cells. *C. neoformans* grew and reproduced in the normal physiological conditions we used for culturing brain slices (37°C; 5% CO_2 and artificial cerebrospinal fluid (CSF) for 24 hours) as is demonstrated by budding magenta fluorescent yeast cells in Figure 3.2B. Additionally, Figure 3.2B shows that when *C. neoformans* is co-cultured with the organotypic brain slices, these fungal cells developed a thick capsule as occurs when it enters the human host. Successful infection of brain slices with *C. neoformans* was achieved when 10 µl of 10^6 cells/ml of *C. neoformans* were applied directly onto the brain slice and incubated for 24 hours post-infection. Figure 3.2C shows a hippocampal OBSC slice (63X) that had been successfully infected with *C. neoformans* over a 24-hour period. Using an Iba1 stain, we observed clusters of GFP^+ *C. neoformans* within green microglial cell bodies.

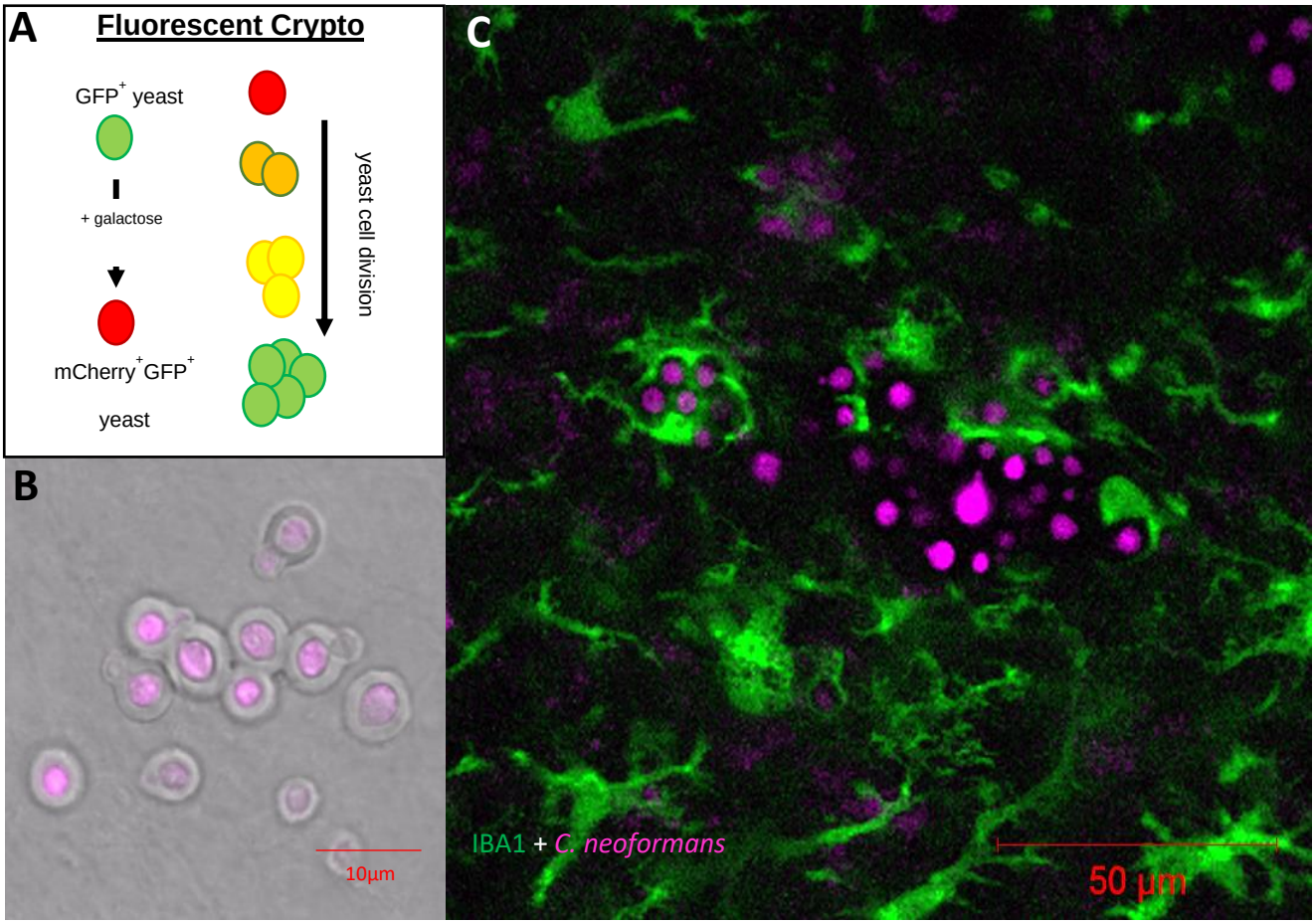

Figure 3.2: Infection of mouse hippocampal tissue with *C. neoformans*

A A schematic of the GFP/mCherry-expressing *C. neoformans* strain used in this study to infect mouse hippocampal organotypic brain slices. **B** A confocal microscope image of magenta fluorescent *C. neoformans* under transmitted light that shows the presence of a thick capsule surrounding the fungal cells (scale bar = 10 µm). **C** is a confocal microscope image of fluorescent cryptococcal cells (magenta) engulfed by microglial cells stained with Iba1 within a mouse OBSC (green) (scale bar = 50 µm).

3.2. NF-IL6 is a robust marker of tracking neuroinflammation in organotypic brain slice cultures

We used the activation of the inflammatory transcription factor NF-IL6 as a biomarker for neuroimmune activation in our mouse hippocampal organotypic brain slice model. NF-IL6 translocation to the nucleus has been previously used as a marker of neuroinflammation [79]. To measure NF-IL6 activation in our brain slice culture model, we stimulated brain slices with

LPS at 100 ng/ml and used a customised ImageJ script to count the number of cells with nuclear NF-IL6 immunoreactivity. Figure 3.3 shows the results of immunofluorescent staining for both NF-IL6 and total (DAPI-stained) nuclei in the slice. Colocalization of the two stains allowed us to count the number of NF-IL6 positive cells as a percentage of the total number of cells. LPS-treated slices showed a robust and significantly greater inflammatory response with an NF-IL6 immunoreactivity of 54.63 % ± 12.75 % (n = 24) nuclei per slice compared to untreated control slices whose immunoreactivity was 14.46 % ± 15.05 % nuclei per slice (n = 24) (Mann-Whitney test; p = <0.001).

In addition to tracking NF-IL6, neuroinflammation was determined by measuring the amount of pro-inflammatory cytokines released by LPS-treated vs untreated slices. Figure 3.3H-I shows the cytokine response of slices. IL6 release was significantly greater in LPS-treated slices (1.140 ng/ml ± 0.540 ng/ml) than untreated control slices (0.040 ng/ml ± 0.030 ng/ml) (Mann-Whitney test; p = <0.0001). Similarly, TNF-α release was significantly greater in LPS-treated slices (0.430 ng/ml ± 0.060 ng/ml) when compared to untreated controls (0.020 ng/ml ± 0.010 ng/ml) (n = 12) (Mann-Whitney test; p = <0.0001).

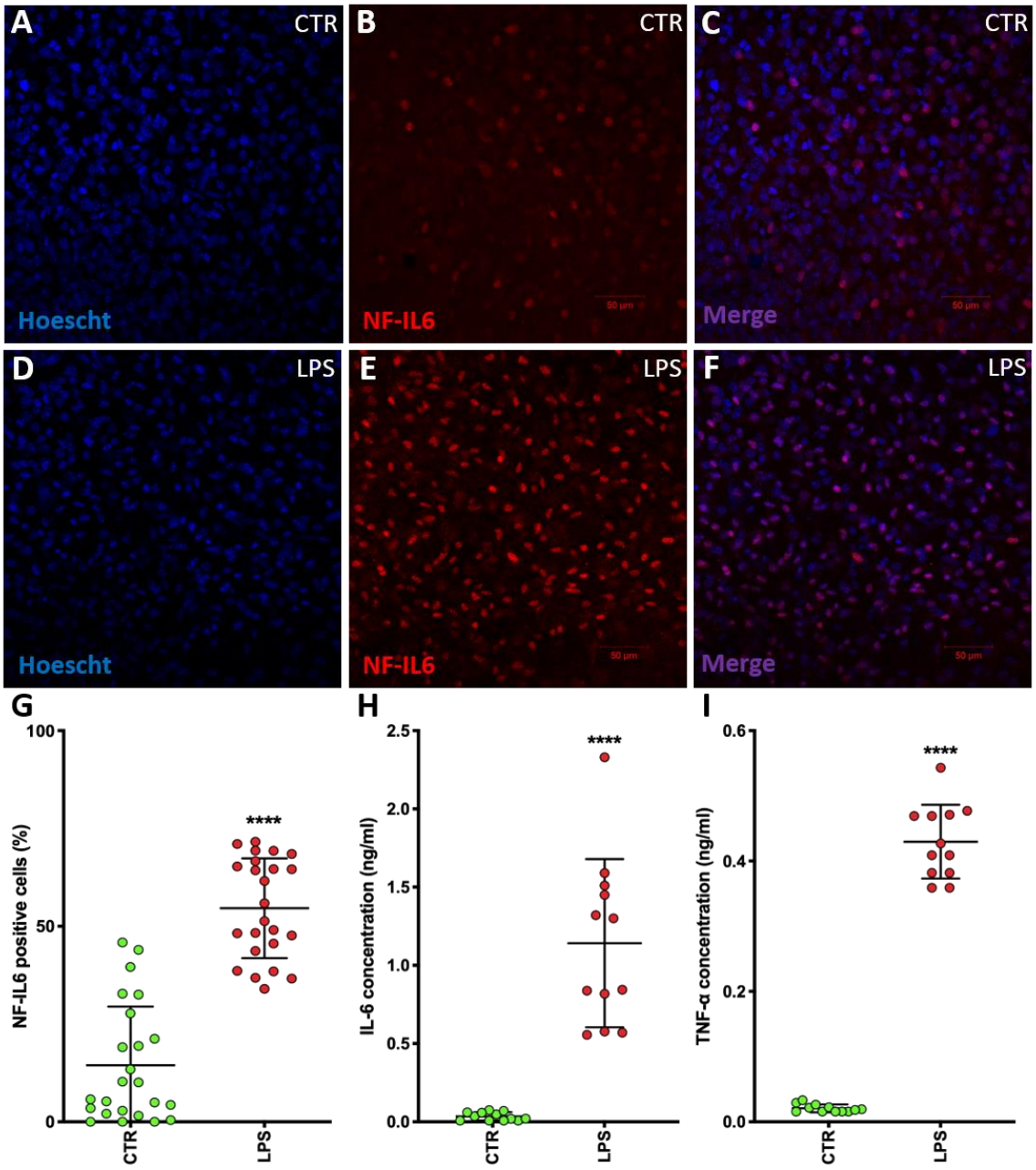

Figure 3.3: The LPS-induced neuroinflammatory response in mouse hippocampal slices

