Cryptococcus gattii isolates induce less protective inflammation than *Cryptococcus neoformans* in a murine model of infection.

by

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ABSTRACT

The fungal pathogen *Cryptococcus neoformans* variety *grubii* is an opportunistic pathogen of immunocompromised people while the related species, *Cryptococcus gattii,* appears to infect people regardless of their immune status. The objective of this study was to investigate the differences between *C. neoformans* and *C. gattii* infections in a mouse model of cryptococcosis in order to better understand why *C. gattii* is able to cause disease in immunocompetent hosts. Normally, protective inflammation mediated by neutrophils and an adaptive Th1-type immune response is required for clearance of *C. neoformans* infections, whereas a Th2-type immune response is inefficient. Furthermore, neutrophils act as important first-responders in innate immunity by initiating the adaptive Th1 immune response during the early stage of infection. We hypothesized that while *C. neoformans* infections are cleared because they elicit strong protective inflammatory immune responses, *C. gattii* infections persist because they do not, thus enabling this species to cause disease in immunocompetent hosts. The results support the hypothesis because we found that *C. gattii* infections induce less protective inflammation than *C. neoformans* infections with respect to leukocyte recruitment to the sites of infection and cytokine induction. Mice infected with the *C. gattii* strains tested had less neutrophil migration into their lungs and had a reduced protective cytokine profile, suggesting that *C. gattii* strains may be able to skew the immune response towards a less efficient response, but not necessarily a Th2 allergic immune response. However, we also observed that the *C. gattii* strains tested varied in virulence, indicating that their ability to limit protective inflammation is not the only factor involved in their pathogenicity.

Overall, these results provide important new insights into the virulence of *Cryptococcus* species; this information may be useful in understanding the outbreak of *C. gattii* infections that is occurring in British Columbia.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
APC	Allophycocyanin
CFU	Colony Forming Unit
DC	Dendritic Cell
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GalXM	Galactoxylomannan
GXM	Glucuronoxylmannan
HIV	Human Immunodeficiency Virus
H&E	Hematoxylin and Eosin
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MCP	Monocyte chemotactic protein
MM	Mayer's Mucicarmine
MPO	Myeloperoxidase
PBS	Phosphate Buffered Saline
SDB	Sabouraud Dextrose Broth
TNF	Tumor Necrosis Factor

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1. INTRODUCTION

1.1 Pathogenic Cryptococcus species: Epidemiology, geographic distribution, and route of infection

Cryptococcus neoformans is an opportunistic fungal pathogen that is capable of infecting humans, domestic animals (e.g., cats and dogs) and wild animals such as marine mammals. In addition, *C. neoformans* is a dimorphic fungus that exhibits a budding, yeast-like morphology during infection and a filamentous morphology during sexual development (Casadevall and Perfect, 1998; Idnurm, *et al.,* 2005; Wickes, *et al.,* 1996). In the environment, the fungus is found in trees, soil and avian excreta where it exists as spores (1.8–2 mm) or desiccated yeast cells (Casadevall and Perfect, 1998; Idnurm, *et al.,* 2005; Wickes, *et al.,* 1996). Inhalation of these cells is the primary means by which infection is initiated, and the fungus can spread throughout the body to cause systemic cryptococcosis. In many cases, the fungus can cross the blood-brain barrier to cause cryptococcal meningitis (Bicanic and Harrison, 2005). This life-threatening disease is difficult to treat and is generally fatal in the absence of antifungal drug therapy. As discussed later, cryptococcosis has become an important disease in the last 30 years because the AIDS epidemic has created a large population of susceptible people.

Isolates of the fungus have traditionally been classified into the varieties *grubii* (serotype A), *neoformans* (serotype D) and *gattii* (serotypes B and C) based on biochemical and molecular markers, and based on antigenic differences in the polysaccharide capsule that is the major virulence factor (Buchanan and Murphy, 1998; Casadevall and Perfect, 1998; Kwon-Chung, *et al.,* 2002; Kwon-Chung and Rhodes,

1986; Meyer, *et al.,* 1999). The classification of *C. neoformans* changed recently in light of new molecular typing data, and *C. neoformans* variety *gattii* is now considered to be a different species rather than a variety of *C. neoformans* (Kwon-Chung, *et al.,* 2002). The work described in this thesis focuses on *C. neoformans* var. *grubii* (serotype A), which infects people with compromised immune systems, and *C. gattii* (serotype B), which infects people regardless of their immune status (Jarvis and Harrison, 2008; Sorrell, 2001a). *C. neoformans* var. *neoformans* (serotype D) also causes infections in immunocompromised patients, but this variety is less prevalent than *C. neoformans* serotype A (Banerjee, *et al.,* 2004; Chen, *et al.,* 2008). Isolates of the *C. gattii* C serotype rarely cause human infections (Escandon, *et al.,* 2006; Lizarazo, *et al.,* 2007). For the purposes of this thesis, *C. neoformans* var. *grubii* (serotype A) will be referred to as *C. neoformans* and *C. gattii* strains of serotype B will be referred to as *C. gattii*.

The epidemiology is different for the *neoformans* and *gattii* species of Cryptococcus. As mentioned earlier, *C. neoformans* is a prominent cause of fungal meningitis as a result of the AIDS epidemic and the fungus is now identified as an AIDS-associated pathogen (Bicanic and Harrison, 2005; Casadevall and Perfect, 1998). The introduction of highly active antiretroviral therapy (HAART) has decreased the number of cases of AIDS-associated *C. neoformans* infections in developed countries, but the pathogen remains a serious problem in other parts of the world. For example, the incidence of cryptococcosis is reported to be as high as 30% in the 25.8 million people with HIV/AIDS in sub-Saharan Africa (www.unaids.org; Bicanic and Harrison, 2005; Casadevall and Perfect, 1998). *C. gattii* typically infects healthy hosts and has a lower incidence in AIDS patients compared to *C. neoformans* (Sorrell, 2001a). *C. gattii*

infections were traditionally only found in tropical and sub-tropical regions of the world such as South America and Australia (MacDougall, *et al.,* 2007). However, since 1999, an outbreak of *C. gattii* infections has been occurring in the temperate climate of Vancouver Island in British Columbia. This outbreak has resulted in at least 200 human cases and eight deaths (Bartlett, *et al.,* 2008; Fyfe, *et al.,* 2008). More recently, cases of *C. gattii* have occurred on the mainland in British Columbia as well as in the states of Washington and Oregon (Byrnes, *et al.,* 2009; Upton, *et al.,* 2007).

1.2 *C. neoformans* and *C. gattii*: virulence factors

C. neoformans and *C. gattii* share all of the major virulence factors, and these include production of a polysaccharide capsule, formation of the pigment melanin in the cell wall, secretion of the enzyme urease, and the unusual ability (among fungi) to grow at 37^{o}C. Much of the research on these virulence factors was performed with *C. neoformans* and less is known about them with respect to *C. gattii*.

The Cryptococcus capsule is the most distinctive feature of the fungus and it is the only human fungal pathogen that is encapsulated (McFadden, *et al.,* 2006). The capsule is composed primarily of glucuronoxylomannan (GXM: 90%) and galactoxylomannan (GalXM: 10%) (Idnurm, *et al.,* 2005). A variety of exported mannoproteins are also associated with the capsule. During infection, a high level of capsule polysaccharide accumulates in the serum and cerebral spinal fluid (CSF) of patients (Casadevall and Perfect, 1998). This accumulation is thought to contribute to neurological symptoms in severely infected people. The capsule of *C. neoformans* is also known to have several immunomodulatory properties, and many of these properties have been attributed to

GXM. For example, GXM has been shown to suppress secretion of pro-inflammatory cytokines from human monocytes (Vecchiarelli, *et al.,* 1995). Furthermore, it has also been shown to suppress T cell proliferation, inhibit dendritic cell activation and maturation, and reduce neutrophil and macrophage killing (Lendvai, *et al.,* 1998; Monari, *et al.,* 2003; Syme, *et al.,* 1999; Vecchiarelli, *et al.,* 1995; Vecchiarelli, *et al.,* 2003). In addition, capsule production within phagocytic cells may also contribute to pathogen survival through toxic effects on the host cells and by aiding fungal escape (Feldmesser, *et al*., 2000; Tucker *et al*., 2002). In this regard, *C. neoformans* is believed to be a facultative intracellular pathogen during at least some stages of infection (Feldmesser, *et al.,* 2000).

Other major cryptococcal virulence factors with immunomodulatory properties include the pigment melanin and the enzyme urease. Both of these virulence factors appear to be involved in inducing ineffective immune responses against Cryptococcus infection. It has been shown, for example, that melanin production is associated with induction of Th2 cytokines such as IL-4 (Mednick, *et al.,* 2005). Furthermore, melanized Cryptococcus cells are more virulent and less prone to phagocytosis compared to non-melanized strains (Casadevall, *et al.,* 2000; Noverr, *et al.,* 2004). Melanized cells also appear to be able to disseminate from the lung more readily than non-melanized cells (Noverr, *et al.,* 2004). The enzyme urease catalyzes the hydrolysis of urea to ammonia and carbamate. In Cryptococcus species, urease-negative strains are significantly less virulent than the wild-type strains (Cox, *et al.,* 2000). Furthermore, this enzyme also appears to have immunomodulatory properties, as evidenced by recent studies showing urease production in Cryptococcus to be associated with highly polarized Th2 immune

responses consisting of high levels of pulmonary eosinophils, immature dendritic cells and high serum IgE and Th2 cytokine levels (Olszewski, *et al.,* 2004; Osterholzer, *et al.,* 2009).

C. gattii and *C. neoformans* share all of the major virulence factors but differ in a number of traits (Idnurm et al., 2005; Lin and Heitman, 2006; Sorrell, 2001). For example, *C. gattii* but not *C. neoformans* can assimilate D-proline, D-tryptophan and L-malic acid, and use creatinine and glycine as nitrogen sources. Furthermore, in a recent study, isolates of *C. neoformans* were shown to be more susceptible to the anti-fungal agents fluconazole and voriconazole compared to *C. gattii* isolates (Khan, *et al.,* 2009). Additionally, *C. gattii* infections appear to be associated with a higher incidence of granulomas in the lungs and brain (cryptococcomas), with increased neurological morbidity and with a slower response to antifungal therapy (Sorrell, 2001b).

1.3 The immune response against *C. neoformans* and *C. gattii*

The mechanisms of the immune response to the Cryptococcal species have been best established for infections caused by *C. neoformans* (using strains of both the A and D serotypes). Little research has been done on the immune response to *C. gattii* infections; thus, the information on the immune response to *C. neoformans* provides the foundation for our investigation of the immune response to infections caused by *C. gattii*. Normally, most of the fungal spores or yeast cells that are inhaled do not even reach the lungs and are cleared due to air turbulence and ciliary action (Shoham and Levitz, 2005). The small numbers of Cryptococcus spores or yeast cells that do reach the lung parenchyma are cleared by a protective inflammatory immune response involving the

production of cytokines such as TNF-α and IFN-γ, and a leukocyte infiltrate consisting of neutrophils, Th1-associated classically activated macrophages, Th1-lymphocytes and dendritic cells (Chen, *et al.,* 2008; Guillot, *et al.,* 2008; Lin and Heitman, 2006; Koguchi and Kawakami, 2002). Furthermore, the timing of the cellular infiltrate is important in determining the outcome of infection, for instance, early neutrophilia has been associated with protective immune responses against *C. neoformans* infection (Guillot, *et al.,* 2008). In contrast, a non-protective response involves the production of high levels of the Th2 cytokine IL-4 as well as a diffuse pulmonary infiltrate consisting of immature dendritic cells and alternatively activated macrophages (Chen, *et al.,* 2008; Guillot, *et al.,* 2008; Koguchi and Kawakami, 2002; Vecchiarelli *et al*., 2003). The deleterious effects of a Th2 type immune response against Cryptococcus infection was illustrated in numerous studies involving different mouse models. For instance, IL-13-overexpressing transgenic mice have a reduced survival time and higher pulmonary fungal load upon *C. neoformans* infection compared to IL-13 knockout or wild-type mice (Muller, *et al.,* 2007). Furthermore, IL-4 knockout mice infected with *C. neoformans* survive longer than wild-type mice and display enhanced pulmonary clearance of the fungus (Blackstock and Murphy, 2004b; Decken, *et al.,* 1998; Hernandez, *et al.,* 2005).

Macrophages and dendritic cells (DCs) play important roles during the initial phase of infection with *C. neoformans*. These cells infiltrate the lung during infection, act as antigen-presenting cells in the lymph nodes, phagocytose the fungus and produce cytokines such as IL-1 and TNF-α that activate CD4+ T cells (Huffnagle *et al.,* 2000; Wozniak, 2006). These CD4+ T cells then initiate an adaptive Th1 type immune response, involving the release of cytokines such as IFN-γ, which activates macrophages

and induces further inflammation. Cryptococci inside these activated macrophages are then killed by lysosomal fusion and enzymatic degradation of fungal components (Shoham and Levitz, 2005). Activated macrophages also release reactive oxygen species, nitric oxide and antifungal peptides that kill extracellular cryptococci (Shoham and Levitz, 2005). Furthermore, activated CD4+ and CD8+ T cells can bind to the cryptococcal cell surface and inhibit replication. Natural killer cells also have a role in killing; they bind to the fungus and release cytolytic compounds and produce additional IFN-γ for the activation of macrophages (Blackstock and Murphy, 2004; Shoham and Levitz, 2005).

Protective inflammation, involving neutrophils is crucial for induction of adaptive Th1 immune responses against cryptococcus infection (Aratani, *et al.,* 2006; Chaturvedi, *et al.,* 1996; Craig *et al*., 2009). The importance of neutrophil involvement was shown in a recent study that found that mice resistant to *C. neoformans* infection exhibited significantly higher neutrophilia compared to mice that were susceptible; this neutrophil response was also accompanied by elevated levels of TNF-α (Guillot, *et al.,* 2008). Additionally, mice deficient in myeloperoxidase, a neutrophil-specific enzyme, produce weak Th1 immune responses and have a significantly reduced survival time upon *C. neoformans* infection compared to wild-type mice (Aratani, *et al.,* 2006). Furthermore, a recent case study showed that an apparently immunocompetent cryptococcus-positive patient actually had defects in neutrophil function, suggesting that neutrophils play an important role in preventing cryptococcosis (Marroni, *et al.,* 2007). Neutrophils are a crucial part of innate immunity against Cryptococcus infection because they induce early production of TNF-α , IFN-γ and important chemokines, which in turn are critical for

development of an effective adaptive immune response for clearance of the fungus (Shoham and Levitz, 2005). The importance of early production of TNF-α was illustrated in a recent study which showed that using an anti-TNF-α antibody to transiently decrease TNF-α levels during the initial phase of infection could permanently impair the ability of the immune system to clear cryptococcal infection in the lungs of infected mice (Herring, *et al.,* 2005).

Although little research has been done on the host immune response to *C. gattii* infections, there is some evidence that *C. gattii* infections induce less protective immune responses than *C. neoformans* infections. For example, a study by Dong and Murphy (1995) showed that culture filtrate antigens from *C. gattii* strains can inhibit neutrophil migration *in vitro* and *in vivo*, whereas culture filtrate antigens from *C. neoformans* stimulate neutrophil migration. Dong and Murphy proposed that *C. gattii* strains are able to cause disease in immunocompetent people because they inhibit neutrophil migration into the lungs during the initial stage of infection. A more recent study by Wright *et al*. (2002) found that even though a *C. gattii* strain was able to inhibit neutrophil migration *in vitro*, neutrophil infiltration into the lungs of infected rats was similar in *C. neoformans* and *C. gattii* infections (Wright, *et al.,* 2002). Furthermore, Wright *et al*. (2002) observed that culture supernatants from a *C. gattii* strain inhibited neutrophil migration through a monolayer of epithelial cells whereas those from *C. neoformans* did not. Additionally, neutrophils incubated with culture supernatants from a *C. gattii* strain displayed significantly decreased superoxide production compared to neutrophils incubated with culture supernatants from a *C. neoformans* strain (Wright, *et al.,* 2002). Thus, Wright *et al*. (2002) suggested that *C. gattii* infections may not be cleared

efficiently by immunocompetent hosts because they do not induce protective inflammation during infection and as a result are not able to elicit adaptive Th1 type immune responses compared to infections with *C. neoformans*. However, this idea has not been explored beyond the differential *C. neoformans* and *C. gattii* effects on neutrophil function.

1.4 Thesis Objectives

The objective of this study was to investigate potential differences in the immune responses to *C. neoformans* and *C. gattii* infections in a mouse model of cryptococcosis. This analysis is a first step toward understanding the ability of *C. gattii* strains to cause disease in immunocompetent hosts. One hypothesis is that *C. gattii* infections do not produce a protective inflammatory immune response and subsequent Th1 immunity and therefore is able to persist to cause disease. For our studies, we used the following strains: WM276, which is a *C. gattii* isolate of the VGI genotype commonly found in Australia and occasionally on Vancouver Island; R265, which is a clinical *C. gattii* isolate of the VGIIa genotype that infected and killed an immunocompetent person on Vancouver Island in 2001; R272 of the VGIIb genotype, which is a less virulent strain of *C. gattii* that was also obtained as a clinical isolate from a patient on Vancouver Island (Fraser *et al*. 2005); and H99, a representative of the *C. neoformans* var. *grubii* strains that commonly infect immunocompromised patients.

Our first objective was to examine the virulence-associated phenotypes of the *C. gattii* strains versus the commonly studied *C. neoformans* strain. Thus, we evaluated the ability of the strains to produce the four major virulence factors that are necessary for

both Cryptococcus species to cause highly lethal disease in animal models. The roles of these factors are demonstrated by the findings that acapsular and temperature sensitive mutants are non-pathogenic while melanin-negative or urease-negative mutants are attenuated in their ability to cause disease (Casadevall, *et al.,* 2000; Cox, *et al.,* 2000; Kwon-Chung and Rhodes, 1986; Wormley, *et al.,* 2005). We also anticipated that if there were differences in the virulence-associated phenotypes in our strains, there might also be differences in their ability to cause disease in an animal host. Thus, our second objective was to examine the virulence of our *C. neoformans* and *C. gattii* strains in a mouse model of cryptococcosis.

Our third and main objective was to determine if there were differences in the immune responses to the *C. neoformans* and *C. gattii* strains. Based on the studies by Dong and Murphy (1995) and Wright *et al*. (2002) described above, there is evidence that *C. gattii* strains may affect neutrophil function and that *C. gattii* infections induce less neutrophil-mediated inflammation than *C. neoformans* infections. Protective immune responses against *C. neoformans* involve early neutrophilia and elevated levels of TNF-α (Guillot, *et al.,* 2008). Other researchers speculated that because neutrophils are important in the early innate immune response to cryptococcal infection, inhibition of neutrophil migration into infected lung tissues might delay the production of TNF-α IFN-γ and important chemokines, thus preventing the induction of an effective Th1 adaptive immune response (Shoham and Levitz, 2005). Given that *C. gattii* can inhibit neutrophil migration *in vitro*, this may indeed be how they are able to infect and cause disease in immunocompetent hosts.

To test our hypothesis, including the idea that *C. gattii* infections persist in

immunocompetent hosts because they elicit an inefficient immune response, we infected mice with *C. neoformans* and *C. gattii* isolates and examined their immune response at specific time points. Specifically, we analyzed the cytokine profiles and pulmonary infiltrates of dendritic cells, macrophages and neutrophils in the infected mice.

In a related project, we also assessed the ability of our isolates of interest to survive during interactions with a macrophage-like cell line in culture (Appendix A). The interaction with macrophages is of interest because, as mentioned earlier, these cells play an important role in phagocytosis and killing of cryptococcal cells. Specifically, we tested our *C. neoformans* and *C. gattii* strains for their ability to survive or grow within macrophages during co-culture.

To date, this is the first study to examine differences in the immune response using *C. gattii* strains from the Vancouver Island outbreak. In support of our main hypothesis, we found that *C. gattii* infections induced less protective immune responses compared to *C. neoformans* infections with respect to leukocyte recruitment to the sites of infection and induction of cytokines. Furthermore, mice infected with *C. gattii* strains had reduced neutrophil infiltration suggesting that *C. gattii* infections may be able to skew the immune response towards a less protective response that is inefficient at clearing the infection.

2. MATERIALS AND METHODS

2.1 Mice

For all studies except histopathology studies, female C57BL/6 mice were obtained from Charles River (Montreal, Quebec, Canada). For histopathology studies, female A/JCr mice were obtained from the NIH animal program (Frederick, Maryland, USA). The mice were 12-14 weeks of age at the time of infection and they were housed under specific-pathogen-free conditions using sterilized cages with a microisolator cage top. Clean food and water were given *ad libitum*. The mice were maintained by the Wesbrook Animal Unit at the University of British Columbia in accordance with the methods and regulations approved by the University of British Columbia's Animal Care Committee.

2.2 Strains of *C. neoformans* and *C. gattii*

The *C. neoformans* H99 strain and the *C. gattii* strains R265, R272 and WM276 were used in this study. Table 2.1 provides the details for all Cryptococcus strains used in this study. R265 and R272 were both obtained in 2001 from the bronchial washings of infected patients from the Vancouver Island outbreak of cryptococcosis. The *C. gattii* strain WM276 is an Australian environmental isolate obtained from Dr. Joseph Heitman (Duke University). The *lac1lac2* double mutant and the *ure1* mutant were both derived from the H99 strain.

