Université de Montréal

Immunopathogenèse de la cryptococcose chez la souris transgénique exprimant le génome du VIH-1

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RESUMÉ

La cryptococcose chez les patients atteints du VIH-1 est principalement causée par *Cryptococcus neoformans var. grubii* tandis que *Cryptococcus gattii* infecte surtout les personnes immunocompétentes. Afin d'élucider les mécanismes causant la susceptibilité différentielle à l'égard de ces deux espèces de *Cryptococcus* dans le contexte de l'infection au VIH-1, nous avons utilisé un modèle novateur de la cryptococcose chez la souris transgénique CD4C/HIV^{MutA}, qui exprime les gènes *nef*, *env* et *rev* du VIH-1. L'expression du transgène VIH-1 a augmenté le recrutement pulmonaire des macrophages alvéolaires mais a diminué celui des lymphocytes T CD4+ et CD8+ en réponse à l'infection par le *C. neoformans* ou le *C. gattii*. La production pulmonaire des chimiokines MCP-1 (CCL2) et RANTES (CCL5) était également réduite chez les souris transgéniques infectées par l'une ou l'autre de ces espèces de *Cryptococcus*. La production pulmonaire de MIP-1α, MIP-1β, TNF-α, TGF-β, IL-2, IL-4 et IL-13 était augmentée chez la souris infectée au *C. neoformans* comparativement à *C. gattii*. *In vitro*, les macrophages alvéolaires prélevés chez la souris Tg et stimulés par des agonistes ont produit davantage de MIP-1β, alors que les chimiokines MCP-1 et RANTES n'ont pas été détectées.

MOTS-CLÉS

Cryptococcose

Cryptococcus neoformans

Cryptococcus gattii

VIH-1

Souris transgénique

Cytokines

SUMMARY

The most common cause of cryptococcosis in HIV-1-infected patients is *Cryptococcus neoformans var. grubii*, while *Cryptococcus gattii* usually infects immunocompetent people. We used a novel inhalation model of cryptococcosis in CD4C/HIV^{MutA} transgenic mice expressing *nef, env,* and *rev* of HIV-1 to examine the mechanisms that cause differential susceptibility to these species of *Cryptococcus* in the context of HIV-1 infection. HIV-1 transgene expression increased alveolar macrophage but decreased pulmonary CD4+ and CD8+ T lymphocyte recruitment. Pulmonary production of the CC chemokines MCP-1 (CCL2) and RANTES (CCL5) was reduced in transgenic mice infected with *C. neoformans* or *C. gattii*, and concentrations were lower after infection with *C. gattii* compared to *C. neoformans*. Production of MIP-1α, MIP-1β, TNF-α, TGF-β, IL-2, IL-4 and IL-13 was increased in mice infected with *C. neoformans* compared to *C. gattii*. Production of MIP-1β by alveolar macrophages harvested from Tg mice was enhanced after agonist exposure *in vitro*, but production of the chemokines MCP-1 and RANTES was undetectable.

KEYWORDS

Cryptococcosis

Cryptococcus neoformans

Cryptococcus gattii

HIV-1

Transgenic mice

Cytokines

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ABBREVIATION LIST

AIDS: *acquired immunodeficiency syndrome*

APC: *antigen-presenting cell*

APP1: *anti-phagocytic protein 1*

AZT: *azidothymidine*

BBB: *blood-brain barrier*

BCCDC: *British Columbia Centre for Disease Control*

BMEC: *brain microvascular endothelial cells*

CAP: *cyclase-associated protein*

CCR2: *chemokine (C-C motif) receptor*

CD: *cluster of differentiation*

CDEA: *Comité de déontologie de l'expérimentation sur les animaux*

CFU: *colony forming unit*

CNA1: *calcineurin A 1*

CNS: *central nervous system*

CR: *complement receptor*

CSF: *cerebrospinal fluid*

CTL: *cytotoxic T lymphocyte*

DC: *dendritic cell*

DTH: *delayed-type hypersensitivity*

FACS: *fluorescence-activated cell sorting*

FIV: *feline immunodeficiency virus*

GalXM: *galactoxylomannan*

GXM: *glucuronoxylomanan*

HAART: *highly active anti-retroviral therapy*

HIV: *human immunodeficiency virus*

HOCl: *hypochlorous acid*

ICAM: *inter-cellular adhesion molecule*

IFN: *interferon*

Ig: *immunoglobulin*

IL: *interleukin*

IV: *intravenous*

JAK/STAT: *janus kinase/ signal transducer and activator of transcription*

KRPG: *Krebs ringer phosphate glucose*

LAC1: *laccase1*

L or D DOPA: *L- or D- 3, 4-dihydroxyphenylalanine*

LPS: *lipopolysaccharide*

LTA: *lipoteichoic acid*

MAIDS: *murine acquired immunodeficiency syndrome*

MALT: *mucosal-associated lymphoid tissue*

MCP: *monocyte chemotactant protein*

MHC: *major histocompatibility complex*

MIP: *macrophage inflammatory protein*

MPO: *myeloperoxidase*

MuLV: *murine leukemia virus*

MyD88: *myeloid differentiation factor 88*

NK: *natural killer*

NO: *nitric oxide*

NOS: *nitric oxide synthase*

OH: *hydroxyl radicals*

PBL: *peripheral blood lymphocytes*

PBMC: *human peripheral blood mononuclear cells*

pDC: *plasmacytoid dendritic cells*

PKA: *protein kinase A*

PKC: *protein kinase C*

PLB: *phospholipase B*

PMN: *polymorphonuclear neutrophil*

Rac1: *ras-related C3 botulinum toxin substrate 1*

RANTES: *regulated upon activation normal T-cell expressed and secreted*

Ras: *rat sarcoma*

ROM2: *RhO1 multicopy 2*

ROS: *reactive oxygen species*

SCID: *severe combined immunodeficiency*

SIV: *Simian immunodeficiency virus*

SOD: *superoxide dismutase*

TCR: *T-cell receptor*

Tg: *transgenic*

TGF: *transforming growth factor*

TLR: *toll like receptor*

TNF: *tumor necrosis factor*

TNFR: *tumor necrosis factor receptor*

UV: *ultraviolet*

WHO: *World Health Organization*

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CHAPTER 1- *CRYPTOCOCCUS* AND CRYPTOCOCCOSIS

Taxonomy and Reproduction

In 1894, an Italian scientist named Francesco Sanfelice isolated a yeast from fermenting peach juice which he called *Saccharomyces neoformans* (Bovers et al. 2008; Dixit et al. 2009; Barnett 2010). In the same year, a German professor, named Otto Busse, observed a pathogen isolated from a woman's tibia and concluded that it resembled organisms from the genus *Saccharomyces* (Barnett 2010). In 1901, Jean-Paul Vuillemin examined these cultures and placed them in the genus *Cryptococcus* because they were unable to produce ascospores, a characteristic of the *Saccharomyces* genus. Both of these cultures later became known as *C. neoformans* (Bovers et al. 2008; Barnett 2010). *C. gattii* was first isolated from a leukemia patient in 1970 (Bovers et al. 2008; Dixit et al. 2009). The genus *Cryptococcus* consists of basidiomycetous yeasts that are part of the order *Tremellales* (Loftus et al. 2005; Byrnes et al. 2009). There are currently 37 recognized species in the genus *Cryptococcus*, but only *C. neoformans, C. gattii, C. laurentii,* and *C. albidus* are pathogenic in humans and animals (Li and Mody 2010). Both *C. neoformans* and *C. gattii* are major human pathogens, while *C. laurentii* and *C. albidus* rarely cause disease in immunocompromised patients. *C. neoformans* was originally classified into four serotypes (A-D) based on capsular agglutination reactions, but due to molecular analysis serotypes B and C have been reclassified as a separate species, *C. gattii* (Li and Mody 2010). *C. neoformans* has been divided into two varieties, *C. neoformans var. grubii* (serotype A) and *C. neoformans var. neoformans* (serotype D) (Dixit et al. 2009; Li and Mody 2010). The third serotype of *C. neoformans* is the hybrid serotype AD (Dixit et al. 2009; Li and Mody 2010; Li et al. 2012). Other hybrids, such as BD and AB,

have been observed, but are extremely rare (Dixit et al. 2009). Both species have been further divided into four major molecular types, VNI-VNIV for *C. neoformans* and VGI-VGIV for *C. gattii* (Li and Mody 2010). The two most prevalent molecular types are VNI and VGI (Dixit et al. 2009). Both in nature and patients, *Cryptococcus* is most commonly found as unicellular budding yeast (Lin and Heitman 2006; Kozubowski and Heitman 2012). It has also been occasionally observed as pseudohyphae, which could serve as a strategy to avoid predators in the environment (Kozubowski and Heitman 2012). *Cryptococcus* can also be found in hyphal form.

Sexual reproduction of *Cryptococcus* has never been observed in nature or within a host, and only specific conditions in the laboratory have been able to trigger mating between compatible yeast cells (Kozubowski and Heitman 2012) (Figure 1). The perfect states of *C. neoformans* and *C. gattii*, discovered by Dr. Kwon-Chung, are named *Filobasidiella neoformans* and *F. bacilispora* respectively (Kwon-Chung 1976; Kwon-Chung 1976). Different serotypes differ in their ability to mate; most of the serotype D strains mate, while the ability of serotype A and *C. gattii* to mate is strain specific. *Cryptococcus* has a bipolar mating system, where there is only a single mating locus called the MAT locus (Kozubowski and Heitman 2012). The MAT locus of *C. neoformans*, which is greater than 100kb and codes for over 20 genes, is longer than the MAT loci of other fungi (Kozubowski and Heitman 2012). The mating type of *Cryptococcus* is called either a or α, depending on the MAT locus (Lin and Heitman 2006; Kozubowski and Heitman 2012). There is an overwhelming predominance of mating type α in the environment (98-99.9%), which could explain why sexual reproduction in nature is rare (Lin and Heitman 2006). Sexual reproduction of *Cryptococcus* begins when fusion occurs between haploid yeast MATa and MATα, to create a dikaryon (Lin and Heitman 2006)

(Figure 1). The dikaryon undergoes a dimorphic transition and becomes dikaryotic hyphae (Lin and Heitman 2006). The hyphal tips swell to create basidia, where nuclear fusion occurs (Lin and Heitman 2006). Meiosis then follows to create four haploid meiotic daughter nuclei (Lin and Heitman 2006). This leads to the production of four chains of basidiospores, which are readily aerosolized (Lin and Heitman 2006).

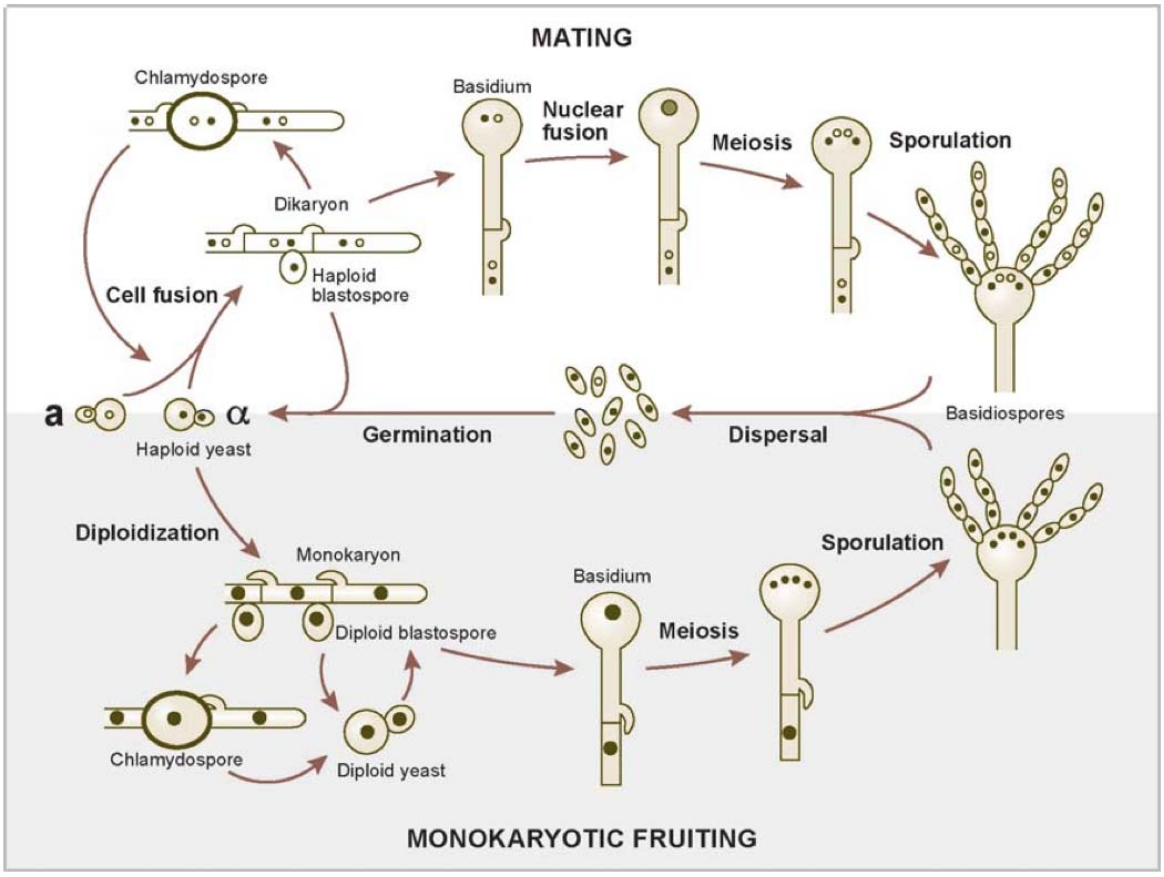

Figure 1. Mating and monokaryotic fruiting of *Cryptococcus* (Lin and Heitman 2006) Used with permission from Annual Review of Microbiology (License number 3172580616034)

C. neoformans can also undergo same-sex mating when exposed to the right conditions, also known as monokaryotic fruiting (Lin and Heitman 2006; Kozubowski and Heitman 2012)

(Figure 1). Although it was originally thought to be asexual haploid fruiting, it has later been shown to be a modified version of sexual reproduction occurring between strains of the same mating type (Lin and Heitman 2006; Kozubowski and Heitman 2012). This can occur with both a and α mating types, and similar to sexual reproduction, monokaryotic fruiting has not been observed in the environment (Kozubowski and Heitman 2012). Monokaryotic fruiting is very similar to sexual reproduction, except the hyphal cells only contain one nucleus (Kozubowski and Heitman 2012). The spores generated from monokaryotic fruiting are also smaller and rounder than those produced from sexual reproduction (Kozubowski and Heitman 2012).

Ecology and Epidemiology

In the environment, *C. neoformans* can be isolated worldwide from avian excreta and the soil surrounding it (Harrison 2000; Litvintseva and Mitchell 2009). Aged avian excreta and the soil surrounding it are more likely to contain *C. neoformans* compared to fresh avian guano (Lin and Heitman 2006; Lin 2009). *C. neoformans* thrives on the nitrogenous components associated with avian excreta (Mitchell and Perfect 1995). Although it is more commonly isolated from avian guano, *C. neoformans* has also been isolated from decaying wood and tree hollows (Harrison 2000; Lin and Heitman 2006; Litvintseva and Mitchell 2009). *Cryptococcus* can infect a wide variety of domestic and wild animals, though no transmission between animals and humans has been reported (Lin and Heitman 2006). However, substantial evidence has shown that birds, specifically pigeons, are directly linked to the worldwide distribution of *C. neoformans* (Mitchell and Perfect 1995; Lin and Heitman 2006; Lin 2009). Most evidence shows that pigeons themselves are not infected and act as carriers

(Lin and Heitman 2006; Lin 2009). Human infection usually occurs without direct contact with birds (Harrison 2000; Warkentien and Crum-Cianflone 2010). Prior to 1955, there had only been less than 300 reported cases of cryptococcosis (Perfect 2005). Currently, *C. neoformans* is responsible for an estimated one million cases a year resulting in approximately 625,000 deaths (Kronstad et al. 2011). The majority of these reported cases occur in Sub-Saharan Africa, where the number of fatal cases can surpass the number of deaths due to tuberculosis in some areas (Kronstad et al. 2011). The increase in incidence of *C. neoformans* infections can be attributed to the increased number of immunocompromised individuals, including HIV/AIDS and transplant patients (Perfect 2005; Li and Mody 2010). Approximately 90% of all cryptococcal infections and 99% of cryptococcosis cases in AIDS patients are attributed to *C. neoformans var. grubii* (serotype A) (Bovers et al. 2008; Litvintseva and Mitchell 2009). Of the patients that are HIV-uninfected, over 90% displayed some form of immunodeficiency (Li and Mody 2010). *C. neoformans var. neoformans* (serotype D) also infects mainly immunocompromised patients, but is less common worldwide and considered less virulent (Litvintseva and Mitchell 2009). In Europe, serotype D is more common and is responsible for 30% of reported cases (Bovers et al. 2008). Hybrid serotype AD has been isolated from patients in North America and Europe, but is uncommon (Litvintseva and Mitchell 2009).