Representative confocal images (40X) showing activation and nuclear translocation of NF-IL6 (red) in control vs LPS-treated slices. **A** is the total DAPI-stained nuclei (Hoescht stain) within a selected area of a control slice, **B** shows the total number of NF-IL6-positive cells (C/EBP β stain) within the same area, while **C** shows colocalization of DAPI and NF-IL6 as confirmation of inflammatory activation. Similarly, **D** shows the total DAPI-stained nuclei within a selected area of an LPS-treated slice, **E** shows the total number of NF-IL6-positive cells in the same area and **F** shows the colocalization of DAPI and NF-IL6 as a confirmation of inflammatory activation. **G** is a graph showing the percentage of NF-IL6 activated cells in LPS-treated and control slices (n = 24), whilst graphs **H** and **I** represent the amount of IL6 and TNF-α released by slices treated with LPS vs untreated control slices (n = 12). Scale bar = 50 μm in all images. **** represents a significant difference between untreated control and LPS-treated slices ($p \leq 0.0001$) (Mann-Whitney test).

3.3. *C. neoformans* does not elicit a neuroinflammatory response in mouse hippocampal tissue

Having established a robust and reliable protocol to measure neuroinflammation in the mouse organotypic brain slice culture system as well as a successful protocol for infecting brain slices with *C. neoformans*, we next examined the neuroimmune response to cryptococcal infection. We compared the number of NF-IL6 activated cells in hippocampal OBSCs treated with whole cell *C. neoformans* to LPS-treated slices and controls. Figure 3.4 shows that slices treated with whole cell *C. neoformans* had an NF-IL6 immunoreactivity of 1.8 % ± 2.83 % which was significantly lower than that of LPS-treated slices (54.63 % ± 12.75 %) ($p = <0.0001$; Kruskal-Wallis test) but not significantly different from control slices (14.46 % ± 15.05 %).

In addition to measuring NF-IL6 immunoreactivity we also measured pro-inflammatory cytokine release as confirmation of neuroinflammation. Figure 3.4E shows that slices treated with whole cell *C. neoformans* released 0.011 ng/ml ± 0.007 ng/ml of IL6, which was significantly lower ($p = <0.0001$; Kruskal-Wallis test) than that of LPS-treated slices (1.542 ng/ml ± 0.154 ng/ml) but not significantly different from control treated slices (0.007 ng/ml ± 0.00 ng/ml). Similarly, slices treated with whole cell *C. neoformans* released significantly lower levels of TNF-α (0.010 ng/ml ± 0.005 ng/ml) as compared to LPS-treated slices that released (0.499 ng/ml ± 0.084 ng/ml) ($p = <0.0001$) and not significantly different than control slices (0.008 ng/ml ± 0.00 ng/ml) as seen in Figure 3.4F.

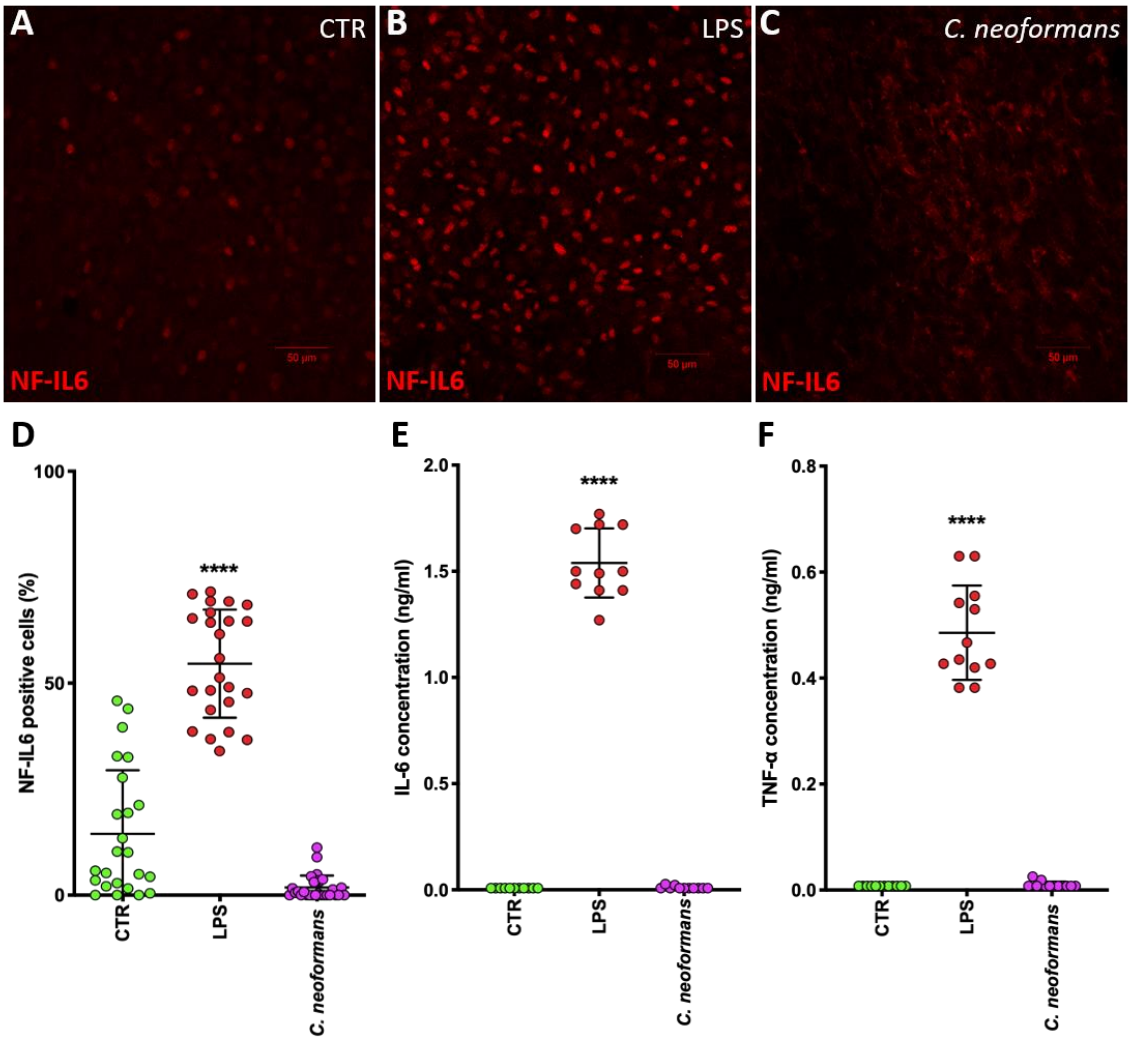

Figure 3.4: The neuroinflammatory response to whole cell *C. neoformans* infection in mouse hippocampal tissue

Representative images showing NF-IL6-immunoreactivity in control, LPS-treated and whole cell *C. neoformans*-infected organotypic brain slices (C/EBP β stain at 40X) (**A-C**). **D** shows the percentage of NF-IL6 activated cells in control, LPS-treated and whole cell *C. neoformans*-infected organotypic brain slices (N = 24), while **E** and **F** show the levels of IL-6 and TNF-α released in each treatment (N = 12). Scale bar = 50 μm in all images. **** represents a significant difference between untreated control and LPS-treated slices ($p \leq 0.0001$) (Kruskal-Wallis test).

3.4. The neuroimmune response to purified cryptococcal capsular extracts

In order to determine the neuroimmune response to the cryptococcal capsule, we treated OBSCs with three, ten-fold concentrations of purified capsule and used immunofluorescent

staining for NF-IL6 activation (C/EBP β stain). Figure 3.5 shows that brain slices treated with all three concentrations of the capsule exhibited low levels of NF-IL6 immunoreactivity. Figure 3.5G shows that slices treated with 0.1 mg/ml (18.50 % ± 20.79 %), 1.0 mg/ml (17.21 % ± 15.23 %) and 10 mg/ml (19.34 % ± 14.44 %) showed significantly lower NF-IL6 immunoreactivity compared to LPS-treated slices (66.74 % ± 16.38 %) ($p = <0.0001$; Kruskal-Wallis test) and were not significantly different from control slices (14.96 % ± 15.44 %) ($n = 24$).

Measuring cytokine release, Figure 3.5H-I shows that slices treated with 0.1, 1.0 and 10 mg/ml capsule released 0.065 ng/ml ± 0.016 ng/ml, 0.062 ng/ml ± 0.010 ng/ml and 0.035 ng/ml ± 0.013 ng/ml of IL-6 respectively, which was significantly lower compared to slices treated with LPS (1.907 ng/ml ± 0.380 ng/ml) ($p = 0.0038$, $p = 0.0058$, $p = <0.0001$; Kruskal-Wallis test) but not significantly different to control slices (0.062 ng/ml ± 0.012 ng/ml) ($N = 12$). We observed a similar trend for TNF-α release with slices treated with 0.1, 1.0 and 10 ng/ml releasing 0.026 ng/ml ± 0.010 ng/ml, 0.016 ng/ml ± 0.001 ng/ml and 0.042 ng/ml ± 0.039 ng/ml which were significantly lower than LPS-treated slices (0.485 ng/ml ± 0.089 ng/ml) ($p = <0.0001$, $p = <0.0001$, $p = 0.0003$; Kruskal-Wallis test) and not significantly different to untreated control slices (0.04 ng/ml ± 0.006 ng/ml) ($n = 12$).