Table 2.1 Strains of Cryptococcus used in this study

Strain	Relevant Characteristics	Source
C. neoformans var. *grubbii* (serotype A) H99	Wild type	Clinical isolate, Dr. Joseph Heitman, Duke University
C. gattii (serotype B) R265	Wild type	Clinical isolate (Kidd, *et al.,* 2004)
C. gattii (serotype B) R272	Wild type	Clinical isolate (Kidd, *et al.,* 2004)
C. gattii (serotype B) WM276	Wild type	Environmental isolate, Dr. Joseph Heitman, Duke University
C. neoformans var. *grubii* (serotype A) H99 *lac1lac2*	Inability to produce laccase activity that is required for melanin synthesis.	(Williamson, 1994)
C. neoformans var. *grubii* (serotype A) H99 *ure1*	Inability to produce urease.	(Cox, *et al.,* 2000)

2.3 Intranasal inoculation

Cryptococcus strains were grown for 24 hours in Sabouraud dextrose broth (SDB) and washed three times with phosphate-buffered saline (PBS), counted in a hemocytometer and resuspended in PBS at a concentration of 1.0×10^6 yeast cells/mL. The intranasal inoculation method has been described elsewhere (Hu, *et al.,* 2008; Jung, *et al.,* 2008). In brief, mice were anesthetized with 82.25 mg/kg ketamine and 5.5mg/kg xylazine by intraperitoneal injection. Anesthetized mice were then suspended by their incisors on a thread to fully extend their necks and 50uL of the yeast suspension (5×10^4 cells) was slowly pipetted into the nares of each mouse. The mice were suspended for an additional 10 minutes and then placed on a heated blanket for recovery. For the virulence assay, mice were euthanized using CO_2 inhalation if they appeared to be in pain or had 20% weight loss (humane endpoint). For all other assays, mice were euthanized on the indicated days using CO_2 inhalation.

2.4 Lung leukocyte isolation

This procedure has been described elsewhere (Milam, *et al.,* 2007). Briefly, whole lungs were excised, minced and enzymatically digested for 1 hour in 10 mL of digestion buffer (RPMI medium, 5% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mg/mL collagenase IV, 30 ug/mL DNase). Cell suspensions were further homogenized by drawing them through the bore of an 18-guage needle attached to a 3 mL syringe. The total cell suspensions were then pelleted and washed with PBS before resuspension in 3 mL of red blood cell lysis buffer (0.9% NH_4Cl in H_2O) for 5 mins. Subsequently, 10 mL of complete medium (RPMI medium, 10% FBS, 2 mM L-

glutamine, 1 mM sodium pyruvate and antibiotics) was added to the suspension to return the solution to isotonicity. The cell suspension was then strained through a 70 μm filter before being pelleted and resuspended in complete medium. Total lung leukocytes were enumerated in the presence of trypan blue using a hemocytometer.

2.5 Neutrophil counting

Leukocyte suspensions were normalized for cell concentration, cytospun onto glass slides and stained using the Hemacolour stain set (Harelco, EMD Chemicals, Gibbstown, NJ, USA). A total of 200 neutrophils from randomly chosen micrographs were visually counted.

2.6 Cytokine assays

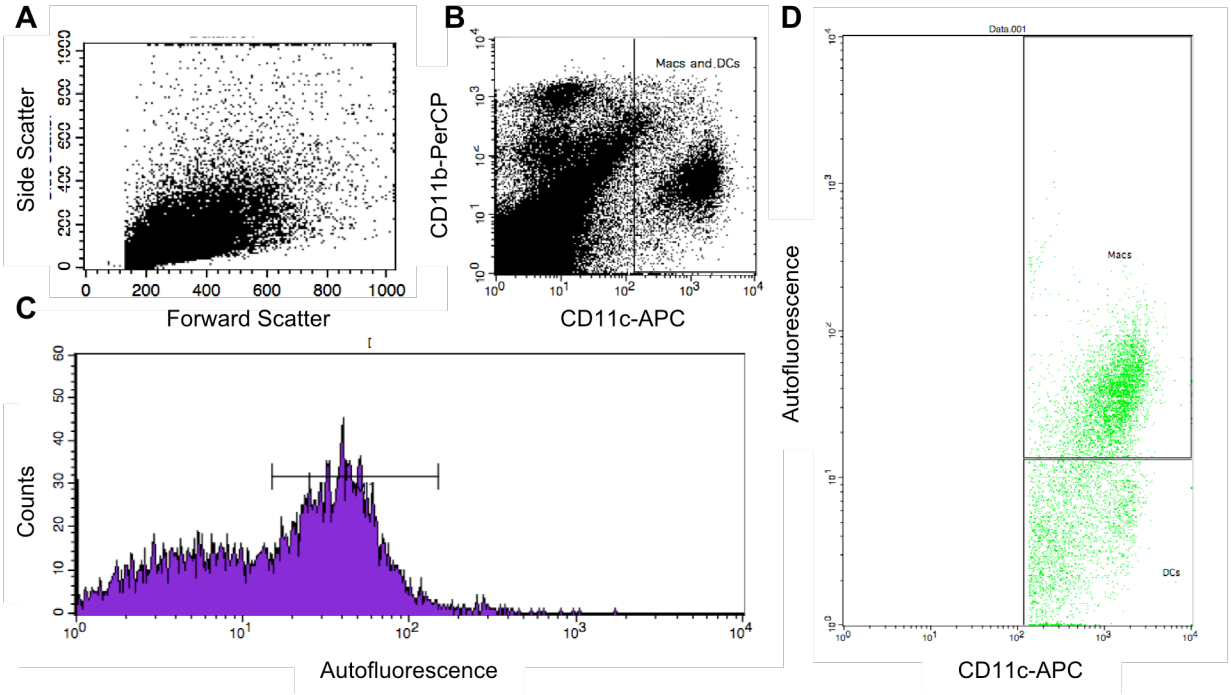
Lung tissue was excised, weighed and homogenized in 5 mL of PBS using a Mixer Mill (MM200, Retsch, Haan, Germany). The tissue homogenates were then clarified by centrifugation and aliquots of the supernatant were stored at -80°C. Cytokine analysis on the undiluted supernatant was performed on a BD FACSCalibur flow cytometer using the BD Cytometric Bead Array (CBA) Mouse Inflammation Kit (Becton Dickinson, San Jose, CA) according to the manufacturer's instructions. The data were analyzed using BD CellQuest, BD CBA software (Becton Dickinson, San Jose, CA) and FlowJo software (Tree Star Inc., San Carlos, CA).

2.7 Antibody staining and flow cytometric analysis

For flow cytometry, 1.0x 10^6 cells from the lung leukocyte suspension were pipetted into wells of a 96-well round bottom plate for staining. Each sample was assayed in duplicate. Cells were washed with 200 uL of buffer (Hanks balanced salt solution (HBSS) + 20 mM Hepes + 0.2% sodium azide + 2% FBS), pelleted and stained in 30 μL of buffer mixed with the following antibodies: fluorescein isothiocyanate (FITC)-labelled anti-Gr.1, PerCP-labelled anti-CD11b and allophycocyanin (APC)-labelled anti-CD11c. Staining was performed on ice for 20 mins, cells were then washed with the buffer, pelleted, resuspended in 0.5% paraformaldehyde in PBS and analyzed on a BD FACSCalibur flow cytometer. A total of 100,000 events were collected per sample. Initial gates were set based on light scatter characteristics to exclude debris, red blood cells and clusters of cells (Figure 2.1 A). Neutrophils were gated based on side-scatter (as a measure for granularity) as well as expression of Gr.1 and CD11b. Pulmonary macrophages are highly autofluorescent, express very low levels of F4/80 and high levels of CD11c and have varying expression of CD11b depending on their activation status (Gonzalez-Juarrero, *et al.,* 2003; Vermaelen and Pauwels, 2004). Dendritic cells (DCs) express high levels of CD11b and CD11c and can be differentiated from macrophages by their low autofluorescence (Gonzalez-Juarrero, *et al.,* 2003; Vermaelen and Pauwels, 2004). Therefore, to differentiate between macrophages and DCs, all cells that were $CD11c^{positive}$ with varying expression of CD11b were gated (Figure 2.1 B) from the total leukocyte population. These $CD11c^{positive}$ cells were then used to draw a histogram showing the autofluorescent populations so that the threshold between $autofluorescence^{negative}$ and $autofluorescence^{positive}$ cells could be determined (Figure 2.1

C). This threshold was used to draw a gate to differentiate between the population of CD11c^{positive} cells that were macrophages or dendritic cells (Figure 2.1 D). All fluorescence activated cell sorting (FACS) data were analyzed using BD Cellquest Pro software (Becton Dickinson, San Jose, CA). The anti-Gr.1 antibody was purchased from Caltag (Cedarlane Laboratories, Burlington, ON, Canada), all other antibodies were purchased from Biolegend (Biolegend Inc., San Diego, CA).

Figure 2.1 Flow cytometry analysis. Total lung leukocytes (A) were examined for dendritic cells (DCs) and macrophages by first gating on all cells that were CD11c^{positive} (B). These CD11c^{positive} cells were then used to draw a histogram showing the autofluorescent populations so that the threshold between autofluorescence^{negative} and autofluorescence^{positive} cells could be determined (C). This threshold was used to draw a gate to differentiate between the population of CD11c^{positive} cells that were macrophages or dendritic cells (D).



2.8 Measurement of myeloperoxidase

Lung tissue was excised, weighed, snap frozen in liquid nitrogen and stored at -80°C until being assayed. This method has been described elsewhere (Webert, *et al.,* 2000). Briefly, frozen tissue was homogenized in hexadecyltrimethylammonium bromide (HTAB) buffer (10mL 50mM KH_2PO_4 pH 6.0, 5 mM EDTA 0.5% HTAB) at 1 mL per 50 mg tissue using a Mixer Mill (MM200, Retsch, Haan, Germany). The tissue homogenates were then clarified by centrifugation and 50 uL of the supernatant was mixed with 1.45 mL of freshly prepared assay buffer (100 mM KH_2PO_4 pH 6.0, 0.005% H_2O_2, 0.005g O-dianisideine dihydroschloride). The change in absorbance at 450 nm was measured every minute for 4 minutes in a spectrophotometer. The results are expressed in units of MPO per gram of tissue (wet weight) where 1 unit of MPO activity is defined as that degrading 1 umol peroxide per min at 25°C (Webert, *et al.,* 2000).

2.9 Phenotyping assays

To examine the production of the main cryptococcal virulence factors, Cryptococcus cells were grown in SDB for 24 hours at 30°C in a shaker, washed twice with PBS and adjusted to a concentration of 2.0×10^8 cells/mL. The cell suspensions were then diluted 10-fold serially and 5 μl of each dilution was spotted onto Sabouraud dextrose agar plates, L-DOPA plates (0.5 mM 3,4-hydroxyl-L-phenylalanine (L-DOPA), 1mM $MgSO_4$ $7H_2O$, 22mM KH_2PO_4, 3 μM thiamine-HCl, 0.1% glucose, 0.1% L-asparagine, pH5.6), or urease plates (333 mM urea, 86 mM NaCl, 15 mM KH_2PO_4, 0.1% peptone, 0.1% glucose, 0.0012% phenol red) to assess growth at 37°C, melanin production and urease production respectively. All incubations were performed at 30°C

unless otherwise specified. To examine capsule formation, strains were grown in low-iron medium for 48 hours at 30°C in a shaker, washed with low-iron water, stained with india ink and examined by differential interference microscopy (DIC).

2.10 Nitric oxide suppression assays

Cryptococcus strains were grown for 24 hours in SDB and washed three times with PBS, counted in a hemocytometer and resuspended in PBS at a concentration of 1.0 × 10^6 yeast cells/mL. RAW macrophages were seeded at 2.0 x 10^5 cells per well in 24-well plates in complete media (DMEM medium, 10% FBS, 4 mM L-glutamine and 100 U/mL penicillin, 100 μg/mL streptomycin) supplemented with 0.1 ug/mL IFN-γ and 1 ug/mL LPS. To initiate the interaction, 100 uL of the fungal cell suspension or PBS (as a control) was then added to each well and plates were incubated at 37°C in an atmosphere of 5% CO_2 for 24 hours. After incubation, supernatants were tested for nitrite concentration using the Greiss assay (Stuehr and Nathan, 1989).

2.11 Histology

A/JCr mice were used for histology experiments. At 2 and 7 days post-infection, lungs were fixed in 10% neutral buffered formalin. The tissue was then embedded in paraffin and cut into 5 μm thick sections, stained with hematoxylin and eosin (H&E) or Mayer's mucicarmine (MM) to visualize cryptococcal capsule and then fixed on slides. Slides were examined by light microscopy.

2.12 Statistics

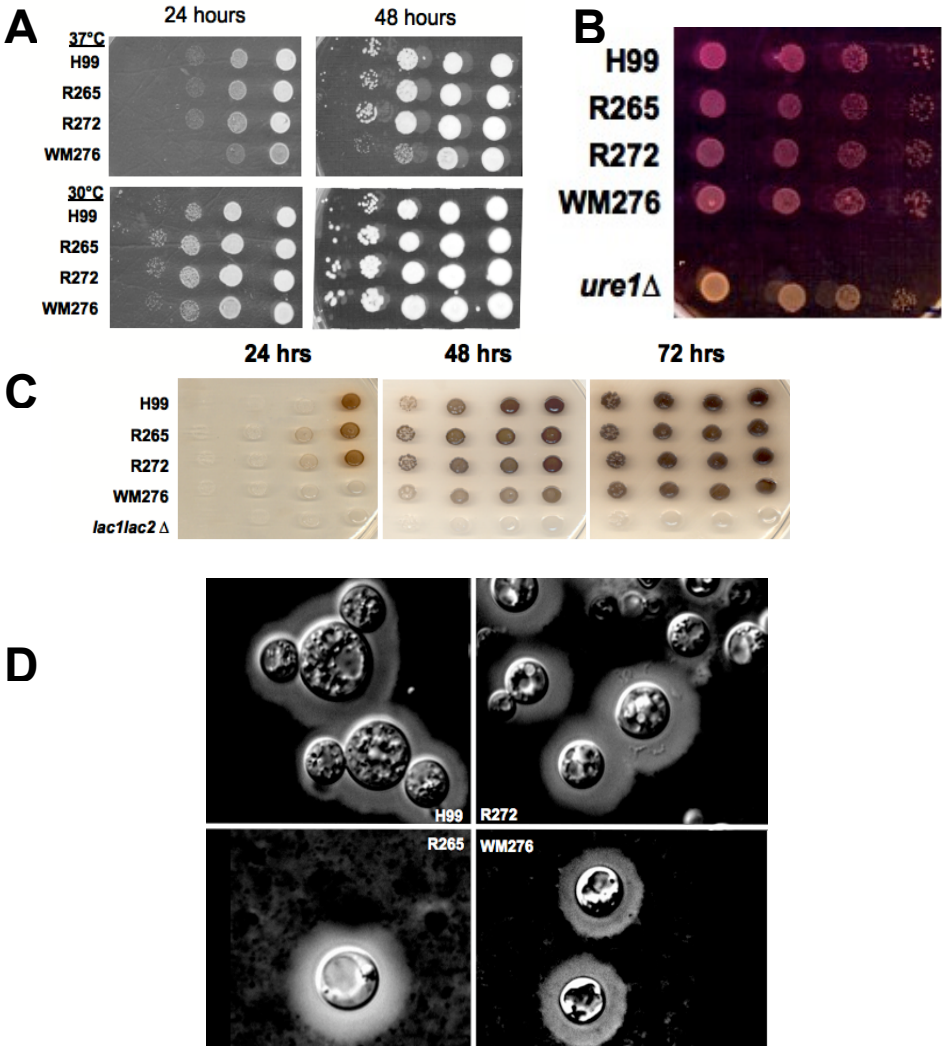
For the virulence assay, the time to mortality was evaluated for statistical significance with Kaplan-Meier survival curves and P-values were obtained from a log-rank test. For all other assays, statistical significance was calculated using one-way analysis of variance (ANOVA) and using the Student-Newman-Keuls post-analysis to obtain P-values. All statistical analyses were done using Graphpad Prism 4.0 software (GraphPad Software Inc). All values are reported as means ± standard error (SEM).

3. RESULTS

3.1 Phenotypic comparisons of virulence factor expression for *C. neoformans* and *C. gattii* strains

C. neoformans and *C. gattii* are known to share the major virulence factors needed to cause disease in mammalian hosts. These factors include capsule and melanin formation, and growth at 37°C. We initially compared the virulence-associated phenotypes of three strains of *C. gattii* with the phenotypes of the commonly studied *C. neoformans* serotype A strain H99. Two of the *C. gattii* strains, R265 and R272, represent the most common molecular subtypes (VGIIa and VGIIb, respectively) from the outbreak on Vancouver Island. The third *C. gattii* strain, WM276, was included as a representative of the other subtype (VGI) that is also found on Vancouver Island. This strain, however, is an environmental isolate from Australia and it was selected as the VGI representative because, like R265, its genome has been sequenced. Our analysis of the virulence factors revealed that the clinical *C. gattii* strains (R265 and R272) and the clinical *C. neoformans* strain (H99) grew well at 37°C, and were similarly able to produce melanin and the polysaccharide capsule (Figure 3.1). However, the environmental *C. gattii* isolate WM276 grew more slowly at 37°C and exhibited a delay in melanin production. We also noted that all four strains were similar in their production of another virulence trait, the enzyme urease (Figure 3.1B). Taken together, these results indicate that all four strains possess the major virulence factors, but WM276 appears to be less robust with regard to growth at 37°C and melanin production.

Figure 3.1. Virulence-associated phenotypes of the *C. gattii* strains R265, R272 and WM276 , and the *C. neoformans* strain H99. The strains were grown at 30ºC overnight and spotted onto different types of media as described in the Materials and Methods (Chapter 2). The photographs demonstrate that the *C. gattii* strain WM276 grew more slowly at 37ºC (A) and had a delay in melanin production (C). We also assessed the strains for their ability to produce urease (B) and capsule (D) and found that there were no major phenotypic differences between the strains. As a control for melanin production, a *lac1 lac2* double mutant derivative of the *C. neoformans* strain H99 was included because this strain lacks laccase function and is unable to produce melanin. As a control for urease production, a *ure1* mutant of strain H99 with a deletion in the urease gene was included because this strain is unable to produce urease. The results are representative of three independent experiments.

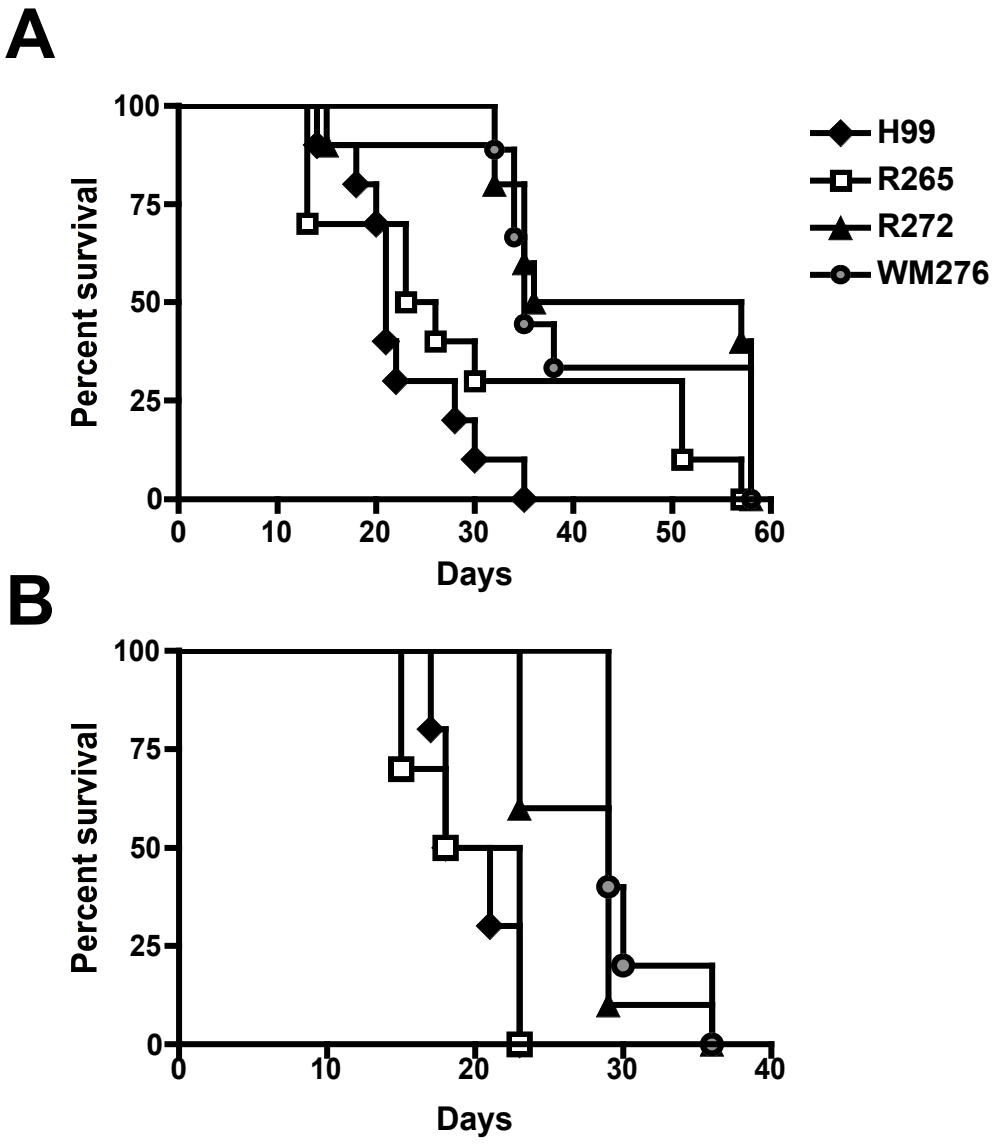


3.2 *C. gattii* isolates from the Vancouver Island outbreak vary in virulence in a mouse model of infection

We next tested the virulence of our selected isolates of *C. neoformans* and *C. gattii* by infecting two different strains of mice using the intranasal inhalation method and assessing survival over a 60 day period. We used C57BL/6 mice because this inbred strain has been commonly used in studies of the immune response to *C. neoformans* and we used A/JCr mice because this strain was used to compare the virulence of *C. neoformans* and *C. gattii* isolates in a previous study (Fraser, *et al.,* 2005). In C57BL/6 mice (Fig 3.2A) and especially in A/JCr mice (Fig 3.2B), we found that the *C. neoformans* strain H99 and the *C. gattii* strain R265 were both significantly more virulent than the *C. gattii* strains R272 and WM276 ($P < 0.001$). Furthermore, there was no significant difference in the virulence of H99 compared to R265, nor was a significant difference observed in the virulence of R272 compared to WM276 in either of these mouse models. In addition to revealing the equivalent virulence of H99 and R265, these results indicate that the differences in growth at 37°C and melanin production between WM276 and R272 were not reflected in their virulence. Furthermore, given that the two clinical isolates from the Vancouver Island outbreak (R265 and R272) are genetically similar (Fraser, *et al.,* 2005; Kidd, *et al.,* 2004) and had identical phenotypes for the major virulence traits, these results indicate that R272 must have differences in other traits that reduce its virulence relative to R265. Interestingly, A/JCr mice were significantly more susceptible to infection with the two *C. gattii* strains with lower virulence (R272 and WM276) ($P < 0.01$) compared to C57BL/6 mice. C57BL/6 mice infected with R272 and WM276 reached the endpoint at 47 days and 35 days post-

infection respectively; whereas A/JCr mice infected with either of these strains reached the endpoint significantly earlier, at 29 days post-infection.