Unlike *C. neoformans*, the most common environmental niche for *C. gattii* is the red gum group of eucalyptus trees, primarily *Eucalyptus camaldulensis* (Mitchell and Perfect 1995; Sorrell 2001; Lin and Heitman 2006; Dixit et al. 2009). It has also been isolated from other tree species including almond, golden shower and Douglas fir (Lin and Heitman 2006; Dixit et al. 2009). Like *C. neoformans*, *C. gattii* has been isolated from decaying trees and tree

hollows (Lin and Heitman 2006). *C. gattii* can also infect both domestic and wild animals including cats, dogs, sheep, rabbits, foxes, and koalas (Lin and Heitman 2006). There are 1.5 times more veterinary cases of *C. gattii* infections compared to human cases (Mak et al. 2010). Although *C. gattii* infections have been occasionally reported in immunocompromised patients, 70-80% of cases are associated with apparently healthy individuals (Sorrell 2001; Lin and Heitman 2006). The AIDS pandemic has not had an effect on the incidence of *C. gattii* infections (Morgan et al. 2006). *C. gattii* is endemic to tropical and subtropical regions, and a majority of reported cases *C. gattii* infections occur in Australia and Papua New Guinea (Dixit et al. 2009; Litvintseva and Mitchell 2009; Mak et al. 2010). Serotype B is more commonly isolated in clinical and environmental samples compared to serotype C (Springer and Chaturvedi 2010). Serotype C has been isolated from clinical samples from India, Africa, and Southern California, but is rarely isolated from the environment (Sorrell 2001; Springer and Chaturvedi 2010).

Beginning in 1999, there was a dramatic increase in the incidence of *C. gattii* infections, in both humans and animals, on the east coast of Vancouver Island in British Colombia, Canada (Mak et al. 2010; Kronstad et al. 2011). British Columbia Centre for Disease Control (BCCDC) recognized the increased incidence of *C. gattii* infections as an outbreak in 2002 (Hoang et al. 2004). The current incidence of *C. gattii* infections in British Columbia is 5 cases per million inhabitants, which is superior to the Australian average (0.94 cases/ million), where *C. gattii* is more common (Hoang et al. 2004; Dixit et al. 2009). The central eastern coast of Vancouver Island has the highest annual incidence rate for both animal and human cases of *C. gattii* infections (Figure 2) (Duncan et al. 2006). In the decade following the initial

outbreak, there have been 236 reported cases of *C. gattii* infections in humans, resulting in 19 deaths, as well as numerous reports of veterinary cases (Kronstad et al. 2011).

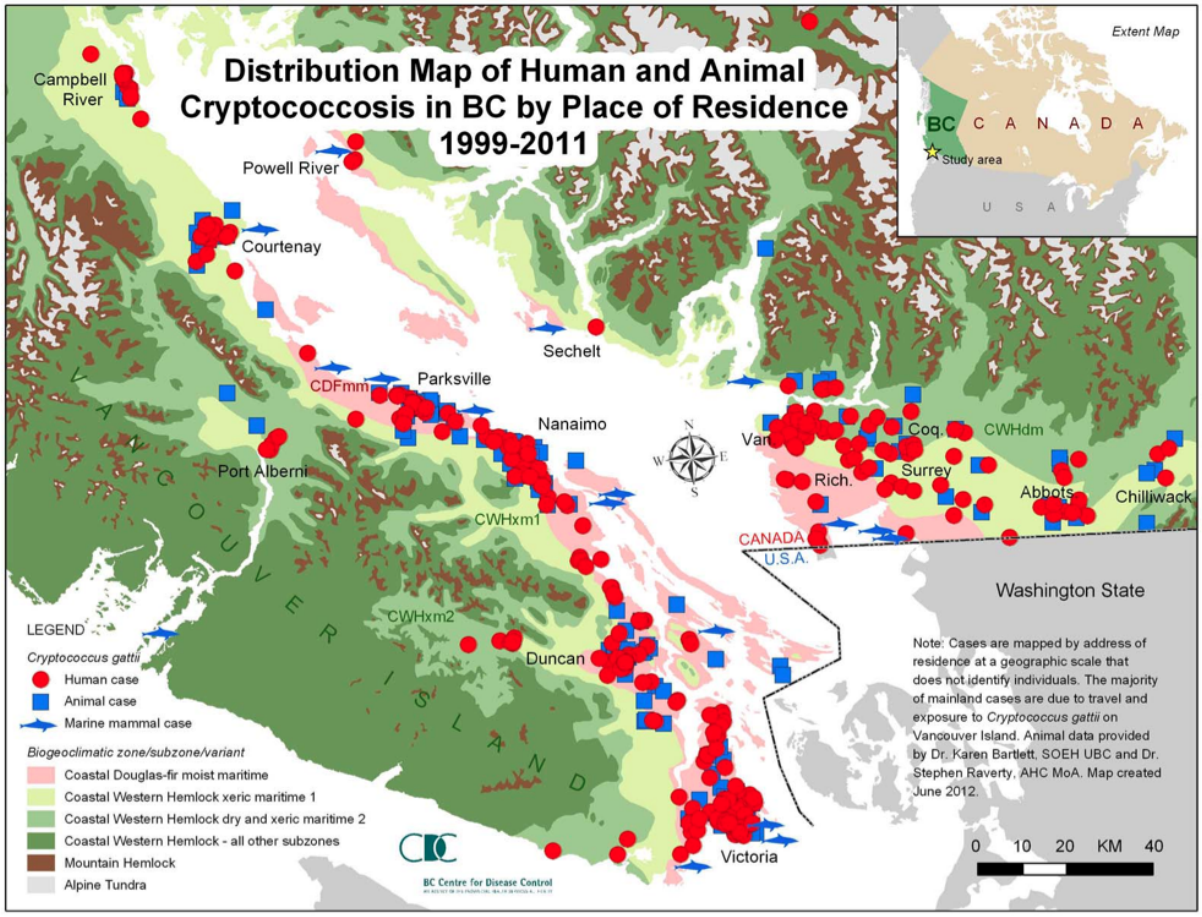

Figure 2. Distribution of reported human and veterinary cases of cryptococcosis in British Columbia between 1999 and 2011. (BC CDC)

The molecular types of *C. gattii* responsible for this outbreak are two sub-genotypes of VGII (Ngamskulrungroj et al. 2011). VGIIa is the predominant sub-genotype in both the environment and in patients, and shows a higher virulence than other *C. gattii* genotypes (Kidd, Bach, et al. 2007; Kronstad et al. 2011; Ngamskulrungroj et al. 2011). VGIIb is the minor sub-genotype and was responsible for a few cases on Vancouver Island (Kidd, Bach, et al. 2007). The original outbreak strain of *C. gattii* is R265 of the VGIIa molecular type

(Ngamskulrungroj et al. 2011). The endemic area for *C. gattii* in the Pacific Northwest has expanded past Vancouver Island; in 2004, cases of *C. gattii* infections were reported from mainland Vancouver, Oregon, and Washington (Kronstad et al. 2011). There have been approximately 60 reported cases in Washington, Oregon, Idaho, and California since 2004 (Kronstad et al. 2011). The molecular type of *C. gattii* most commonly isolated from this region is VGIIa, the same as the major type in British Columbia (Ngamskulrungroj et al. 2011). VGIIc, a new molecular type, has been isolated exclusively from Oregon (Byrnes and Heitman 2009; Datta et al. 2009). The emergence of *C. gattii* in the Pacific Northwest is thought to have partly originated from Australia because the VGIIb strain from Vancouver Island is identical to the Australian VGIIb clinical isolate NT-13 (Dixit et al. 2009). The VGIIa strain has been hypothesized to originate from South America, because VGIIa isolates from both Brazil and Vancouver Island are of mating type α (Dixit et al. 2009). Some possible methods of dispersal of *C. gattii* to the Pacific Northwest include human-mediated spread (*C. gattii* transferred by contact surfaces like shoes), passive transport by wild and domestic animals, and airborne dispersal through deforestation (Kidd, Bach, et al. 2007). *C. gattii* has been observed to be able to survive on shoes for over 144 days, but the active usage of the shoes reduced the levels of viable *C. gattii* (Kidd, Bach, et al. 2007). Samples taken from cutting down and chipping of a Red alder (*Alnus rubra*) and a Douglas fir (*Pseudotsuga menziesii*) resulted in high levels (up to 53,125 CFU/m^3) of *C. gattii* 0-15 meters above the ground (Kidd, Bach, et al. 2007).

Pathogenesis

Natural *Cryptococcus* infections begin with the inhalation of infectious propagules (Botts and Hull 2010; Kronstad et al. 2011). Desiccated yeast cells or spores are considered to be the infectious propagule for *Cryptococcus*, because their small size, 1-3 μm, allows them to be deposited more easily deep in the alveoli of the lung (Botts et al. 2009; Giles et al. 2009; Kronstad et al. 2011). Spores have been more generally accepted as the infectious propagules of *Cryptococcus* because they are more resistant to environmental stress compared to desiccated yeast cells, making them better suited for air dispersal and survival (Giles et al. 2009; Botts and Hull 2010; Kronstad et al. 2011). Purified spores have thick cell walls, which allow them to have a greater resistance to desiccation, oxidative stress, and temperature compared to desiccated yeast (Botts et al. 2009; Kronstad et al. 2011). *Cryptococcus* spores have been shown to be very infectious, having a lethal dose of as few as 500 cells in a murine model (Velagapudi et al. 2009; Kronstad et al. 2011). In the lungs, *Cryptococcus* is phagocytized by alveolar macrophages through interactions between fungal β- (1,3)- glucan and host receptors Dectin-1 and CD11b (Giles et al. 2009). *Cryptococcus* is well adapted to survive and reproduce in an acidic environment, such as the microenvironment of macrophage phagolysosomes (Levitz et al. 1999). *Cryptococcus* is able to produce extracellular vesicles, named "virulence factor delivery bags", that allow it to export protein components important to virulence outside of the cell wall (Rodrigues et al. 2007; Rodrigues et al. 2008; Oliveira et al. 2010; Kronstad et al. 2011). The major capsular polysaccharide, glucuronoxylomanan (GXM) is exported in these vesicles which are necessary for the formation of the capsule (Rodrigues et al. 2008). The enzymes laccase, which synthesizes melanin, phospholipase B, and urease are also transported in the "delivery bags" (Rodrigues et al. 2008; Kronstad et al.

2011). In mice, these "delivery bags" induce production of TNF-α, IL-10, TGF-β, and nitric oxide (NO), and alternatively activate macrophage antimicrobial activity (Oliveira et al. 2010). *Cryptococcus* is a facultative intracellular pathogen and has developed a variety of strategies to avoid being killed by macrophages (Garcia-Rodas and Zaragoza 2012). These mechanisms are separated into two groups, capsule-dependent and capsule-independent (Garcia-Rodas and Zaragoza 2012). The capsule itself acts as a physical barrier that successfully inhibits mannose-binding lectin, binding and conceals surfactant protein A binding sites, which can opsonize fungal cells (Seider et al. 2010; Garcia-Rodas and Zaragoza 2012). It is also able to bind to CD14 and both TLR2 and TLR4, which translocate NF-κB to the nucleus, inhibiting the secretion of TNF-α; this inhibition causes a deficient activation of macrophages (Garcia-Rodas and Zaragoza 2012). Glucuronoxylomannan (GXM) affects neutrophils by reducing expression of L-selectin (CD62L), thus impairing neutrophil migration; restraining neutrophil rolling on the endothelium; and inducing the loss of tumor necrosis factor receptor (TNFR), which inhibits neutrophil activation by TNF- α (Urban et al. 2006). *C. neoformans* is also able to produce enlarged "titan" cells (Garcia-Rodas and Zaragoza 2012; Okagaki and Nielsen 2012). Titan cells are 5 to 10 times larger than normal yeast cells and are characterized by an altered capsule structure, thickened cell wall, and increased ploidy (Okagaki and Nielsen 2012). Titan cells represent approximately 20% of the cryptococcal cell population during pulmonary infections (Okagaki and Nielsen 2012). These titan cells are more resistant to phagocytosis as well as oxidative stress and nitrosative antimicrobial mechanisms (Okagaki and Nielsen 2012). *Cryptococcus* is able to secrete antiphagocytic protein 1 (APP1) which binds to the complement receptors CR2 and CR3, thereby inhibiting phagocytosis mediated by these receptors (Stano et al. 2009; Garcia-Rodas and Zaragoza 2012). Recently, the

pleiotropic virulence determinant Gat201 has been shown to be important in the antiphagocytic activity of *Cryptococcus* (Garcia-Rodas and Zaragoza 2012). Mutants lacking Gat201 had a basal capsule and were more readily phagocytized than acapsular mutants (Garcia-Rodas and Zaragoza 2012).

In vitro and *in vivo, Cryptococcus* has been observed to be able to exit macrophages through phagosomal extrusion, avoiding pathogen and host cell death, as quickly as 2 hours after phagocytosis (Alvarez and Casadevall 2006). This process occurs when a mature phagosome containing *Cryptococcus* fuses with the plasma membrane and the yeast cells are expulsed into the extracellular space (Ma et al. 2006; Casadevall 2010). This mechanism is used by both *C. neoformans* and *C. gattii*, but they differ slightly from one another (Alvarez and Casadevall 2006). *C. neoformans var. grubii* has been observed being ejected as individual cells, while *C. neoformans var. neoformans* and *C. gattii* are expulsed as yeast cells accumulated in a polysaccharide matrix (Alvarez and Casadevall 2006).

Systemic dissemination of *Cryptococcus* from the lungs can occur though different mechanisms. *In vitro*, *Cryptococcus* has been shown to adhere and be internalized by pulmonary epithelial cells (Filler and Sheppard 2006). Also, human type II pneumocytes, which cover approximately 5% of the surface area of alveoli, have a receptor for GXM, which allows them to internalize *Cryptococcus* (Filler and Sheppard 2006; Zhao et al. 2010). Another mechanism *Cryptococcus* can use to disseminate from the lungs is a "Trojan horse" approach (Figure 3), in which *Cryptococcus* uses an infected phagocyte as transportation (Casadevall 2010). Infected phagocytes can then migrate through blood vessels and cross the blood-brain barrier (BBB) carrying *Cryptococcus* (Casadevall 2010). The second strategy that *Cryptococcus* can use to cross the BBB is by direct transcytosis of free yeast cells (Casadevall

2010). This is achieved when cryptococcal cells stop suddenly, without rolling or tethering, in the capillaries adjacent to the meninges, most likely due to their inability to pass through the narrow capillary (Casadevall 2010; Shi et al. 2010). Following this microembolic event, cryptococcal cells are observed crossing the capillary wall in a manner that requires the deformation of cell morphology, and is urease dependent (Casadevall 2010; Shi et al. 2010). Cryptococcal hyaluronic acid interacts with CD44 of brain microvascular endothelial cells (BMEC), which activates protein kinase C α (PKCα) (Jong, Wu, Prasadarao, et al. 2008; Jong, Wu, Shackleford, et al. 2008). PKCα in turn regulates actin rearrangement in BMEC, facilitating yeast internalization (Jong, Wu, Prasadarao, et al. 2008). It has also been shown that dual specificity tyrosine phosphorylation-regulation kinase 3 is required for internalization of *Cryptococcus*, suggesting that *Cryptococcus* may use the endocytic signaling pathway to facilitate transcytosis of BMEC (Huang et al. 2011).

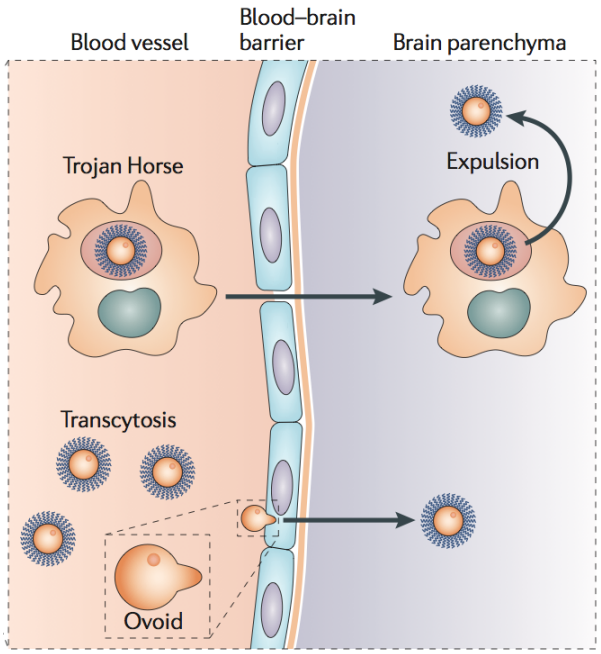

Figure 3. Cryptococcal mechanisms of crossing the blood-brain barrier (Kronstad et al. 2011). Used with permission from Nature Publishing Group (License number 3172560360717)

Virulence Factors

The ability to grow at 37°C, melanin synthesis, and the capsule are the three major virulence factors of *Cryptococcus*.