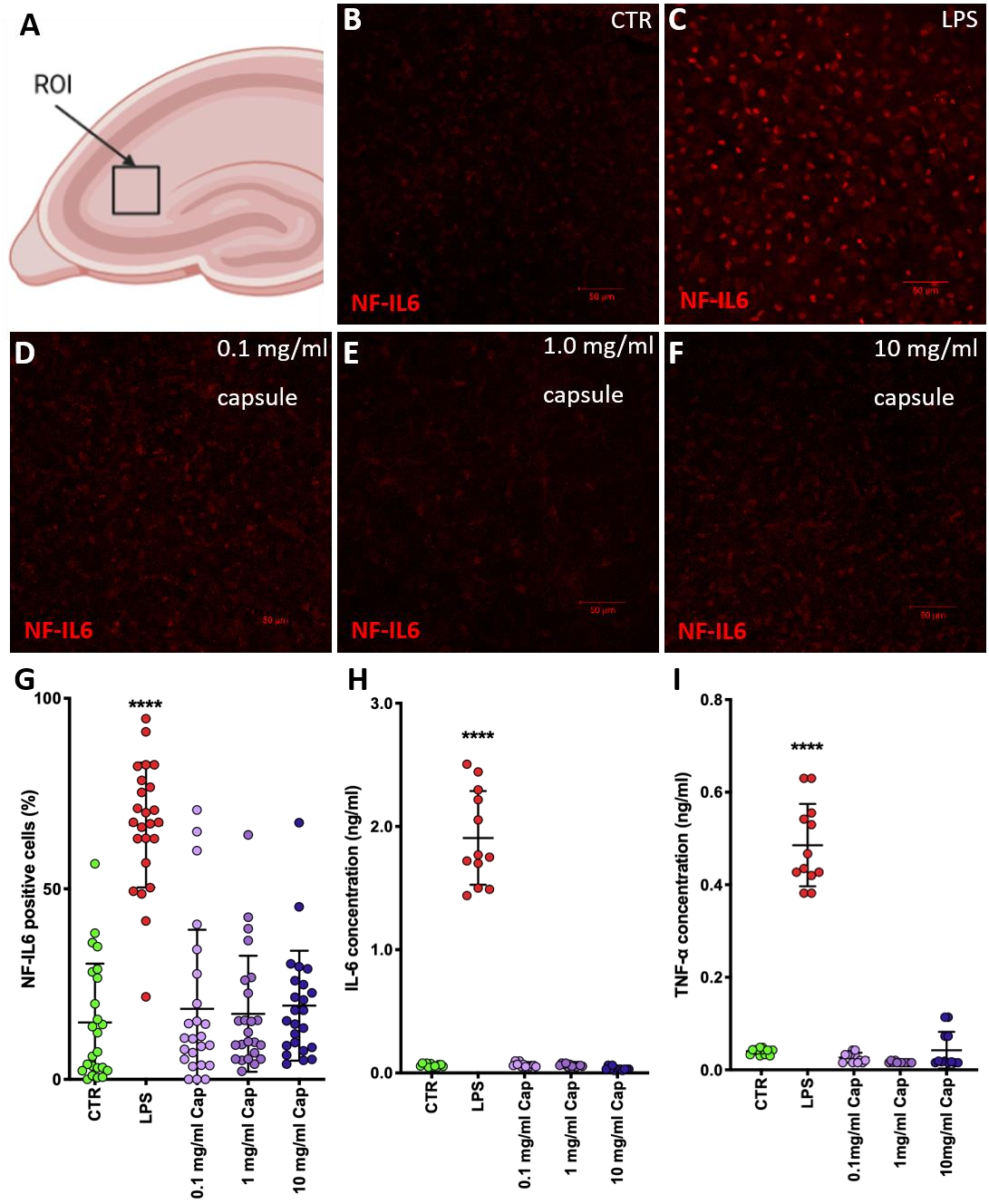

Figure 3.5 The neuroinflammatory response to stimulation with purified cryptococcal capsule

A is a schematic diagram showing the CA3 region; region of interest (ROI) in the hippocampus where confocal images were taken from. **B-F** are representative confocal images showing the level of NF-IL6 activation (C/EBP β stain at 40X) in untreated control slices **(B)**, slices treated with LPS **(C),** slices treated with 0.1 mg/ml capsule (**D)** slices treated with 1.0 mg/ml capsule **(E),** and slices treated with 10.0 mg/ml capsule **(F)**. **G** shows the number of NF-IL6-positive cells across treatment groups (N = 24), while **H** and **I** show the levels of IL-6 and TNF-α across treatments (n = 12). Scale bar = 50 μm in all images. **** represents a significant difference between untreated control and LPS-treated slices ($p \leq 0.0001$) (Kruskal-Wallis test).

3.5. Whole cell *C. neoformans* and its purified capsule do not have an anti-inflammatory effect in mouse hippocampal tissue

Following our observation that neither whole cell *C. neoformans* nor its purified capsule have an inflammatory effect on the innate immune system of the brain, we tested the possibility that they may exhibit anti-inflammatory effects. In order to measure a possible anti-inflammatory effect, we co-stimulated mouse hippocampal OBSCs with either whole cell *C. neoformans* or its purified capsule and LPS. Figure 3.6 shows representative images of the level of NF-IL6 activation in slices co-stimulated slices (C/EBP β stain), compared to slices treated with only LPS and untreated control slices. Slices co-stimulated with both LPS and *C. neoformans* showed significantly greater NF-IL6 immunoreactivity (68.100% ± 18.110%) when compared to untreated control slices (14.46 % ± 15.05 %) ($p = <0.0001$; Kruskal-Wallis test), but not significantly different to LPS-treated slices (54.63 % ± 12.75 %) (N = 24). IL6 release was significantly greater in brain slices co-stimulated with LPS and *C. neoformans* (1.057 ng/ml ± 0.428 ng/ml), than in untreated control slices (0.034 ng/ml ± 0.026 ng/ml) ($p = <0.0001$; Kruskal-Wallis test) but not significantly different to slices treated with LPS only (1.142 ng/ml ± 0.537 ng/ml) (N = 12). Similarly Figure 3.6F, shows that slices co-stimulated with LPS and *C. neoformans* released 0.450 ng/ml ± 0.136ng/ml of TNF-α, which was significantly greater than untreated control slices (0.020 ng/ml ± 0.006 ng/ml) ($p = <0.0001$; Kruskal-Wallis test) but not significantly different from LPS-treated slices (0.4297 ng/ml ± 0.056 ng/ml) (N = 12).

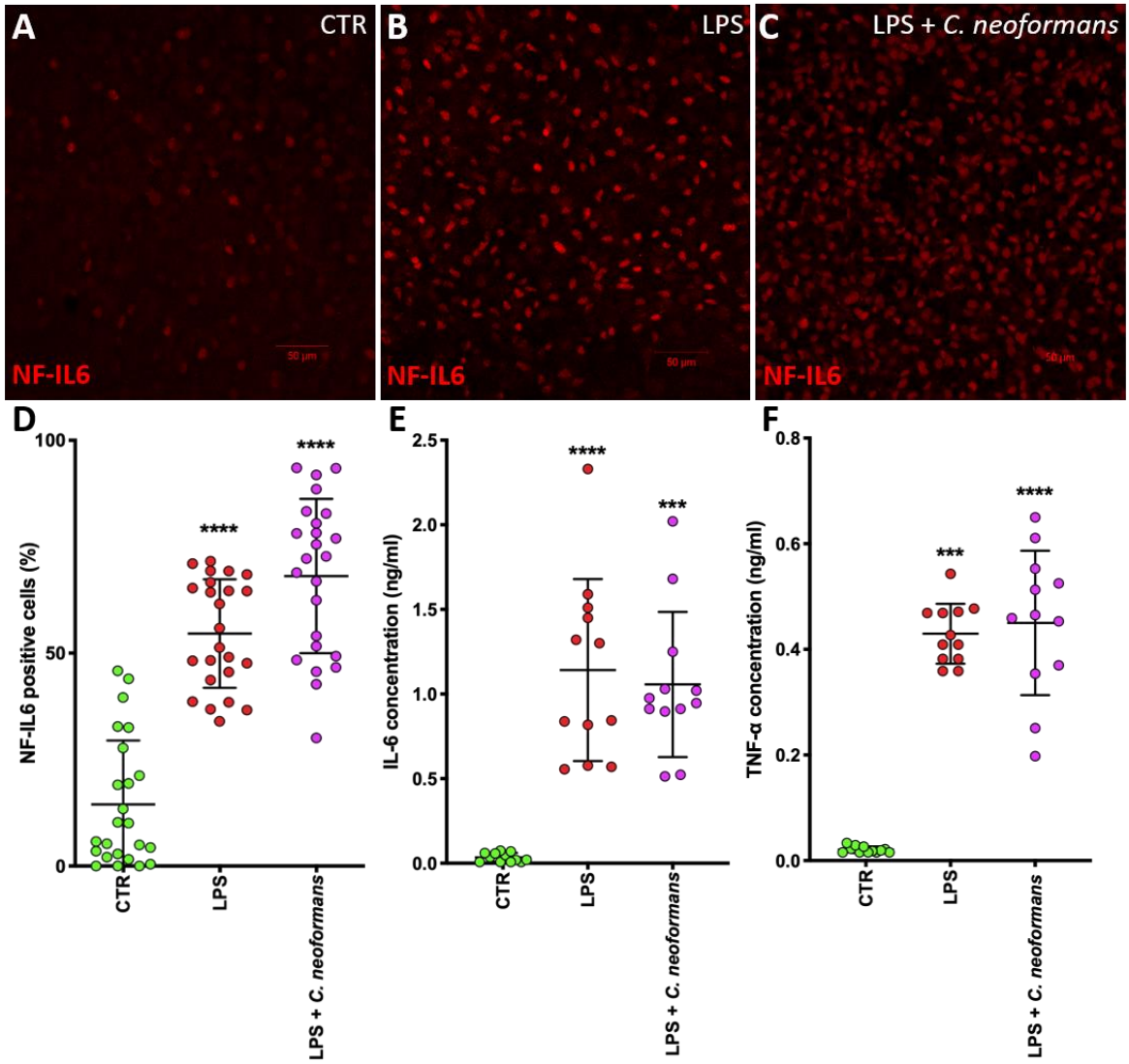

Figure 3.6: Whole cell *C. neoformans* does not significant alter LPS-induced neuroinflammation.