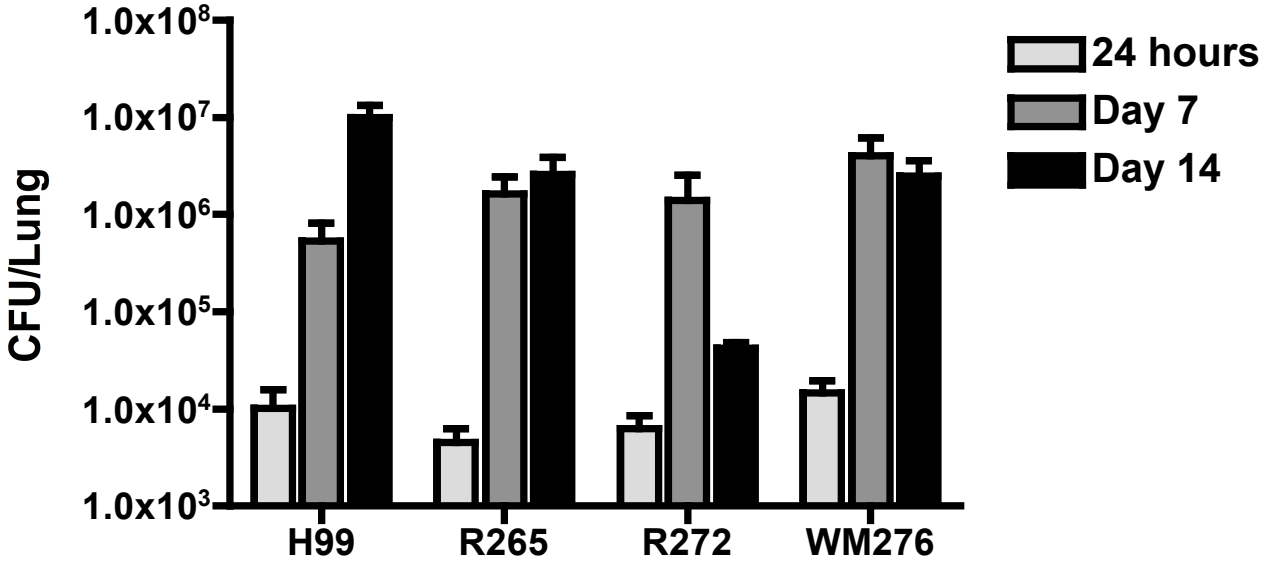
Figure 3.2. Virulence of the *C. neoformans* strain H99 and three strains of *C. gattii* in a mouse model of cryptococcosis. As described in the Materials and Methods, mice were infected via intranasal inhalation with 5×10^4 CFU fungal cells. Infected animals were observed until 58 and 38 days post-infection in C57BL/6 (A) and A/JCr (B) mice respectively. The experiment in B was performed by Anita Sham. These virulence assays were performed once. The *C. neoformans* strain H99 and the *C. gattii* strain R265 were significantly more virulent than the *C. gattii* strains R272 and WM276 ($P < 0.001$) in both animal models. Statistical analysis was performed using the log-rank test to obtain P-values. (n = 10 except for WM276 infection of C57BL/6 mice where n =9).



3.3 Growth of *C. neoformans* and *C. gattii* strains in pulmonary tissue

Having observed significant differences in virulence among our isolates of interest, we wanted to determine if there were any differences in the pulmonary fungal load at different times of the infections. Previous studies have shown that mice infected with more virulent strains of *C. neoformans* have significantly higher pulmonary fungal loads throughout infection compared to mice infected with strains of lower virulence (Wormley, *et al.,* 2005). As shown in Figure 3.3, all isolates were able to grow in the lungs during infection. The fungal loads in mice infected with the more virulent isolates, H99 and R265, increased steadily throughout infection. By 14 days post-infection, the fungal load in these mice was 1000 times higher than the initial fungal load at 24 hours and mice in these two groups began losing weight and reaching the endpoint (Fig 3.2). These results are consistent with previous studies on H99-infected mice, which exhibit a significant increase in pulmonary fungal load from days 3 to 14 post-infection (Wormley, *et al.,* 2005). These results indicate that not only is the *C. gattii* strain R265 as virulent as the *C. neoformans* strain H99, but R265 is also able to grow to levels similar to H99 in the lungs of infected mice. The fungal load in mice infected with the less virulent isolates, R272 and WM276, were similar to those in mice infected with the more virulent isolates at 7 days post-infection.

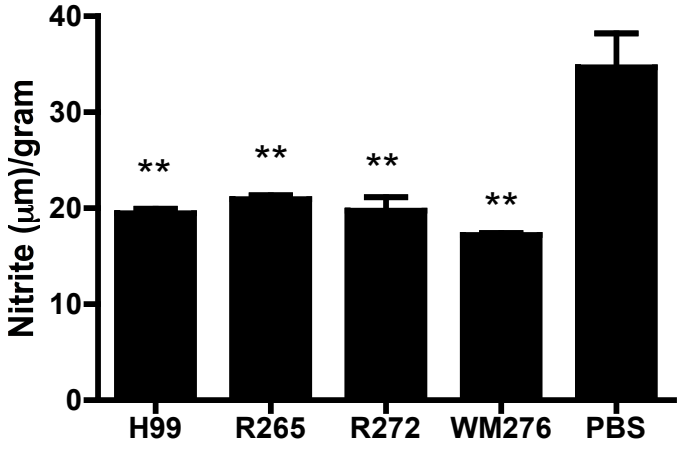
Figure 3.3 Pulmonary fungal load in *C. neoformans*- and *C. gattii*-infected mice. C57BL/6 mice were infected with 5×10^4 CFU of the indicated strains. At the specified time points, lungs were excised, homogenized and plated on media to determine the pulmonary fungal load. Data for the 7 days post-infection results are representative from 3 separate experiments, data for the 24 hours post-infection results are representative from 2 separate experiments and data for the 14 days post-infection results are representative of 1 experiment. Results are expressed as mean ± SEM (n = 3 mice/group).



3.4 Suppression of nitric oxide production

Macrophages play an important role in the inflammatory immune response and they produce large amounts of microcidal agents such as nitric oxide upon activation. Furthermore, this nitric oxide production in macrophages is associated with control of the pulmonary fungal load during Cryptococcus infection (Goldman, *et al.,* 1996; Rossi, *et al.,* 1999). Given that we observed differences in the pulmonary fungal load of mice infected with our *C. neoformans* and *C. gattii* isolates, we wanted to determine whether there were any differences among the strains in their ability to inhibit nitric oxide production by macrophages *in vitro*. However, as shown in Figure 3.4, each *C. neoformans* and *C. gattii* strain that we tested was able to suppress nitric oxide production of macrophages to a similar extent.

Figure 3.4 *C. neoformans* and *C. gattii* isolates can suppress nitric oxide production in LPS-stimulated macrophages. Macrophages were stimulated with LPS and IFN-γ and co-cultured with fungal cells for 24 hours. Nitrite was measured in the supernatants using the Greiss Assay. The data are representative of three separate experiments. Results are expressed as mean ± SEM. The symbol ** $P < 0.001$ represents the statistical comparison to uninfected mice.



3.5 Histopathology of *C. neoformans* and *C. gattii* infections

In order to obtain a gross morphological view of the lungs during cryptococcal infection, the lungs of A/JCr mice infected with the *C. neoformans* strain H99 and the *C. gattii* strain R265 were examined histologically at 2 and 7 days post-infection. These Cryptococcus strains were selected because they have equivalent virulence in this mouse model (Figure 3.2B). Furthermore, A/JCr mice were used for this study because this mouse strain is also commonly used for virulence studies with *C. neoformans* and we wanted to include studies with these mice to allow comparisons with published work. Sections were stained with hematoxylin and eosin (H&E) to visualize lung structures, and with Mayer's mucicarmine (MM) to view the Cryptococcus capsular polysaccharide (magenta colour) (Wright, *et al.,* 2002). At 2 days post-infection, the lungs of infected mice appeared healthy with no obvious differences in the bronchovascular infiltrate surrounding the capillaries and airways compared to uninfected mice (Figure 3.5). Furthermore, cryptococci were present in the lungs, but were mostly confined to the bronchiolar and alveolar airspaces (Figure 3.6). At 7 days post-infection, the lungs of infected mice showed signs of differential immune responses. Specifically, the bronchovascular infiltrate surrounding small blood vessels and airways in the lungs of H99-infected mice (Figure 3.7C and D) appeared to be densely packed with leukocytes, suggesting the development of protective immune responses as others have shown in previous studies (Muller, *et al.,* 2007; Osterholzer, *et al.,* 2008). In contrast, the bronchovascular infiltrate in R265-infected mice (Figure 3.7B) appeared to be more diffuse. Diminished leukocyte infiltration and the presence of YM1 crystals has been associated with non-protective Th2 immune responses to Cryptococcus infections (Arora,

et al., 2005; Osterholzer, *et al.,* 2008). However, we did not observe the presence of YM1 crystals in the histopathology of these *C. gattii*-infected mice. These results indicate that a less protective immune response is developed against *C. gattti* R265 infection compared to *C. neoformans* H99 infections, at 7 days post-infection. Additionally, the presence of Cryptococcus cells and mucus development was also observed in the vicinity of the bronchovascular infiltrates at this time point (Figure 3.7). Furthermore, Mayer's mucicarmine staining revealed cryptococci invading the tissues around the bronchiolar and alveolar airspaces and multiplying in the surrounding tissues (Figure 3.8 A and B). Interestingly, these cryptococci appeared to vary in size and the *C. neoformans* H99 cryptococci appeared to have larger capsules (diameter 2-14 μm) (Figure 3.8 C) compared to the *C. gattii* R265 cryptococci (diameter 2-5 μm) (Figure 3.8 D); this was also observed at 16 days post-infection (Figure 3.9 C and D), but not at 2 days post-infection. At 16 days post-infection, mucus production was mostly observed in the bronchiolar airspace of *C. neoformans*-infected mice while in *C. gattii*-infected mice, mucus production was mostly in alveolar airspaces (Fig 3.9 A and B).

It should be noted that our histopathology studies were performed in A/JCr mice rather than in the C57BL/6 mice that we used for our other studies. We do not believe that there are major differences between these two mouse strains in the context of our study because, as we showed in our virulence assay, this histopathology analysis, and our other analyses below, the effect of our *C. neoformans* and *C. gattii* isolates on the survival and immune responses of these mice are similar between the two animal models

Figure 3.5 Histopathology of Cryptococcus infection at 2 days post-infection. A/JCr mice were infected with 5×10^4 CFU of Cryptococcus cells and lungs were harvested 2 days post-infection and prepared as described in the Materials and Methods. Photomicrographs (hematoxylin and eosin staining, ×200 magnification, scale bar is 50 μm) of lung sections from uninfected mice (A), mice infected with *C. neoformans* strain H99 (B) and mice infected with the *C. gattii* strain R265 (C). The lungs of infected mice appeared healthy with a normal degree of bronchovascular infiltrates surrounding the capillaries and air spaces.

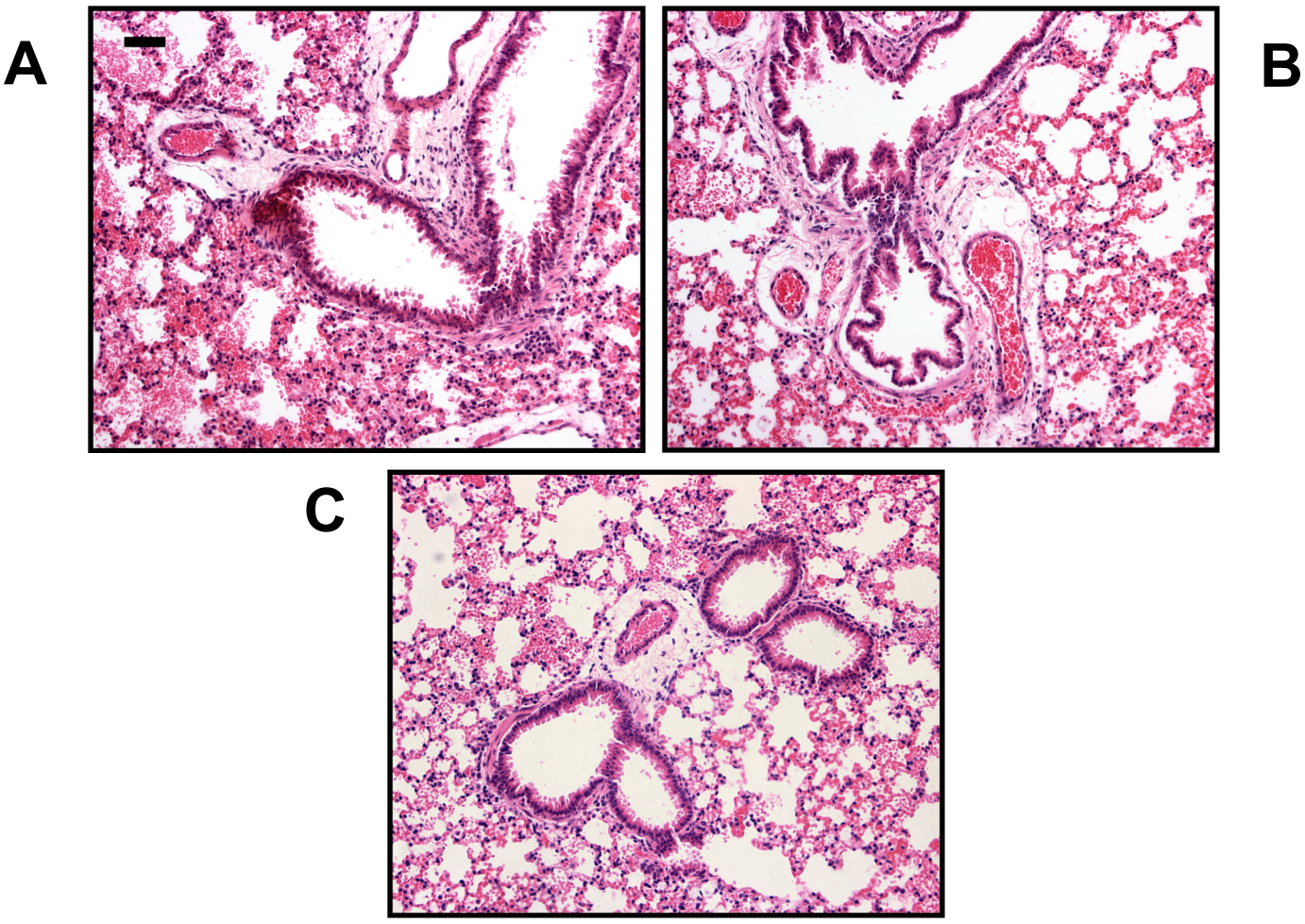


Figure 3.6 Histopathology of Cryptococcus infection at 2 days post-infection. A/JCr mice were infected with 5×10^4 CFU of Cryptococcus cells and lungs were harvested 2 days post-infection and prepared as described in the Materials and Methods. Photomicrographs (Mayer's mucicarmine staining, ×400 magnification, scale bar is 40 μm) of lung sections from mice infected with *C. neoformans* strain H99 (A) and mice infected with the *C. gattii* strain R265 (B). Mayer's mucicarmine allows visualization of the cryptoccocal polysaccharide capsule by staining it a magenta colour. Note that at 2 days post-infection, cryptococci are present in the lungs, but are confined to the alveolar and bronchiolar airspaces.

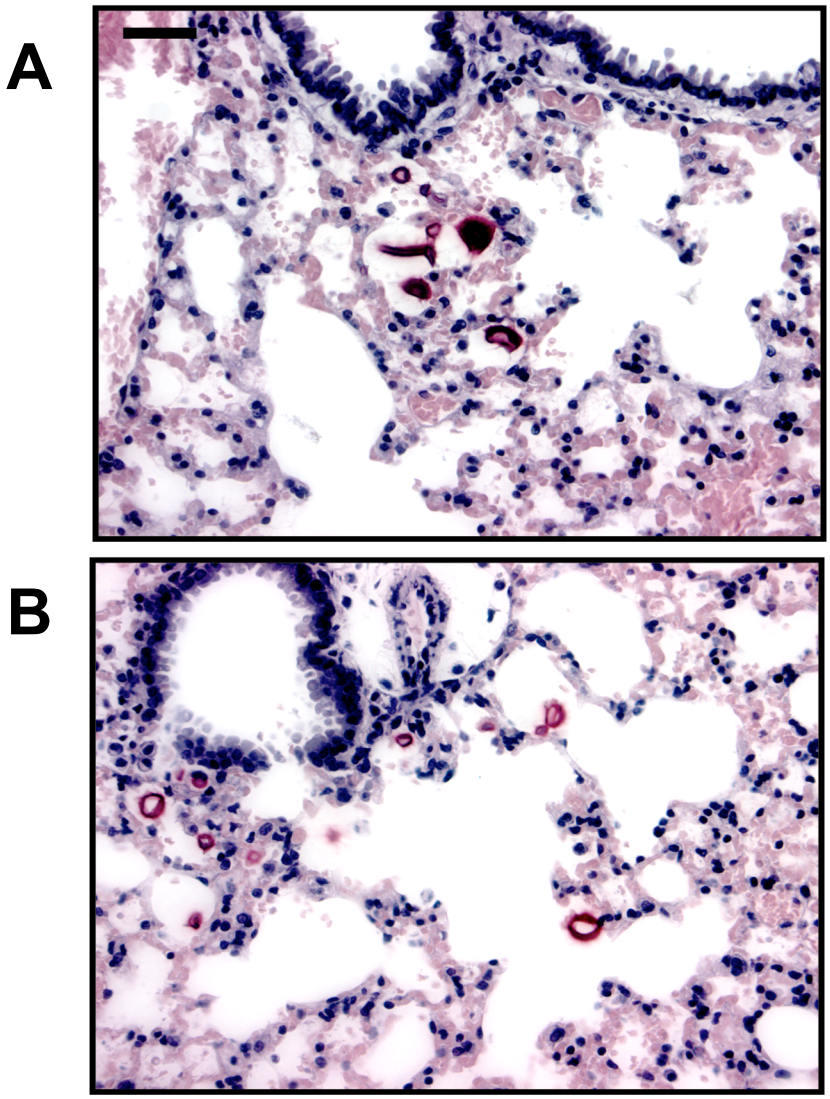


Figure 3.7 Histopathology of Cryptococcus infection at 7 days post-infection. A/JCr mice were infected with 5×10^4 CFU of Cryptococcus cells and lungs were harvested 7 days post-infection and prepared as described in the Materials and Methods. Photomicrographs (hematoxylin and eosin staining, ×200 magnification, scale bar is 50 μm) of lung sections from uninfected mice (A), mice infected with the *C. gattii* strain R265 (B) and mice infected with the *C. neoformans* strain H99 (C and D). Note that at this time point, the bronchovascular infiltrate (brackets) in *C. neoformans*-infected mice appeared more densely packed with leukocytes compared to those in the uninfected or the *C. gattii*-infected mice. Also note the appearance of cryptococci (indicated by an asterisk) in the vicinity of the bronchovascular infiltrate and mucus production in the airways (indicated by arrowheads).