The only *Tremellales* that are capable of growing optimally at temperatures superior to 30°C are *C. neoformans* and *C. gattii* (Bovers et al. 2008). This allows *Cryptococcus* to grow in the environment of the human body which is at 37°C (Perfect 2005). Mutants of *Cryptococcus* that are unable to grow at temperatures above 30°C have been shown to be avirulent in mammalian models (Perfect 2005). Over 15 genes have been shown to be associated with high temperature growth of *Cryptococcus*, but these most likely represent a fraction of the genes necessary for growth at 37°C (Perfect 2005). Calcineurin A (CNA1) gene has been shown to

be necessary for survival at 37°C, and regulates pathogenicity (Buchanan and Murphy 1998; Alspaugh et al. 2000; Perfect 2005). Ras proteins serve as molecular switches and are implicated in the activation of many signaling pathways; *Cryptococcus* with a mutated RAS1 gene is not viable at 37°C, has a severe defect in mating and poorly adheres to the surface of the agar (Alspaugh et al. 2000). The guanine nucleotide exchange factor Cdc24, a RAS1 effector, functions in a RAS1 signaling cascade and is important for *Cryptococcus* growth at 37°C (Nichols et al. 2007). Rac1, a small G protein, interacts with Ste20, a PAK kinase, and acts downstream of Ras proteins to control both growth at elevated temperatures and cellular differentiation (Vallim et al. 2005). ROM2 is necessary for growth at 37°C because it is involved in actin and microtubule organization specifically at high temperatures (Fuchs et al. 2007).

The production of melanin, a brown or black pigment, protects *Cryptococcus* from oxidative host defenses, phagocytosis, ionizing radiation, heavy metals and UV light (Mitchell and Perfect 1995; Buchanan and Murphy 1998; Langfelder et al. 2003; Eisenman et al. 2009). Melanized *Cryptococcus* cells are also less susceptible to the antifungal drugs amphotericin B and caspofungin (van Duin et al. 2002; Walton et al. 2005). Melanin is produced in intracellular vesicles and transported to the cell wall where melanin granules are incorporated into the cell wall (Eisenman et al. 2007; Eisenman et al. 2009). Melanogenesis is accomplished when cryptococcal laccase catalyzes phenol components, such as both L- and D- 3, 4-dihydroxyphenylalanine (L- or D- DOPA) but not tyrosine, to dopaquinone (Buchanan and Murphy 1998; Eisenman et al. 2007; Frases et al. 2007). Dopaquinone is then rearranged to dopachrome and polymerized to melanin, both of which are spontaneous events (Buchanan and Murphy 1998). The brain is a tissue that is rich in phenol components, which could partly

explain why the brain is a target of *Cryptococcus* (Buchanan and Murphy 1998; Nosanchuk et al. 2000). Melanization is also dependent on other factors such as the copper transporter CC2, the copper chaperone Atx1, the chitin synthase Chs3, the transcriptional coactivator Mbf1, and the chromatin-remodeling enzyme Snf5 (Walton et al. 2005). Melanin reduces the production of TNF-α from alveolar macrophages and shields the yeast from microbicidal proteins (Jacobson 2000). Since melanin is negatively charged it effectively neutralizes neutrophil defensins as well as other cationic antimicrobial peptides (Liu and Nizet 2009). It can also reduce the amount of ferric iron, by converting Fe^{3+} to Fe^{2+}, thus improving survival of *Cryptococcus in vivo* (Liu and Nizet 2009).

The polysaccharide capsule is the most important virulence factor of *Cryptococcus*. It is responsible for inhibition of phagocytosis, alterations in cytokine secretion by leukocytes, impairment of complement recognition, resistance to NO and reactive oxygen species (ROS), reduction of antibody production and leukocyte migration, down-regulation of MHC I, II, and CD83, inducing the shedding of L-selectin from neutrophils and a non-protective Th2 response to *Cryptococcus* (Kozel et al. 1991; Buchanan and Murphy 1998; Lupo et al. 2008; Zaragoza et al. 2008; De Jesus et al. 2009). Cryptococcal polysaccharides interact with CD18 on neutrophils, inhibiting them from adhering to endothelial cells, thus inhibiting their migration to the site of infection (Dong and Murphy 1997). The capsule can also increase the expression of CTLA-4 on CD4+ T cells, which reduces the production of IFN-γ and IL-2, and inhibits T cell proliferation (Pietrella, Perito, et al. 2001). It has the ability to mask C3b and C3bi deposits, which facilitate binding of *Cryptococcus* to CR3 on leukocytes, and block the Fc fragment on antibodies, which binds to the Fc receptor on phagocytes (Buchanan and Murphy 1998).

The capsule is composed of approximately 90% glucuronoxylomannan (GXM), 7% galactoxylomannan (GalXM), and the remaining 3% is composed of mannoprotein (Bose et al. 2003). CAP genes (CAP59, CAP64, CAP60, CAP10) are all individually necessary for capsule biosynthesis (Janbon 2004; Zaragoza et al. 2009). GXM is responsible for many different pathological properties that are attributed to the capsule (Perfect 2005). GXM can interfere with E-selectin binding, as well as down-regulate C5aR on neutrophils, which inhibit migration (Ellerbroek et al. 2004; Monari, Kozel, et al. 2006). GXM is handled differently by PMNs and macrophages; in macrophages there is continuous intracellular accumulation of GXM while in PMNs it is expulsed from the cell (Zaragoza et al. 2009). This differential handling of GXM is reflected on cytokine production, where in macrophages GXM induces IL-10, IL-8, TNF-β, and IL-6; while TNF-α, IL-1β, IL-6 and IL-8 production is increased in neutrophils (Zaragoza et al. 2009). The production of TNF-β inhibits T cell proliferation and down-regulates MHC-II and B7 expression (Monari, Bistoni, et al. 2006). GXM can also induce macrophage apoptosis by dissociating the tetramers of 6-phosphofructo-1-kinase (PFK), therefore inhibiting the glycolytic pathway (Grechi et al. 2011). GXM also interacts with CD18 and FcγII, which down-regulates caspase-3 activity, promoting NO-dependent apoptosis of macrophages (Chiapello et al. 2008). GalXM is located in discrete pockets on the outer edge of the capsule, and is a transient component of the capsule (De Jesus et al. 2009). It strongly induces the production of TNF-α and NO, as well as the production of IL-6, IL-10, and IFN-γ (Pericolini et al. 2006; Villena et al. 2008; Zaragoza et al. 2009). GalXM is also able to induce Fas/ FasLigand expression, which leads to macrophage apoptosis (Villena et al. 2008). Increased Fas/FasL expression also induces apoptosis of T lymphocytes by activating caspase-8 (Pericolini et al. 2006). Mannoprotein induces an increase in TNF-α production,

and regulates the expression of other cytokines in monocytes such as IL-12, IL-6, IL-8, IFN-γ, and IL-10 (Zaragoza et al. 2009). Mannoprotein 4 reduces the expression of L-selectin and TNF receptor on neutrophils (Coenjaerts et al. 2001).

The ability of *Cryptococcus* to secrete extracellular enzymes, laccase, phospholipase, and urease, is an important contribution to its virulence (Kronstad et al. 2011). Only *C. neoformans* and *C. gattii* produce laccase (Chan and Tay 2010). Laccase is important to virulence because it facilitates the production of melanin (Buchanan and Murphy 1998; Frases et al. 2007). The two conditions that induce LAC1 are a low concentration of glucose or a high concentration of copper (Zhu and Williamson 2004). IPC1, GPA1, MET3, and STE12 are all involved in the regulation of laccase (Noverr et al. 2004). Laccase is able to oxidize phagosomal iron in macrophages, limiting the formation of hydroxyl radicals (Liu et al. 1999). *Cryptococcus* also secrets phospholipases, including lysophospholipase, lysophospholipase transacylase, and phospholipase B (PLB), which are most active between 25°C and 40°C (Santangelo et al. 1999). The activation of SEC14 is required for the secretion of PLB (Chayakulkeeree et al. 2011). PLB can hydrolyze the phospholipids PG and DPPC, the most common components of lung surfactant, which facilitates adherence of *Cryptococcus* to lung epithelial cells and dissemination (Santangelo et al. 1999; Djordjevic 2010). In macrophages, the disruption of the phagolysosome membrane by PLB1 is required for non-lytic extrusion of *Cryptococcus* (Djordjevic 2010; Chayakulkeeree et al. 2011). The enzyme urease is a key virulence factor, because it has been shown to increase the accumulation of immature dendritic cells and induce a non-protective Th2 response (Osterholzer, Surana, et al. 2009; Li and Mody 2010). Urease also promotes microvascular sequestration, enhancing the ability of *Cryptococcus* to invade the central nervous system (CNS) (Olszewski et al. 2004).

Cryptococcus also has other elements that play a minor role in virulence. *Cryptococcus* encounters a hostile nutrient environment in the phagosomes of macrophages, and in order to survive *Cryptococcus* undergoes autophagy (Hu, Hacham, et al. 2008). Autophagy is the recycling of the cells` cytoplasm and defective organelles in order to survive in high stress conditions (Hu, Hacham, et al. 2008; Palmer et al. 2008). PI3K signaling is required in *Cryptococcus* to survive during nutrient-deprived conditions; it also plays a role in vesicular transportation of vacuolar hydrolases (Hu, Hacham, et al. 2008; Palmer et al. 2008). The interconversion of CO_2 and HCO_3 is catalyzed by carbon anhydrases, which allow *Cryptococcus* to regulate CO_2 levels (Elleuche and Poggeler 2010). Adenylyl cyclase helps regulate CO_2 concentrations by acting as a CO_2 chemosensor (Klengel et al. 2005). Glucosylceramide has been shown to be associated with the cell wall, and allows *Cryptococcus* to survive in alkaline conditions (Rhome et al. 2007). Gcn5, a histone acetyltranferase, facilitates survival at high temperatures, decreases sensitivity to oxidative stress, and is important in capsule attachment to the cell surface (O'Meara et al. 2010). The sexual mating type of *Cryptococcus* plays a role in virulence, and the majority of clinical samples are MATα (Nielsen et al. 2005; Li and Mody 2010). The STE12α gene exists only in MATα cells, and is involved in capsule and melanin production (Chang et al. 2000). Ctr2 regulates copper homeostasis and is important in the production of the polysaccharide capsule and inhibition of phagocytosis (Chun and Madhani 2010). The PKC1 protein and its downstream components are essential for cryptococcal defense against nitrosative and oxidative stresses, as well as playing a role in temperature sensitivity, capsule production, and the synthesis of melanin (Gerik et al. 2008). *C. neoformans* produces D-mannitol, which protects the yeast cells from free radicals (Niehaus and Flynn 1994; Guimaraes et al. 2010).

Superoxide dismutase (SOD) is an enzyme that has the ability to neutralize toxic levels of superoxide radicals by converting them into hydrogen peroxide and oxygen (Cox et al. 2003). Copper and zinc SODs are vital for the survival of *C. gattii* in neutrophils, and in the expression of laccase, urease, and phospholipase (Narasipura et al. 2003). The trehalose synthesis pathway, controlled by synthesis genes TPS1 and TPS2, regulates protein secretion, mating, and cell wall integrity in *C. gattii* (Ngamskulrungroj et al. 2009).

Clinical Manifestations

The majority of humans have already been exposed to *Cryptococcus* before the age of 5 years (Bovers et al. 2008). Humans frequently come in contact with *Cryptococcus*, but immunocompetent individuals are able to either clear it or it remains latent (Bovers et al. 2008). The incubation period for *C. neoformans* infection is on average 110 months, while the incubation of *C. gattii* from Vancouver Island was determined to have a shorter incubation period of between 2 and 11 months, with the average being 6 or 7 months (MacDougall and Fyfe 2006; Kidd, Chow, et al. 2007). A majority of cryptococcosis patients are immunodeficient, including patients with AIDS, organ transplant recipients (primarily kidney and liver), patients receiving immunosuppressive medications, and patients with diabetes, or an autoimmune disease (Shirley and Baddley 2009; Li and Mody 2010; Pfaller and Diekema 2010; Warkentien and Crum-Cianflone 2010). Interestingly, it is extremely rare for a patient with either cancer or bone marrow transplant to be infected with *Cryptococcus* (Pukkila-Worley and Mylonakis 2008). The age of the patient also seems to be a factor in *Cryptococcus* infections. Children are rarely affected by *Cryptococcus*, and the incidence of cryptococcosis in children with AIDS is extremely low at around 1% (Subramanian and

Mathai 2005; Severo et al. 2009; Pfaller and Diekema 2010). The mean age of *Cryptococcus*-infected HIV-negative individuals is ≥45 years (Pfaller and Diekema 2010). The sex of the individual also seems to be an important risk factor as males are 3 times more likely to be infected by *Cryptococcus* than females (Chen et al. 2000; Subramanian and Mathai 2005; Li and Mody 2010). A retrospective study done by Subramanian et al. showed that, out of the 105 cryptococcosis cases in their center, 90% of the patients were male (Subramanian and Mathai 2005).

Cryptococcosis is caused by *C. neoformans* and *C. gattii* and most commonly affects the lungs and the brain (Sorrell 2001). It is considered to be the most common cause of fungal meningitis (Sorrell 2001). The site, degree of severity of cryptococcal infection, and the health status of the patient can affect the clinical manifestations of cryptococcosis, ranging from being asymptomatic or a cough to meningoencephalopathy or even death (Li and Mody 2010). There are numerous signs and symptoms of cryptococcal infection, and they do not all necessarily manifest in every case (Li and Mody 2010). These include: cough, headache, fever, nausea, chest pain, loss of weight, profound hearing or visual loss, altered mental state, and coma (Pappalardo and Melhem 2003; Black and Baden 2007; Baddley et al. 2008; Costa et al. 2009; Li and Mody 2010). Chest X-rays are performed when the patient displays pulmonary disease. Chest X-ray findings in cryptococcosis include solitary or multiple small nodules (60-80% of cases), which resemble those of tuberculosis (Subramanian and Mathai 2005; Shirley and Baddley 2009). The diagnosis is then confirmed through biopsy, cultures of bronchoalveolar lavage and cerebrospinal fluid (CSF), and detection of *Cryptococcus* polysaccharide in serum or CSF (Goldman et al. 1995; Subramanian and Mathai 2005). The lungs are primarily infected by *Cryptococcus* since they are the portal of entry (Dixit et al.

2009; Li and Mody 2010; Kronstad et al. 2011). Immunocompetent patients are normally able to contain *Cryptococcus* infection in the lungs (Shankar et al. 2007). Dissemination through the bloodstream most commonly leads to infection of the CNS, but *Cryptococcus* can also infect other organs including the skin, eyes, prostate, liver, urinary tract, bones, mucus membranes (mouth, larynx and anal region), and joints (Subramanian and Mathai 2005; Dixit et al. 2009; Li and Mody 2010). Dissemination from the lungs to other organs occurs in 50% of immunocompromised patients (Shankar et al. 2007). A meningoencephalitis occurs when *Cryptococcus* disseminates to the brain, and predominantly occurs in AIDS patients (Li and Mody 2010; Pfaller and Diekema 2010). Dissemination to the brain is more common with *C. gattii* than with *C. neoformans* (Galanis et al. 2009). On chest X-rays, *C. gattii* appears as large inflammatory masses while *C. neoformans* presents as small pulmonary lesions (Severo et al. 2009). *C. gattii* can also be differentiated from *C. neoformans* because of its slightly greenish coloration when growing on creatinine dextrose bromothymol blue thymine medium, while *C. neoformans* develops as either bright red colonies (serotype D) or pale colonies (serotype A) (Irokanulo et al. 1994).