Representative data showing the lack of an anti-inflammatory response to whole cell cryptococcal infection in mouse hippocampal OBSCs. **A-C** are representative images of the level of NF-IL6-immunoreactivity in control, LPS-treated and slices co-stimulated with both whole cell *C. neoformans* and LPS (C/EBP β stain at 40X). Graph **D** shows the level of NF-IL6 immunoreactivity in control slices, slices treated with LPS and slices co-stimulated with LPS and whole cell *C. neoformans* (N = 24). Graphs **E** and **F** show the levels of IL-6 and TNF-α released by control, LPS-treated and slices co-stimulated with LPS and *C. neoformans* (N =12). Scale bar = 50 μm in all images. An asterisk (*) represents a significant difference between untreated control slices and LPS-treated slices or between control slices and slices co-stimulated with LPS and whole cell *C. neoformans*. *** = $p \leq 0.001$, and **** = $p \leq 0.0001$ (Kruskal-Wallis test).

To investigate the potential anti-inflammatory effects of the cryptococcal capsule, we treated mouse hippocampal OBSCs with a combination of LPS and purified capsule extracts at three different tenfold concentrations. We observed that slices co-stimulated with LPS and 0.1 mg/ml capsule, 1.0 mg/ml capsule and 10 mg/ml capsule had NF-IL6 immunoreactivity (C/EBP β stain) of 61.30 % ± 21.98 %, 55.13 % ± 23.40 % and 49.04 % ± 24.18 % respectively, which was significantly higher than control slices (14.96 % ± 15.44 %) ($p = <0.0001$, $p = <0.0001$, $p = 0.0005$; Kruskal-Wallis test), but not significantly different to slices only treated with LPS (66.74 % ± 16.38 %) (N = 24). Slices co-stimulated with LPS and 0.1, 1.0 and 10 mg/ml capsule released 2.555 ng/ml ± 0.605 ng/ml, 1.970 ng/ml ± 0.483 ng/ml and, 1.807 ng/ml ± 0.324 ng/ml of IL-6, which was not significantly different from LPS-treated slices (2.309 ng/ml ± 0.229 ng/ml), but significantly higher than untreated control slices (0.062 ng/ml ± 0.012 ng/ml) ($p = <0.0001$, $p = <0.0001$, $p = 0.05$; Kruskal-Wallis test) (N = 12) (Figure 3.7H). Similarly in Figure 3.7I, slices co-stimulated with LPS and with 0.1 mg/ml capsule, 1.0 mg/ml capsule and 10 mg/ml capsule released 0.661 ng/ml ± 0.100 ng/ml, 0.572 ng/ml ± 0.112 ng/ml and 0.5787 ng/ml ± 0.038 ng/ml of TNF-α, which was not significantly different from LPS-treated slices (0.646 ng/ml ± 0.040 ng/ml), but significantly higher than untreated control slices (0.050 ng/ml ± 0.033 ng/ml) ($p = <0.0001$, $p = 0.0329$, $p = 0.0068$; Kruskal-Wallis test) (N = 12).

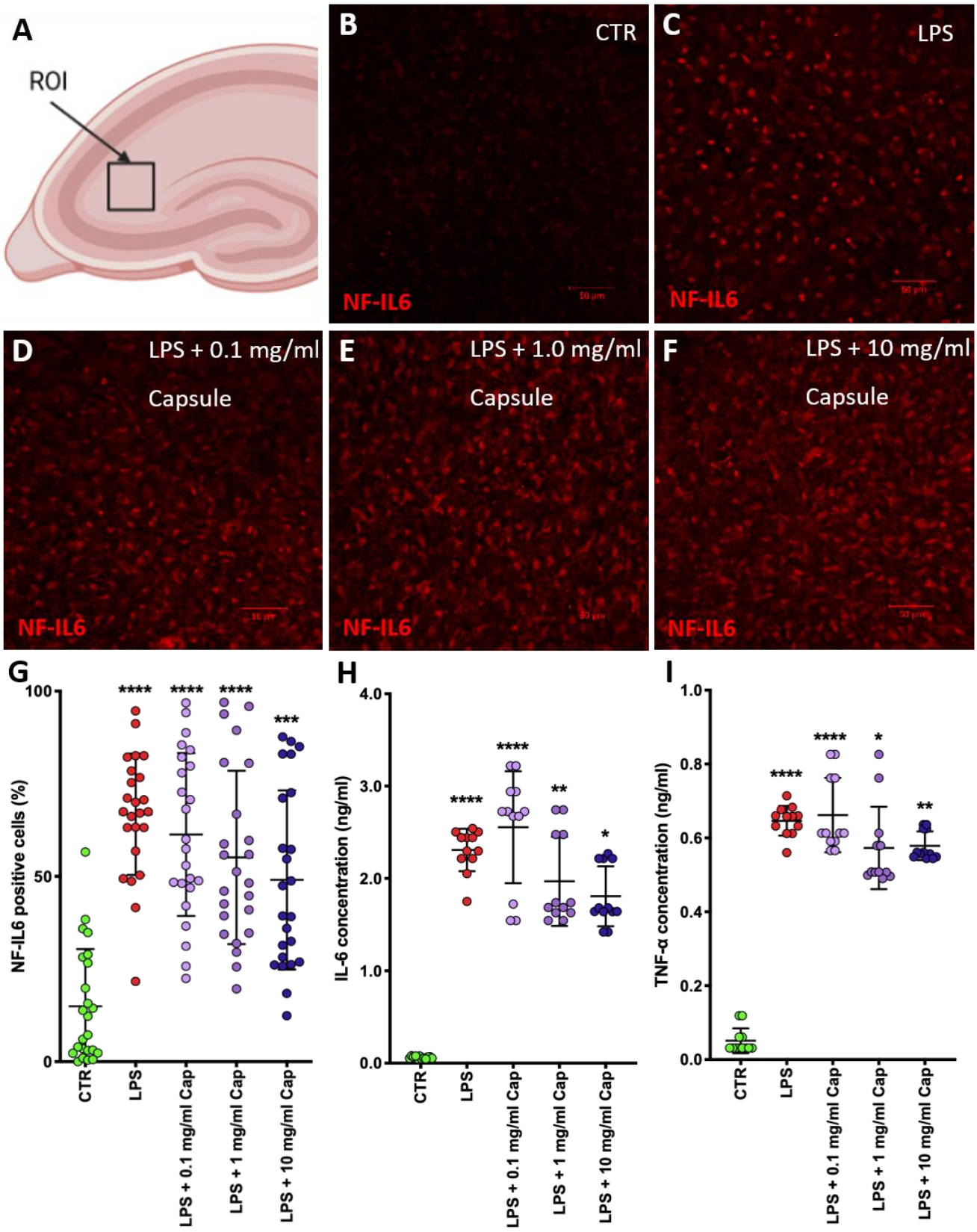

Figure 3.7 The cryptococcal capsule does not significantly alter LPS-induced neuroinflammation.

A is a schematic diagram showing the CA3 region; region of interest (ROI) in the hippocampus where confocal images were taken from. **B-F** are representative confocal images (40X) showing the level of NF-IL6 activation (C/EBP β stain) in untreated control slices **(B)**, slices treated with LPS (**C),** slices treated with LPS and 0.1 mg/ml capsule (**D)** slices treated with LPS and 1.0 mg/ml capsule (**E),** and slices treated with LPS and 10.0 mg/ml capsule (**F)**. **G** shows the number of NF-IL6-positive cells across treatment groups (N = 24) while **H** and **I** show the levels of IL-6 and TNF-α across treatments (n = 12). Scale bar = 50 μm in all images. An asterisk (*) represents a significant difference between untreated control slices and LPS-treated slices or slices co-stimulated with LPS and three tenfold concentrations of purified capsular extract. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, and **** = $p \leq 0.0001$ (Kruskal-Wallis test).

3.6. The role of microglia in mediating the neuro-immune response to *C. neoformans* infection

Using dual immunofluorescence staining we observed that the GFP^+ whole cell *C. neoformans* was engulfed by microglial cells (Iba1 stain) within the slices, therefore we investigated the microglial involvement during whole cell cryptococcal infection or to its purified capsule extract in isolation. Figure 3.8A-D shows representative images of control, LPS-treated, and slices treated with whole cell *C. neoformans* or its purified capsular extract (C/EBP β and Iba1 stain).

We observed that slices treated with whole cell *C. neoformans* had a significantly lower number of NF-IL6 positive microglia (11.44 % ± 16.14 %) when compared to LPS-treated slices (82.64 % ± 14.04 %) (p = <0.0001; Kruskal-Wallis). However, there was no significant difference between *C. neoformans* treated slices and untreated controls (18.10 % ± 25.02 %), as seen in Figure 3.8E (n = 24). Slices treated with all three doses of the cryptococcal capsule (9.46 % ± 16.84 % for 0.1 mg/ml, 10.04 % ± 13.95 % for 1.0 mg/ml and 9.03 % ± 8.86 % for 10 mg/ml) had significantly lower microglial activation than LPS-treated slices (80.17 % ± 20.26 %) (p = <0.0001; Kruskal-Wallis test) although they showed no significant difference from untreated control slices (80.17 % ± 20.26 %) (p = <0.0001; Kruskal-Wallis test) (n = 24).