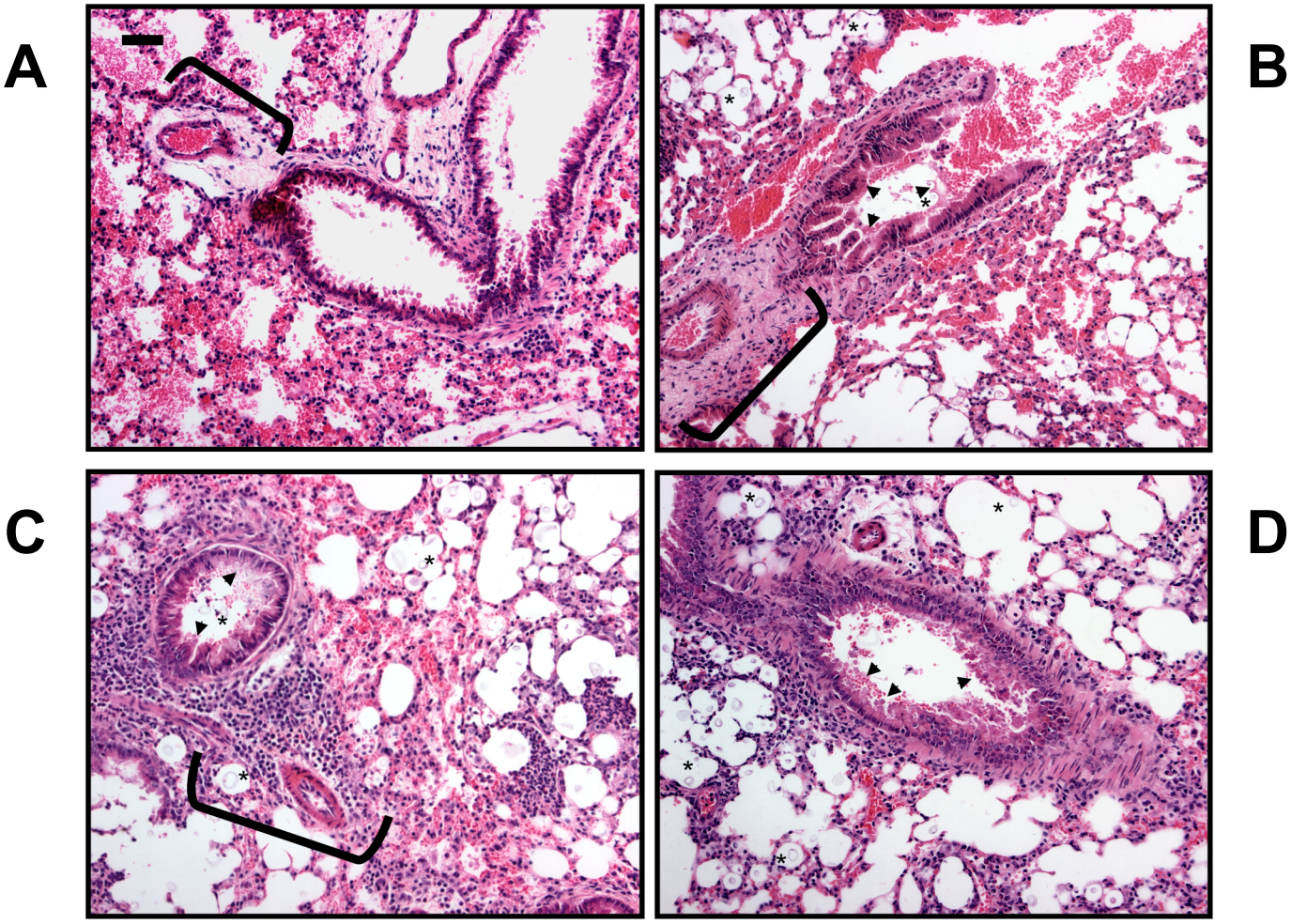


Figure 3.8 Histopathology of Cryptococcus infection at 7 days post-infection. A/JCr mice were infected with 5×10^4 CFU of Cryptococcus cells and lungs were harvested 7 days post-infection and prepared as described in Materials and Methods. Photomicrographs (Mayer's mucicarmine staining, ×200 magnification, scale bar is 50 μm for A and B; ×630 magnification, scale bar is 10 μm for C and D) of lung sections from mice infected with *C. neoformans* strain H99 (A) and mice infected with the *C. gattii* strain R265 (B) show cryptococci invading the tissues around the bronchiolar and alveolar airspaces and multiplying in the surrounding tissues. Closer examination of the cryptococci showed that *C. neoformans* cells appear to have a larger capsule (C) than *C. gattii* cells (D).

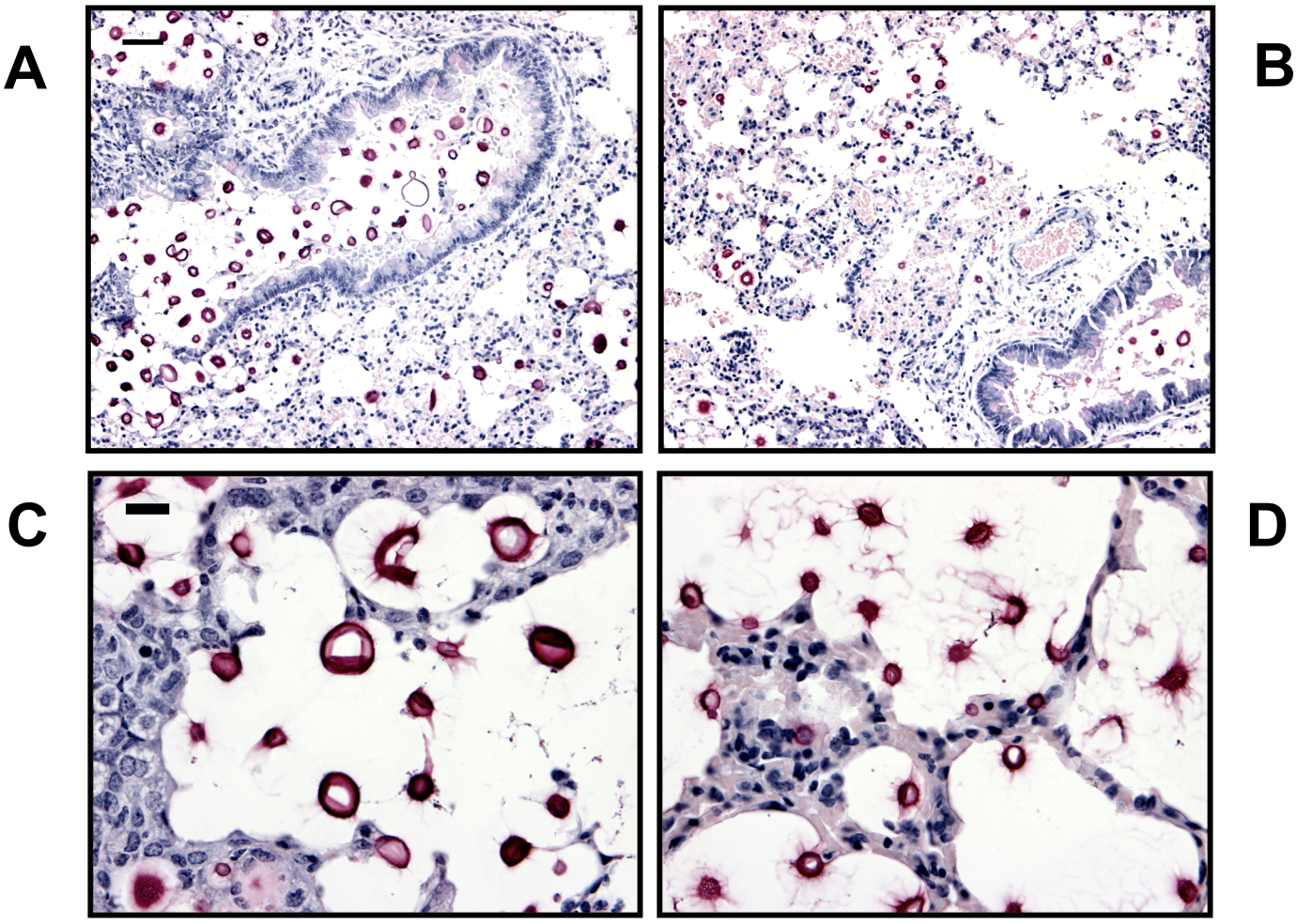
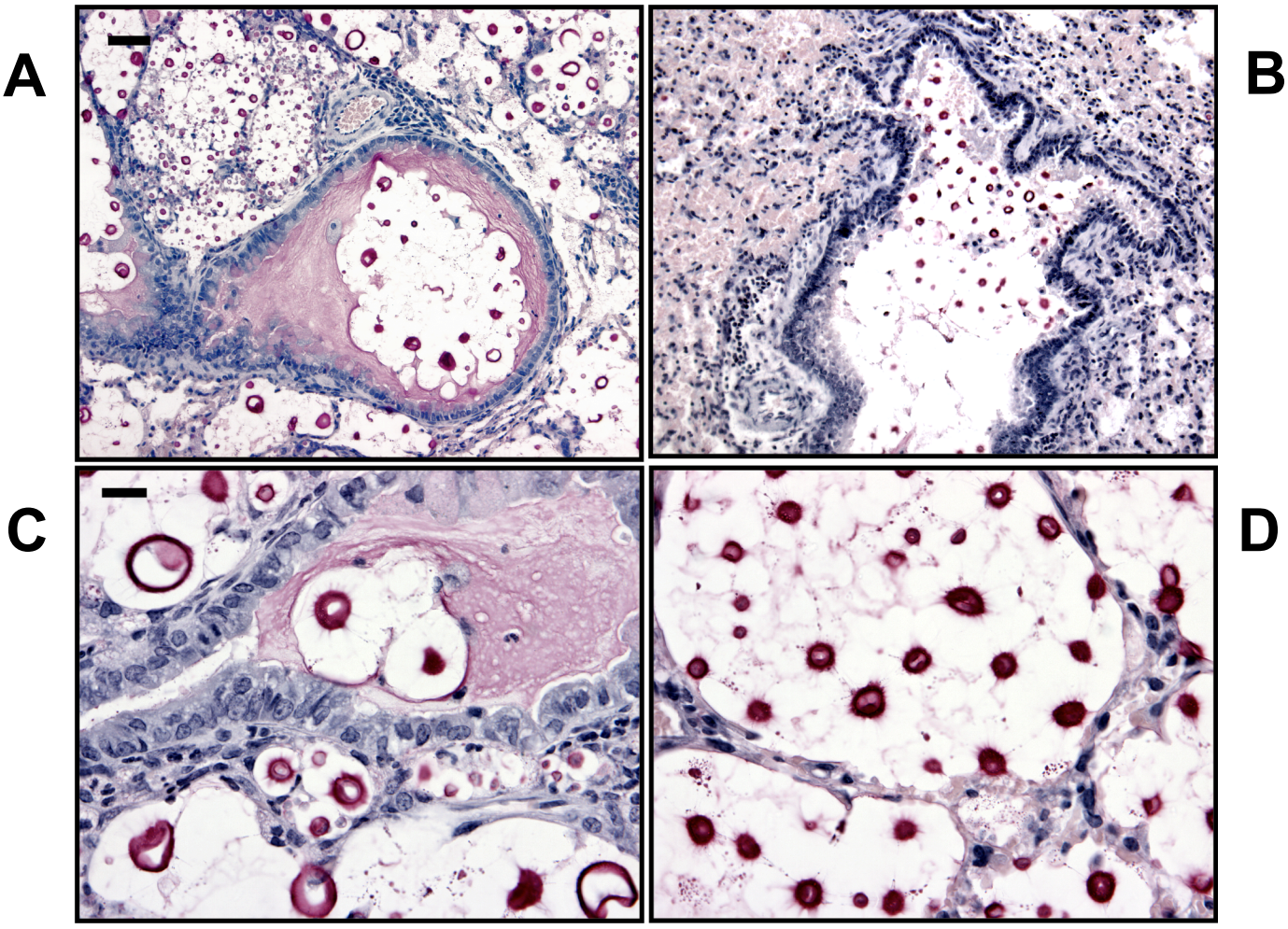


Figure 3.9 Histopathology of Cryptococcus infection at 16 days post-infection. A/JCr mice were infected with 5×10^4 CFU of Cryptococcus cells and lungs were harvested 16 days post-infection and prepared as described in Materials and Methods. Photomicrographs (Mayer's mucicarmine staining, ×200 magnification, scale bar is 50 μm for A and B; ×630 magnification, scale bar is 10 μm for C and D) of lung sections from mice infected with *C. neoformans* strain H99 (A) show abundant mucus production in bronchiolar airspaces while in mice infected with the *C. gattii* strain R265 (B), mucus is mostly in the alveolar airspaces. Closer examination of the cryptococci showed that *C. neoformans* cells appear to have a larger capsule (C) than *C. gattii* cells (D).

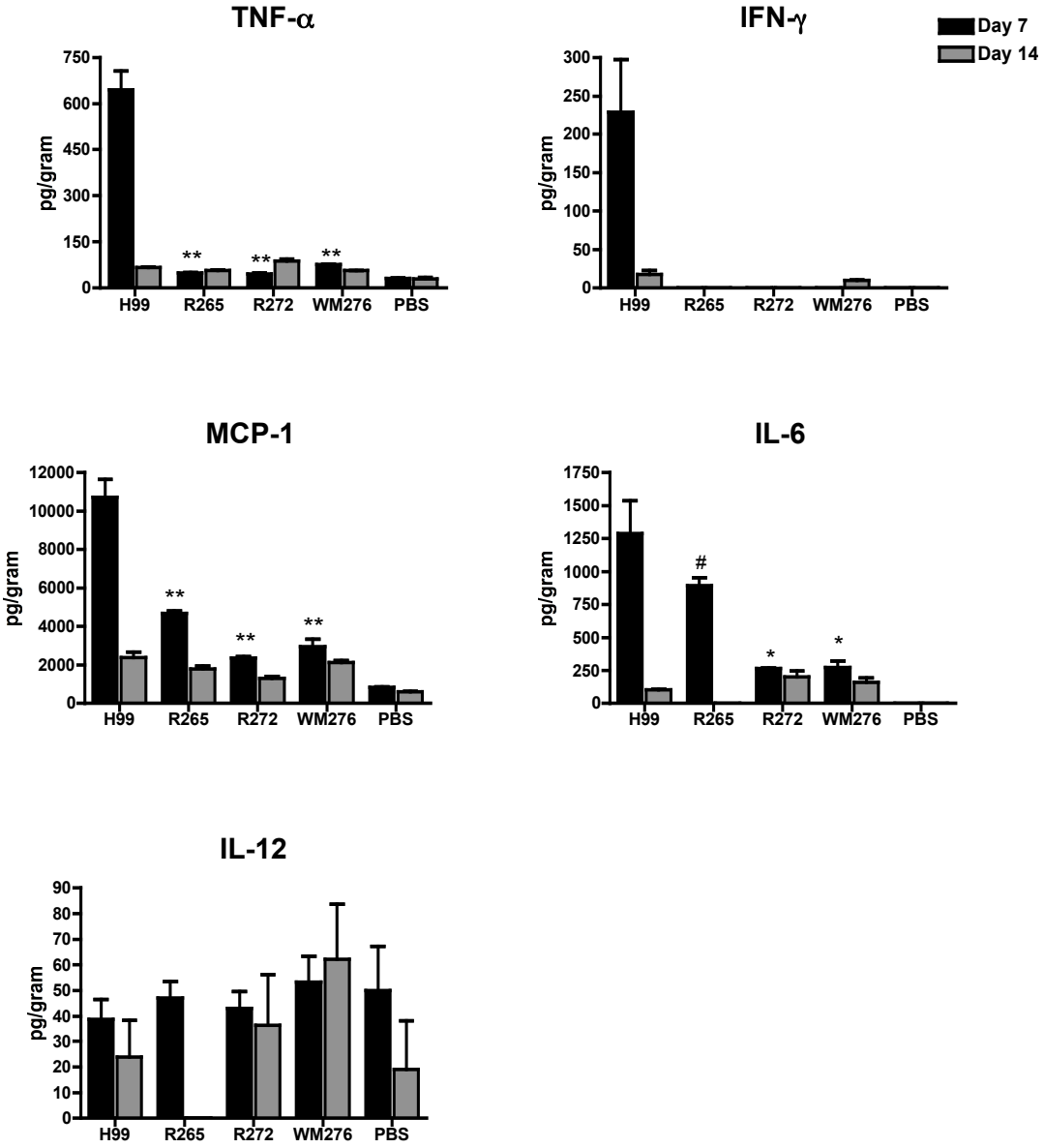


3.6 Cytokine profiles during pulmonary infections with *C. neoformans* and *C. gattii*.

Previous investigators suggested that *C. gattii* infections may be less able to induce Th1-associated inflammation than *C. neoformans* infections and that this may be the reason why *C. gattii* strains are able to infect immunocompetent people and cause disease (Wright, *et al.,* 2002). However, to date, there have not been any studies to test this idea, such as determining the cytokine profiles of *C. gattii*-infected mice to examine their immune status. Furthermore, there have not been any immunological studies that specifically focus on the *C. gattii* isolates from the Vancouver Island outbreak. We therefore evaluated the cytokine profiles of infected C57BL/6 mice to address these gaps in our knowledge and to specifically determine whether the *C. gattii* isolates from the outbreak induce less protective immunity than *C. neoformans* isolates during infection. We chose to use C57BL/6 mice in these immunological studies because this mouse strain has commonly used in many other previous studies on the immune response against *C. neoformans* infection. We focused our analysis on the cytokines TNF-α, IFN-γ, MCP-1, IL-6, the Th1 cytokine IL-12 (p70 subunit), which all have been associated with protective immune responses against *C. neoformans infection*, and the Th2 cytokine IL-10, which has been associated with non-protective immune responses. We performed all of our measurements on lung homogenates from infected C57BL/6 mice collected at 24 hours, 7 days and 14 days post-infection. We found that there were no significant differences in cytokine expression among mice infected with the different strains of Cryptococcus at 24 hours post-infection (data not shown). At 7 days post-infection, the cytokine measurements showed that the *C. gattii* infections indeed induced lower levels

of protective cytokines compared to the *C. neoformans* H99 infection (Figure 3.10). Specifically, mice infected with the *C. gattii* strains had significantly lower levels of TNF-α, ($P < 0.001$), MCP-1 ($P < 0.001$) and IL-6 ($P < 0.05$) compared to mice infected with the *C. neoformans* H99 strain. The levels of MCP-1 and IL-6 in mice infected with *C. gattii* isolates were slightly elevated at this time point compared to the uninfected mice, especially in the case of R265, indicating that there was some induction of an immune response in these mice. However, the levels of TNF-α in all of the *C. gattii*-infected mice were not significantly different than those from the uninfected mice and the levels of IFN-γ in these *C. gattii*-infected mice were below the limit of detection of the assay (2.5 pg/mL); indicating the absence of a significant immune response, especially in comparison to *C. neoformans*-infected mice. Overall, our cytokine results indicate that infections with these particular *C. gattii* strains are consistent with a less protective profile than infection with the *C. neoformans* strain H99. We also detected very low levels of the Th1 cytokine IL-12, in infected mice; however, these levels were not significantly different from that in the uninfected mice at any of the time points tested. The absence of significantly elevated levels of IL-12 in the *C. neoformans* strain H99-infected mice suggests that the immune response in these mice was not due to a Th1 type immune response. After 14 days of infection, the levels of protective cytokines in the *C. neoformans*-infected mice decreased such that they were no longer significantly different than those in the *C. gattii*-infected mice, indicating a dampening effect. We did not detect the Th2 cytokine IL-10 in any samples at any of the time points and the lower limit of detection for this cytokine was 17.5 pg/mL under our assay conditions.

Figure 3.10 Cytokine expression in mice infected with *C. neoformans* and *C. gattii* isolates. Lung homogenates from mice infected with 5×10^4 CFU of the indicated Cryptococcus isolates were prepared by mechanical disruption and assayed for the production of cytokines at 7 and 14 days post-infection. Results are expressed as the mean ± SEM (n = 3 mice/group/time point). The data are representative of three separate experiments. The symbols (** P < 0.001, * P < 0.01, # P < 0.05) represent the statistical analysis based on comparisons to mice infected with *C. neoformans* strain H99.