Treatment

Untreated cases of cryptococcal meningitis have a mortality rate of 100% (Pfaller and Diekema 2010). In the 1950's, the introduction of amphotericin B monotherapy (0.4 mg/kg/day), given intravenously (IV) for six weeks, improved the cure rate of cryptococcal meningitis to over 50%, but dose-related nephrotoxicity was a frequent adverse event (Subramanian and Mathai 2005; Pfaller and Diekema 2010). To decrease toxicity, liposomal amphotericin B was developed, and has been shown to be safe and effective, although more

costly than regular amphotericin B (Subramanian and Mathai 2005). Flucytosine monotherapy has been previously used, but usually results in *Cryptococcus* developing resistance (Subramanian and Mathai 2005). The combination of amphotericin B (0.5-1 mg/kg/day) or liposomal amphotericin B (3-4 mg/kg/day) with oral flucytosine (150 mg/kg/day) for 2 weeks (induction phase) followed by oral fluconazole (400 mg/day) maintenance therapy for 10 weeks is now the standard treatment regimen for cryptococcal meningitis (Subramanian and Mathai 2005; Perfect et al. 2010; Pfaller and Diekema 2010). This combination therapy has shown sustained clearance of *Cryptococcus* from the CNS (60%) compared to amphotericin B monotherapy (51%) (Subramanian and Mathai 2005). In the case of mild to moderate pulmonary infections the recommended treatment is fluconazole (200-400 mg/day) for up to 36 months (Subramanian and Mathai 2005; Ritter and Goldman 2009; Perfect et al. 2010). Certain patients cannot tolerate fluconazole; these patients are then treated with itraconazole (200-400 mg/day) for 4-12 months (Subramanian and Mathai 2005; Ritter and Goldman 2009). Itraconazole is hydrophobic, thus the drug is accumulated in the host cells and reduces its penetration of the CNS (Subramanian and Mathai 2005; Gomez-Lopez et al. 2008). AFR1 efflux pumps of *C. neoformans* have been shown to be important to its ability to become resistant to azoles (Morschhauser 2010). Cryptococcosis patients with AIDS are given chronic suppressive therapy with fluconazole (200 mg/day) to prevent relapse (Subramanian and Mathai 2005). In Africa, amphotericin B is not readily available, thus patients are treated with fluconazole monotherapy, with a clinical cure rate of 63% in AIDS patients (Subramanian and Mathai 2005). Dosage of fluconazole can be increased to 800 mg/day for patients who show no improvement (Subramanian and Mathai 2005). If the infection is persistent, then the induction phase can be reinstituted for a longer period, as well

as increasing the dosage for amphotericin B and flucytosine (Perfect et al. 2010). Also, recombinant IFN-γ treatment (100 μl/m^2 for adults who weigh over 50 kg) 3 times a week for 10 weeks can be added to the patients' treatment (Perfect et al. 2010). Dexamethasone, a corticosteroid, has been used to treat persistent *C. gattii* infections (75%), where patients present with worsening mental status and/or inflammatory lesions on brain images (Phillips et al. 2009). Interestingly, radioimmunotherapy has been shown to be more effective than amphotericin B by almost completely eliminating *Cryptococcus* from the lungs and brain in mice (Bryan et al. 2010).

CHAPTER 2- HOST IMMUNE RESPONSE TO *CRYPTOCOCCUS*

Immune cell response to *Cryptococcus*

Alveolar Macrophages

The first immune cells exposed to *Cryptococcus* following its inhalation into the lungs are alveolar macrophages (Osterholzer, Milam, et al. 2009; Garcia-Rodas and Zaragoza 2012). Macrophages are derived from granulocyte/macrophage progenitor cells in the bone marrow (Goldsby and Goldsby 2003). Progenitor cells differentiate into pro-monocytes and enter the circulation, where they mature into monocytes (Goldsby and Goldsby 2003). Monocytes leave the circulation and then differentiate into different phenotypes according to their location: osteoclasts in bones, Kupffer cells in the liver, microglia in neural tissue, histiocytes in connective tissue, and alveolar macrophages in the lungs (Goldsby and Goldsby 2003). Phagocytosis by macrophages can be initiated through adherence to a microorganism, viral particles, or through the use of antibodies or complement particles that act as opsonins (Goldsby and Goldsby 2003).

Macrophage recognition normally occurs through antibodies that are bound to *Cryptococcus* and bind to the Fcγ receptors, or by complement component C3b which binds to CR3 on macrophages; however, it has been shown that alveolar macrophages do not need opsonins to phagocytize *Cryptococcus* (Casadevall and Pirofski 2005). Macrophages have been shown to recognize GXM of the cryptococcal capsule (Chang et al. 2006). Chitosan, which is present in the cell wall of *Cryptococcus*, can also be recognized by macrophages and this interaction induces an inflammatory response (Gorzelanny et al. 2010). Other than direct antifungal activity, macrophages have an assortment of roles, which include antigen presentation, polysaccharide sequestration, and cytokine and chemokine production (He et al. 2003). There

is an increased production of MCP-1, which stimulates recruitment of monocytes and T cells to the site of inflammation, and TNF-α, which promotes DC cell migration from tissues to lymph nodes, induces chemokines, and up-regulates antigen presentation by macrophages in response to *Cryptococcus* (Herring et al. 2002; He et al. 2003). Macrophages can be classically activated by IFN-γ, which induces an anti-cryptococcal Th1 response, or they can be alternatively activated by IL-4, prompting a non-protective Th2 response (Arora et al. 2011). If the concentrations of both IL-4 and IFN-γ are relatively equal, macrophages are intermediately activated and express both nitric oxide synthase (Th1 response) and arginase (Th2 response) (Arora et al. 2011). The activation phenotype of macrophages is important, because it predicts fungal clearance or persistence (Hardison, Ravi, et al. 2010). Classically activated macrophages also produce IL-6 and IL-23, which are important for differentiating naïve CD4+ T lymphocytes into the Th17 subset (Hardison, Wozniak, et al. 2010). Activation markers, MHC-II, adhesion molecule ICAM-1 and FcγR, are all up-regulated when macrophages are exposed to *Cryptococcus* (Kawakami et al. 1994). In both rat and rabbit models, alveolar macrophages showed increased levels of oxidative metabolism, phagocytosis, as well as lower pH in phagolysosomes as soon as 24 h after exposure to *Cryptococcus* (Gross et al. 1997; Nessa et al. 1997). Macrophages are also able to produce reactive oxygen intermediates and nonoxidative mediators, which can kill *Cryptococcus* (Schop 2007). After phagocytosis of *Cryptococcus*, macrophages fail to respond as well to chemokines, which allows the host to contain the infected macrophages and reduce dissemination (Luo et al. 2009).

Dendritic Cells

Dendritic cells (DC) are, along with alveolar macrophages, one of the first immune cells to interact with *Cryptococcus* (Osterholzer, Milam, et al. 2009). There are two major types of DCs: myeloid DCs, which are important for antigen presentation and the induction of the adaptive immune response, and plasmacytoid DCs, which play a role in antiviral immunity and produce type I IFN (Ueno et al. 2010). Immature DCs capture foreign antigens through phagocytosis, receptor-mediated endocytosis, or pinocytosis; they then mature and migrate to secondary lymphoid organs in order to present the antigen to T lymphocytes (Goldsby and Goldsby 2003; Andersson et al. 2008). All dendritic cell populations constitutively express MHC class I and II as well as the co-stimulatory molecules, CD80 and CD86 (Goldsby and Goldsby 2003). They also express CD40, which activates T and B lymphocytes by interaction with CD40L (Goldsby and Goldsby 2003).

DCs have the capacity to perform phagocytosis and kill *Cryptococcus*, but require the use of opsonins, either complement or antibodies (Wozniak and Levitz 2008). Following phagocytosis, *Cryptococcus* is processed via the endocytic pathway for presentation with MHC-II (Wozniak and Levitz 2008). In a murine model, DCs have been shown to be necessary for survival to a cryptococcal infection (Osterholzer, Milam, et al. 2009). In DC-depleted mice, death was caused by a massive accumulation of neutrophils and B lymphocytes that causes neutrophil bronchopneumonia, cyst formation, and alveolar damage (Osterholzer, Milam, et al. 2009). The co-stimulation of CD40 and CD40L is necessary for an efficient immune response to *Cryptococcus*, because it regulates cytokine production, T cell activation, CD28 co-stimulatory molecule expression, and NO_2^- production (Chen et al. 2010). Pulmonary recruitment of DC is dependent on CCR2, and up-regulation of its ligands MCP-1

and MCP-3 (Osterholzer et al. 2008). Both macrophages and DCs have mannose receptors and FcγR II, required for the uptake of *Cryptococcus*, but DCs are more efficient at presenting antigens to T lymphocytes (Syme et al. 2002). The interaction of cryptococcal mannoproteins with mannose receptor CD206, CD209 (DC-SIGN), and FcγRII of dendritic cells induces differentiation of naïve T lymphocytes towards a Th1 response, through the increased expression of maturation markers CD80, CD86, MHC II, and co-stimulatory molecule CD40, which increases IL-12 production (Mansour et al. 2002; Syme et al. 2002; Dan et al. 2008). In a murine model, DCs originating from the bone marrow can recognize *C. neoformans* with TLR9 and TLR2, but not TLR4, and activate the MyD88 pathway, leading to the production of IL-12 and IL-23 (Netea et al. 2004; Yauch et al. 2004; Biondo et al. 2005). GXM can associate with TLR4 and CD14, but DC activation is incomplete and does not stimulate TNF-α production (Shoham et al. 2001).

Neutrophils

Neutrophils are formed in the bone marrow through hematopoiesis and circulate in the peripheral blood (Goldsby and Goldsby 2003). Upon inflammatory stimulation, neutrophils rapidly migrate to infected tissue sites (Zhang et al. 2005). The lung vasculature contains approximately 40% of the total body's polymorphonuclear neutrophil (PMN) population, although only a few PMNs can be observed in the alveolar space in normal states (Zhang et al. 2005).

PMN recruitment is vital to the protective immune response to *Cryptococcus* infections (Ye et al. 2001). *Cryptococcus* stimulates the production of IL-17 by T lymphocytes, which recruits PMNs to the site of infection and enhances neutrophil phagocytosis and antimicrobial

respiratory burst (Ye et al. 2001; Hardison, Wozniak, et al. 2010). PMNs have been shown to be able to phagocytize *Cryptococcus* (Kozel et al. 1987). Mannose binding lectin, a carbohydrate-binding protein, activates the lectin pathway of complement, as well as increases PMN phagocytosis of acapsular strains of *Cryptococcus* (van Asbeck et al. 2008). PMNs can also directly kill *Cryptococcus* through the use of oxidative and non-oxidative mechanisms (Mambula et al. 2000). Myeloperoxidase (MPO) is found in PMNs and, in the presence of hydrogen peroxide, produces hypochlorous acid (HOCl) and hydroxyl radicals (OH), which can kill *Cryptococcus* but requires a higher concentration than bacteria (Chaturvedi et al. 1996; Mambula et al. 2000; Aratani et al. 2006). PMNs have three major elements that have antimicrobial activity: primary and secondary granules and cytoplasm (Mambula et al. 2000). Granules fuse with phagosomes, after phagocytosis, and release their contents on the pathogen (Mambula et al. 2000). Primary granules contain the antimicrobial substances: defensins, elastase, cathespsin G, collagenase, proteinase 3, bacterial permeability factor, and azurocidin (Mambula et al. 2000). Lysozyme and lactoferrin are the antimicrobial proteins in secondary granules. The cytoplasm contains the zinc-binding protein, calprotectin (Mambula et al. 2000). Of these substances, calprotectin, lysozyme, lactoferrin, and defensins inhibit or kill *Cryptococcus* (Mambula et al. 2000).

CD4+ T lymphocytes

CD4+ T lymphocytes circulate in the blood and lymph nodes until they are activated through contact with an antigen by antigen-presenting cells (Goldsby and Goldsby 2003). CD4+ T lymphocytes can then differentiate into either Th1, Th2, Th17, or Treg sub-populations (Goldsby and Goldsby 2003). The Th1 sub-population secretes IL-12, IFN-γ, and TGF-β; it is

also responsible for activating CD8+ cytotoxic lymphocytes and delayed type hypersensitivity (DTH) (Goldsby and Goldsby 2003). Th2 lymphocytes secrete IL-4, IL-5, Il-6, and IL-10 and also activate B lymphocytes (Goldsby and Goldsby 2003). The Th17 sub-population is involved in inflammatory responses, auto-immune diseases, and resistance to pathogens (Goldsby and Goldsby 2003; Harrington et al. 2005). Tregs have a strong immunosuppressive activity (Honda et al. 2011). CD4+ T cells located in secondary lymphoid tissue are specialized for proliferation, but are poor effectors, while CD4+ T lymphocytes from the site of infection readily produce effector cytokines, but lack proliferative capacities (Lindell et al. 2006). In pulmonary *C. neoformans* infections, CD4+ T lymphocytes are recruited to the lungs through MCP-1 (Huffnagle et al. 1995). CD4+ T lymphocytes are able to kill *C. neoformans* through granulysin, which is dependent on IL-2, STAT5, and PI3K (Zheng et al. 2007; Xing et al. 2010). Activated CD4+ T lymphocytes that are present in the CNS play an important role in leukocyte accumulation at this site (Buchanan and Murphy 1998). CD4+ T lymphocytes are involved in the formation of granulomas, which traps *Cryptococcus* in multinucleated giant cells, and helps prevent dissemination (Hill 1992).

Th1, Th2, Th17 response

Th1 cell-mediated immunity is driven by the production of IFN-γ, TNF-α, and IL-12 (Herring et al. 2002; Arora et al. 2011). The Th1 response results in leukocyte recruitment and the production of granulomas, containing classically activated macrophages, promoting cryptococcal clearance (Huffnagle et al. 1995; Jain et al. 2009). CCR2 is required for the promotion of Th1 differentiation in the lymph nodes in response to *C. neoformans* (Traynor et al. 2002). TNF-α, one of the first cytokines produced by activated macrophages, is necessary

for driving the production of IFN-γ by APCs and IL-12 by T and NK cells, which induces a protective Th1 response (Herring et al. 2002). Higher concentrations of IFN-γ and TNF-α, as well as G-CSF and IL-6, in the cerebrospinal fluid (CSF) resulted in a faster decline in cryptococcal CFUs in the CSF (Siddiqui et al. 2005).

Contrary to Th1 responses, Th2 immune responses are non-protective (Jain et al. 2009). Th2 responses are driven by the production of IL-4, IL-5, and IL-13 and are characterized by pulmonary eosinophilia, alternatively activated macrophages, increased mucus production, and elevated airway hyperactivity (Muller et al. 2007; Jain et al. 2009). Th2-activated macrophages have significantly lowered anticryptococcal activities, and they also have a lower rate of cryptococcal expulsion, compared to Th1- or Th17-activated macrophages (Voelz et al. 2009). GM-CSF, which activates macrophages and increases their antifungal and antibacterial activities, plays a dual role in the immune response to *C. neoformans* (Chen et al. 2007). It induces the production of Th2 cytokines, producing a nonprotective Th2 response, but also stimulates the production of Th1 cytokines, producing a protective Th1 response (Chen et al. 2007). Th2 responses are responsible for inhibiting anticryptococcal functions, thus promoting a chronic *Cryptococcus* infection (Voelz et al. 2009; Voelz and May 2010).

The Th17 immune responses are characterized by the secretion of IL-17, IL-6, and TNF-α, which leads to an increased inflammatory response and clearance of *C. neoformans* in the lungs (Kleinschek et al. 2006; Kleinschek et al. 2010). IL-23, secreted by macrophages and DCs, stabilizes the differentiation of Th17 CD4+ T lymphocytes during the immune response (Kleinschek et al. 2010). IL-13 expression down-regulates the Th17 response and induces a non-protective Th2 immune response (Muller et al. 2007). Both Th1 and Th17 cells are essential to the anticryptococcal immune response by decreasing cryptococcal intracellular

proliferation and expulsion, but have no effect on dissemination (Voelz et al. 2009; Zhang et al. 2009).

CD8+ T lymphocytes

CD8+ T lymphocytes are antigen specific cytotoxic cells that must first be activated by an antigen-presenting cell (Goldsby and Goldsby 2003). The two mechanisms that CD8+ T lymphocytes use to kill a pathogen are by either mediating cell death through the Fas/FasL pathway, or through the production of cytotoxic proteins, such as perforin and granulysin (Waring and Mullbacher 1999; Goldsby and Goldsby 2003). Anticryptococcal activity due to CD8+ T lymphocytes, in humans, is attributed to the secretion of granulysin (Ma et al. 2002). Granulysin secretion is dependent on presentation of cryptococcal mitogen to CD4+ T lymphocytes, which activates accessory cells such as monocytes or macrophages (Ma et al. 2002). The accessory cells produce IL-15, activating CD8+ T lymphocytes to produce granulysin (Ma et al. 2002). The JAK/STAT pathway was also found to be necessary for granulysin production in response to IL-15 and IL-21 (Oykhman and Mody 2010). Granulysin causes cell death by interacting with lipids in the cell membrane and also by activating lipid-degrading enzymes (Ma et al. 2002; Oykhman and Mody 2010).

Natural killer cells

Natural killer (NK) cells are important during the early immune response to *Cryptococcus* due to their ability to directly kill *Cryptococcus* without the help of accessory cells (Marr et al. 2006). After the direct contact of NK cells and *Cryptococcus*, NK cells are activated and undergo degranulation and release granules containing perforin and granzyme A, B, and H

(Marr et al. 2009). Perforin is necessary for NK cell anticryptococcal activity (Marr et al. 2009). Following direct contact with *Cryptococcus*, NK cells produce IFN-γ inducing the transcription of perforin (Marr et al. 2006; Marr et al. 2009). Perforin secretion is dependent on activation of ERK1 and ERK2 through the PI3K pathway (Wiseman et al. 2007). The interaction between NK cells and *Cryptococcus* also inhibits the production of GM-CSF and TNFα (Murphy et al. 1997). NKT cells, which express both T cell and NK cell receptors, interact with *C. neoformans* and produce IFN- γ, increasing the protective Th1 response (Kawakami 2004).