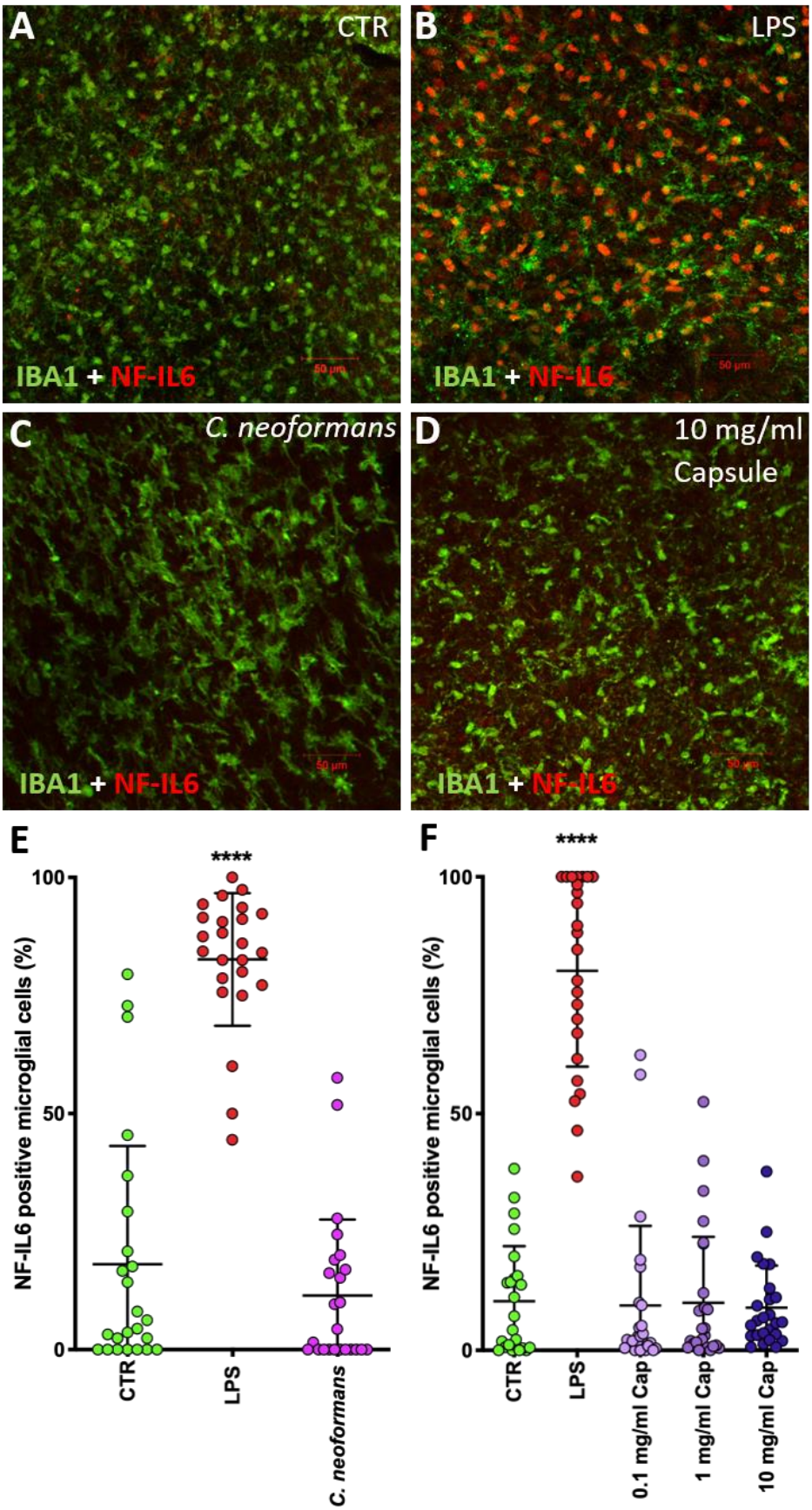

Figure 3.8: The microglial response to cryptococcal infection of hippocampal slices

(A-D) Representative confocal images showing activation and nuclear translocation of NF-IL6 (red) co-localised with microglial cell bodies (green) (C/EBP β and Iba1 stain). **A** Activated microglial cells in an untreated control slice, **B** an LPS-treated slice, **C** a slice infected with whole cell *C. neoformans* and **D** a slice treated with of 10 mg/ml of the purified cryptococcal capsule (Images of the two lower concentrations of the cryptococcal capsule are not shown). **E** shows the level of microglial NF-IL6 immunoreactivity in control, LPS-treated and whole cell *C. neoformans* infected slices (n = 24), whilst **F** shows the microglial NF-IL6 immunoreactivity in control, LPS-treated slices and slices treated 0.1, 1.0 and 10 mg/ml of purified cryptococcal capsule (n = 24). Scale bar = 50 μm in all images. **** represents a significant difference between untreated control and LPS-treated slices ($p \leq 0.0001$; Kruskal-Wallis test).

Stimulation with whole cell *C. neoformans* or its purified capsule does not result in an anti-inflammatory response in microglial cells. Figure 3.9E shows that mouse hippocampal OBSCs co-stimulated with LPS and whole cell *C. neoformans* resulted in an NF-IL6 activation of 95.86 % ± 6.24 %, which is significantly higher than control slices (18.10 % ± 25.02 %) ($p = <0.0001$; Kruskal-Wallis test), but not significantly different from LPS-treated slices (82.64 % ± 14.04 %) (n = 24). Similarly, slices co-stimulated with LPS and 0.1 mg/ml, 1.0 mg/ml and 10 mg/ml capsule had a microglial NF-IL6 immunoreactivity of 94.63 % ± 9.63 %, 94.62 % ± 10.65 % and 87.99 % ± 15.81 % respectively. All three concentrations were significantly higher than control slices at 10.39 % ± 11.61 % ($p = <0.0001$ at all three concentrations; Kruskal-Wallis test), but not significantly different to LPS-treated slices (88.75 % ± 14.74 %) (n = 24).

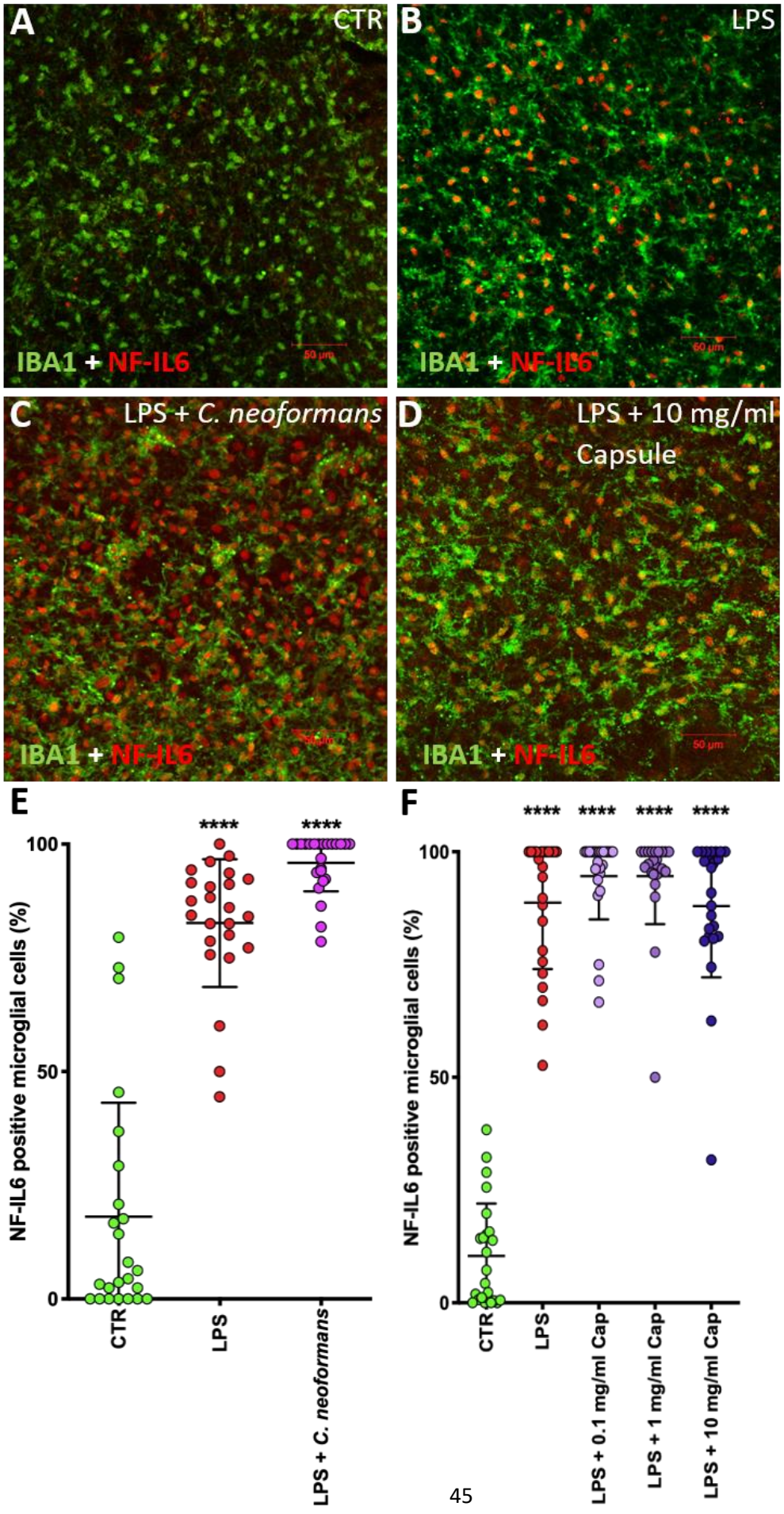
A
CTR
IBA1 + NF-IL6
B
LPS
IBA1 + NF-IL6
C
LPS + C. neoformans
IBA1 + NF-IL6
D
LPS + 10 mg/ml Capsule
IBA1 + NF-IL6
E
NF-IL6 positive microglial cells (%)
100
50
0
****
****
CTR
LPS
LPS + C. neoformans
F
NF-IL6 positive microglial cells (%)
100
50
0
****
****
****
****
CTR
LPS
LPS + 0.1 mg/ml Cap
LPS + 1 mg/ml Cap
LPS + 10 mg/ml Cap

Figure 3.9 The anti-inflammatory microglial response in cryptococcal brain infection.

(A-D) Representative confocal images showing activation and nuclear translocation of NF-IL6 (red) co-localised with microglial cell bodies (green)(C/EBP β and Iba1 stain). **A** shows an untreated control slice, **B** shows an LPS-treated slice, **C** shows a slice co-stimulated with LPS and whole cell *C. neoformans* and **D** showing a slice co-stimulated with LPS and 10 mg/ml of the purified cryptococcal capsule (Images of the two lower concentrations of the cryptococcal capsule are not shown). **E** shows the level of microglial NF-IL6 immunoreactivity in untreated control, LPS-treated and slices co-stimulated with LPS and whole cell *C. neoformans* (n = 24), whilst **F** shows the microglial NF-IL6 immunoreactivity in untreated control, LPS-treated slices and slices co-stimulated with LPS and 0.1, 1.0 and 10 mg/ml of purified capsular extract (n = 24). Scale bar = 50 μm in all images. An asterisk (*) represents a significant difference between untreated control slices and LPS-treated slices or slices co-stimulated with LPS and either whole cell *C. neoformans* or its purified capsular extract. **** = $p \leq 0.0001$; Kruskal-Wallis test.