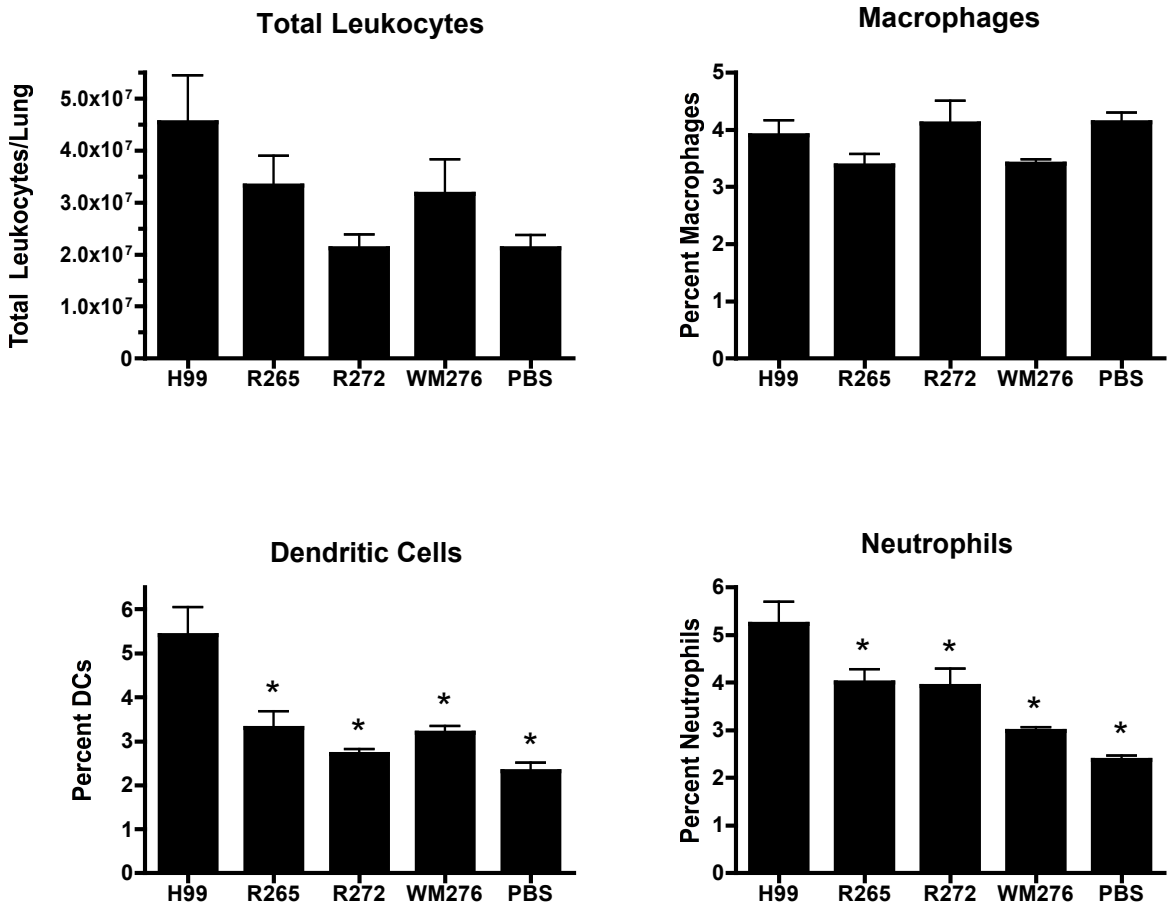


3.7 Pulmonary leukocyte infiltration during infections with *C. neoformans* and *C. gattii*.

Seven days post-infection is the onset of cell-mediated immunity this mouse model of cryptococcosis (Wormley, *et al.,* 2005). As discussed previously (section 3.5), at this timepoint, we observed a more diffuse bronchopulmonary infiltrate in A/JCr mice infected with the *C. gattii* strain R265 in our histopathology study. Furthermore, having observed differences in the cytokine profiles of *C. neoformans*- and *C. gattii*-infected C57BL/6 mice at this time point, we next measured the proportions of dendritic cells (DCs), macrophages and neutrophils in the lungs of infected mice using flow cytometry to determine if the lack of protective cytokine responses we observed in *C. gattii*-infected mice was mirrored in the population of cells in the pulmonary infiltrate. Pulmonary leukocyte infiltration during Cryptococcus infection is an important part of the immune response against this pathogen. Previous studies have shown that a protective response involves increased levels of neutrophils, classically-activated macrophages and lymphocytes in the lungs (Chen, *et al.,* 2008; Guillot, *et al.,* 2008). For this study, C57BL/6 mice were infected intranasally with 5×10^4 CFU of Cryptococcus cells and leukocyte infiltration levels were examined at 7 days post-infection (Figure 3.11). We observed significantly lower levels of dendritic cells and neutrophils in the lungs of mice infected with the *C. gattii* strains compared to mice infected with the *C. neoformans* H99 strain ($P < 0.05$). Furthermore, the total lung leukocyte levels were lower in mice infected with *C. gattii* compared to mice infected with the *C. neoformans* strain, but this result was not statistically significant. Additionally, there were no significant differences in the levels of pulmonary macrophages in the infected mice. These results confirm our

finding that at 7 days post-infection, the immune response to the *C. gattii* strains is consistent with a less protective profile than the immune response to the *C. neoformans* infection.

Figure 3.11 Pulmonary leukocyte infiltration. C57BL/6 mice were infected with 5×10^4 CFU of the indicated Cryptococcus isolates for 7 days, lungs were excised and leukocytes were isolated from whole lungs by enzymatic digestion. As described in Materials and Methods, leukocytes were labeled with anti-CD11b, anti-CD11c, anti-Gr.1 and analyzed by flow cytometry. Results are represented as the mean ± SEM (n = 3 mice/group). The data are representative of three separate experiments, except in the cases of R272 and WM276, which are representative of two separate experiments. The symbol (* $P < 0.01$) indicates the statistical differences compared to mice infected with *C. neoformans* strain H99.



3.8 Neutrophil infiltration during pulmonary infections with *C. neoformans* and *C. gattii*.

Neutrophils are important mediators of protective immune responses against *C. neoformans* infection. We therefore measured neutrophil infiltration in the lungs of infected mice at 24 hours and 7 days post-infection by cytological and enzymatic methods to further investigate the differences in neutrophil accumulation we observed in our flow cytometry analysis. As described in Chapter 1, a report from Dong and Murphy (1995) showed that culture filtrate antigens from *C. gattii* strains inhibited neutrophil migration and function *in vitro*, whereas those from *C. neoformans* strains stimulated neutrophil migration and function. Another study found that even though a *C. gattii* strain was able to inhibit neutrophil migration *in vitro*, neutrophil infiltration into the lungs of infected rats are similar in *C. neoformans* and *C. gattii* infections (Wright, *et al.,* 2002). However, rats and mice differ widely in their susceptibility to Cryptococcus infection and have been shown to have differential immune responses against infection (Goldman, *et al.,* 1994; Shao, *et al.,* 2005). Thus, the role of neutrophils in *C. gattii* infection is unclear. Some researchers speculate that because neutrophils are important in the early innate immune responses to *C. neoformans* infection, inhibition of neutrophil migration into infected lung tissues may delay the production of TNF-α, IFN-γ and important chemokines. This delay would potentially prevent the induction of an effective adaptive immune response for clearance of the pathogen (Shoham and Levitz, 2005). This latter scenario could potentially explain the lack of protective cytokines that we observed in the *C. gattii*-infected mice. Thus, using cytological methods, we examined the *in vivo* neutrophil response in C57BL/6 mice to our *C. gattii* strains from the

Vancouver Island outbreak in comparison to the response against a *C. neoformans* infection to determine whether there were any differences. At 24 hours post-infection, there were significantly lower numbers of neutrophils in all of the *C. gattii*-infected mice compared to mice infected with the *C. neoformans* strain H99 ($P < 0.001$) (Figure 3.12). Furthermore, the levels of pulmonary neutrophils in mice infected with the *C. gattii* strains were not significantly different from those in the uninfected mice ($P > 0.05$), indicating that these *C. gattii* strains failed to provoke the migration of neutrophils into the sites of infection.

Myeloperoxidase (MPO) is a neutrophil-specific enzyme that is a useful indicator for neutrophil granulocyte sequestration (Klebanoff, 2005). MPO was therefore measured to further assess neutrophil accumulation in the lung tissues of infected mice. Measurement of MPO activity in the lung tissue of infected C57BL/6 mice at 24 hours and 7 days post-infection showed that mice infected with the *C. gattii* strains had significantly lower levels of pulmonary neutrophil activity compared to the *C. neoformans*-infected mice (Figure 3.13). These results therefore confirmed our cytology and flow cytometry analysis. Furthermore, at all three time points analyzed, neutrophil activities in the lungs of *C. gattii*-infected mice were not significantly different than the activity in uninfected mice at the corresponding time point ($P > 0.05$). Additionally, *C. neoformans*-infected mice showed a significant increase in neutrophil activity from 24 hours to 7 days post-infection ($P < 0.001$); this activity was also significantly greater than that in uninfected mice at 7 days post-infection ($P < 0.001$), indicating continued induction of the neutrophil response. We also observed an increase in neutrophil activity in the *C. gattii*-infected mice from 24 hours post-infection to 7 days post-infection,

indicating that there was some neutrophil migration into the lungs between these time points. However, because these neutrophil activity levels at 7 days post-infection were not significantly different from those in the uninfected mice at this time point, this increase is likely not relevant. By 14 days post-infection, neutrophil activity levels in *C. neoformans*-infected mice were slightly reduced compared to those at 7 days post-infection. Overall, our findings demonstrate that there are significantly lower levels of neutrophil activity in mice infected with the *C. gattii* isolates, thus providing further evidence that these *C. gattii* infections induce less protective immune responses than infection with the *C. neoformans* strain H99.

Figure 3.12 Pulmonary neutrophil infiltration in *C. neoformans*- and *C. gattii*-infected mice at 24 hours post-infection. As described in Materials and Methods, lung leukocytes were isolated by enzymatic digestion of tissue and cytospun onto glass slides for staining. Neutrophils were counted by microscopy and frequencies are expressed as percentages of the total leukocytes present in the sample. The data are representative of two separate experiments. Results are expressed as the mean ± SEM (n = 5 mice/group). The symbol ** $P < 0.001$ and * $P < 0.01$ represents statistical comparisons to mice infected with H99.

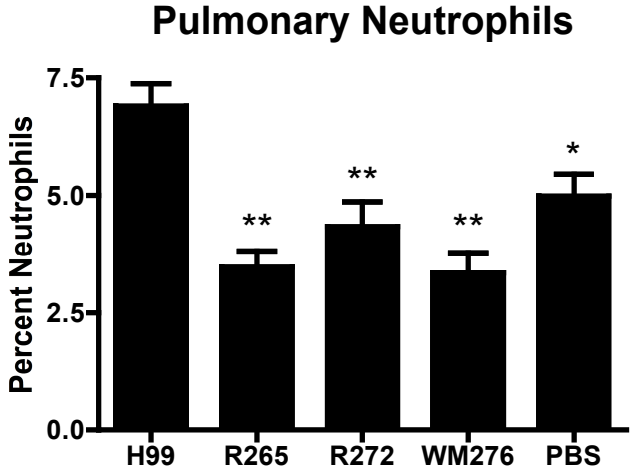
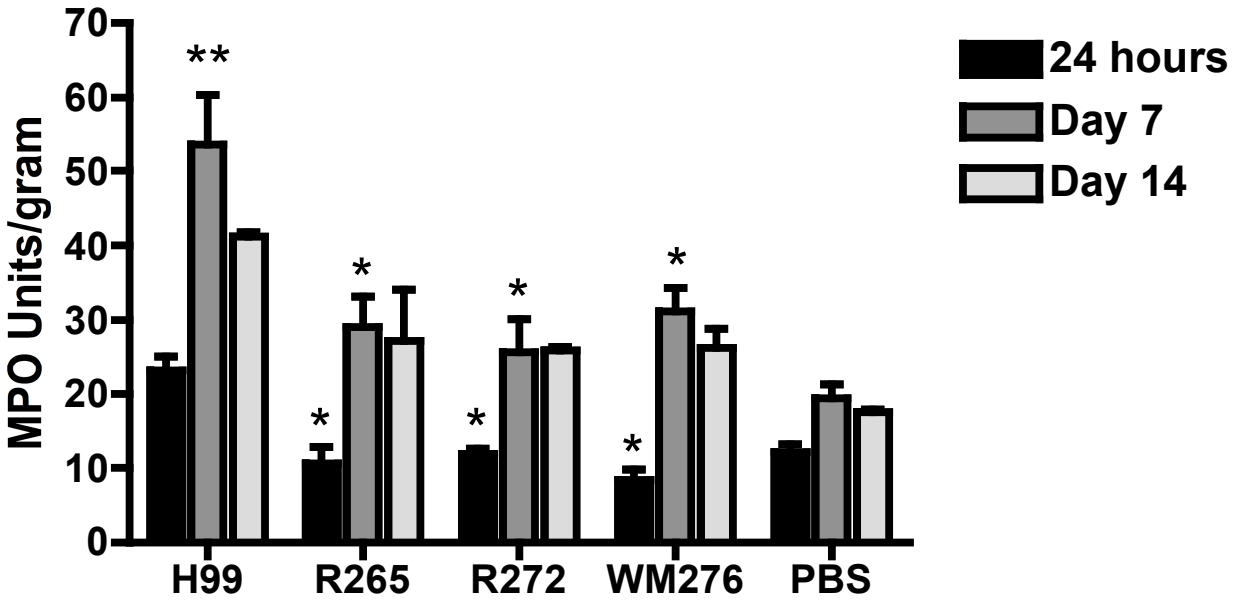


Figure 3.13 Neutrophil infiltration as represented by myeloperoxidase activity in *C. neoformans*- and *C. gattii*–infected mice at 24 hours, 7 and 14 days post-infection. Myeloperoxidase (MPO) activity was employed as a measure of neutrophil infiltration. Lungs were excised from infected mice, snap frozen with liquid nitrogen and measured for MPO activity as described in the Materials and Methods. The data are representative of three separate experiments. Results are expressed as mean ± SEM (n = 3 mice/group). The symbol * P <0.01 represents statistical comparisons to mice infected with H99 at the respective time points. The symbol ** P<0.001 represents statistical comparison of mice infected with *C. neoformans* H99 at 7 days post-infection to mice infected with H99 at 24 hours post-infection and to uninfected mice at 7 days post-infection.



4. DISCUSSION

C. neoformans infections have commonly been associated with people with compromised immune systems whereas *C. gattii* infections are thought to occur in people regardless of their immune status. To understand the mechanisms of the immune response that might begin to explain how *C. gattii* is able to cause disease in immunocompetent people, we examined the immune response elicited by selected strains of these species in a mouse model of cryptococcosis. Overall, we found that *C. gattii* isolates caused infections with less protective characteristics compared to a representative *C. neoformans* strain. This observation raises the possibility that *C. gattii* isolates may actively suppress or may lack factors that provoke a protective inflammatory immune response during infection. Furthermore, it is possible that these differences may contribute to the ability of *C. gattii* strains to cause disease in immunocompetent people.

4.1 *C. gattii* infections induce less protective inflammation than *C. neoformans* infections

A protective inflammatory immune response against cryptococcus infection involves the elevated production of cytokines such as TNF-α and IFN-γ as well as increased levels of pulmonary leukocyte infiltration (Chen, *et al.,* 2008; Guillot, *et al.,* 2008). There is little reseach on the immune response against *C. gattii* infections, but previous studies indicated that *C. gattii* strains may induce less protective inflammation than *C. neoformans* strains *in vitro* (Dong and Murphy, 1995; Wright, *et al.,* 2002). Our results suggest that this may also be the case *in vivo*. Early events in the immune

response to *C. gattii* infection indicated little induction of protective inflammation. At 24 hours post-infection, the levels of neutrophil infiltration in the lungs of these mice were significantly lower than those in *C. neoformans*-infected mice. 7 days post-infection is the onset of cell-mediated immunity in this mouse model (Wormley, *et al.,* 2005). At this time point, we observed a minor increase in the levels of MCP-1 and IL-6 in the lungs of *C. gattii*-infected mice. However, this immune response was very weak as evidenced by the absence of IFN-γ and similarity in the levels of pulmonary TNF-α, dendritic cells and neutrophils in these *C. gattii*-infected mice and the uninfected mice. Additionally, the histopathology of A/JCr mice infected with the *C. gattii* strain R265 showed a bronchovascular infiltrate that was diffuse compared to that in the *C. neoformans*-infected mice, further indicating weak inflammation at 7 days post-infection, which is similar to what others have shown in mice producing ineffective immune responses against Cryptococcus infection (Chen, *et al.,* 2007; Osterholzer, *et al.,* 2008). Furthermore, these results were consistent at 14 days post-infection, indicating a continued lack of protective inflammation. Since these *C. gattii*-infected mice did not trigger a Th1 type inflammatory immune response, we considered the possibility that they may skew the immune response toward a Th2 profile. However, we did not detect the Th2 cytokine IL-10 in these mice at any of the time points tested. Nor did we observe the formation of YM1/YM2 crystals, which are indicative of Th2 responses (Arora, *et al.,* 2005; Chen, *et al.,* 2005; Chen, *et al.,* 2007), in the histopathology of R265-infected mice at 7 days post-infection. However, our investigation was limited in terms of the cytokines that were tested, and further studies may be necessary to entirely rule out a Th2

immune response in these mice, including testing for IL-4, IL-13 and histopathology at other time points.

In contrast to our observations in *C. gattii*-infected mice, *C. neoformans* infection appeared to induce a strong protective inflammatory immune response. Specifically, in the *C. neoformans*-infected mice, we observed a significant influx of neutrophils early during the immune response with a subsequent increase in production of the protective cytokines TNF-α and IFN-γ and chemokines as well as an increase in pulmonary neutrophils and dendritic cells at 7 days post-infection. Furthermore, at this time point, we observed a dense bronchovascular infiltrate in the histopathology of A/JCr mice infected with the *C. neoformans* H99 strain, which is indicative of a protective inflammation. These results are consistent with previous studies involving H99 and other strains of *C. neoformans* that showed that they elicit a protective inflammatory immune responses in mice and other animal models (Chen, *et al.,* 2008; Wormley, *et al.,* 2005). However, in contrast with previous studies involving *C. neoformans* infection, we did not observe significant increases in the hallmark Th1 cytokine, IL-12 in these mice. The lack of IL-12 induction, together with the presence of pulmonary neutrophils and IL-6, highlights the possibility that an inflammatory Th17 type immune response was induced in these *C. neoformans*-infected mice; this possibility is discussed further in section 4.4.1.

By 14 days post-infection, the significant differences in the immune response against *C. neoformans* and *C. gattii* infections were not observed and there was little production of cytokines detected in mice infected with any strain, indicating a decrease in protective immune responses. We speculate that by 14 days post-infection, while the immune response in *C. gattii*-infected mice was consistently weak, the immune response

in the *C. neoformans*-infected mice is dampened due to the uncontrolled replication of cryptococci in the lungs. This decrease in the protective immune response may also be related to the shedding of GXM, as the cryptococcal infection gets firmly established and GXM titers rise, as described in the next section. Furthermore, our results confirm those from other studies, which have also shown that mice infected with the *C. neoformans* strain H99 initially display protective inflammation that is dampened after 7 days post-infection (Wormley, *et al.,* 2005).

It is important to note that while the immune response (associated with protective immunity in other studies) did not clear *C. neoformans* H99 infection in our study, this does not mean that this type of inflammatory immune response is unable to clear *C. neoformans* infection. Other studies have also shown that the *C. neoformans* strain H99 is highly virulent and while it does elicit protective inflammation during infection, mice infected with this strain always succumb to infection (Fraser, *et al.,* 2005; Wormley, *et al.,* 2005). The importance of protective inflammatory immune responses in *C. neoformans* infection has been shown in numerous studies, as outlined in chapter 1. Rather, the use of inbred mouse strains and the high inoculum likely resulted in the lethal outcome for these *C. neoformans*-infected mice. It is important to also note that while we cannot make the claim that the lack of protective inflammation in *C. gattii*-infected mice resulted in their outcome, we have shown that *C. gattii* infections induce less Th1-associated inflammation than *C. neoformans* infections. As discussed in section 4.4.3, further studies will be necessary in order to determine what entails a protective immune response against *C. gattii* infection.

We also note that our observations are different from another study in which there were no differences in neutrophil accumulation in a rat model of cryptococcocis (Wright, *et al.,* 2002). One possible explanation for this discrepancy is the choice and history of the *C. gattii* strains. We tested three different *C. gattii* strains, two of which were clinical isolates from the Vancouver Island outbreak. Wright *et al*. (2002) tested one strain, an environmental *C. gattii* isolate that was obtained from a eucalyptus tree. Although one of our isolates was originally an environmental isolate, it is possible that the *C. gattii* isolates that we used were better adapted to causing disease due to previous passage through an animal or a human host. Furthermore, we employed a mouse model of cryptococcosis whereas Wright *et al*. (2002) chose a rat model. Recent studies have revealed fundamental differences in these models, especially with regard to neutrophil infiltration during lung injury (Bermudez, *et al.,* 2004). Furthermore, rats and mice differ widely in their susceptibility to Cryptococcus infection and have been shown to have differential immune responses against infection (Goldman, *et al.,* 1994; Shao, *et al.,* 2005). In addition, Wright *et al*. (2002) used histological methods to enumerate neutrophils in the lungs during infection, which only gives a partial view of pulmonary immune responses depending on which part of the organ is sectioned. This approach is not as accurate as flow cytometric methods and myeloperoxidase measurement, which take the whole organ into account.

The differences we observed in the immune responses against *C. neoformans* and *C. gattii* may provide a clue to understanding how *C. gattii* infections are able to infect immunocompetent people. Specifically, it may be that *C. gattii* infections induce less protective inflammation in humans, as we have shown in mice, and this may be how they

are able to persist in the host. However, the interplay between host and pathogen is very complex and involves a multitude of factors on both sides. Therefore, the situation in this case may not be as simple as differences in the immune response since *C. neoformans* and *C. gattii* are different in many other ways (e.g., metabolic capabilities). Further studies are necessary in order to determine the molecular mechanisms of pathogenesis that lead to the observed differences. It is interesting to point out that a recently published case study suggests that our observations in mice may be relevant for human cases of cryptococcosis. Specifically, an HIV-seronegative patient with *C. gattii* meningitis had reduced levels of the cytokines TNF-α, IFN-γ and IL-6 associated with protective immune responses in his blood compared to patients with HIV-related *C. neoformans* infections (Brouwer, *et al.,* 2007). Although this is only a single case, the observations suggest that the patient had a maladapted immune response to *C. gattii* infection. It is likely that our study will prompt further analyses of cytokine levels in patients with *C. gattii* infections including future cases that arise from the ongoing outbreak in British Columbia.