B lymphocytes

B lymphocytes have been shown to play an important role against *Cryptococcus* in the absence of T lymphocytes, or T lymphocyte impairment, in the brain (Aguirre and Johnson 1997). The *Cryptococcus* capsule has been shown to stimulate B lymphocytes, but this interaction is not accompanied by increased antibody production (Rodrigues et al. 2005). IgM+ memory B lymphocytes are essential to encapsulated pathogens, due to their production of TNF-α, IFN-γ, and IL-12 (Subramaniam et al. 2009). Antibody production by B lymphocytes plays a role in the cryptococcal immune response, but its protective effect is dependent on the quantity, specificity, and isotype composition of the antibody, and also the susceptibility of *Cryptococcus* (Casadevall and Pirofski 2005). Antibodies can be specific to the capsule, proteins, and mannoproteins of *Cryptococcus* (Casadevall and Pirofski 2005; Robertson and Casadevall 2009). Antibodies specific to GXM play a role in the inhibition of biofilm formation (Robertson and Casadevall 2009). They can also bind to extracellular GXM, preventing GXM from inhibiting leukocyte recruitment (Casadevall and Pirofski 2005).

IgG1 bound to the cell wall of *C. neoformans* caused changes in lipid metabolism and produced specific differences in the pattern of phosphorylated proteins, which resulted in increased susceptibility to amphotericin B (McClelland et al. 2010). Antibodies also act as opsonins, with complement components, due to the fact that macrophages phagocytize *Cryptococcus* only in the presence of opsonins (Macura et al. 2007).

Protective and Non-protective Cytokine Response to *Cryptococcus*

IL-1β

The expression of IL-1β is induced by the activation of NF-κB, and is synthesized by monocytes, macrophages, and dendritic cells as an inactive precursor molecule (proIL-1β) (Fettelschoss et al. 2011; Contassot et al. 2012). ProIL-1β is processed by a protease, usually caspase-1, to the active form of IL-1β (Fettelschoss et al. 2011). IL-1β plays a critical role in the inflammatory response (Fettelschoss et al. 2011; Contassot et al. 2012). GXM induces the production of IL-1β in both monocytes and neutrophils (Zaragoza et al. 2009).

IL-2

IL-2 is produced by recently activated T-cells (Goldsby and Goldsby 2003; Malek 2003). The main function of IL-2 is to induce the production of Tregs and the differentiation of CD4+ T lymphocytes to effector T subsets following antigen-mediated activation (Malek 2003; Boyman and Sprent 2012). IL-2 also plays a role in NK cell activation and proliferation, and B cell proliferation (Goldsby and Goldsby 2003).

IL-4

IL-4 is produced by both Th2 lymphocytes and mast cells (Goldsby and Goldsby 2003). It induces the differentiation of naïve CD4+ T lymphocyte to the Th2 subset (Goldsby and Goldsby 2003). IL-4 is also the first signal in the induction of IgE synthesis by B cells (Jabara et al. 1991). The secretion of IL-4 also induces the development of alternatively activated macrophages (Byers and Holtzman 2011).

IL-6

IL-6 is a pleiotropic cytokine that has a wide range of activities in inflammation, hematopoiesis, immune regulation, and ontogenesis (Furuya et al. 2010). It was originally known as B cell differentiation factor, because it induces the final maturation of B cells into antibody-producing B cells (Kishimoto et al. 1995). It is produced by both macrophages and endothelial cells (Goldsby and Goldsby 2003). The production of IL-6 can be induced by all three capsule components of *Cryptococcus* (Zaragoza et al. 2009).

IL-10

IL-10 is an anti-inflammatory cytokine produced by both myeloid cells and lymphocytes, that can have effects on both the innate and adaptive immune responses (Trinchieri 2007; Bolpetti et al. 2010). IL-10 has the ability to inhibit the pro-inflammatory cytokine production of APCs, which can suppress the function of NK cells and T lymphocytes (Trinchieri 2007). All three components of the *Cryptococcus* capsule have the ability to induce IL-10 production (Zaragoza et al. 2009).

IL-12

Macrophages and DCs produce IL-12 (Goldsby and Goldsby 2003). IL-12 is a heterodimer and is essential for an immune response against cancer cells and microbial pathogens (Minkis et al. 2008). It can induce a Th1 response, as well as enhance the cytotoxic activities of NK cells and CD8+ T lymphocytes (Wang et al. 2000; Minkis et al. 2008). The mannoprotein component of the capsule of *Cryptococcus* can induce the production of IL-12 (Zaragoza et al. 2009).

IL-13

IL-13 closely resembles IL-4, in that they both are important factors in up-regulating Th2 responses, and they both use IL-4Rα-chain as a receptor (de Vries 1998; Muller et al. 2007). IL-13 also down-regulates the Th17 response, alternatively activates macrophages, and modulates goblet cell function (Muller et al. 2007). IL -13 is mainly secreted by CD4+ Th2 lymphocytes, but can also be produced by other T-cell subsets and DCs (de Vries 1998; Zhu et al. 1999)

IL-17A

IL-17A is mostly produced by Th17 lymphocytes, but can also be produced by γδ and CD8+ T lymphocytes and NKT cells (He et al. 2006; Xu and Cao 2010; Pappu et al. 2011). Both IL-21 and IL-23 play important roles in the maintenance of Th17 lymphocytes and IL-17A production (Pappu et al. 2011). IL-21 stimulation of Th17 cells leads to the upregulation of the expression of IL-23R; IL-23 then interacts with IL-23R on Th17 lymphocytes and induces the production of IL-17A as well as other effector cytokines (Pappu et al. 2011; Siakavellas

and Bamias 2012). IL-17A acts as a pro-inflammatory cytokine, and can induce the production of other cytokines, chemokines, antimicrobial peptides, thereby regulating neutrophil mobilization (Xu and Cao 2010; Pappu et al. 2011). *In vitro* experiments have shown that treatment with IL-17A reduced *C. neoformans* proliferation and expulsion from macrophages, compared to IL-4 or IL-13 treated macrophages (Wozniak et al. 2011).

IL-21

IL-21 is mainly produced by CD4+ T lymphocytes, but also by NKT and CD8+ T lymphocytes under certain conditions (Yi et al. 2010). IL-21 affects both the innate and adaptive immune responses by activating macrophages, CD8+ T lymphocytes, and NK cells, development of Th17 lymphocytes, and inducing the differentiation of B cells to plasma cells (Yi et al. 2010).

TNF-α

The primary source of tumor necrosis factor alpha (TNF-α) is activated macrophages, but it can also be produced by activated T lymphocytes (Goldsby and Goldsby 2003; De Paepe et al. 2012). TNF-α activates macrophages, T and B lymphocytes, and neutrophils (Goldsby and Goldsby 2003; De Paepe et al. 2012). It also induces the production of other cytokines and cell adhesion molecules, and upregulates NF-κB signaling pathways (De Paepe et al. 2012). *Cryptococcus* inhibits the activation of neutrophils by TNF-α by inducing the loss of TNFR on neutrophils (Urban et al. 2006). The capsule components of *Cryptococcus* induce production of TNF-α (Zaragoza et al. 2009). Production of TNF-α is also induced in macrophages when

they are incubated with cryptococcal "virulence factor delivery bags" *in vitro* (Oliveira et al. 2010).

TGF-β

Transforming growth factor β (TGF-β) is produced by many cell types including macrophages and T lymphocytes (Goldsby and Goldsby 2003; Kubiczkova et al. 2012). TGF-β is one of the most potent regulators of immune cells (Kubiczkova et al. 2012). It is involved in B lymphocyte maturation and differentiation including stimulating isotype switching to IgA, inhibiting the transferrin receptor, and inducing MHC-II expression (Lebman and Edmiston 1999; Goldsby and Goldsby 2003; Kubiczkova et al. 2012). TGF-β also serves to differentiate naïve CD4+ T lymphocytes to the Th17 and Treg subsets (Jin et al. 2009). In macrophages and monocytes, TGF-β plays a suppressive role, and can inhibit cell proliferation and down-regulate the production of NO and ROS (Kubiczkova et al. 2012). Cryptococcal extracellular vesicles induce the production of TGF-β when incubated with macrophages *in vitro* (Oliveira et al. 2010). Macrophages activated through the alternate pathway are also able to produce TGF-β (Arora et al. 2011).

IFN-γ

Interferon-γ (IFN- γ) is secreted by Th1 lymphocytes, CD8+ T lymphocytes, and NK cells (Szabo et al. 2002; Goldsby and Goldsby 2003). IFN- γ is a hallmark Th1 cytokine and it activates macrophages through the classical pathway (Szabo et al. 2002). It also increases the expression of MHC I and II, which increase antigen presentation (Goldsby and Goldsby 2003). It has been shown that IFN- γ knockout mice have a higher fungal burden than wild

type mice infected with *C. neoformans* (Voelz et al. 2009; Voelz and May 2010). Both GalXM and mannoproteins can induce production of IFN- γ (Zaragoza et al. 2009).

MIP-1α (CCL3)

Macrophage inflammatory protein 1α (MIP-1α) is produced mainly by macrophages, but can also be produced by monocytes, DCs, and lymphocytes (Cook 1996; Maurer and von Stebut 2004). MIP-1α has a wide variety of effects on both the adaptive and innate immune responses, and induces the recruitment of CD8+ T and B lymphocytes, monocytes, eosinophils, and NK cells (Cook 1996). It can also stimulate basophils to release histamines, the degranulation of mast cells, and the production of TNF-α, IL-1, and IL-6 (Cook 1996). Human microglial cells have been shown to produce MIP-1α when they are exposed to *C. neoformans*, *in vitro* (Goldman et al. 2001). Sixteen days after being infected with *C. neoformans*, MIP-1α knockout mice have seven times the organ fungal burden compared to wild type mice (Olszewski et al. 2000).

MIP-1β (CCL4)

Macrophages are mostly responsible for the production of macrophage inflammatory protein-1β (MIP-1β), but it can also be secreted by DCs, monocytes and lymphocytes (Maurer and von Stebut 2004). It has some of the same effects as MIP-1α, for example, they both induce the recruitment of monocytes and NK cells (Cook 1996; Maurer and von Stebut 2004). MIP-1β also induces the recruitment of CD4+ T lymphocytes (Cook 1996). Exposure to *C. neoformans* can induce MIP-1β production in both HIV- positive and negative human peripheral blood mononuclear cells (PBMC) (Huang and Levitz 2000).

MCP-1 (CCL2)

Monocyte chemotactant protein 1 (MCP-1) can be produced by epithelial cells, endothelial cells, fibroblasts and alveolar macrophages in the lungs (Huffnagle et al. 1995). Monocytes, astrocytes, mesangial, and microglial cells can also produce MCP-1 (Deshmane et al. 2009). MCP-1 induces the recruitment of monocytes, T lymphocytes, and NK cells to the site of infection (Huffnagle et al. 1995; He et al. 2003; Deshmane et al. 2009). Rats infected with *C. neoformans* had increased amounts of MCP-1 in the lungs (He et al. 2003). There are increased levels of MCP-1 in the bronchoalveolar lavage fluid of mice infected with *C. neoformans*, which correlate with elevated levels of CD4+ T lymphocytes (Huffnagle et al. 1995).

RANTES (CCL5)

Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) was originally considered to be secreted only by T lymphocytes, but it is now known that epithelial cells, platelets, macrophages, and monocytes also produce RANTES (Crawford et al. 2011; Qian et al. 2011). RANTES induces the recruitment of Th1 lymphocytes, macrophages, eosinophils, DCs, and NK cells (Murooka et al. 2008; Crawford et al. 2011). HIV-positive PBMC infected with *C. neoformans* showed an increase in the production of RANTES, while HIV-negative PBMC had no detectable production of RANTES (Huang and Levitz 2000).

Toll-like receptors 2 and 4

Toll-like receptors (TLR) are innate immune-pattern recognition receptors that recognize molecules that are broadly shared by pathogens (Goldsby and Goldsby 2003). Signals that are transduced through TLRs usually result in the activation of transcription, synthesis, and secretion of pro-inflammatory cytokines (Goldsby and Goldsby 2003). TLR2 and TLR4 activate macrophages and DCs, and can induce the production of several cytokines (Goldsby and Goldsby 2003). LPS is recognized by both TLR2 and TLR4, but lipoproteins, cell-wall components of Gram-positive bacteria, and the yeast cell wall component zymosan are recognized by TLR2 (Goldsby and Goldsby 2003). Myeloid differentiation factor 88 (MyD88) is a TLR-associated protein that, when activated, leads to the activation NF-κB and MAPK (Biondo et al. 2005). The activation of NF-κB and MAPK subsequently leads to the production of cytokines and increased expression of MHC and co-stimulatory molecules (Shoham et al. 2001; Biondo et al. 2005). The cryptococcal capsule component GXM has been shown to bind to both TLR2 and TLR4 (Shoham et al. 2001; Roeder et al. 2004; Yauch et al. 2004). In C57BL/6 mice, it has been shown that both TLR2 and MyD88, but not TLR4, critically contribute to the anti-cryptococcal immune response by inducing the production of TNF-α, IFN-γ, and IL-12 (Biondo et al. 2005).

CHAPTER 3- HIV AND HIV INFECTION

According to World Health Organization`s (WHO) Global HIV/AIDS Response Progress Report 2011, at the end of 2010, there were an estimated 34 million people infected with HIV globally, of whom 3.4 million were children less than 15 years of age. Approximately two-thirds of all people infected with HIV reside in Sub-Saharan Africa. Annually, the number of newly infected individuals has decreased, from 3.1 million in 2001 to 2.7 million in 2010. AIDS, caused by HIV-1, was first reported in 1981 in Los Angeles, San Francisco and New York. HIV-1 is most commonly transmitted through sexual contact, but can also be transmitted by receiving contaminated blood, blood products, and drug injections, and from mother to infant (Goldsby and Goldsby 2003; Forsman and Weiss 2008).

Human Immunodeficiency Virus type 1 (HIV-1)

HIV-1 is a member of the genus *Lentivirus*, part of the *Retroviridae* family, which are capsular viruses containing two copies of single-stranded RNA (Goldsby and Goldsby 2003) (Figure 4). There are three known groups of HIV-1: major (M), outlier (O), and non-M and non-O (N), and >90% of infections worldwide are caused by group M subtypes (clades A-J) (Vasan et al. 2006). Clade B is responsible for the majority of cases that occur in North America and Europe (Forsman and Weiss 2008). The 9.2-kb ssRNA of HIV-1 contains 9 different genes (Goldsby and Goldsby 2003). The three major genes are *gag*, *env*, and *pol*, which encode polyprotein precursors that are cleaved to produce the nucleocapsid core proteins, envelope glycoproteins, and enzymes required for replication. *Gag* encodes the precursor gag polyprotein that is processed during maturation by viral protease to create p17, which forms the outer core-protein layer; p24, which forms the inner core-protein layer; p9, a component of

the nucleoid core; and p7, which binds directly to the viral genomic RNA (Goldsby and Goldsby 2003). The *env* gene encodes a 160-kDa precursor, and is cleaved by cellular protease to create gp41, a transmembrane protein associated with gp120 and required for fusion; and gp120, a surface lipoprotein that binds to CD4 (Goldsby and Goldsby 2003). The pol precursor, encoded by *pol* gene, is also cleaved by viral protease and produces the following enzymes: p64, which has reverse transcriptase and RNase activity; p51, that also has reverse transcriptase activity; p10, a protease that cleaves the gag precursor; and p32, an integrase (Goldsby and Goldsby 2003). The *tat*, *rev*, and *nef* genes all encode regulatory proteins that play a vital role in controlling viral gene expression (Goldsby and Goldsby 2003). P14, encoded by the *tat* gene, strongly activates the transcription of proviral DNA (Goldsby and Goldsby 2003; Pugliese, Vidotto, Beltramo, Petrini, et al. 2005). The *rev* gene encodes p19 that allows the export of unspliced and singly spliced mRNAs from the nucleus of the target cell. *Nef* encodes p27 which downregulates both host-cell MHC class I, class II and CD4 (Goldsby and Goldsby 2003). *Vif* and *vpu* both encode proteins that are required for maturation of HIV-1. *Vif* encodes p23, which promotes maturation and the infectivity of the virus, while *vpu* encodes p16 (Goldsby and Goldsby 2003). P16 is necessary for viral assembly and budding, promotes the extracellular release of viral particles, and degrades CD4 in the endoplasmic reticulum (Goldsby and Goldsby 2003). Finally, *vpr* encodes p15, a protein that promotes nuclear localization of preintegration complex and inhibits cell division (Goldsby and Goldsby 2003).