CHAPTER FOUR

4. Discussion

The pathogenesis of cryptococcal meningitis is grossly understudied and the response of resident immune cells of the brain to the fungus that causes this fatal infection is not clearly defined. The aim of this study was to investigate the neuroimmune response to *C. neoformans* and its purified capsule using a novel brain slice culture system. Our results show that neither whole cell *C. neoformans* nor its purified capsule elicit an inflammatory response, nor do they possess anti-inflammatory properties in cultured mouse hippocampal brain slices. Further findings show that *C. neoformans* is phagocytosed by microglial cells but the microglial cells are not classically activated.

4.1. Organotypic brain slice cultures are a good model for studying the pathogenesis of neurocryptococcosis

Organotypic slice cultures have been successfully used in other studies to study brain-specific innate immune responses to viral, bacterial and parasitic pathogens [80, 81]. Our study is the first study to employ the organotypic slice culture model for studying the neuroimmune response to cryptococcal infection. Our results show that the brain slice cultures proffer several advantages for studying host-fungus interactions at the cellular level. First, the brain slices have a full complement of resident immune cells of the brain therefore the interaction of the fungal pathogen and each cell type can be studied. Second, the brain slices maintain their intercellular connections as they would be *in vivo* thus, are more representative of what happens in a living brain than does single-cell cultures. Lastly, the slices are responsive to inflammatory insults

and we were able to measure detectable changes of cytokines in slices challenged with LPS (See Figure 3.3).

Although the brain slice culture system is not new, its application to study neuroinflammatory responses to pathogens is quite novel. We measured inflammatory activation in cells by tracking the activation of NF-IL6; a transcription factor that can translocate to the nucleus to activate IL6 production [63]. The activation of this signalling molecule has been reliably used for detection of inflammation in bacterial and fungal pathogens but not for *C. neoformans* [79]. However, *C. neoformans* PAMPs including the major cell capsule component GXM are recognised by TLR4, the PRR that recognises LPS [56]. An ability to robustly track inflammatory signalling in organotypic brain slices presents a good opportunity to investigate the neuroinflammatory response to *C. neoformans* and to determine the cell types that mediate this response.

Overall, the organotypic brain slice culture model is a powerful platform to study how *C. neoformans* interacts with different neuroimmune cells without the interference of the peripheral immune system and could potentially address the lack of experimental models that has hindered cryptococcal brain research as described in a recent review [3].

4.2. Infection with whole cell *C. neoformans* or its purified fungal capsule does not elicit an inflammatory response in organotypic brain slices

Our findings show that neither whole cell *C. neoformans* nor its purified fungal capsule elicit an inflammatory immune response in OBSCs (Figure 3.4 and 3.5). We observed that slices treated with whole cell *C. neoformans* or its capsule did not show significant activation of inflammatory signalling molecules such as NF-IL6 nor the activation of downstream release of the proinflammatory cytokines IL-6 and TNF-α. Paradoxically, clinical *C. neoformans*

infection is associated with meningoencephalitis, an inflammatory response in the parenchyma and meninges [13]. Our findings suggest that recognition of *C. neoformans* by resident immune cells of the brain may not directly invoke a pro-inflammatory response. These findings tie in well with prior studies that have shown that live, encapsulated and acapsular *C. neoformans* did not elicit a proinflammatory response in BV2 microglial cells [71]. Others have also shown that GXM a major component of the cryptococcal capsule is associated with immunomodulation and the production of anti-inflammatory cytokines [70, 82-84]. In the periphery, GXM has also been shown to skew immune responses towards a Th2-type response which is associated with the release of anti-inflammatory cytokines [35]. Contrary to our observations, some researchers have reported that in pulmonary cryptococcosis, *C. neoformans* has been shown to induce the production of proinflammatory cytokines including IFNγ and IL12 [70, 82-84]. A plausible explanation that the initial peripheral response of the immune system to *C. neoformans* is a proinflammatory response that is aimed at clearing the fungus but if the immune system is compromised or if the fungus develops virulence mechanisms it will switch the response to a Th2-type response that then allows it to be disseminated to the brain. In our model of fungal infection of brain slices, *C. neoformans* cells developed a thick capsule (Figure 3.2), similar to what is seen when fungal cells enter the host [21]. This may explain why the resident immune cells of the brain do not mount an inflammatory response to the pathogen.

Although usually immunologically quiescent, the CNS is known to recruit peripheral immune cells to help fight pathogens and the recruitment of monocytes, neutrophils and T cells [36, 37]. Recruitment of peripheral cells has often been associated with an exacerbated inflammatory response [37] thus it is possible that meningoencephalitis in CM may be the result of invasion of the CNS by inflammatory cells from the periphery as opposed to the

activation of the resident immune cells by the fungus. The influence of peripheral cells on the neuroimmune response was beyond the scope of this study and cannot be easily studied with our model as it lacks blood supply from the periphery [85].

4.3. Whole cell *C. neoformans* and its purified fungal capsule do not evoke an anti-inflammatory response in organotypic brain slices

Our findings showed that neither whole cell *C. neoformans* or its purified capsule reduced the inflammatory response induced by LPS in brain slices. These results suggest that neither the whole cell nor the capsular fractions of *C. neoformans* have an anti-inflammatory effect. In a previous study done using BV2 microglial cell cultures that were treated with LPS, the addition of *C. neoformans* or its purified capsule decreased the amount of TNF-α released [71]. In comparison to the study that used single cell cultures, our model uses tissue that has all the resident cell types in the and hence presents are a representative response to what occurs *in vivo*. Other cells have been shown to participate in anti-cryptococcal defences e.g., astrocytes are known to induce M2 microglia states upon undergoing astrogliosis [86] and have been proposed to potentially activate protective T-cell responses during *C. neoformans* infection [12, 52, 53].

4.4. Microglial cells phagocytose *C. neoformans* but are not classically activated

The *C. neoformans* strain we used for infecting slices has fluorescent reporters that allows for tracking fungal cells and their interaction with specific cell types in the brain. Our results showed that microglial cells are the main cell that phagocytosed *C. neoformans* cells but contrary to our expectations, they were not classically activated i.e., did not show increased NF-IL6 translocation, nor the release of IL-6 and TNF-α. Previous research also reports little

to no increase of inflammatory gene activation and pro-inflammatory cytokine release in microglia challenged by *C. neoformans* and its capsule [71]. Both acapsular and capsular variants of *C. neoformans* have been reported a failure to elicit TNF-α expression and secretion in BV-2 microglia [71]. The failure to elicit a TNF-α response could possibly be a protective measure by *C. neoformans* as previous research has identified an increased clearance of cryptococcal cells in the presence of higher levels of TNF-α and IFN-γ [87, 88].

CHAPTER FIVE

5. Conclusions

5.1. Conclusion

The burden of CM is a pertinent issue in the developing world especially in SSA where a significant proportion of the population is immunocompromised and vulnerable to opportunistic infections. Effective treatment strategies for CM are inaccessible and unaffordable to most affected individuals and as a result mortality is unacceptably high. Currently, there is very little understanding of the pathogenesis of CM especially in the CNS. In this study we used the organotypic brain slice culture system as a novel model for investigating the neuroimmune response to *C. neoformans* infection. This is the first time that this model has been used for studying host-fungal interactions and here we have demonstrated that this model provides a powerful platform to study cell-type specific antifungal defences at the cellular and molecular levels.

Our findings contribute to an on-going debate concerning the underlying cause for the neuroinflammatory symptoms observed in clinical CM. We have shown that recognition and phagocytosis of *C. neoformans* by resident immune cells of the brain does not on its own elicit an inflammatory response. We have also shown that the *C. neoformans* capsule does not elicit an inflammatory response in microglia. Lastly, this study also explored potential anti-

inflammatory effects of *C. neoformans* and most importantly its purified capsule and we have shown that they do not abolish an existing inflammatory response (induced by LPS).

5.2. Limitations

While mice and humans share a large similarity in terms of their immune system, murine models cannot model human disease completely because host-pathogen interactions are often species-specific. Thus, the current study would be better suited to serve as a precursor to human models. Although the OBSC system is good for studying the innate immune response to cryptococcal infection, it lacks blood supply form the periphery therefore cannot be used to study peripheral recruitment of immune cells as occurs in the clinical form of the disease. However, because CM mainly affects immunocompromised individuals, the contribution of cell-mediated defences is significantly reduced and patients rely on innate responses for anti-cryptococcal defences.

While organotypic cultures give a good understanding of how the innate resident immune cells of the brain respond to *C. neoformans*, the contribution of peripheral immunity to this response is not fully recapitulated by this model. Future studies could compliment this model with *in vivo* studies and this would offer us the ability to study the influence of the peripheral immune system on the CNS response to the fungus.