4.2 Possible mechanisms of *C. gattii* immunomodulation

The mechanism(s) by which *C. gattii* interferes with or fails to trigger the host inflammatory immune response is unknown, but it is interesting to speculate about possible explanations for our observations. Neutrophils play an important role in the innate inflammatory immune response against infections of the lower respiratory tract by killing microbes and by producing cytokines to trigger adaptive immunity (Craig, *et al.,* 2009). Neutrophils modulate the immune response by producing cytokines such as TNF-

α and IL-6 (Shoham, *et al.,* 2001). Furthermore, neutrophil migration into the lungs during infection helps protect mice against progressive cryptococcal infection (Guillot, *et al.,* 2008). Previous studies showed that *C. gattii* strains can inhibit neutrophil migration *in vitro* (Dong and Murphy, 1995; Wright, *et al.,* 2002). Our results suggest that *C. gattii* strains may also inhibit neutrophil migration *in vivo*. Specifically, we observed that mice infected with the *C. gattii* strains had significantly lower levels of pulmonary neutrophil infiltrates compared to *C. neoformans*-infected mice and, furthermore, these neutrophil levels were not significantly different than those from uninfected mice at 24 hours, 7 and 14 days post-infection. We also observed a concurrent weak cytokine response in these *C. gattii*-infected mice. Lack of early neutrophil migration into infected lung tissues may delay the production of TNF-α, IFN-γ and important chemokines, thus preventing the induction of an effective adaptive immune response for clearance of the pathogen (Shoham and Levitz, 2005). It is possible that the lack of pulmonary neutrophil infiltration during the initial immune response may have led to the lack of protective adaptive immunity in these *C. gattii*-infected mice. Our study revealed that *C. gattii*-infected mice had significantly lower levels of pulmonary dendritic cells and neutrophils compared to *C. neoformans* infected mice. However, we did not observe corresponding differences in the levels of macrophages. Further studies are required in order to understand the significance of these results with respect to dendritic cells due to their mobile nature (trafficking from the lungs to lymph nodes) and because their maturation status is critical for determining the type of immune response they elicit. However, because there is evidence that *C. gattii* isolates have an effect on neutrophils (Wright, *et*

al., 2002), we speculate that the *C. gattii* isolates from Vancouver Island may inhibit induction of adaptive immunity via neutrophil-specific mechanisms.

4.2.1 Differences in capsule size may result in differences in the immune response.

One possible explanation for our observations involves the cryptococcal capsule. Our *in vitro* studies did not reveal any phenotypic differences among our isolates with respect to capsule size. However, we observed that cryptococci in the lungs of mice infected with the *C. neoformans* strain H99 were larger than those in mice infected with the *C. gattii* strain R265. Interestingly, this difference was only observed at 7 days post-infection and not at 2 days post-infection. A previous study by Retini et al. (1996) showed that encapsulated strains of *C. neoformans* can promote the secretion of the protective cytokines TNF-α, IL-6, IL-8 and IL-1β from human neutrophils and the magnitude of this response is dependent on capsule size (Retini, *et al.,* 1996). The idea that a larger capsule may induce higher levels of protective cytokine secretion from neutrophils suggests that the significantly higher levels of protective cytokines we observed in *C. neoformans*-infected mice at 7 days post-infection may be due to the larger cryptococci capsule observed in these mice at this time point. Furthermore, this also suggests that the lack of protective inflammation we observed in *C. gattii*-infected mice may be due to their smaller capsule size *in vivo*.

Another possible explanation for the observed differences in the cytokine response in infected mice may be related to the differential ability of *C. neoformans* and *C. gattii* strains to activate complement (Young and Kozel, 1993). Specifically, *C. gattii* does not activate the alternative complement cascade as strongly as *C. neoformans* and

one study, for example, found that *C. gattii* binds fewer C3 molecules than *C. neoformans* (Washburn, *et al.,* 1991). This difference in complement activation between *C. gattii* and *C. neoformans* may be responsible for the lack of protective cytokine production observed in *C. gattii*-infected mice because capsule-induced activation of the alternative complement pathway generates C3a and C5a. These proteins have been shown to induce cytokine production by neutrophils (Vecchiarelli, *et al.,* 1998). In other words, if there is less complement deposition on the surface of *C. gattii* cryptococci due to the smaller size of the capsule (as we observed in our studies with R265) and the capsule of these cryptococci do not activate the alternative complement pathway as vigorously as those of *C. neoformans* strains, this would result in less C3a and C5a and less neutrophil-induced protective cytokine production. Furthermore, another concurrent possibility could be that the lower levels of C3 deposition on *C. gattii* isolates *in vivo* may result in less complement-mediated killing and reduced protective inflammation. These strategies could potentially explain the lack of protective inflammation we observe in these *C. gattii*-infected mice. This strategy of immune evasion is also observed in other pathogens; for example, pathogenic strains of *Fransicella tularensis* escape complement-mediated lysis by limiting the deposition of C3 molecules on their cells (Clay, *et al.,* 2008).

4.2.2 Capsular GXM may be involved in inhibition of neutrophil migration

Cryptococcal capsule has been shown to have many immunomodulatory effects on the host immune response. In particular, capsule has been shown to inhibit neutrophil migration *in vitro*. Inhibition of neutrophil migration as a mechanism for immune

evasion is employed by many other respiratory pathogens (Craig, *et al.,* 2009). For example, the causative agent of whooping cough, *Bordetella pertussis*, produces a toxin that inhibits pulmonary neutrophil recruitment, resulting in inhibition of protective immunity against this pathogen (Kirimanjeswara, *et al.,* 2005). Previous studies have shown that the capsular components of *C. gattii* isolates are able to inhibit neutrophil migration through cell monolayers and bare filters (Dong and Murphy, 1995; Wright, *et al.,* 2002). Our results suggest that this may also be the case *in vivo*. Other studies have shown that this ability of Cryptococcus capsule to inhibition neutrophils may be due to the presence of O-acetyl groups on the major component of the capsule, glucuronoxylmannan (GXM) (Ellerbroek, *et al.,* 2004; Fujihara, *et al.,* 1997). Specifically, one study showed that de-O-acetylation of isolated fractions of GXM reduces its ability to inhibit neutrophil migration *in vitro* (Ellerbroek, *et al.,* 2004). Furthermore, free GXM has been shown to promote L-selectin shedding from neutrophils and this may interfere with migration (Dong and Murphy, 1996). These studies on the effects of O-actylation and GXM were all done on *C. neoformans* strains. Further studies are necessary in order to determine the relevance of O-acetylation in relation to the ability of *C. gattii* isolates to inhibit neutrophil migration, although it is possible that the GXM of *C. gattii* isolates has similar properties which enable it to inhibit neutrophil migration *in vivo*.

Free GXM of *C. neoformans* has different immunoregulatory properties compared with GXM assembled in the capsule structure (Vecchiarelli, 2000), which may explain how the capsule of *C. neoformans* strains may promote the production of protective cytokines as discussed in the previous section, while capsular material from these same

strains may inhibit neutrophil migration. The deleterious effects of capsular GXM on the host immune response may not be apparent until after 7 days post-infection, when the fungal load of cryptococci reaches a critical point such that GXM begins to shed from the cryptococcus capsule. This idea has been proposed by others as well to explain the inhibitory effect of free GXM on T cell maturation *in vitro* (Yauch, *et al.,* 2006). Indeed, GXM is present in the blood and cerebrospinal fluid at high concentrations in patients with cryptococcosis (Chuck and Sande, 1989). Our results support and extend these ideas. At 14 days post-infection, cytokine levels and pulmonary neutrophil levels in the *C. neoformans*-infected mice are decreased, indicating a decline in protective immune responses. Other studies have also shown that mice infected with the *C. neoformans* strain H99 initially display a protective immune response that is dampened after 7 days post-infection (Wormley, *et al.,* 2005). This dampened immune response could be due to the uncontrolled replication of cryptococci in the lungs, as observed by the increased pulmonary fungal load from 7 days to 14 days post-infection. As the cryptococcal infection is established, GXM is shed from the capsule resulting in decreased immune responses.

The idea that free GXM has an opposite effect on neutrophils compared to capsule GXM may not be true in the case of *C. gatti* isolates because our observations along with those from others show that *C. gattii* and as well as its capsule components can inhibit or fail to provoke neutrophil migration *in vitro* and *in vivo*. Further studies are required in order to understand the differences in capsule composition among the isolates of *C. gattii* and *C. neoformans* and how they may differentially influence the immune response.

4.3 *C. gattii* isolates from the Vancouver Island outbreak vary in virulence in a mouse model of infection

As part of our study, we observed differences in the virulence of *C. neoformans* and *C. gattii* strains in the C57BL/6 and A/JCr mouse models of cryptococcosis. Specifically, the *C. neoformans* strain H99 and the *C. gattii* strain R265 were similar in virulence and were both significantly more virulent than the *C. gattii* strains R272 and WM276. We also observed that in C57BL/6 mice infected with the more virulent isolates, H99 and R265, the pulmonary fungal load steadily increased from 24 hours post-infection to 14 days post-infection. These observations and our histopathology results showing cryptococcus invasion in all areas of the lung indicate that these mice likely succumbed to infection due to uncontrolled cryptococcal replication. As we discussed in the previous section, this outcome in the *C. neoformans* strain H99-infected mice was most likely due to a dampening of the immune response we observed after 7 days post-infection. As for the outcome of R265-infected mice, it may be that failure to induce a protective inflammatory response eventually resulted in uncontrolled cryptococcal replication. In contrast, the fungal load of C57BL/6 mice infected with the isolates of lower virulence (*C. gattii* strains R272 and WM276) decreased slightly after 7 days post-infection which is consistent with their lower virulence.

We also note that while A/JCr mice did not show a significant difference in susceptibility to the more virulent H99 and R265 strains, these mice appeared to be significantly more susceptible to R272 and WM276 compared to C57BL/6 mice. Host genetic background plays an important role in determining susceptibility to Cryptococcus infection (Guillot, *et al.,* 2008; Zaragoza, *et al.,* 2007), thus it may be that A/JCr mice

differ from C57BL/6 mice in some critical property which makes them more susceptible to infection with R272 and WM276.

It is interesting that these *C. gattii* isolates show different degrees of virulence *in vivo* despite not showing significant differences in their phenotypes *in vitro* or immune responses *in vivo*. We did observe delayed melanin production and weaker growth at 37°C in the *C. gattii* strain WM276. However, given that we did not observe these phenotypes in the similarly virulent *C. gattii* strain R272, these apparent defects may not play a role in the ability of WM276 to suppress protective inflammation or cause disease in our mouse model. Furthermore, the *C. gattii* strain WM276 is genetically different compared to the more closely related isolates from the Vancouver Island outbreak R272 and R265 (Kidd, *et al.,* 2004), which makes it difficult to compare it to the other *C. gattii* strains individually without further analysis. It may be that our *in vitro* screening methods of the virulence-associated phenotypes limited detection of subtle differences among the isolates. Furthermore, screening for phenotypes on laboratory media may not have revealed differences that exist during infection, which others have shown previously (Blackstock, *et al.,* 1999) and as evidenced by our observation that capsule size of *C. neoformans* cryptococci are increased during infection. Thus, we will focus on the difference in virulence observed between the two *C. gattii* isolates R265 and R272 in further discussion in the following paragraph.

The *C. gattii* strains R265 and R272 are genetically similar and represent the major and minor genotypes of isolates from the Vancouver Island outbreak, respectively (Fraser, *et al.,* 2005; Kidd, *et al.,* 2004). Previous studies have proposed that the *C. gattii* strain from the less pathogenic minor genotype (represented by R272) mated with an

unknown strain to produce the more pathogenic major genotype (represented by R265), which was introduced onto Vancouver Island and emerged as a pathogen due to its improved fitness in the new environment (Fraser, *et al.,* 2005). Based on comparative genome hybridization studies performed in our laboratory, there are approximately seven regions of difference between the R265 and R272 genomes (unpublished data). One of these regions includes a gene encoding phospholipase B (PLB1), which is present in the R265 genome, but not in the R272 genome. Furthermore, a previous study showed that *PLB1* in *C. neoformans* is required for virulence in a mouse model of cryptococcosis (Noverr, *et al.,* 2003). It is possible therefore that the reduced virulence we observed in the R272 strain of *C. gattii* can be due in part to its lack of *PLB1*. Further studies to examine these genetic regions of difference may aid in revealing the underlying mechanism of virulence in these *C. gattii* isolates.

WM276 is an environmental *C. gattii* strain originally isolated from a eucalyptus tree in Australia. We observed that in addition to its phenotypic differences on laboratory media, it also appeared to survive better in macrophage co-culture assays compared to any of the other strains tested (Appendix Figure A.1). Furthermore, colonies of this particular *C. gattii* strain showed a mucoid morphology whereas those from the other *C. gattii* strains and the *C. neoformans* strain appeared dull (Figure A.2). It is possible that mucoidy may have contributed to the ability of WM276 to survive within macrophages. A previous study showed that a clinical *C. gattii* strain was able to switch between its mucoid and non-mucoid (dull) variant and that the mucoid variant had enhanced survival within macrophages (Jain, *et al.,* 2006). Furthermore, our tests on other *C. gattii* isolates with a mucoid phenotype also confirmed this idea (Figure A.3). Studies have shown that

more virulent strains of Cryptococcus are able to replicate within macrophages and cause disease more readily than less virulent strains (Zaragoza, *et al.,* 2007). Furthermore, intracellular survival may be linked to the ability of the infection to disseminate from the lungs and spread to other organs, resulting in increased virulence (Shoham and Levitz, 2005). Thus, we speculate that in the case of WM276, its mucoid phenotype may have allowed it to survive within macrophages and disseminate to other body sites and this ability may be in part related to its virulence in our animal model. However, these results are preliminary and further studies will be needed to verify these ideas.

We note that our virulence analyses are different than those in a previous study that found that the *C. gattii* strain R265 was significantly more virulent than the *C. neoformans* strain H99 (Fraser, *et al.,* 2005). A possible explanation for this discrepancy is the use of different isolates of the strains or ages of mice because we used C57BL/6 and A/JCr mice at 12-14 weeks old whereas Fraser et al. (2005) used A/JCr mice of an unspecified age. Studies have shown that the outcome of cryptococcal infection varies greatly with respect to the age, strain and source of mice, and this may explain differences among various studies of virulence (Blackstock and Murphy, 2004a; Chen, *et al.,* 2008; Guillot, *et al.,* 2008; Zaragoza, *et al.,* 2007a).

4.4 Proposed further studies to examine the immune response to Cryptococcal infections

4.4.1 The role of Th17 immune responses in Cryptococcal infections

The Th1/Th2 paradigm has long been used to explain adaptive immunity. However, new studies have begun to shed light on a third member of the CD4+ T helper cells, the IL-17-producing Th17 cells. A Th17 immune response involves neutrophils and the production of the cytokines IL-17 and IL-23 (Aujla, *et al.,* 2007; Dubin and Kolls, 2008). We observed a strong inflammatory immune response (associated with protective immunity) in mice infected with the *C. neoformans* strain and this involved a significant increase in pulmonary neutrophils and production of cytokines such as TNF-α, IFN-γ, IL-6 and MCP-1. However, in contrast with previous studies involving *C. neoformans* infection, we did not observe significant increases in the hallmark Th1 cytokine IL-12 in these mice. The lack of IL-12 induction, together with the presence of pulmonary neutrophils and IL-6, highlights the possibility that a Th17 type immune response was induced in these *C. neoformans*-infected mice. Furthermore, one recent study showed that splenic cultures from mice resistant to *C. neoformans* infection produce elevated levels of IL-17 upon stimulation with Cryptococcus antigen (Muller, *et al.,* 2007). The Th17 cytokine IL-17A is critical for pulmonary neutrophil recruitment because it regulates CXC chemokines and has been shown to act in synergy with TNF-α in recruitment during pulmonary immune responses (Dubin and Kolls, 2008). Furthermore, in the presence of IL-6, Th0 cells differentiate into Th17 cells (Basso, *et al.,* 2009). Given that the Th17 immune response plays an important role in mediating host mucosal immunity in a variety of respiratory pathogens (Dubin and Kolls, 2008), it

would be interesting to determine if it is also important in immunity to cryptococcal infections. Understanding the type of immune response that is occurring during infection may aid in understanding how *C. gattii* strains are able to inhibit or fail to provoke protective inflammation. In order to define these immune responses in greater detail, we propose further cytokine profiling to measure the Th17 cytokines IL-17 and IL-23, as well as the Th2 cytokines IL-4 and IL-13. The role of Th17 immunity in cryptococcal infection is as yet unknown but the fact that it was recently revealed to be important in protective immunity against a number of pulmonary pathogens such as *Klebsiella pneumoniae*, *Mycoplasma pulmonis*, *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* (Dubin and Kolls, 2008) necessitates further studies into its role during cryptococcosis.

4.4.2 Further analysis of the pulmonary infiltrate in *C. gattii*-infected mice

We observed differences in dendritic cell accumulation but no differences in macrophage infiltration in the lungs of infected mice. In order to further explore the significance of this result, additional studies should be carried out to identify the maturation status of the dendritic cells and the activation status of the macrophages. Previous studies have shown that a protective immune response involves the maturation of dendritic cells and subsequent trafficking into lung-associated lymph nodes where T cell priming takes place (Lindell, *et al.,* 2006; Wozniak, *et al.,* 2006). Furthermore, inhibiting dendritic cell maturation during Cryptococcus infection can lead to abrogation of effective T cell-mediated inflammation (Vecchiarelli, *et al.,* 2003). To determine if the lack of protective inflammation we observed in *C. gattii*-infected mice is related to

the maturation status of dendritic cells, these cells from the lungs and lung-associated lymph nodes of infected mice can be stained for dendritic cell maturation markers such as CD80, CD86 and MHC-II, and analyzed by flow cytometry. Classically activated macrophages are associated with a protective immune response in Cryptococcus infection whereas alternatively activated macrophages are not (Chen, *et al.,* 2008; Olszewski, *et al.,* 2004; Osterholzer, *et al.,* 2009). Determining the activation status of macrophages by tissue sectioning of infected mouse lungs and staining for arginase will aid in the identification of the types of macrophages involved in *C. neoformans* and *C. gattii* infection. Furthermore, enumerating eosinophils in the lungs of infected mice may clarify the type of immune response elicited by *C. neoformans* and *C. gattii* infections. It may be that *C. gattii* infections induce less protective immune responses because they alter the maturation status of dendritic cells and/or they promote the development of alternatively activated macrophages and the subsequent development of a non-protective allergic eosinophilic immune response, as observed during infection of susceptible mice with highly-virulent *C. neoformans* strains (Chen, *et al.,* 2008; Osterholzer, *et al.,* 2009).

4.4.3 Protective immune responses against *C. gattii* infection

Based on our results, we concluded that *C. gattii* strains might be able to cause disease in immunocompetent people because they may actively suppress or lack factors that provoke protective inflammation during infection. The corollary would be that in the presence of a protective inflammatory immune response, a *C. gattii* infection would be cleared. In order to test this idea, mice could be pre-treated with bleomycin to induce neutrophil-mediate inflammation and then infected with *C. gattii* to determine if this type

of protective inflammation can promote clearing of the infection. The bleomycin lung injury model is well established and has been used for the study of pulmonary fibrosis (Matute-Bello, *et al.,* 2008). Intratracheal delivery of bleomycin in mice results in neutrophil accumulation in the lungs and Th1-associated cytokine production. If subsequent intranasal inoculation with *C. gattii* does not cause disease, this would support the idea that *C. gattii* strains may lack factors that provoke protective inflammation and thus are able to establish infections in immunocompetent people. Of course, the host-pathogen interaction is complex and thus the outcome of this proposed experiment would have to be cautiously interpreted and confirmed with additional work.

4.4.4 Further studies to determine the mechanisms of *Cryptococcus* immunomodulation – a biochemical approach

Previous studies showed that culture filtrate antigens from *C. gattii* can inhibit neutrophil migration *in vitro* (Dong and Murphy, 1995). However, this result has only been demonstrated so far for one strain of *C. gattii*. Studies have also shown that heat-inactivated supernatants from *C. gattii* can reduce the neutrophil superoxide response compared to those from *C. neoformans* (Wright, *et al.,* 2002). We observed that *C. gattii* infections induce significantly lower numbers of neutrophils *in vivo* compared to the *C. neoformans* infections. Determining the molecular components of *C. gattii* that may be responsible for interfering with pulmonary neutrophil accumulation will aid in understanding the overall mechanisms of *C. gattii* pathogenesis. Recent studies have shown that the presence of O-acetyl groups on the GXM of *C. neoformans* are responsible for the ability of GXM to inhibit neutrophil migration *in vitro* (Ellerbroek, *et*

al., 2004). Furthermore, previous studies have shown that the O-acetyl groups are more abundant on the GXM of *C. neoformans* isolates than those of *C. gattii* isolates, but these studies were done more than 20 years ago (Bhattacharjee, *et al.,* 1984), before the emergence of the Vancouver Island outbreak and the discovery of the *C. gattii* isolates that caused the outbreak. To determine if the concentration of O-acetyl groups on the GXM of these Vancouver Island isolates of *C. gattii* is related to our observations *in vivo*, nuclear magnetic resonance studies to analyze the acetyl content of GXM preparations from these isolates may be informative. Overall, pinpointing molecular determinants of immune evasion may aid in the development of new therapies for treating *C. gattii* infections.