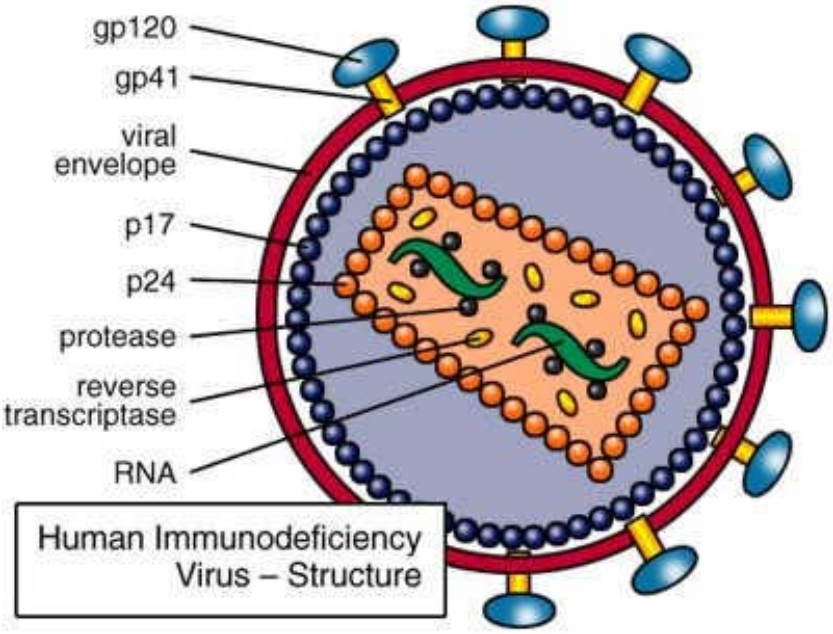

Figure 4. Human Immunodeficiency Virus (HIV) structure (from www.avert.org/hiv-virus.htm)

HIV-1 Replication

To complete replication, HIV-1 must first attach itself to the target cell (Goldsby and Goldsby 2003). HIV-1 attaches to CD4 T lymphocytes through the CXCR4 receptor, and to macrophages and dendritic cells through the CCR5 receptor (Goldsby and Goldsby 2003; Vasan et al. 2006). Gp120 of HIV-1 must also be engaged to CD4 on the target cell in order to initiate fusion of the viral envelope with the host cell membrane (Goldsby and Goldsby 2003). Gp41 from HIV-1, and either CXCR4 or CCR5 from the target cell, mediate fusion allowing the nucleocapsid, containing the viral genome and enzymes, to enter the target cell (Goldsby and Goldsby 2003). The viral genome and enzymes are released following the dissolution of the capsid. The viral reverse transcriptase then initiates reverse transcription of the ssRNA creating a RNA-DNA hybrid (Goldsby and Goldsby 2003). The RNA is partially

degraded by ribonuclease H, allowing for the synthesis of the second strand of DNA (Goldsby and Goldsby 2003). The double-stranded HIV DNA is translocated and integrated into the host chromosomal DNA by p32, a viral integrase enzyme, thus forming a provirus (Goldsby and Goldsby 2003). The provirus can remain latent in the host cell genome until events in the cell trigger its activation. Transcription of the proviral DNA leads to the formation of several mRNAs and genomic ssRNA (Goldsby and Goldsby 2003). Viral RNA is exported to the cytoplasm where host ribosomes catalyze the synthesis of viral precursor proteins. Viral protease cleaves these precursor proteins into viral proteins, which assemble beneath the host cell membrane(Goldsby and Goldsby 2003). Gp41 and gp120 are inserted into the host membrane and bud out during viral egress to form part of the viral envelope (Goldsby and Goldsby 2003). The budded viron is still immature until the gag precursor polyprotein is cleaved to create the matrix, capsid, and nucleocapsid proteins (Goldsby and Goldsby 2003). These structural components can then assemble to create a mature HIV-1 virus that is capable of infecting another target cell (Goldsby and Goldsby 2003).

Natural Course of HIV Infection

The natural progression of HIV infection towards AIDS is separated into three different phases: the acute, chronic, and AIDS phases (Forsman and Weiss 2008) (Figure 5). The acute phase of HIV infection (primary infection) is characterized by high viral load and a depletion of CD4+ T cells, and only lasts a few months (Forsman and Weiss 2008). HIV-1 infects the mucosal-associated lymphoid tissue (MALT) at the portal of entry, damaging the integrity of the gut epithelium, thus allowing commensal gut bacteria to penetrate into the MALT and cause an inflammatory response mediated by their bacterial LPS (Forsman and Weiss 2008).

Depletion of CD4+ T cells is also more clearly seen in the MALT compared to peripheral blood (Forsman and Weiss 2008). Due to vigorous HIV-1 replication in the MALT, the viral load in peripheral blood reaches a peak before sharply falling after seroconversion (Forsman and Weiss 2008). This sharp fall is associated with the appearance of HIV-specific CD8+ cytotoxic T cells and the lack of CD4+ T cells is necessary to maintain a high viral load (Forsman and Weiss 2008). The acute phase of HIV-1 infection is usually asymptomatic, but a minority of patients present with fever, lymphadenopathy, and rash lasting no more than a few weeks (Forsman and Weiss 2008). The chronic phase of HIV-1 infection commences after seroconversion has occurred (Forsman and Weiss 2008). The viral load in peripheral blood settles down to a "set point" which is predictive of the rate of progression to AIDS (Forsman and Weiss 2008). A high set point is associated with a more rapid progression towards AIDS. On average, the progression of HIV-1 infection towards AIDS takes approximately 9 years, but is variable (Forsman and Weiss 2008). Five years is considered a rapid progression and long-term nonprogressors show little or no decrease in CD4+ T cells for 15 or more years (Forsman and Weiss 2008). During this period, active replication of HIV-1 and the destruction of CD4+ T cells continue in the MALT and lymph nodes, while the viral load in plasma remains fairly stable (Forsman and Weiss 2008). For the physician, a decreasing number of CD4+ T cells in peripheral blood is an important marker of when to initiate antiretroviral therapy (Le et al. 2013). Progression to the AIDS phase of HIV-1 infection begins when the CD4+ T lymphocyte count falls below 200 cells/mm^3 in peripheral blood (Goldsby and Goldsby 2003; Forsman and Weiss 2008). A high HIV-1 viral load, decreased or absent delayed-type hypersensitivity (DTH), and an increase in opportunistic infections, such as cryptococcosis and candidiasis, also accompany the progression to AIDS

(Goldsby and Goldsby 2003). Opportunistic neoplasms, such as Kaposi`s sarcoma, or non-Hodgkin`s lymphoma, can also occur during AIDS and are mainly caused by latent oncogenic viruses (Forsman and Weiss 2008). The most frequent cause of death in AIDS patients is attributed to opportunistic infections and not AIDS itself (Forsman and Weiss 2008).

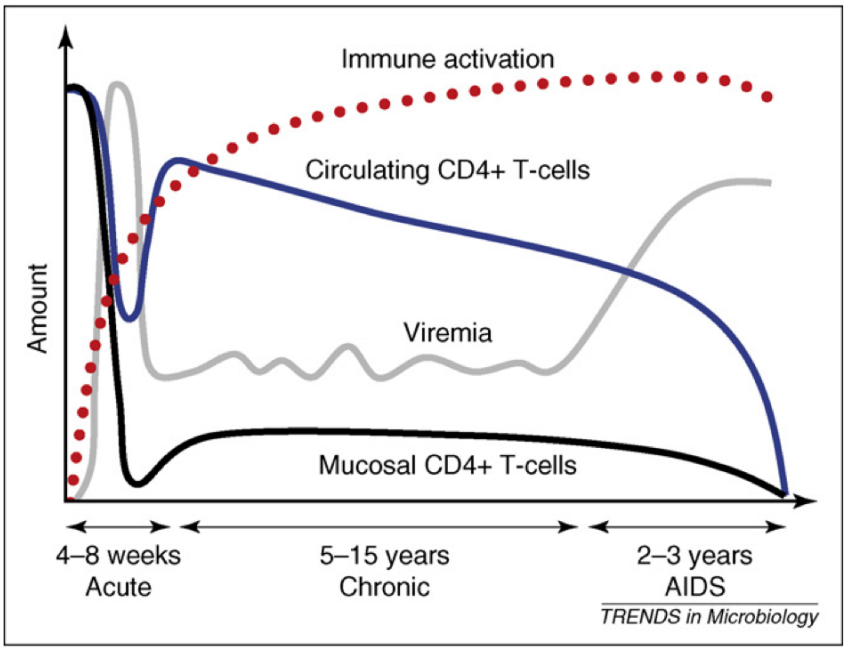

Figure 5. Progression of HIV-1 infection. HIV viral load (grey line) and circulating CD4+ T lymphocytes (blue line) (Forsman and Weiss 2008) Used with permission from Elsevier (License number 3172570874300)

HIV Treatment

Although the highest priority of researchers is to develop a vaccine to prevent the spread of HIV/AIDS, it is also critical to develop effective antiretroviral drugs and therapies that can reverse the effects of HIV-1 infection. The objective of these drugs or therapies is to target HIV-1 specifically and not to interfere with normal cellular processes (Goldsby and Goldsby 2003). Three different strategies have been used to target susceptible steps in the life cycle of

HIV-1. The first successful strategy was to target reverse transcriptase of viral RNA to cDNA (Goldsby and Goldsby 2003). The prototype for this kind of drug is Zidovudine or azidothymidine (AZT) (Goldsby and Goldsby 2003). AZT introduces itself into the growing cDNA chain of retroviruses and causes the termination of the chain (Goldsby and Goldsby 2003). One of the problems with AZT is that it can also be used by human DNA polymerase and can be inserted into the host DNA, killing the cell (Goldsby and Goldsby 2003). Precursor red blood cells are susceptible to AZT, causing anemia in some patients as well as nausea, headache, weakness, insomnia, and neutropenia (Goldsby and Goldsby 2003). Other drugs, such as Nevirapine, target the reverse transcriptase directly (Goldsby and Goldsby 2003). The second strategy is to use protease inhibitors, which inhibit the cleavage of precursor polyproteins (Goldsby and Goldsby 2003). This method has been proven effective when combined with AZT or other reverse transcriptase inhibitors. This combination therapy has been designated HAART (highly active anti-retroviral therapy) (Goldsby and Goldsby 2003). HAART has been shown to be an effective method that appears to overcome the ability of the virus to rapidly produce mutants that are drug resistant (Goldsby and Goldsby 2003). There are many serious side effects that are associated with HAART such as blurred vision, metallic taste in the mouth, abnormal distribution of fat, and elevated triglyceride and cholesterol levels (Goldsby and Goldsby 2003). The last strategy, and the newest, is to inhibit intergrase, thus inhibiting the integration of viral DNA into the host genome and the formation of a provirus (Goldsby and Goldsby 2003; Savarino 2006). The first FDA-approved integrase inhibitor was Raltegravir, in 2007 (Savarino 2006). There are also some drugs that are being tested that act at the stage of viral attachment to the host cell (Goldsby and Goldsby 2003). The main disadvantages to these drugs or therapies are the cost (around $15,000/year) and the possibility

of side effects that range from nausea to peripheral neuropathy (Goldsby and Goldsby 2003). Currently, the best option to stop the spread AIDS is the discovery of a safe, inexpensive vaccine that prevents infection and progression of the disease (Goldsby and Goldsby 2003).

Immunological Effects of HIV-1

Several immunological abnormalities are associated with HIV-1 infection, the most evident being the severe depletion of CD4+ T lymphocytes (Wahl et al. 2003; Forsman and Weiss 2008). The inability of lymph nodes to trap antigens or support the activation of T and B cells, a decrease in T helper cells, a shift in cytokine production from the Th1 to the Th2 subset, the elimination of DTH response, and a reduced activity of cytotoxic T lymphocytes (CTL), are just some of the other abnormalities that occur during HIV-1 infection (Goldsby and Goldsby 2003). Nef protein (p27) affects intracellular signaling pathways, inducing AP-1 and NF-κB in lymphocytes (Percario et al. 2003). Additionally, *nef* activates T cell receptor chain (TCR) signaling as well as calcium-dependent signaling in a TCR-independent manner (Percario et al. 2003). HIV-1 disrupts signaling events in the JAK/STAT pathway, which suppresses granulysin expression in CD8+ T lymphocytes (Hogg et al. 2009). HIV-1 infected macrophages resist HIV-1 mediated apoptotic death and play an important role in long-term persistence of the virus by acting as a reservoir (Wahl et al. 2003). Infected macrophages induce chemotaxis and activation of resting T lymphocytes, facilitating a more productive HIV-1 infection (Swingler et al. 1999; Wahl et al. 2003). *Nef* induces an activation state in macrophages, which leads to an increase in transcription and release of MIP-1α, MIP-1β, IL-1β, IL-6, and TNF-α (Olivetta et al. 2003; Percario et al. 2003). The *nef*-dependent release of inflammatory cytokines is also accompanied by an activation of NF-κB transcription factor

(Olivetta et al. 2003). STAT3 is also activated by *nef* through mechanisms mediated by the release of MIP-1α and MIP-1β (Percario et al. 2003). Gp120 impairs lysosome-phagosome fusion in phagocytes and down-regulates the production of IL-12 and oxidative burst in macrophages (Pugliese, Vidotto, Beltramo and Torre 2005). Neutrophils from HIV-infected patients have been shown to be impaired in neutrophil fungal killing and cytokine production (Vecchiarelli et al. 2000). IL-8 production is reduced due to decreased expression of CD88 (complement component 5a receptor) in neutrophils (Monari et al. 1999). Lower levels of dendritic cells have been observed in HIV-1 infected patients (de Repentigny et al. 2004). Both the viral matrix and *nef* have been shown to cause only partial maturation of plasmacytoid dendritic cells (pDC); they also acquire a migratory phenotype, facilitating travel to the lymph nodes (Coleman and Wu 2009). These dendritic cells (DC) fail to express an increase of the maturation markers MHC-II, CD80, and CD86 (Coleman and Wu 2009). HIV-1 also suppresses the activation of antiviral Toll-like receptors TLR7 and TLR8, by blocking the release of antiviral IFN-α in pDCs (Coleman and Wu 2009).

Altered Innate and Adaptive Immune Response to Cryptococcus

Cryptococcosis is one of the leading fungal causes of morbidity and mortality among AIDS patients (Mitchell and Perfect 1995). *C. neoformans var. neoformans* serotype A is responsible for almost all of cryptococcosis cases in AIDS patients globally, while it is responsible for only 75% of cryptococcosis cases in patients without AIDS (Mitchell and Perfect 1995). There are many differences in clinical manifestations between cryptococcosis in immunocompromised and immunocompetent patients. The sites of cryptococcal infections in AIDS patients usually contain a higher fungal burden and a low inflammatory cell response

(Mitchell and Perfect 1995). In an immunocompromised host, cryptococcal pneumonia tends to have a more rapid clinical course. *C. neoformans* will usually disseminate rapidly from the lungs to establish an infection in the CNS (Huang and Crothers 2009). In some cases, dissemination to the meninges occurred 2-20 weeks after being diagnosed with pulmonary cryptococcosis (Mitchell and Perfect 1995). At the time of CNS cryptococcosis diagnosis, HIV-1 infected patients commonly develop a second site of infection, for example cutaneous cryptococcosis (Mitchell and Perfect 1995; Kovarik and Barnard 2009). In immunocompetent patients, chest radiographs of cryptococcal pneumonia reveal well-defined, noncalcified, single or multiple lung nodules; in immunocompromised patients, chest radiographs reveal alveolar or interstitial infiltrates, single or multiple lesions, masses, cavitary lesions, and pleural effusions (Mitchell and Perfect 1995; Huang and Crothers 2009). In AIDS patients, the prostate and CNS represent potential reservoirs for clinical relapse of cryptococcosis (Mitchell and Perfect 1995).

HIV-1 infection can reduce the anti-cryptococcal host response, and increase the virulence of *Cryptococcus* (Lortholary et al. 2005). Gp120 of HIV-1 has many effects that facilitate *Cryptococcus* infections. It has been shown to inhibit the anti-cryptococcal activity of human alveolar macrophages. Gp120 also inhibits the production of IL-12 and the expression of IL-12 receptor and induces the production of IL-10, inhibiting the translocation of CD40, which inhibits the generation of a Th1 response against *Cryptococcus* (Pietrella, Kozel, et al. 2001; Lortholary et al. 2005). Gp120 can also inhibit the protective Th1 response by decreasing the surface expression of CD86 and MHC-II, inhibiting the production of IFN-γ, and promoting the induction of IL-4 release (Pietrella et al. 1999; Pietrella, Kozel, et al. 2001). The

decreased expression of CD88 on neutrophils and IL-8 production due to HIV-1 infection reduces the activation of the complement system (Monari et al. 1999). *Cryptococcus* also promotes HIV-1 replication (Harrison et al. 1997). *C. neoformans* causes an increase in production of TNF-α in monocytes, which increases the production of HIV-1 by stimulating transcription from the HIV long terminal repeat (Harrison et al. 1997). The secretion of MIP-1β by microglial cells can inhibit HIV-1, but *C. neoformans* is able to inhibit microglial cell production of MIP-1β (Harrison et al. 1997). So, HIV-1 and *Cryptococcus* can mutually increase their virulence.

CHAPTER 4- MODELS OF CRYPTOCOCCOSIS AND HIV-1 INFECTION

Invertebrate Models of Cryptococcosis

Invertebrates can be excellent models of disease because they have certain advantages that include reduced maintenance costs, fewer ethical restrictions, shorter reproduction times, and large brood sizes (Sabiiti et al. 2012). Invertebrates also lack an adaptive immune system, providing an excellent method to study the innate immune response to diseases without the potential confusion of the adaptive immune response (Sabiiti et al. 2012).