5.3. Recommendations for future studies

This study has shown the potential of organotypic brain slice cultures in studying neuroimmune interactions during cryptococcal infection and opens up an opportunity for similar studies in other fungal infections that affect the CNS e.g., candidiasis and aspergillosis. While this study focussed mainly on microglia, future studies could explore the interactions of other cell types in the brain e.g., astrocytes, neurons and blood vessels. Learning more about the role of astrocytes in *C. neoformans* infection will be beneficial in understanding the pathogenesis of the disease. Similarly, a better understanding of the interaction of *C. neoformans* with neurons

may help understand the mechanisms underlying the milieu of neurological impairments that CM patients suffer [19, 37]. Finally, this model can be translated to a human brain tissue-based model, allowing us to study a species-specific interaction between human neuroimmune cells and *C. neoformans.*

References

1. Derbie, A., et al., *Cryptococcal antigenemia and its predictors among HIV infected patients in resource limited settings: a systematic review.* BMC Infect Dis, 2020. **20**(1): p. 407.
2. Rajasingham, R., et al., *The global burden of HIV-associated cryptococcal infection in adults in 2020: a modelling analysis.* Lancet Infect Dis, 2022. **22**(12): p. 1748-1755.
3. Dangarembizi, R., S. Wasserman, and J.C. Hoving, *Emerging and re-emerging fungal threats in Africa.* Parasite Immunol, 2023. **45**(2): p. e12953.
4. Rajasingham, R., et al., *Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis.* Lancet Infect Dis, 2017. **17**(8): p. 873-881.
5. Iyer, K.R., et al., *Treatment strategies for cryptococcal infection: challenges, advances and future outlook.* Nat Rev Microbiol, 2021. **19**(7): p. 454-466.
6. Girois, S.B., et al., *Adverse effects of antifungal therapies in invasive fungal infections: review and meta-analysis.* Eur J Clin Microbiol Infect Dis, 2006. **25**(2): p. 138-49.
7. Bicanic, T., et al., *Toxicity of Amphotericin B Deoxycholate-Based Induction Therapy in Patients with HIV-Associated Cryptococcal Meningitis.* Antimicrob Agents Chemother, 2015. **59**(12): p. 7224-31.
8. Rodrigues, M.L., *Funding and Innovation in Diseases of Neglected Populations: The Paradox of Cryptococcal Meningitis.* PLoS Negl Trop Dis, 2016. **10**(3): p. e0004429.
9. Molloy, S.F., et al., *Cryptococcal meningitis: A neglected NTD?* PLoS Negl Trop Dis, 2017. **11**(6): p. e0005575.
10. Bicanic, T. and T.S. Harrison, *Cryptococcal meningitis.* Br Med Bull, 2004. **72**: p. 99-118.
11. Maziarz, E.K. and J.R. Perfect, *Cryptococcosis.* Infect Dis Clin North Am, 2016. **30**(1): p. 179-206.
12. Drummond, R.A., *Neuro-Immune Mechanisms of Anti-Cryptococcal Protection.* J Fungi (Basel), 2017. **4**(1).
13. Liu, T.B., D.S. Perlin, and C. Xue, *Molecular mechanisms of cryptococcal meningitis.* Virulence, 2012. **3**(2): p. 173-81.
14. Chang, Y.C., et al., *Cryptococcal yeast cells invade the central nervous system via transcellular penetration of the blood-brain barrier.* Infect Immun, 2004. **72**(9): p. 4985-95.
15. Chretien, F., et al., *Pathogenesis of cerebral Cryptococcus neoformans infection after fungemia.* J Infect Dis, 2002. **186**(4): p. 522-30.
16. Charlier, C., et al., *Evidence of a role for monocytes in dissemination and brain invasion by Cryptococcus neoformans.* Infect Immun, 2009. **77**(1): p. 120-7.
17. Shi, M., et al., *Real-time imaging of trapping and urease-dependent transmigration of Cryptococcus neoformans in mouse brain.* J Clin Invest, 2010. **120**(5): p. 1683-93.
18. Strickland, A.B. and M. Shi, *Mechanisms of fungal dissemination.* Cell Mol Life Sci, 2021. **78**(7): p. 3219-3238.
19. Dangarembizi, R., *Reimagining the future of African brain health: Perspectives for basic research on the pathogenesis of cryptococcal meningitis.* Brain Behav Immun Health, 2021. **18**: p. 100388.
20. Antinori, S., et al., *AIDS-associated cryptococcosis: a comparison of epidemiology, clinical features and outcome in the pre- and post-HAART eras. Experience of a single centre in Italy.* HIV Med, 2009. **10**(1): p. 6-11.
21. Mukaremera, L., et al., *Titan cell production in Cryptococcus neoformans reshapes the cell wall and capsule composition during infection.* Cell Surf, 2018. **1**: p. 15-24.
22. Okagaki, L.H., et al., *Cryptococcal cell morphology affects host cell interactions and pathogenicity.* PLoS Pathog, 2010. **6**(6): p. e1000953.
23. Zaragoza, O., et al., *Fungal cell gigantism during mammalian infection.* PLoS Pathog, 2010. **6**(6): p. e1000945.
24. Casadevall, A., et al., *The capsule of Cryptococcus neoformans.* Virulence, 2019. **10**(1): p. 822-831.
25. Zaragoza, O., et al., *The capsule of the fungal pathogen Cryptococcus neoformans.* Adv Appl Microbiol, 2009. **68**: p. 133-216.

26. McFadden, D., O. Zaragoza, and A. Casadevall, *The capsular dynamics of Cryptococcus neoformans*. Trends Microbiol, 2006. **14**(11): p. 497-505.
27. Pietrella, D., et al., *Role of mannoprotein in induction and regulation of immunity to Cryptococcus neoformans.* Infect Immun, 2001. **69**(5): p. 2808-14.
28. Caballero Van Dyke, M.C. and F.L. Wormley, Jr., *A Call to Arms: Quest for a Cryptococcal Vaccine.* Trends Microbiol, 2018. **26**(5): p. 436-446.
29. Wiesner, D.L., et al., *Chitin recognition via chitotriosidase promotes pathologic type-2 helper T cell responses to cryptococcal infection.* PLoS Pathog, 2015. **11**(3): p. e1004701.
30. Mukaremera, L. and K. Nielsen, *Adaptive Immunity to Cryptococcus neoformans Infections.* J Fungi (Basel), 2017. **3**(4).
31. Voelz, K. and R.C. May, *Cryptococcal interactions with the host immune system.* Eukaryot Cell, 2010. **9**(6): p. 835-46.
32. Miller, M.F. and T.G. Mitchell, *Killing of Cryptococcus neoformans strains by human neutrophils and monocytes.* Infect Immun, 1991. **59**(1): p. 24-8.
33. Chaturvedi, V., B. Wong, and S.L. Newman, *Oxidative killing of Cryptococcus neoformans by human neutrophils. Evidence that fungal mannitol protects by scavenging reactive oxygen intermediates.* J Immunol, 1996. **156**(10): p. 3836-40.
34. Chen, Y., et al., *Cryptococcus neoformans Infection in the Central Nervous System: The Battle between Host and Pathogen.* J Fungi (Basel), 2022. **8**(10).
35. Ellerbroek, P.M., et al., *Effects of the capsular polysaccharides of Cryptococcus neoformans on phagocyte migration and inflammatory mediators.* Curr Med Chem, 2004. **11**(2): p. 253-66.
36. Barker, R.A. and H. Widner, *Immune problems in central nervous system cell therapy.* NeuroRx, 2004. **1**(4): p. 472-81.
37. Klein, R.S. and C.A. Hunter, *Protective and Pathological Immunity during Central Nervous System Infections.* Immunity, 2017. **46**(6): p. 891-909.
38. Weller, R.O., et al., *Lymphatic drainage of the brain and the pathophysiology of neurological disease.* Acta Neuropathol, 2009. **117**(1): p. 1-14.
39. Aspelund, A., et al., *A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules.* J Exp Med, 2015. **212**(7): p. 991-9.
40. Louveau, A., et al., *Structural and functional features of central nervous system lymphatic vessels.* Nature, 2015. **523**(7560): p. 337-41.
41. de Vries, H.E., et al., *The influence of cytokines on the integrity of the blood-brain barrier in vitro.* J Neuroimmunol, 1996. **64**(1): p. 37-43.
42. Laflamme, N., S. Lacroix, and S. Rivest, *An essential role of interleukin-1beta in mediating NF-kappaB activity and COX-2 transcription in cells of the blood-brain barrier in response to a systemic and localized inflammation but not during endotoxemia.* J Neurosci, 1999. **19**(24): p. 10923-30.
43. Tschen, S.I., et al., *CNS viral infection diverts homing of antibody-secreting cells from lymphoid organs to the CNS.* Eur J Immunol, 2006. **36**(3): p. 603-12.
44. Griffin, D.E., *Immune responses to RNA-virus infections of the CNS.* Nat Rev Immunol, 2003. **3**(6): p. 493-502.
45. Scharton-Kersten, T.M., et al., *Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen Toxoplasma gondii.* J Exp Med, 1997. **185**(7): p. 1261-73.
46. Deckert-Schluter, M., et al., *Crucial role of TNF receptor type 1 (p55), but not of TNF receptor type 2 (p75), in murine toxoplasmosis.* J Immunol, 1998. **160**(7): p. 3427-36.
47. Nansen, A., et al., *Role of interferon-gamma in the pathogenesis of LCMV-induced meningitis: unimpaired leucocyte recruitment, but deficient macrophage activation in interferon-gamma knock-out mice.* J Neuroimmunol, 1998. **86**(2): p. 202-12.
48. Sofroniew, M.V., *Astrocyte barriers to neurotoxic inflammation.* Nat Rev Neurosci, 2015. **16**(5): p. 249-63.
49. Horng, S., et al., *Astrocytic tight junctions control inflammatory CNS lesion pathogenesis.* J Clin Invest, 2017. **127**(8): p. 3136-3151.
50. Liddelow, S.A. and B.A. Barres, *Reactive Astrocytes: Production, Function, and Therapeutic Potential.* Immunity, 2017. **46**(6): p. 957-967.