4.4.5 Further studies to determine the mechanisms of *Cryptococcus* immunomodulation – a genetic approach.

A genetic approach to determining to the molecular mechanisms responsible for *C. gattii* immunomodulation may also yield interesting results. As mentioned above, previous studies have shown that the presence of acetyl groups on the GXM component of capsule from *C. neoformans* is required for interference with neutrophil migration; furthermore, the acetylation of GXM is dependent on *CAS1*, which encodes a putative glycosyltransferase (Ellerbroek, *et al.,* 2004; Janbon, *et al.,* 2001; Kozel, *et al.,* 2003). Thus far, there have not been any studies done on the *CAS1* gene in *C. gattii* isolates. It may be that differences in the expression of *CAS1* may be related to the differential ability of the *C. neoformans* and *C. gattii* isolates to inhibit neutrophil infiltration *in vivo*. Thus, we suggest analyzing the expression levels of the *CAS1* gene in different isolates of

C. neoformans and *C. gattii* by quantitative PCR. Furthermore, deletion and complementation with different copies of *CAS1* from different isolates and testing the transformants in neutrophil migration assays *in vitro* may yield more clues as to how the *CAS1* genes from *C. neoformans* and *C. gattii* can affect neutrophil migration.

4.5 Conclusions

In summary, the present study is the first to examine the immune responses elicited by infection of *C. gattii* strains from the Vancouver Island outbreak. The differences we observed in the immune responses against *C. neoformans* and *C. gattii* may provide a clue to understanding how *C. gattii* infections are able to infect immunocompromised people. Specifically, it may be that *C. gattii* infections induce less protective inflammatory responses in humans as we have shown in mice and this is how they are able to evade an effective immune response and persist in the host. Our observations, combined with those from other studies, indicate that *C. gattii* may be interfering with protective inflammation by inhibiting or failing to provoke the migration of neutrophils to the site of infection. Studies are currently in progress to further elucidate potential mechanisms for this lack of a neutrophil response.

REFERENCES

Aratani, Y., Kura, F., Watanabe, H., Akagawa, H., Takano, Y., Ishida-Okawara, A., Suzuki, K., Maeda, N., and Koyama, H. (2006). Contribution of the myeloperoxidase-dependent oxidative system to host defence against *Cryptococcus neoformans*. J. Med. Microbiol. *55,* 1291-1299.

Arora, S., Hernandez, Y., Erb-Downward, J.R., McDonald, R.A., Toews, G.B., and Huffnagle, G.B. (2005). Role of IFN-γ amma in regulating T2 immunity and the development of alternatively activated macrophages during allergic bronchopulmonary mycosis. J. Immunol. *174,* 6346-6356.

Aujla, S.J., Dubin, P.J., and Kolls, J.K. (2007). Interleukin-17 in pulmonary host defense. Exp. Lung Res. *33,* 507-518.

Banerjee, U., Datta, K., and Casadevall, A. (2004). Serotype distribution of Cryptococcus neoformans in patients in a tertiary care center in India. Med. Mycol. *42,* 181-186.

Bartlett, K.H., Kidd, S.E., and Kronstad, J.W. (2008). The emergence of *Cryptococcus gattii* in British Columbia and the Pacific Northwest. Curr. Infect. Dis. Rep. *10,* 58-65.

Basso, A.S., Cheroutre, H., and Mucida, D. (2009). More stories on Th17 cells. Cell Res. *19*, 399-411.

Bermudez, E., Mangum, J.B., Wong, B.A., Asgharian, B., Hext, P.M., Warheit, D.B., and Everitt, J.I. (2004). Pulmonary responses of mice, rats, and hamsters to subchronic inhalation of ultrafine titanium dioxide particles. Toxicol. Sci. *77,* 347-357.

Bhattacharjee, A.K., Bennett, J.E., and Glaudemans, C.P. (1984). Capsular polysaccharides of *Cryptococcus neoformans*. Rev. Infect. Dis. *6,* 619-624.

Bicanic, T., and Harrison, T.S. (2005). Cryptococcal meningitis. Br. Med. Bull. *72,* 99-118.

Blackstock, R., Buchanan, K.L., Cherniak, R., Mitchell, T.G., Wong, B., Bartiss, A., Jackson, L., and Murphy, J.W. (1999). Pathogenesis of *Cryptococcus neoformans* is associated with quantitative differences in multiple virulence factors. Mycopathologia *147,* 1-11.

Blackstock, R., and Murphy, J.W. (2004a). Age-related resistance of C57BL/6 mice to *Cryptococcus neoformans* is dependent on maturation of NKT cells. Infect. Immun. *72,* 5175-5180.

Blackstock, R., and Murphy, J.W. (2004b). Role of interleukin-4 in resistance to *Cryptococcus neoformans* infection. Am. J. Respir. Cell Mol. Biol. *30,* 109-117.

Brouwer, A.E., Siddiqui, A.A., Kester, M.I., Sigaloff, K.C., Rajanuwong, A., Wannapasni, S., Chierakul, W., and Harrison, T.S. (2007). Immune dysfunction in HIV-seronegative, *Cryptococcus gattii* meningitis. J. Infect. *54,* e165-8.

Buchanan, K.L., and Murphy, J.W. (1998). What makes Cryptococcus neoformans a pathogen? Emerg. Infect. Dis. *4,* 71-83.

Byrnes 3rd, E.J., Bildfell, R.J., Frank, S.A., Mitchell, T.G., Marr, K.A., and Heitman, J. (2009). Molecular evidence that the range of the Vancouver Island outbreak of *Cryptococcus gattii* infection has expanded into the Pacific Northwest in the United States. J. Infect. Dis. (in press).

Casadevall, A., and Perfect, J.R. (1998). *Cryptococcus neoformans* American Society for Microbiology Press, Washington, D.C.).

Casadevall, A., Cleare, W., Feldmesser, M., Glatman-Freedman, A., Goldman, D.L., Kozel, T.R., Lendvai, N., Mukherjee, J., Pirofski, L.A., Rivera, J. *et al*. (1998). Characterization of a murine monoclonal antibody to Cryptococcus neoformans polysaccharide that is a candidate for human therapeutic studies. Antimicrob. Agents Chemother. *42,* 1437-1446.

Casadevall, A., Rosas, A.L., and Nosanchuk, J.D. (2000). Melanin and virulence in Cryptococcus neoformans. Curr. Opin. Microbiol. *3,* 354-358.

Chaturvedi, V., Wong, B., and Newman, S.L. (1996). Oxidative killing of Cryptococcus neoformans by human neutrophils. Evidence that fungal mannitol protects by scavenging reactive oxygen intermediates. J. Immunol. *156,* 3836-3840.

Chen, G.H., McDonald, R.A., Wells, J.C., Huffnagle, G.B., Lukacs, N.W., and Toews, G.B. (2005). The gamma interferon receptor is required for the protective pulmonary inflammatory response to *Cryptococcus neoformans*. Infect. Immun. *73,* 1788-1796.

Chen, G.H., McNamara, D.A., Hernandez, Y., Huffnagle, G.B., Toews, G.B., and Olszewski, M.A. (2008). Inheritance of immune polarization patterns is linked to resistance versus susceptibility to *Cryptococcus neoformans* in a mouse model. Infect. Immun. *76,* 2379-2391.

Chen, G.H., Olszewski, M.A., McDonald, R.A., Wells, J.C., Paine, R.,3rd, Huffnagle, G.B., and Toews, G.B. (2007). Role of granulocyte macrophage colony-stimulating factor in host defense against pulmonary *Cryptococcus neoformans* infection during murine allergic bronchopulmonary mycosis. Am. J. Pathol. *170,* 1028-1040.

Chen, J., Varma, A., Diaz, M.R., Litvintseva, A.P., Wollenberg, K.K., and Kwon-Chung, K.J. (2008). *Cryptococcus neoformans* strains and infection in apparently immunocompetent patients, China. Emerg. Infect. Dis. *14,* 755-762.

Chuck, S.L., and Sande, M.A. (1989). Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. N. Engl. J. Med. *321,* 794-799.

Clay, C.D., Soni, S., Gunn, J.S., and Schlesinger, L.S. (2008). Evasion of complement-mediated lysis and complement C3 deposition are regulated by *Francisella tularensis* lipopolysaccharide O antigen. J. Immunol. *181,* 5568-5578.

Cox, G.M., Mukherjee, J., Cole, G.T., Casadevall, A., and Perfect, J.R. (2000). Urease as a virulence factor in experimental cryptococcosis. Infect. Immun. *68,* 443-448.

Craig, A., Mai, J., Cai, S., and Jeyaseelan, S. (2009). Neutrophil recruitment to the lungs during bacterial pneumonia. Infect. Immun. *77,* 568-575.

Decken, K., Kohler, G., Palmer-Lehmann, K., Wunderlin, A., Mattner, F., Magram, J., Gately, M.K., and Alber, G. (1998). Interleukin-12 is essential for a protective Th1 response in mice infected with *Cryptococcus neoformans*. Infect. Immun. *66,* 4994-5000.

Dong, Z.M., and Murphy, J.W. (1996). Cryptococcal polysaccharides induce L-selectin shedding and tumor necrosis factor receptor loss from the surface of human neutrophils. J. Clin. Invest. *97,* 689-698.

Dong, Z.M., and Murphy, J.W. (1995). Effects of the two varieties of *Cryptococcus neoformans* cells and culture filtrate antigens on neutrophil locomotion. Infect. Immun. *63,* 2632-2644.

Dubin, P.J., and Kolls, J.K. (2008). Th17 cytokines and mucosal immunity. Immunol. Rev. *226,* 160-171.

Ellerbroek, P.M., Lefeber, D.J., van Veghel, R., Scharringa, J., Brouwer, E., Gerwig, G.J., Janbon, G., Hoepelman, A.I., and Coenjaerts, F.E. (2004). O-acetylation of cryptococcal capsular glucuronoxylomannan is essential for interference with neutrophil migration. J. Immunol. *173,* 7513-7520.

Escandon, P., Sanchez, A., Martinez, M., Meyer, W., and Castaneda, E. (2006). Molecular epidemiology of clinical and environmental isolates of the *Cryptococcus neoformans* species complex reveals a high genetic diversity and the presence of the molecular type VGII mating type a in Colombia. FEMS Yeast Res. *6,* 625-635.

Fan, W., Kraus, P.R., Boily, M.J., and Heitman, J. (2005). *Cryptococcus neoformans* gene expression during murine macrophage infection. Eukaryot. Cell. *4,* 1420-1433.

Feldmesser, M., Kress, Y., Novikoff, P., and Casadevall, A. (2000). Cryptococcus neoformans is a facultative intracellular pathogen in murine pulmonary infection. Infect. Immun. *68,* 4225-4237.

Fraser, J.A., Giles, S.S., Wenink, E.C., Geunes-Boyer, S.G., Wright, J.R., Diezmann, S., Allen, A., Stajich, J.E., Dietrich, F.S., Perfect, J.R., and Heitman, J. (2005). Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. Nature *437,* 1360-1364.

Fujihara, H., Kagaya, K., and Fukazawa, Y. (1997). Anti-chemotactic activity of capsular polysaccharide of *Cryptococcus neoformans in vitro*. Microbiol. Immunol. *41,* 657-664.

Fyfe, M., MacDougall, L., Romney, M., Starr, M., Pearce, M., Mak, S., Mithani, S., and Kibsey, P. (2008). *Cryptococcus gattii* infections on Vancouver Island, British Columbia, Canada: emergence of a tropical fungus in a temperate environment. Can. Commun. Dis. Rep. *34,* 1-12.

Goldman, D., Cho, Y., Zhao, M., Casadevall, A., and Lee, S.C. (1996). Expression of inducible nitric oxide synthase in rat pulmonary *Cryptococcus neoformans* granulomas. Am. J. Pathol. *148,* 1275-1282.

Goldman, D., Lee, S.C., and Casadevall, A. (1994). Pathogenesis of pulmonary *Cryptococcus neoformans* infection in the rat. Infect. Immun. *62,* 4755-4761.

Gonzalez-Juarrero, M., Shim, T.S., Kipnis, A., Junqueira-Kipnis, A.P., and Orme, I.M. (2003). Dynamics of macrophage cell populations during murine pulmonary tuberculosis. J. Immunol. *171,* 3128-3135.

Guillot, L., Carroll, S.F., Homer, R., and Qureshi, S.T. (2008). Enhanced innate immune responsiveness to pulmonary *Cryptococcus neoformans* infection is associated with resistance to progressive infection. Infect. Immun. *76,* 4745-4756.

Hernandez, Y., Arora, S., Erb-Downward, J.R., McDonald, R.A., Toews, G.B., and Huffnagle, G.B. (2005). Distinct roles for IL-4 and IL-10 in regulating T2 immunity during allergic bronchopulmonary mycosis. J. Immunol. *174,* 1027-1036.

Herring, A.C., Falkowski, N.R., Chen, G.H., McDonald, R.A., Toews, G.B., and Huffnagle, G.B. (2005). Transient neutralization of tumor necrosis factor alpha can produce a chronic fungal infection in an immunocompetent host: potential role of immature dendritic cells. Infect. Immun. *73,* 39-49.

Hu, G., Cheng, P.Y., Sham, A., Perfect, J.R., and Kronstad, J.W. (2008). Metabolic adaptation in *Cryptococcus neoformans* during early murine pulmonary infection. Mol. Microbiol. *69,* 1456-1475.

Idnurm, A., Bahn, Y.S., Nielsen, K., Lin, X., Fraser, J.A., and Heitman, J. (2005). Deciphering the model pathogenic fungus *Cryptococcus neoformans*. Nat. Rev. Microbiol. *3,* 753-764.

Jain, N., and Fries, B.C. (2008). Phenotypic switching of *Cryptococcus neoformans* and *Cryptococcus gattii*. Mycopathologia *166,* 181-188.

Jain, N., Li, L., McFadden, D.C., Banarjee, U., Wang, X., Cook, E., and Fries, B.C. (2006). Phenotypic switching in a *Cryptococcus neoformans* variety *gattii* strain is associated with changes in virulence and promotes dissemination to the central nervous system. Infect. Immun. *74,* 896-903.

Janbon, G., Himmelreich, U., Moyrand, F., Improvisi, L., and Dromer, F. (2001). Cas1p is a membrane protein necessary for the O-acetylation of the *Cryptococcus neoformans* capsular polysaccharide. Mol. Microbiol. *42,* 453-467.

Jarvis, J.N., and Harrison, T.S. (2008). Pulmonary cryptococcosis. Semin. Respir. Crit. Care. Med. *29,* 141-150.

Jung, W.H., Sham, A., Lian, T., Singh, A., Kosman, D.J., and Kronstad, J.W. (2008). Iron source preference and regulation of iron uptake in *Cryptococcus neoformans*. PLoS Pathog. *4,* e45.

Khan, Z.U., Randhawa, H.S., Chehadeh, W., Chowdhary, A., Kowshik, T., and Chandy, R. (2009). *Cryptococcus neoformans* serotype A and *Cryptococcus gattii* serotype B isolates differ in their susceptibilities to fluconazole and voriconazole. Int. J. Antimicrob. Agents. (in press).

Kidd, S.E., Hagen, F., Tscharke, R.L., Huynh, M., Bartlett, K.H., Fyfe, M., Macdougall, L., Boekhout, T., Kwon-Chung, K.J., and Meyer, W. (2004). A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). Proc. Natl. Acad. Sci. U. S. A. *101,* 17258-17263.

Kirimanjeswara, G.S., Agosto, L.M., Kennett, M.J., Bjornstad, O.N., and Harvill, E.T. (2005). Pertussis toxin inhibits neutrophil recruitment to delay antibody-mediated clearance of *Bordetella pertussis*. J. Clin. Invest. *115,* 3594-3601.

Klebanoff, S.J. (2005). Myeloperoxidase: friend and foe. J. Leukoc. Biol. *77,* 598-625.

Koguchi, Y., and Kawakami, K. (2002). Cryptococcal infection and Th1-Th2 cytokine balance. Int. Rev. Immunol. *21,* 423-438.

Kozel, T.R., Levitz, S.M., Dromer, F., Gates, M.A., Thorkildson, P., and Janbon, G. (2003). Antigenic and biological characteristics of mutant strains of *Cryptococcus neoformans* lacking capsular O acetylation or xylosyl side chains. Infect. Immun. *71,* 2868-2875.

Kwon-Chung, K.J., Boekhout, T., Fell, J.W., and Diaz, M. (2002). Proposal to conserve the name *Cryptococcus gattii* against *C. hondurianus* and *C. bacillisporus* (Basidiomycota, Hymenomycetes, Tremellomycetidae). Taxon *51,* 804.

Kwon-Chung, K.J., and Rhodes, J.C. (1986). Encapsulation and melanin formation as indicators of virulence in *Cryptococcus neoformans*. Infect. Immun. *51,* 218-223.

Lendvai, N., Casadevall, A., Liang, Z., Goldman, D.L., Mukherjee, J., and Zuckier, L. (1998). Effect of immune mechanisms on the pharmacokinetics and organ distribution of cryptococcal polysaccharide. J. Infect. Dis. *177,* 1647-1659.

Lin, X., and Heitman, J. (2006). The biology of the *Cryptococcus neoformans* species complex. Annu. Rev. Microbiol. *60,* 69-105.

Lindell, D.M., Moore, T.A., McDonald, R.A., Toews, G.B., and Huffnagle, G.B. (2006). Distinct compartmentalization of CD4+ T-cell effector function versus proliferative capacity during pulmonary cryptococcosis. Am. J. Pathol. *168,* 847-855.

Lizarazo, J., Linares, M., de Bedout, C., Restrepo, A., Agudelo, C.I., Castaneda, E., and Grupo Colombiano para el Estudio de la Criptococosis. (2007). Results of nine years of the clinical and epidemiological survey on cryptococcosis in Colombia, 1997-2005. Biomedica *27,* 94-109.

MacDougall, L., Kidd, S.E., Galanis, E., Mak, S., Leslie, M.J., Cieslak, P.R., Kronstad, J.W., Morshed, M.G., and Bartlett, K.H. (2007). Spread of *Cryptococcus gattii* in British Columbia, Canada, and detection in the Pacific Northwest, USA. Emerg. Infect. Dis. *13,* 42-50.

Macura, N., Zhang, T., and Casadevall, A. (2007). Dependence of macrophage phagocytic efficacy on antibody concentration. Infect. Immun. *75,* 1904-1915.

Marroni, M., Pericolini, E., Cenci, E., Bistoni, F., and Vecchiarelli, A. (2007). Functional defect of natural immune system in an apparent immunocompetent patient with pulmonary cryptococcosis. J. Infect. *54,* e5-8.

Matute-Bello, G., Frevert, C.W., and Martin, T.R. (2008). Animal models of acute lung injury. Am. J. Physiol. Lung Cell. Mol. Physiol. *295,* L379-99.

McFadden, D., Zaragoza, O., and Casadevall, A. (2006). The capsular dynamics of *Cryptococcus neoformans*. Trends Microbiol. *14,* 497-505.

Mednick, A.J., Nosanchuk, J.D., and Casadevall, A. (2005). Melanization of *Cryptococcus neoformans* affects lung inflammatory responses during cryptococcal infection. Infect. Immun. *73,* 2012-2019.

Meyer, W., Marszewska, K., Amirmostofian, M., Igreja, R.P., Hardtke, C., Methling, K., Viviani, M.A., Chindamporn, A., Sukroongreung, S., John, M.A., Ellis, D.H., and Sorrell, T.C. (1999). Molecular typing of global isolates of *Cryptococcus neoformans* var. *neoformans* by polymerase chain reaction fingerprinting and randomly amplified polymorphic DNA-a pilot study to standardize techniques on which to base a detailed epidemiological survey. Electrophoresis *20,* 1790-1799.

Milam, J.E., Herring-Palmer, A.C., Pandrangi, R., McDonald, R.A., Huffnagle, G.B., and Toews, G.B. (2007). Modulation of the pulmonary type 2 T-cell response to *Cryptococcus neoformans* by intratracheal delivery of a tumor necrosis factor alpha-expressing adenoviral vector. Infect. Immun. *75,* 4951-4958.

Monari, C., Retini, C., Casadevall, A., Netski, D., Bistoni, F., Kozel, T.R., and Vecchiarelli, A. (2003). Differences in outcome of the interaction between *Cryptococcus neoformans* glucuronoxylomannan and human monocytes and neutrophils. Eur. J. Immunol. *33,* 1041-1051.

Muller, U., Stenzel, W., Kohler, G., Werner, C., Polte, T., Hansen, G., Schutze, N., Straubinger, R.K., Blessing, M., McKenzie, A.N., Brombacher, F., and Alber, G. (2007). IL-13 induces disease-promoting type 2 cytokines, alternatively activated macrophages and allergic inflammation during pulmonary infection of mice with *Cryptococcus neoformans*. J. Immunol. *179,* 5367-5377.

Noverr, M.C., Cox, G.M., Perfect, J.R., and Huffnagle, G.B. (2003). Role of PLB1 in pulmonary inflammation and cryptococcal eicosanoid production. Infect. Immun. *71,* 1538-1547.