Amoeboid models are useful because amoebas feed through phagocytosis in a method that is similar to phagocytosis of microbes by macrophages in humans (Sabiiti et al. 2012). Thus, amoebas provide a simple model to investigate phagocytosis of microorganisms like *Cryptococcus.* Two amoeboid models, *Dictyostelium discoideum* and *Acantamoeba castellanii*, have been used to study *Cryptococcus neoformans* infection (Sabiiti et al. 2012). *A. castellanii* is advantageous because the amoebae are viable above 25°C, which better simulates the conditions of a human infection compared to *D. disoideum* (Sabiiti et al. 2012). *D. disoideum* is useful because it is more thoroughly genetically characterized, and is more receptive to genetic manipulation than *A. castellanii.* Both of these models have been used to show that an acapsular mutant of *C. neoformans* is eliminated when consumed by these amoebas, while the capsular strain was able to replicate inside the amoebae just like in macrophages (Sabiiti et al. 2012). Also, it was shown that there is a significant increase in growth when *C. neoformans* is incubated with the amoebae compared to when it is incubated in PBS alone (Sabiiti et al. 2012). These results suggest the characteristics that contribute to

the virulence of *C. neoformans* in mammals are a result of its adaptations to survive in the environment(Sabiiti et al. 2012).

Caenorhabditis elegans is a nematode that has been used for immunological study of many pathogens, and was established as a model for *Cryptococcus* by Mylonakis et al. in 2002 (Mylonakis et al. 2002). They showed that *C. elegans* was able to ingest *C. neoformans*, *C. laurentii*, and *C. kuetzingii*; but only *C. neoformans* was virulent and able to kill *C. elegans*. The ability of *C. neoformans* to kill *C. elegans* is dependent on the presence of its polysaccharide capsule, the MATα mating type, laccase production (LAC1), and genes associated with signal transduction pathways (GPA1, PKA1, BRK1, and RAS1) (Mylonakis et al. 2002; Sabiiti et al. 2012). These factors have been previously shown to be important in the ability of *C. neoformans* to be virulent in mammals (Sabiiti et al. 2012). The largest problem of *C. elegans* as a method to investigate mammalian pathogenesis is that the mode of infection is completely different in nematodes compared to mammals (Sabiiti et al. 2012). *C. elegans* ingests *C. neoformans*, and it is restricted to the intestines, while in mammals it is inhaled and in humans can subsequently disseminate (London et al. 2006; Sabiiti et al. 2012).

Galleria mellonella, or the greater wax moth larva, has been used to examine either whole-organism virulence or antifungal activity of several species of pathogenic fungi (Mylonakis et al. 2005; London et al. 2006; Sabiiti et al. 2012). Some advantages that *G. mellonella* has over other invertebrate models are its ability to live at mammalian body temperature, that it is easy to inoculate, and the injections are minimally invasive due to the fact that the haemocoel does not have to be pierced to inject *Cryptococcus* (Sabiiti et al. 2012). Mylonakis et al. developed

the first model of *G. mellonella* for *Cryptococcus* in 2005, and they showed that all tested strains of *Cryptococcus* were virulent and caused larval death (Mylonakis et al. 2005). They then showed the beneficial effects of combining the antihistamine Astemizole, and a closely related analog (A2) to fluconazole for *G. mellonella* survival (Mylonakis et al. 2005; Sabiiti et al. 2012).

Another insect model that has been used as a model for *Cryptococcus* infection is *Droshophila melanogaster* (London et al. 2006; Sabiiti et al. 2012). The immune signaling pathways are highly conserved between flies and humans making this model useful (Sabiiti et al. 2012). The relevance of this, with regards to fungal diseases, is the capability of antifungal peptide production from the downstream activation of Toll receptors by *D. melanogaster* (Sabiiti et al. 2012). Three *Cryptococcus* species have been studied with *D. melanogaster*: *C. neoformans*, *C. laurentii*, and *C. keutzingii* (Apidianakis et al. 2004). When these species were injected into *D. melanogaster*, causing a systemic infection, none were virulent; once ingested, only *C. neoformans* was virulent (Apidianakis et al. 2004). The death of the flies after ingestion of *C. neoformans* implicates several virulence factors, such as the polysaccharide capsule, the MATα mating type, and laccase production, shown previously with *Caenorhabditis elegans* (Apidianakis et al. 2004). Toll-like receptors (TLR) do not play a role when *Cryptococcus* is ingested by *D. melanogaster* (Apidianakis et al. 2004; Sabiiti et al. 2012). When *Cryptococcus* is injected, TLRs are activated and are crucial for host resistance against *Cryptococcus* and other fungal pathogens such as *Aspergillus fumigatus* or *Candida albicans* (Apidianakis et al. 2004; Sabiiti et al. 2012). This suggests that *Cryptococcus* triggers different

responses in either systemic or digestive related immunity in *D. melanogaster* (Apidianakis et al. 2004; Sabiiti et al. 2012).

Mammalian Models of Cryptococcosis

The ability of *Cryptococcus* to naturally infect mammals and cause a disease similar to humans makes mammals relevant to study *Cryptococcus* infection (Carroll et al. 2007). The advantage of vertebrate over invertebrate models is that they are more anatomically, physiologically, and immunologically similar to humans, allowing a more accurate method to model *Cryptococcus* infections (Carroll et al. 2007).

The first mammal used to model *Cryptococcus* infections is *Cavia porcellus,* or guinea pig (Carroll et al. 2007; Sabiiti et al. 2012). The guinea pig has been used to model many other invasive fungal infections caused by *Zygomycota*, *Candida*, and *Aspergillus* (Carroll et al. 2007). The docile nature, and medium body size and susceptibility to *Cryptococcus* make the guinea pig a suitable host for *Cryptococcus* infection (Carroll et al. 2007; Sabiiti et al. 2012). It was shown that female guinea pigs are more resistant to *Cryptococcus* infections compared to male animals; the same gender effect can be observed in human cryptococcal disease susceptibility (Carroll et al. 2007). Administration of corticosteroids to infected guinea pigs revealed the important role of cell-mediated immunity in host resistance to *C. neoformans*, which is consistent with findings from studies with rabbits, rats, and mice (Carroll et al. 2007). Unlike murine models and humans, guinea pig alveolar macrophages are unable to eliminate acapsular *C. neoformans*, and capsular *C. neoformans* is able to completely inhibit phagocytosis by alveolar macrophages (Carroll et al. 2007). Other disadvantages of the guinea

pig model are that there are only a few inbred strains of guinea pigs available, and there is a lack of immunologic reagents and genetic tools available for analysis of disease pathogenesis (Carroll et al. 2007).

Oryctolagus cuniculus, or rabbits are naturally resistant to *Cryptococcus* infection, which is partially due to their high normal body temperature (39.3-39.5° C) (Carroll et al. 2007). The high body temperature of rabbits inhibits fungal replication and dissemination through the respiratory tract; to overcome this problem rabbits are immunosuppressed with corticosteroids (Carroll et al. 2007). The dosages of corticosteroid closely match those in patients with organ transplants (Carroll et al. 2007). Rabbits are mostly used to study cryptococcal meningoencephalitis, because their large size allows multiple samplings of cerebrospinal fluid (CSF) and better access to study *Cryptococcus* at the site of infection (Carroll et al. 2007; Sabiiti et al. 2012). The large size of the rabbit has also allowed researchers to investigate the effectiveness of various antifungal medications for the treatment of cryptococcal meningitis (Carroll et al. 2007). Limitations of rabbit models for *Cryptococcus* infections include high purchase and maintenance costs, large infectious dose, requirement of immunosuppression, and the limited repertoire of immunologic reagents and genetic information available (Carroll et al. 2007).

The *Rattus norvegicus*, or rat, is mainly used to study chronic or latent pulmonary *Cryptococcus* infections (longer than 18 months) (Carroll et al. 2007; Sabiiti et al. 2012). Intratracheal infection of *C. neoformans* in the rat reproduces many histopathological and serological features of human cryptococcal pneumonia, and unlike the rabbit establishment of

infection does not require the use of corticosteroids (Carroll et al. 2007). Immunocompetent rats show no dissemination from pulmonary infection after intratracheal inoculation, indicating that rats may be more resistant to progressive cryptococcal disease. The rats' ability to contain the infection in the lungs may be due to macrophage-derived MCP-1 and inducible nitric oxide synthase (NOS) that mediates cellular recruitment to the lungs after cryptococcal infection (Carroll et al. 2007). Intracellular persistence and long-term survival of *Cryptococcus* is due to a downregulation of cellular and humoral immunity in the host (Carroll et al. 2007). An advantage that rats have is that they are large enough for intratracheal infections that are less invasive and do not require surgery (Carroll et al. 2007). In order to study cryptococcal meningitis in rats, intracisternal inoculation is used. Compared to mice, the main disadvantages of rat models of *Cryptococcus* infection are high acquisition costs and the limited number of immunologic reagents available (Carroll et al. 2007).

The most widely used animal for *Cryptococcus* infection is *Mus musculus*, or mouse (Carroll et al. 2007; Sabiiti et al. 2012). Mice are highly susceptible to *Cryptococcus* infection by many different routes including intranasal, intratracheal, intravenous, and intraperitoneal routes, without the need for immunosuppression (Carroll et al. 2007; Sabiiti et al. 2012). The route of infection and genetic background of the mice are of critical importance to the outcome of the host-pathogen interaction (Carroll et al. 2007). For example, BALB/c mice are more resistant to intratracheal inoculation of *C. neoformans* compared to CBA/J mice, but there is no difference in resistance after intravenous inoculation (Zaragoza et al. 2007). One of the greatest advantages to using a murine model is that *Cryptococcus* infection in mice closely resembles *Cryptococcus* infection in humans, where susceptible mice strains develop

disseminated disease after experimental pulmonary infection (Carroll et al. 2007). Some other advantages of the murine model are the relatively inexpensive costs of purchase and maintenance (Carroll et al. 2007). The ease of handling, the availability of numerous inbred strains, as well as extensive immunologic and genetic resources available are also notable advantages (Carroll et al. 2007; Sabiiti et al. 2012). The main disadvantage in using a murine model of *Cryptococcus* is the small size of mice, which could hinder some procedures (Carroll et al. 2007). The overall conclusion arising from the studies conducted with murine models is that robust innate and cell-mediated immunity interacting with humoral host defenses is essential for protection and clearance of *Cryptococcus* infections (Carroll et al. 2007).

Murine Models of HIV-infection and AIDS

Many models of retroviral infections have been employed, including primate models using Simian immunodeficiency virus (SIV) or a SIV/HIV chimera, and a feline model using feline immunodeficiency virus (Borkow 2005). Some of the disadvantages of these models are the high costs to purchase and maintain the animals, lengthy primate/feline maturation, uncertain value in predicting human immune responses, and the molecular and immunogenetic diversity of SIV, SIV/HIV, FIV, and HIV (Borkow 2005). Murine models provide certain unique advantages. The ready availability of mice, shorter time periods required for the experiments, the ability to increase the number of subjects to achieve higher statistical significance, and the relative ease of transgenic manipulations are just some of the advantages to using a murine model (Borkow 2005).

Murine acquired immunodeficiency syndrome (MAIDS) is a disease that can be induced in sensitive strains of mice using the retroviral mixture designated LP-BM5 murine leukemia virus (MuLV) (Fredrickson et al. 2010; Jones et al. 2012). LP-BM5 is comprised of the replication competent helper virus BM5e, the mink cell cytopathic focus-inducing virus, and the replication-defective BM5def (Jones et al. 2012). There are many similarities between MAIDS and human AIDS including hypergammaglobulinemia, lymphadenopathy, severely depressed T- and B-cell responses to mitogens, increased susceptibility to infections, disease progression, and the development of B-cell lymphomas and splenomegaly (Jolicoeur 1991; Jones et al. 2012). Due to the similarities between LP-BM5 and HIV transmission and disease outcome, it is a good model for examining sexual transmission of HIV and has been used for the initial evaluation of new drugs (Jolicoeur 1991; Jones et al. 2012). The main problems with the MAIDS model are that, unlike human AIDS, the mice may die of lymphadenopathy, respiratory failure, or from extensive lymphoid infiltration and associated dysfunction of the liver or the kidneys (Fredrickson et al. 2010). Nevertheless, mice with MAIDS have been successfully used to investigate the immunopathogenesis of oral candidiasis (Deslauriers et al. 1997).

Another murine model that has been used to examine HIV-1 is a humanized mouse model that has been infected with HIV-1 (Brady et al. 1994). The two main strategies that are used to humanize mice are to either intraperitoneally inject human peripheral blood lymphocytes (PBL) into severely combined immunodeficiency (SCID) mice (hu-PBL-SCID model), or to implant fragments of fetal human thymus and liver under the kidney capsule of SCID mice (SCID-hu (Thy/Liv) model) (Borkow 2005). These mice can then be infected with HIV-1

which causes a rapid depletion of CD4+ T-cells within a few weeks of infection (Borkow 2005). The hu-PBL-SCID-HIV-1 mouse model has been used to study HIV-1 infection, pathogenesis, viral fitness, and different approaches to generate effective anti-HIV-1 responses (Borkow 2005). Some of the disadvantages associated with the hu-PBL-SCID-HIV-1 mouse model are that the infection can only persist up to 16 weeks, a progressive restriction of T- and B-cell repertoires occurs, the engrafted T-cells become anergic and unresponsive to T-cell receptor stimulation, and the inability to create cytotoxic T-lymphocytes against viral antigens (Borkow 2005). The SCID-hu (Thy/Liv)-HIV-1 mouse model has been used mainly to study HIV-1 infection, tropism, cellular pathogenesis, and to investigate gene therapy and the effects of antiviral treatment on the renewal of thymopoiesis (Borkow 2005). The two main disadvantages to this model are the lack of humoral and cellular responses to the viral load, and that the implanted tissues are of fetal origin which may not accurately reflect the structure and function of their adult counterparts (Borkow 2005).

There have been many different transgenic mouse models developed to study HIV-1 infection, but most of these models differ too much from HIV-1 infections in humans (Brady et al. 1994). Since mice are resistant to HIV-1, it is essential to create a transgenic mouse model that closely resembles HIV-1 infection in humans.

CD4C/HIV^{Mut} Mice

The laboratory of Dr. Paul Jolicoeur (IRCM) created and characterized transgenic (Tg) mice (on a C3H background) using the whole coding, or a partially mutated sequence of HIV-1, along with the regulatory sequences of the human CD4 promoter, and the murine CD4

enhancer (Hanna, Kay, Cool, et al. 1998). This allowed the HIV-1 genome (partial or whole) to be expressed in the targeted cells normally infected in HIV-1-positive individuals, including CD4+ T lymphocytes, immature CD4+CD8+ thymic T lymphocytes, dendritic cells, and macrophages (Hanna, Kay, Cool, et al. 1998). These Tg mice developed a severe AIDS-like disease characterized by thymic atrophy, loss of CD4+ lymphocytes and mature dendritic cells, an accumulation of immature dendritic cells, loss of architecture of lymphoid organs, muscle wasting, weight loss, diarrhea, interstitial lymphocytic pneumonitis, and tubular-interstitial nephritis eventually leading to premature death that can occur as early as one month of age (Hanna, Kay, Cool, et al. 1998). These Tg mice also display an increase in apoptosis of T lymphocytes due to an overexpression of Fas and FasL, which is also observed among patients infected with HIV-1 (Priceputu et al. 2005). The severity of the AIDS-like disease in these mice is directly associated with the level of expression of the transgene in the target cells, which is similar to an increased replication of HIV-1 leading to a faster progression to AIDS in humans (Hanna, Kay, Cool, et al. 1998).

Five different mutants (CD4C/HIV$^{MutA, B, C, G, H}$) were constructed from the HIV-1 genome (Hanna, Kay, Rebai, et al. 1998) (Figure 6). It was shown that only *nef* is required and sufficient to cause an AIDS-like disease phenotype in CD4C/HIV^{MutG} Tg mice (Hanna, Kay, Rebai, et al. 1998). CD4C/HIV^{MutH} mice have a mutated *nef* gene to make it inactive, express all the other HIV-1 genes, and do not develop the AIDS-like phenotype (Hanna, Kay, Rebai, et al. 1998). These results demonstrate that *nef* alone is required to cause an AIDS-like phenotype in these transgenic mice. CD4C/HIV^{MutA} mice, which express *nef, env,* and *rev,* also develop an AIDS-like disease phenotype (Hanna, Kay, Rebai, et al. 1998).