51. Shinozaki, Y., et al., *Transformation of Astrocytes to a Neuroprotective Phenotype by Microglia via P2Y(1) Receptor Downregulation.* Cell Rep, 2017. **19**(6): p. 1151-1164.
52. Huang, S.H., et al., *Cryptococcus neoformans-derived microvesicles enhance the pathogenesis of fungal brain infection.* PLoS One, 2012. **7**(11): p. e48570.
53. Olave, M.C., et al., *Infective capacity of Cryptococcus neoformans and Cryptococcus gattii in a human astrocytoma cell line.* Mycoses, 2017. **60**(7): p. 447-453.
54. Erny, D., et al., *Host microbiota constantly control maturation and function of microglia in the CNS.* Nat Neurosci, 2015. **18**(7): p. 965-77.
55. Saijo, K. and C.K. Glass, *Microglial cell origin and phenotypes in health and disease.* Nat Rev Immunol, 2011. **11**(11): p. 775-87.
56. Biondo, C., et al., *MyD88 and TLR2, but not TLR4, are required for host defense against Cryptococcus neoformans.* Eur J Immunol, 2005. **35**(3): p. 870-8.
57. Nakamura, K., et al., *Dectin-1 is not required for the host defense to Cryptococcus neoformans.* Microbiol Immunol, 2007. **51**(11): p. 1115-9.
58. Cherry, J.D., J.A. Olschowka, and M.K. O'Banion, *Are "resting" microglia more "m2"?* Front Immunol, 2014. **5**: p. 594.
59. Carta, A.R. and A. Pisanu, *Modulating microglia activity with PPAR-gamma agonists: a promising therapy for Parkinson's disease?* Neurotox Res, 2013. **23**(2): p. 112-23.
60. Choi, S.H., et al., *Inhibition of NADPH oxidase promotes alternative and anti-inflammatory microglial activation during neuroinflammation.* J Neurochem, 2012. **120**(2): p. 292-301.
61. Orihuela, R., C.A. McPherson, and G.J. Harry, *Microglial M1/M2 polarization and metabolic states.* Br J Pharmacol, 2016. **173**(4): p. 649-65.
62. Bonizzi, G. and M. Karin, *The two NF-kappaB activation pathways and their role in innate and adaptive immunity.* Trends Immunol, 2004. **25**(6): p. 280-8.
63. Akira, S., et al., *A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family.* EMBO J, 1990. **9**(6): p. 1897-906.
64. Colonna, M. and O. Butovsky, *Microglia Function in the Central Nervous System During Health and Neurodegeneration.* Annu Rev Immunol, 2017. **35**: p. 441-468.
65. Tang, Y. and W. Le, *Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases.* Mol Neurobiol, 2016. **53**(2): p. 1181-1194.
66. Adami, C., et al., *S100B expression in and effects on microglia.* Glia, 2001. **33**(2): p. 131-42.
67. Trajkovic, V., et al., *Cryptococcus neoformans neutralizes macrophage and astrocyte derived nitric oxide without interfering with inducible nitric oxide synthase induction or catalytic activity - possible involvement of nitric oxide consumption.* Scand J Immunol, 2000. **51**(4): p. 384-91.
68. Woo, Y.H. and L.R. Martinez, *Cryptococcus neoformans-astrocyte interactions: effect on fungal blood brain barrier disruption, brain invasion, and meningitis progression.* Crit Rev Microbiol, 2021. **47**(2): p. 206-223.
69. Campuzano, A. and F.L. Wormley, *Innate Immunity against Cryptococcus, from Recognition to Elimination.* J Fungi (Basel), 2018. **4**(1).
70. Lipovsky, M.M., et al., *Cryptococcal glucuronoxylomannan induces interleukin (IL)-8 production by human microglia but inhibits neutrophil migration toward IL-8.* J Infect Dis, 1998. **177**(1): p. 260-3.
71. Barluzzi, R., et al., *Role of the capsule in microglial cell-Cryptococcus neoformans interaction: impairment of antifungal activity but not of secretory functions.* Med Mycol, 1998. **36**(4): p. 189-97.
72. Pericolini, E., et al., *Cryptococcus neoformans capsular polysaccharide component galactoxylomannan induces apoptosis of human T-cells through activation of caspase-8.* Cell Microbiol, 2006. **8**(2): p. 267-75.
73. Pericolini, E., et al., *Involvement of glycoreceptors in galactoxylomannan-induced T cell death.* J Immunol, 2009. **182**(10): p. 6003-10.
74. Pericolini, E., et al., *Role of CD45 signaling pathway in galactoxylomannan-induced T cell damage.* PLoS One, 2010. **5**(9): p. e12720.
75. Humpel, C., *Organotypic brain slice cultures: A review.* Neuroscience, 2015. **305**: p. 86-98.

76. De Simoni, A. and L.M. Yu, *Preparation of organotypic hippocampal slice cultures: interface method.* Nat Protoc, 2006. **1**(3): p. 1439-45.
77. Wozniak, K.L. and S.M. Levitz, *Isolation and purification of antigenic components of Cryptococcus.* Methods Mol Biol, 2009. **470**: p. 71-83.
78. Sun, Y., et al., *Interleukin-10 inhibits interleukin-1beta production and inflammasome activation of microglia in epileptic seizures.* J Neuroinflammation, 2019. **16**(1): p. 66.
79. Rummel, C., *Inflammatory transcription factors as activation markers and functional readouts in immune-to-brain communication.* Brain Behav Immun, 2016. **54**: p. 1-14.
80. Guldimann, C., et al., *Ruminant organotypic brain-slice cultures as a model for the investigation of CNS listeriosis.* Int J Exp Pathol, 2012. **93**(4): p. 259-68.
81. Welsch, J.C., et al., *Organotypic Brain Cultures: A Framework for Studying CNS Infection by Neurotropic Viruses and Screening Antiviral Drugs.* Bio Protoc, 2017. **7**(22): p. e2605.
82. Hoag, K.A., et al., *Early cytokine production in pulmonary Cryptococcus neoformans infections distinguishes susceptible and resistant mice.* Am J Respir Cell Mol Biol, 1995. **13**(4): p. 487-95.
83. Hoag, K.A., et al., *IL-12 and IFN-gamma are required for initiating the protective Th1 response to pulmonary cryptococcosis in resistant C.B-17 mice.* Am J Respir Cell Mol Biol, 1997. **17**(6): p. 733-9.
84. Eisenman, H.C., A. Casadevall, and E.E. McClelland, *New insights on the pathogenesis of invasive Cryptococcus neoformans infection.* Curr Infect Dis Rep, 2007. **9**(6): p. 457-64.
85. Lyman, M., et al., *Neuroinflammation: the role and consequences.* Neurosci Res, 2014. **79**: p. 1-12.
86. Kim, S. and Y. Son, *Astrocytes Stimulate Microglial Proliferation and M2 Polarization In Vitro through Crosstalk between Astrocytes and Microglia.* Int J Mol Sci, 2021. **22**(16).
87. Redlich, S., et al., *Toll-like receptor stimulation increases phagocytosis of Cryptococcus neoformans by microglial cells.* J Neuroinflammation, 2013. **10**: p. 71.
88. Uicker, W.C., et al., *Cytokine and chemokine expression in the central nervous system associated with protective cell-mediated immunity against Cryptococcus neoformans.* Med Mycol, 2005. **43**(1): p. 27-38.

Appendix A: Ethics clearance

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Animal Ethics Committee

Room G50 Old Main Building
Groote Schuur Hospital
Observatory 7925
Website: www.health.uct.ac.za/fhs/research/animalethics/forms

14 March 2022

Dr Joseph Raimondo
Department of Human Biology
Faculty of Health Sciences
University of Cape Town

Dear Dr Raimondo

PROTOCOL TITLE: ***Harvesting of brain tissue immediately after death from rats and mice for neurological studies***

FHS AEC REF NO: 021_026 PREV 018_010

Thank you for submitting your amended protocol to the Faculty of Health Sciences (FHS) Animal Ethics Committee (AEC) for review.

I am pleased to inform you that the FHS AEC has **authorised** your protocol, which will terminate on **31 March 2025.**

Number of animals & species:

- 520 Mice

Please quote the FHS AEC REF NO (above) in all future correspondence.

Please note that the authorisation of this protocol imposes the following obligations on the principal investigator (PI):

1. To submit an annual mandatory progress report. The first annual report for this protocol is due on **28 February 2023**. The forms can be accessed from http://www.health.uct.ac.za/fhs/research/animalethics/forms
2. To submit a final mandatory report on the **31 March 2025**, please access the final report form from: http://www.health.uct.ac.za/fhs/research/animalethics/forms
3. Ensuring that all study participants perform within the confines of the procedures and experimental design of the protocol as authorised, or as amended.

4. Ensuring that all study participants comply with all applicable national legislation, UCT policies, FHS AEC policies and standard operating procedures (SOPs) and national standards (SANS 10386: 2008).
5. Ensuring compliance with DAFF Section 20 requirements.
6. Ensuring that you as the PI immediately alert the FHS AEC to any event involving the welfare of the animals which has occurred during the course of the study, as well as the actions that were taken to respond to these events.
7. Ensuring that you as the PI alert the FHS AEC to any new or unexpected ethical issues that arose during the course of the study, and how these issues were addressed.
8. Ensuring that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals or will be performing the procedures under the direct and continuous supervision of SAVC-registered veterirary professionals or SAVC-registered para-veterinary professionals.
9. If the PI or any study participant is in any way uncertain how to respond to any of these obligations or deal with any of the issues referred to above, they must consult with FHS AEC.
10. All animals found dead must be reported to the RAF on the appropriate form: http://www.health.uct.ac.za/fhs/research/animalethics/forms
11. All animals found in distress must be reported to the RAF on the appropriate form.

My best wishes for successful research and /or teaching endeavour.

Yours sincerely

Signed by candidate

PROF G. LOUW
CHAIR, FHS AEC

AEC REF# 021_026

Appendix B: Published work and Conference abstracts

1. Second author in a methods paper written by our lab. Citation: Awala, A.N. *et al.* (2023). Mouse Organotypic Brain Slice Cultures: A Novel Model for Studying Neuroimmune Responses to Cryptococcal Brain Infections. In: Drummond, R.A. (eds) Antifungal Immunity. Methods in Molecular Biology, vol 2667. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-3199-7_3
2. Presented "The neuroimmune response to *Cryptococcus neoformans* capsule fractions" at the UCT HUB research day and SANS 2022 symposium: Cryptococcal meningitis (CM) is a fatal fungal infection of the brain that kills roughly 181 000 immunocompromised people per year in Sub-Saharan Africa (SSA). With between 70-100% of CM patients succumbing to infection, it is surprising how very little is known about the pathogenesis of CM, particularly the interaction of the fungus with the resident immune cells of the brain. Therefore, the aim of this study is to identify neuroimmune responses to the cryptococcal capsule (CCap) which is the major cell surface component of the fungal cell wall and its main virulent factor. To investigate this, we stimulated organotypic brain slices (OBSs) with a low (0.1 mg/ml), medium (1 mg/ml) and high (10 mg/ml) dose of a purified extract of the cryptococcal capsule for 24 hours. In addition, we had a positive control of 10 ng/ml Lipopolysaccharide (LPS) a strong inflammatory causing agent. We used immunofluorescence staining to identify activated microglial cells by tracking the activation of the nuclear factor for interleukin 6 (NF-IL6). In addition, we performed enzyme-linked immunosorbent assays to measure the levels of IL6 and tumor necrosis factor (TNF) released by the treated slices. The stained slices were imaged using confocal microscopy and image analysis was performed in ImageJ. Data analysis was done in GraphPad Prism. Our data shows that, at all three doses, the CCap does not activate inflammatory signaling in microglial cells. In contrast, we observed a robust activation of microglial cells and the release of TNF and IL6 from slices treated with LPS.