Noverr, M.C., Williamson, P.R., Fajardo, R.S., and Huffnagle, G.B. (2004). CNLAC1 is required for extrapulmonary dissemination of *Cryptococcus neoformans* but not pulmonary persistence. Infect. Immun. *72,* 1693-1699.

Olszewski, M.A., Noverr, M.C., Chen, G.H., Toews, G.B., Cox, G.M., Perfect, J.R., and Huffnagle, G.B. (2004). Urease expression by *Cryptococcus neoformans* promotes microvascular sequestration, thereby enhancing central nervous system invasion. Am. J. Pathol. *164,* 1761-1771.

Osterholzer, J.J., Curtis, J.L., Polak, T., Ames, T., Chen, G.H., McDonald, R., Huffnagle, G.B., and Toews, G.B. (2008). CCR2 mediates conventional dendritic cell recruitment and the formation of bronchovascular mononuclear cell infiltrates in the lungs of mice infected with *Cryptococcus neoformans*. J. Immunol. *181,* 610-620.

Osterholzer, J.J., Surana, R., Milam, J.E., Montano, G.T., Chen, G.H., Sonstein, J., Curtis, J.L., Huffnagle, G.B., Toews, G.B., and Olszewski, M.A. (2009). Cryptococcal urease promotes the accumulation of immature dendritic cells and a non-protective T2 immune response within the lung. Am. J. Pathol. *174,* 932-943.

Retini, C., Vecchiarelli, A., Monari, C., Tascini, C., Bistoni, F., and Kozel, T.R. (1996). Capsular polysaccharide of *Cryptococcus neoformans* induces proinflammatory cytokine release by human neutrophils. Infect. Immun. *64,* 2897-2903.

Rossi, G.R., Cervi, L.A., Garcia, M.M., Chiapello, L.S., Sastre, D.A., and Masih, D.T. (1999). Involvement of nitric oxide in protecting mechanism during experimental cryptococcosis. Clin. Immunol. *90,* 256-265.

Shao, X., Mednick, A., Alvarez, M., van Rooijen, N., Casadevall, A., and Goldman, D.L. (2005). An innate immune system cell is a major determinant of species-related susceptibility differences to fungal pneumonia. J. Immunol. *175,* 3244-3251.

Shoham, S., Huang, C., Chen, J.M., Golenbock, D.T., and Levitz, S.M. (2001). Toll-like receptor 4 mediates intracellular signaling without TNF-α lpha release in response to *Cryptococcus neoformans* polysaccharide capsule. J. Immunol. *166,* 4620-4626.

Shoham, S., and Levitz, S.M. (2005). The immune response to fungal infections. Br. J. Haematol. *129,* 569-582.

Sorrell, T.C. (2001b). *Cryptococcus neoformans* variety *gattii*. Med. Mycol. *39,* 155-168.

Stuehr, D.J., and Nathan, C.F. (1989). Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J. Exp. Med. *169,* 1543-1555.

Syme, R.M., Bruno, T.F., Kozel, T.R., and Mody, C.H. (1999). The capsule of *Cryptococcus neoformans* reduces T-lymphocyte proliferation by reducing phagocytosis, which can be restored with anticapsular antibody. Infect. Immun. *67,* 4620-4627.

Tucker, S.C., and Casadevall, A. (2002). Replication of *Cryptococcus neoformans* in macrophages is accompanied by phagosomal permeabilization and accumulation of vesicles containing polysaccharide in the cytoplasm. Proc. Natl. Acad. Sci. U. S. A. *99,* 3165-3170.

Upton, A., Fraser, J.A., Kidd, S.E., Bretz, C., Bartlett, K.H., Heitman, J., and Marr, K.A. (2007). First contemporary case of human infection with Cryptococcus gattii in Puget Sound: evidence for spread of the Vancouver Island outbreak. J. Clin. Microbiol. *45,* 3086-3088.

Vecchiarelli, A. (2000). Immunoregulation by capsular components of *Cryptococcus neoformans.* Med. Mycol. *38,* 407-417.

Vecchiarelli, A., Pietrella, D., Lupo, P., Bistoni, F., McFadden, D.C., and Casadevall, A. (2003). The polysaccharide capsule of *Cryptococcus neoformans* interferes with human dendritic cell maturation and activation. J. Leukoc. Biol. *74,* 370-378.

Vecchiarelli, A., Retini, C., Casadevall, A., Monari, C., Pietrella, D., and Kozel, T.R. (1998). Involvement of C3a and C5a in interleukin-8 secretion by human polymorphonuclear cells in response to capsular material of *Cryptococcus neoformans*. Infect. Immun. *66,* 4324-4330.

Vecchiarelli, A., Retini, C., Pietrella, D., Monari, C., Tascini, C., Beccari, T., and Kozel, T.R. (1995). Downregulation by cryptococcal polysaccharide of tumor necrosis factor alpha and interleukin-1 beta secretion from human monocytes. Infect. Immun. *63,* 2919-2923.

Vermaelen, K., and Pauwels, R. (2004). Accurate and simple discrimination of mouse pulmonary dendritic cell and macrophage populations by flow cytometry: methodology and new insights. Cytometry A. *61,* 170-177.

Washburn, R.G., Bryant-Varela, B.J., Julian, N.C., and Bennett, J.E. (1991). Differences in *Cryptococcus neoformans* capsular polysaccharide structure influence assembly of alternative complement pathway C3 convertase on fungal surfaces. Mol. Immunol. *28,* 465-470.

Webert, K.E., Vanderzwan, J., Duggan, M., Scott, J.A., McCormack, D.G., Lewis, J.F., and Mehta, S. (2000). Effects of inhaled nitric oxide in a rat model of *Pseudomonas aeruginosa* pneumonia. Crit. Care Med. *28,* 2397-2405.

Wickes, B.L., Mayorga, M.E., Edman, U., and Edman, J.C. (1996). Dimorphism and haploid fruiting in *Cryptococcus neoformans*: association with the alpha-mating type. Proc. Natl. Acad. Sci. U. S. A. *93,* 7327-7331.

Williamson, P.R. (1994). Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase. J. Bacteriol. *176,* 656-664.

Wormley, F.L.,Jr, Cox, G.M., and Perfect, J.R. (2005). Evaluation of host immune responses to pulmonary cryptococcosis using a temperature-sensitive *C. neoformans* calcineurin A mutant strain. Microb. Pathog. *38,* 113-123.

Wozniak, K.L., Vyas, J.M., and Levitz, S.M. (2006). *In vivo* role of dendritic cells in a murine model of pulmonary cryptococcosis. Infect. Immun. *74,* 3817-3824.

Wright, L., Bubb, W., Davidson, J., Santangelo, R., Krockenberger, M., Himmelreich, U., and Sorrell, T. (2002). Metabolites released by *Cryptococcus neoformans* var. *neoformans* and var. *gattii* differentially affect human neutrophil function. Microbes Infect. *4,* 1427-1438.

Yauch, L.E., Lam, J.S., and Levitz, S.M. (2006). Direct inhibition of T-cell responses by the Cryptococcus capsular polysaccharide glucuronoxylomannan. PLoS Pathog. *2,* e120.

Young, B.J., and Kozel, T.R. (1993). Effects of strain variation, serotype, and structural modification on kinetics for activation and binding of C3 to *Cryptococcus neoformans*. Infect. Immun. *61,* 2966-2972.

Zaragoza, O., Alvarez, M., Telzak, A., Rivera, J., and Casadevall, A. (2007). The relative susceptibility of mouse strains to pulmonary *Cryptococcus neoformans* infection is associated with pleiotropic differences in the immune response. Infect. Immun. *75,* 2729-2739.

APPENDIX A. Cryptococcal survival during macrophage co-culture

A.1 Introduction

C. neoformans has been shown to survive within macrophages (Feldmesser, Kress, *et al.,* 2000; Tucker, and Casadevall, 2002). Studies have shown that more virulent strains of Cryptococcus are able to replicate within macrophages and cause disease more readily than less virulent strains (Zaragoza, *et al*., 2007). Furthermore, intracellular survival may be linked to the ability of the infection to disseminate from the lungs and spread to other organs, resulting in increased virulence (Shoham and Levitz, 2005). Additionally, studies have shown that a phenomenon known as phenotypic switching may be involved in intracellular macrophage survival, as demonstrated by the enhanced survival of the mucoid variant of a *C. gattii* strain within macrophages (Jain, *et al.*, 2006). Because we observed differences in the virulence of our strains of *C. neoformans* and *C. gattii*, we wanted to determine if these strains had differential ability to survive within macrophages and if survival was associated with phenotypic switching. To do these assays, we used the techniques that have been commonly employed in other studies to examine intracellular survival of Cryptococcus strains in macrophages.

A.2 Materials and Methods

This assay has been described elsewhere (Casadevall, *et al.,* 1998; Fan, *et al.,* 2005; Feldmesser, *et al.,* 2000; Macura, *et al.,* 2007; Tucker and Casadevall, 2002; Zaragoza, *et al.,* 2007b). Briefly, Cryptococcus strains were grown for 24 hours in SDB and washed three times with PBS, counted in a hemocytometer and resuspended in PBS

at a concentration of 1.0×10^6 yeast cells/mL. In addition to the *C. neoformans* and *C. gattii* strains mentioned in the body of the thesis, additional *C. gattii* strains which were previous observed to have a mucoid morphology were used. The fungal suspensions were then opsonized with 1 ug/mL of mAB18B7 (gift from Arturo Casadevall of the Albert Einstein College of Medicine), which binds to the capsular polysaccharide and is opsonic (Casadevall, *et al.,* 1998). After 20 mins of opsonization at 37°C, the fungal suspensions were washed with PBS and resuspended in complete media (DMEM medium, 10% FBS, 4 mM L-glutamine and 100 U/mL penicillin, 100 μg/mL streptomycin) at a concentration of 1.0×10^6 yeast cells/mL. RAW macrophages were seeded at 2.0×10^5 cells per well in 24-well plates in complete medium supplemented with 0.1 ug/mL IFN-γ and 1 ug/mL LPS. Three wells were assayed for each strain. To initiate the interaction, 100 uL of the fungal cell suspension or PBS (as a control) was then added to each well and plates were incubated at 37°C in an atmosphere of 5% CO_2. After 1 hour of incubation, all wells were gently washed twice with pre-warmed PBS and 1 mL of complete media was added to each well. At each time point, Cryptococcus cells were harvested by gently washing each well with distilled deionized water and macrophages were removed by gently scraping with a cell scraper, this suspension was then plated onto Sabouraud dextrose agar plates to determine CFU and colony morphology. Shiny colonies were labeled as mucoid and dull colonies labeled as non-mucoid (or dull) (Jain, *et al.,* 2006).

A.3 Results and Discussion

Surprisingly, the *C. gattii* strain WM276 appeared to survive to a greater extent during the interaction with macrophages over a 24-hour period, compared to any of the other strains tested (Figure A.1). This result was interesting because WM276 was the only strain to show differences for any of the four major cryptococcal virulence factors (Figure 3.1). However, these differences in intracellular survival did not account for the observed differences in virulence between the more virulent isolates (H99 and R265) and the less virulent ones (R272 and WM276). Furthermore, we noticed that the colony morphology of the WM276 isolates after passage through macrophages was more shiny compared to the appearance of the other strains (Figure A.2). This colony morphology has been described before as part of a phenomenon known as phenotypic switching in which a strain can switch between mucoid and dull variants; these variants are distinguished by a number of different properties (Jain and Fries, 2008). It is possible that mucoidy may have contributed to the ability of WM276 to survive within macrophages. A previous study showed that a clinical *C. gattii* strain was able to switch between its mucoid and non-mucoid (dull) variant and that the mucoid variant had enhanced survival within macrophages (Jain, Li, *et al.,* 2006). We therefore further tested our strains of interest in the macrophage co-culture assay along with other *C. gattii* isolates that had previously been shown to display a mucoid colony morphology. Our results show that the *C. gattii* strains WM276 and RV6608, both of which are environmental isolates with a mucoid colony morphology, displayed a significant enhancement in survival in macrophages compared to H99 and R265, both of which have a dull colony morphology (Figure A.3). The environmental strain with mucoid colony

morphology, KB3864, also showed enhanced survival in macrophages; however, this did not reach statistical significance. Studies have shown that more virulent strains of Cryptococcus are able to replicate within macrophages and cause disease more readily than less virulent strains (Zaragoza, *et al.,* 2007). Furthermore, intracellular survival may be linked to the ability of the infection to disseminate from the lungs and spread to other organs, resulting in increased virulence (Shoham and Levitz, 2005). Thus, we speculate that in the case of WM276, its mucoid phenotype may have allowed it to survive within macrophages and disseminate to other body sites and this ability may be in part related to its virulence in our animal model. However, given that we observed that some mucoid strains (R794) do not display enhanced survival in macrophages, mucoidy may not strictly correlate with survival in the presence of macrophages. These results are preliminary and further studies will be needed to verify these ideas.

Figure A.1 Cryptococcus growth within macrophages. Macrophages were seeded into 24-well plates and infected with *Cryptococcus* cells at an MOI of 1:2 as described in the Materials and Methods. Colony forming units (CFU) in each well after 24 hours of incubation were divided by the number of macrophage-ingested/associated *Cryptococcus* cells after 1 hour of co-incubation to obtain a fold growth number. Data are representative of three separate experiments. Results are expressed as mean ± SEM. The asterisk * $P < 0.001$ indicates the statistical difference in survival of WM276 compared to all other isolates.

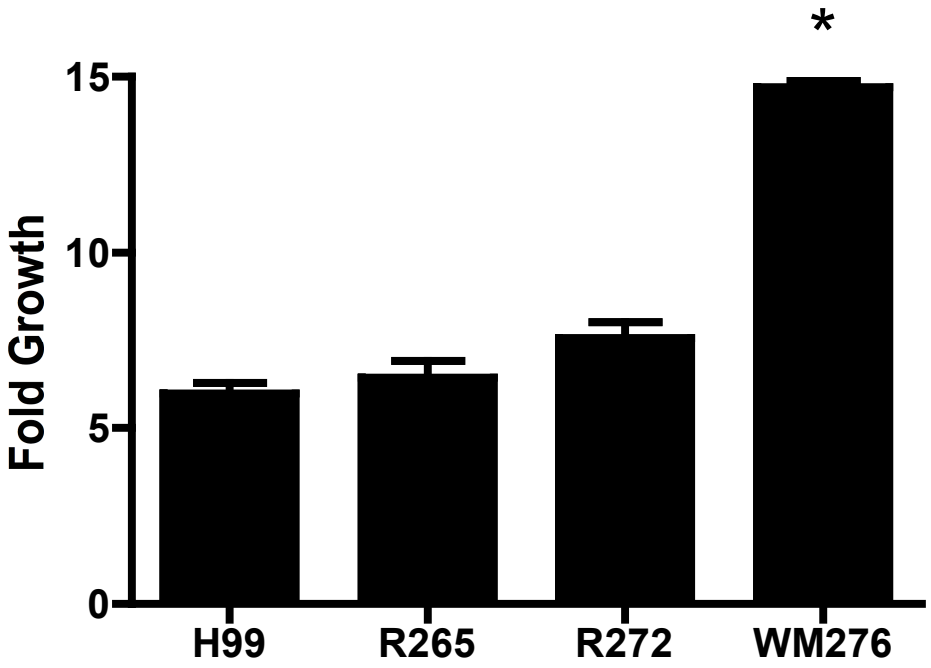


Figure A.2 Colony morphologies of *C. neoformans* and *C. gattii* strains after passage through macrophages. Macrophages were seeded into 24-well plates and infected with *Cryptococcus* cells at an MOI of 1:2 as described in the Materials and Methods. Colony morphology was examined after co-culture with macrophages for 24 hours. Note the dull colony morphology of the H99 and R265 strains compared to the shiny colony morphology of the KB3864, WM276, R794 and RV6608 strains. The results are representative of two separate experiments.

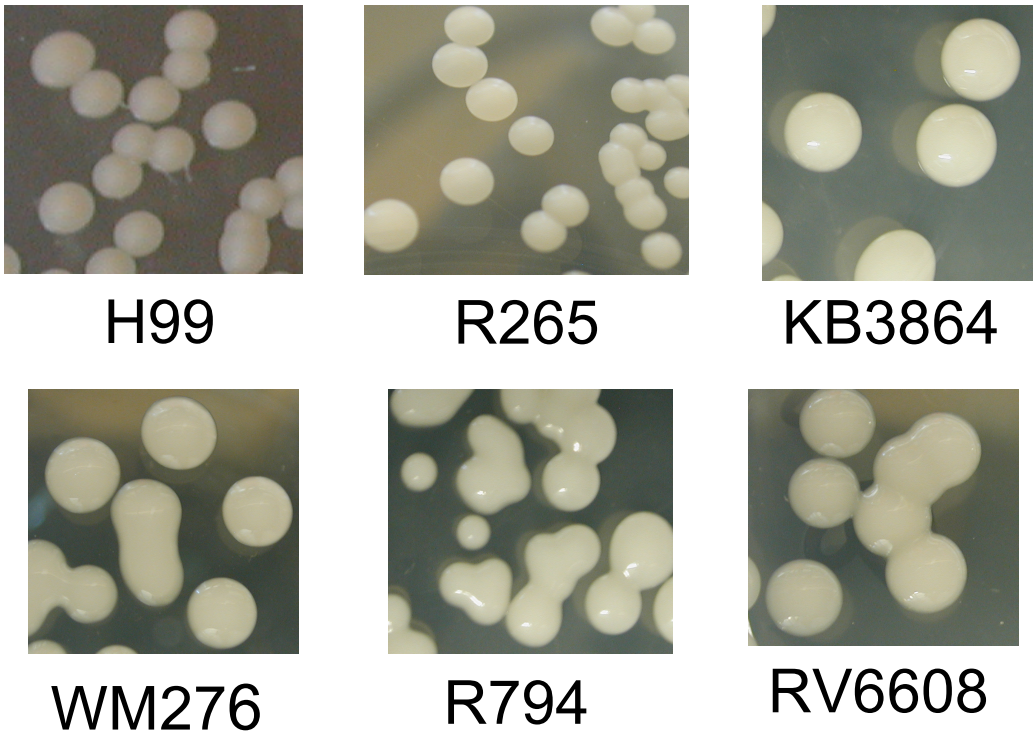
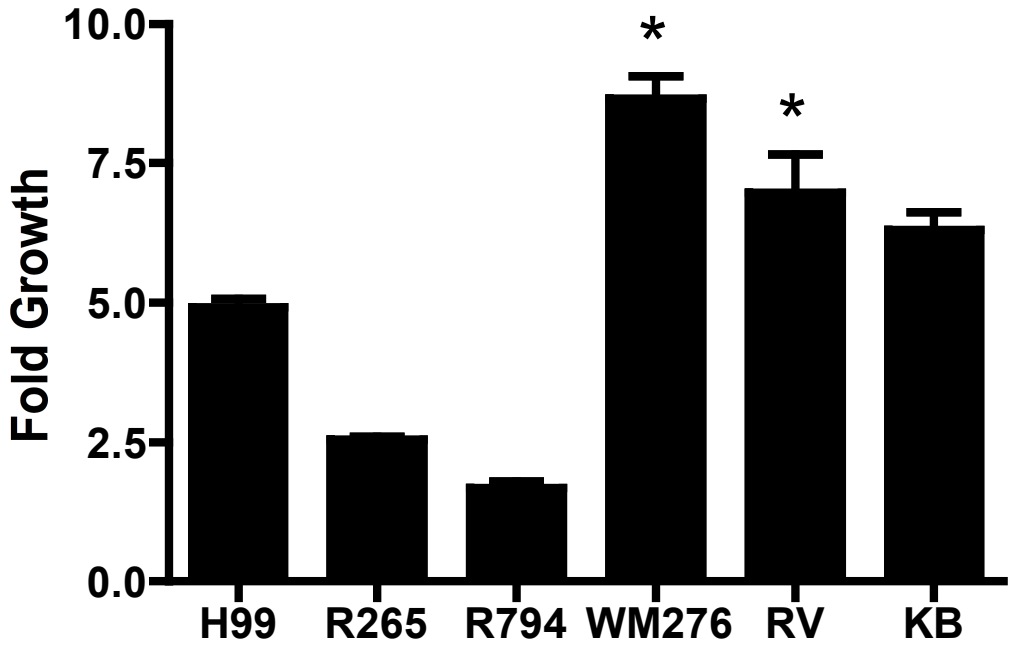


Figure A.3 Cryptococcus growth within macrophages. Macrophages were seeded into 24-well plates and infected with *Cryptococcus* cells at an MOI of 1:2 as described in the Materials and Methods. Colony forming units (CFU) in each well after 24 hours of incubation were divided by the number of macrophage-ingested/associated *Cryptococcus* cells after 1 hour of co-incubation to obtain a fold growth number. KB indicates strain KB3864, and RV indicates strain RV6608. The data are representative of two separate experiments. Results are expressed as mean ± SEM. The asterisk * $P < 0.05$ indicates the statistical difference in survival of the indicated strain compared to H99, R265 and R794.



APPENDIX B.

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0364

Investigator or Course Director: Jim W. Kronstad

Department: Michael Smith Laboratories

Animals:

Mice C57BL/6 246

Start Date: **October 1, 2007** **Approval Date:** **September 12, 2008**

Funding Sources:

Funding Agency: British Columbia Lung Association

Funding Title: Emergence of Cryptococcus gatti in British Columbia: evaluation of the immune response during pulmonary infection

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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