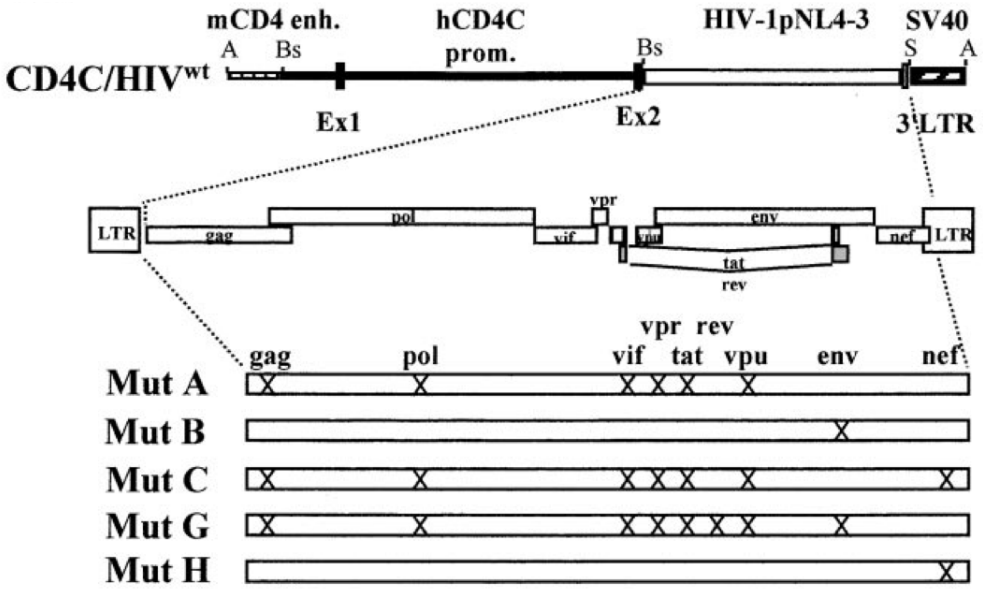

Figure 6. Genetic construction of CD4C/HIV^{Mut} transgenic mice. The X`s represent mutated genes (Hanna, Kay, Rebai, et al. 1998). Used with permission from Elsevier (License number 3172571069951)

Different founders of CD4C/HIV^{MutA} mice developed AIDS-like disease between 30 days and 16 months of age and had a much higher life expectancy compared to CD4C/HIV^{WT} mice (Hanna, Kay, Rebai, et al. 1998). In the laboratory of Dr. Louis de Repentigny, CD4C/HIV^{MutA} Tg mice have previously been used to analyze the immunopathogenesis of persistent oropharyngeal candidiasis, which closely resembles those found in patients infected with HIV-1 (de Repentigny et al. 2004). CD4C/HIV^{MutA} Tg mice provide an ideal candidate to develop a model of *C. neoformans* and *C. gattii* infections, in the context of HIV-1 infection.

HYPOTHESIS

Cryptococcosis caused by *C. neoformans* usually infects immunocompromised patients, in contrast to *C. gattii,* which mostly infects immunocompetent people. We formulate the hypothesis that CD4C/HIV^{MutA} transgenic mice present functional and/or quantitative immune alterations that selectively augment their susceptibility to *C. neoformans* but not to *C. gattii* infection.

OBJECTIVES

We aim to analyze potential differences in pulmonary immune cell recruitment and cytokine production, in transgenic mice and non-transgenic controls infected with either *C. neoformans* or *C. gattii*.

SPECIFIC AIMS

To quantitate:

1. The recruitment of dendritic cells, interstitial and alveolar macrophages, CD4+ and CD8+ T lymphocytes, and Gr-1+ cells by flow cytometry, in both Tg and non-Tg mice 14 days after infection with *C. neoformans* or *C. gattii*.

2. Pulmonary production of cytokines, in both Tg and non-Tg mice, 7 and 14 days after infection with *C. neoformans*.

3. Alveolar macrophage production of MIP-1α, MIP-1β, MCP-1, and RANTES *in vitro*, 24 and 48 hours after exposure to *C. neoformans* (viable or heat-killed), *C. gattii* (viable or heat-killed), lipopolysaccharide, and lipoteichoic acid, using macrophages harvested from both Tg and non-Tg mice.

CHAPTER 5- MATERIALS AND METHODS

CD4C/HIV^{MutA} Transgenic mice

CD4C/HIV^{MutA} Tg mice, from founder mouse F31388, express *nef, env,* and *rev* from the HIV-1 genome at moderate levels (Hanna, Kay, Rebai, et al. 1998). Both Tg and control mice are from the C3H lineage. The mice are certified pathogen-free. They are housed in sterile microisolation cages in the G-5 animal facility at Université de Montréal, where the temperature, humidity, and light cycles are controlled. Mice are supplied with sterile water and are fed sterile mouse chow. Francine Aumont and Mathieu Goupil, from the laboratory of Dr. Louis de Repentigny, maintained the colony. Male Tg mice from the colony are placed with two C3H females from Harlan Laboratories for reproduction. Males used for reproduction must show no signs of infection. Littermates of both sexes used for experimentation were between the ages of 42 to 65 days. It is also important to note that the room designated for experimentation is separate from the room for reproduction.

The presence of the transgene was determined by using REDExtract-N-Amp Tissue PCR kit, from Sigma-Aldrich, to extract DNA from a tail tissue sample of the mouse according to the manufacturer's instructions. Briefly, an extraction and tissue preparation mixture was added to the tail sample and incubated at room temperature for 10 minutes, the mixture was then incubated at 95°C for three minutes, and finally a neutralization solution was added. PCR was then performed using the REDExtract-N-Amp PCR reaction mix and specific oligonucleotides for either the functional gene, or the mutated gene due to the insertion of the transgene. The presence or absence of the transgene was observed by polyacrylamide gel

electrophoresis. All experiments were approved by the Comité de déontologie de l'expérimentation sur les animaux (CDEA) of the Université de Montréal.

Preparation of *C. neoformans* and *C. gattii* inocula

The H99 strain of *C. neoformans var. grubii* (serotype A), molecular type VNI, was isolated from the CSF of an HIV positive patient. The R265 strain of *C. gattii*, molecular type VGIIa, was isolated from the bronchoalveolar lavage of an infected patient from Vancouver Island in 2001. Both of the strains were provided by Dr. James Kronstad of the University of British Columbia.

Aliquots of the strains were kept frozen at -80°C. Prior to inoculation, the yeast were thawed and grown for 48 hours at 30°C on yeast extract-peptone-dextrose (YPD) agar. After incubation, several colonies were transferred from the agar into 10 mL of YPD broth and agitated for 18 hours at 35°C. They were then washed twice with sterile PBS and centrifuged and pelleted at 200 x g for 10 minutes. After resuspension in 10 mL of PBS the yeast were counted using a hemacytometer. Different inocula were prepared, dependent on the species of *Cryptococcus* and/or the experiment being performed. The suspension of *Cryptococcus* in PBS, or KRPG for *in vitro* experiments, was contained in a 1 mL volume in an Eppendorf tube. 50 µL of the suspension was used to inoculate one mouse. In the *in vitro* experiments, 200 µL of the suspension was deposited in individual wells of a 96-well plate.

Intranasal inoculation of mice

Intranasal inoculation of mice was performed under sterile conditions in a biological safety cabinet at the G-5 animal facility. The mice were anesthetized with an intraperitoneal

injection of 100 to 150 µL, depending on the weight of the mouse, of a ketamine/xylazine solution (1.5 mL of ketamine 100 mg/mL (Bioniche, Belleville, ON), 0.1 mL of xylazine 100 mg/mL (Bimeda-MTC, Cambridge, ON), and 8.5 mL of sterile saline solution). Anesthetized mice were then suspended by their incisors on a sterile nylon thread attached across the opening of a polystyrene box. This is done to ensure that the airway of the mouse is fully open and to prevent leakage of the inoculum from the nares. With the use of a pipette, 50 µL of inoculum is taken from the Eppendorf tube and is slowly pipetted into the nares of each mouse. The mice are then left suspended for 10 minutes in order to ensure optimal aspiration of yeast into the lungs. The mice are then returned to their microisolation cages until they are euthanized 7 or 14 days postinfection.

Flow cytometry analysis of lung immune cell populations

All manipulations were performed under sterile conditions in a biological safety cabinet. Groups of six CD4C/HIV^{MutA} and non-Tg littermates were infected intranasally with 1.25 X 10^4 CFU of H99, 1.25 X 10^5 CFU of R265, or PBS for control mice and euthanized 14 days postinfection. After the lethal dose of ketamine/xylazine, the mice were exsanguinated by cutting the left atrium and injecting a 0.9% NaCl solution into the right ventricle. The lungs were then excised and mechanically disrupted in 3 mL of sterile PBS using a mortar and pestle. The lung cell suspension was then placed in a 15 mL conical tube and 1 mL of 1% collagenase (Sigma-Aldrich) in RPMI 1640 medium (Wisent) supplemented with 5% heat-inactivated fetal bovine serum (Wisent), 100 U/mL penicillin-streptomycin, and 50 µg/mL gentamicin was added. This suspension was incubated for 1 hour on a rotating platform at 37°C, and every 15 minutes the suspension was manually agitated. The solution was then

filtered with a 10 mL syringe equipped with a sterile nylon filter with a pore size of 80 μm. The suspension was centrifuged at 200 g for 10 minutes and the supernatant was removed. The cells were resuspended with 3 mL of sterile PBS, and 250 μL of the cell suspension was added to each of 11 FACS tubes. These cell suspensions were then ready to be labeled with fluorochrome-conjugated antibodies. Analysis of immune cell populations was performed with different panels of antibodies (Table I).

Table I. Antibodies used to analyze immune cell populations by flow cytometery

Antibody	Fluorochrome	Manufacturer	Cat. No.
CD45	PE	Biolegend	103106
Macrophages and Dendritic Cells			
CD11b	APC	Biolegend	101211
CD11c	PE/Cy7	Biolegend	117318
F4/80	FITC	Biolegend	123108
isotype	APC	Biolegend	400612
isotype	PE/Cy7	Biolegend	400922
isotype	FITC	Biolegend	400506
Gr1+ cells			
CD3	FITC	Biolegend	100204
Gr-1	PE/Cy7	Biolegend	108416
isotype	FITC	Biolegend	400606
isotype	PE/Cy7	Biolegend	400618
CD4+/ CD8+ T Lymphocytes			
CD4	FITC	Biolegend	100406
CD8	PE/Cy7	Biolegend	100722
isotype	FITC	Biolegend	400606
isotype	PE/Cy7	Biolegend	400522

The antibodies and their isotypes were placed in their correct tube and incubated at 4°C for 30 minutes (Table II). In order to lyse all the red blood cells and to fix immune cells, 2 mL of lysing solution (BD Biosciences) was then added to each tube for 10 minutes in the dark at room temperature. Then, 1 mL of PBS was added to each tube and the tubes were centrifuged

at 200 g for 10 minutes. The supernatant was removed and the cells were resuspended in either 400 μL of PBS for the calibration tubes or 250 μL of PBS for the experimental tubes. Tubes 1 to 6, 8, and 10 are used to calibrate the FACS, while tubes 7, 9, and 11 are experimental tubes. The labeled cells were then analyzed on a FACSCalibur flow cytometer (BD Biosciences) equipped with CellQuest software. Labeled immune cell populations were first gated as $CD45^+$, and further gated according to the expression of specific markers: interstitial ($CD45^+$, $CD11b^+$, $CD11c^-$, $F4/80^+$) and alveolar ($CD45^+$, $CD11b^+$, $CD11c^{hi}$, $F4/80^{low}$) macrophages; dendritic cells ($CD45^+$, $CD11b^{hi}$, $CD11c^{hi}$); CD4+ T lymphocytes ($CD45^+$, $CD4^+$, $CD8^-$); CD8+ T lymphocytes ($CD45^+$, $CD4^-$, $CD8^+$); and Gr-1+ cells ($CD45^+$, $CD3^-$, $Gr\text{-}1^+$). Data were acquired for 30,000 $CD45^+$ events, and the immune cell populations were calculated as a percentage of $CD45^+$ cells.

Table II. Combinations of antibodies used in flow cytometery analysis of lung immune cell populations

Tubes	Antibodies
1	No Antibody
2	CD45
3	CD45 + CD11b
4	CD45 + CD11c
5	CD45 + F4/80
6	CD45 + isotypes (Macrophages and DCs)
7	CD45 + CD11b + CD11c + F4/80
8	CD45 + isotypes (CD4 and CD8 T lymphocytes)
9	CD45 + CD4 + CD8
10	CD45 + isotypes (Gr-1+ cells)
11	CD45 + CD3 + Gr-1

Production of cytokines in the lungs 7 and 14 days postinoculation

Manipulations were performed in a sterile biological safety cabinet. Mice were infected at the same inoculum as for analysis of immune cell populations, but were euthanized at fixed times 7 or 14 days postinfection. After the mice were given a lethal dose of ketamine/xylazine, they were exsanguinated and the lungs were excised. The lungs for each mouse were mechanically disrupted with a mortar and pestle after adding 1.5 mL of PBS. The lung homogenates were then filtered using a 5 mL syringe equipped with a nylon filter with a pore size of 80 μm, and the filter was washed with 0.5 mL of PBS. The filtered suspension was centrifuged at 2000 g for 10 minutes, and the supernatant was collected and stored at -80°C. The cytokines in the supernatants were then assayed using the BD Flex cytometric bead array set (BD Biosciences) according to the manufacturer's instructions. Briefly, Flex set standards are prepared and 50 μL of standard or sample is added to each FACS tube. Fifty μL of a mixture containing all of the capture beads was added to a FACS tube and incubated for 1 hour at room temperature (Table III). Fifty μL of mixed PE detection reagent was added to each tube, and incubated for 1 hour for mouse assays, or 2 hours for human-based assays (Human TGF-β bead kit was employed as it is cross-reactive with mouse TGF-β). One mL of wash buffer was added to each tube and centrifuged at 200 g for 5 minutes. The supernatant was aspirated and the beads were resuspended in 300 μL of wash buffer. The tubes were read on a FACSCalibur flow cytometer equipped with CellQuest software. The data was analyzed using BD FCAP array 3.0 software.

Table III. Cytometric bead array beads used to analyze pulmonary production of cytokines by *Cryptococcus*-infected mice

Cytokine	Bead Position	Manufacturer	Cat. No.
IL-1β	E5	BD Biosciences	560232
IL-2	A5	BD Biosciences	558297
IL-4	A7	BD Biosciences	558298
IL-6	B4	BD Biosciences	558301
IL-10	C4	BD Biosciences	558300
IL-12p70	D7	BD Biosciences	558303
IL-13	B8	BD Biosciences	558349
IL-17A	C5	BD Biosciences	560283
IL-21	B6	BD Biosciences	560160
TNF-α	C8	BD Biosciences	558299
IFN-γ	A4	BD Biosciences	558296
MIP-1α	C7	BD Biosciences	558449
MIP-1β	C9	BD Biosciences	558343
MCP-1	B7	BD Biosciences	558342
RANTES	D8	BD Biosciences	558345
Human TGF-β	B6 (Single plex)	BD Biosciences	560429

Cytokine analysis of alveolar macrophage supernatants

Groups of 7 Tg and 7 non-Tg uninfected mice were euthanized with a lethal dose of ketamine/xylazine. Bronchoalveolar lavages were performed as previously described (Zaragoza et al. 2007). Briefly, euthanized mice were exsanguinated, and their tracheas were cannulated with PE (0.030-inch) tubing. The lungs were lavaged 10 times with sterile ice cold PBS. The lavage fluid was centrifuged at 200 g for 10 minutes and resupended with 2 mL of KRPG supplemented with 100 U/mL penicillin-streptomycin, and 50 μg/mL gentamicin. Alveolar macrophages were counted using a hemacytometer, and 1 X 10^5 macrophages were added to the wells of a 96-well plate. The plate was then incubated at 37°C with 5% CO_2 for 2 hours to promote macrophage adherence (Goupil 2009). The supernatant was discarded and

the wells were washed with 100 μL of KRPG. Two hundred microliters of KRPG, H99 (1 X 10^6 CFU), R265 (1 X 10^6 CFU), heat-killed H99 (1 X 10^6 CFU), heat-killed R265 (1 X 10^6 CFU), LPS from *E. coli* (100 ng/mL), or LTA from *S. aureus* (10 μg/mL) were added to the wells (Grunfeld et al. 1999). Heat-killed *Cryptococcus* was prepared by incubating the cultures at 65°C for 30 minutes (Luo et al. 2005). The plate was then incubated for 24 or 48 hours at 37°C with 5% CO_2, and the supernatants were collected and stored at -80°C. MCP-1, RANTES, MIP-1α, and MIP-1β concentrations in the supernatants were analyzed using BD Flex cytometric bead array set (BD Biosciences) according to the manufacturer's instructions, as described above (Table III). The tubes were read on a BD FACSCanto II flow cytometer equipped with FACSDiva software. The data was analyzed with BD FACP array 3.0 software.

Statistical Analysis

Statistical analysis for both immune cell populations and cytokine production was performed by analysis of variance using SPSS software (SPSS, Chicago, IL). Differences were considered significant at a P value <0.05.

CHAPTER 6- RESULTS

Quantification of pulmonary immune cell populations 14 days postinfection in Tg and non-Tg mice

To determine if expression of the HIV-1 transgene alters immune cell recruitment in mice infected with *C. neoformans* or *C. gattii*, CD4C/HIV^{MutA} Tg and non-Tg mice were inoculated with either control PBS, *C. neoformans* H99, or *C. gattii* R265 and lung immune cell populations were quantified 14 days later (Figure 7).

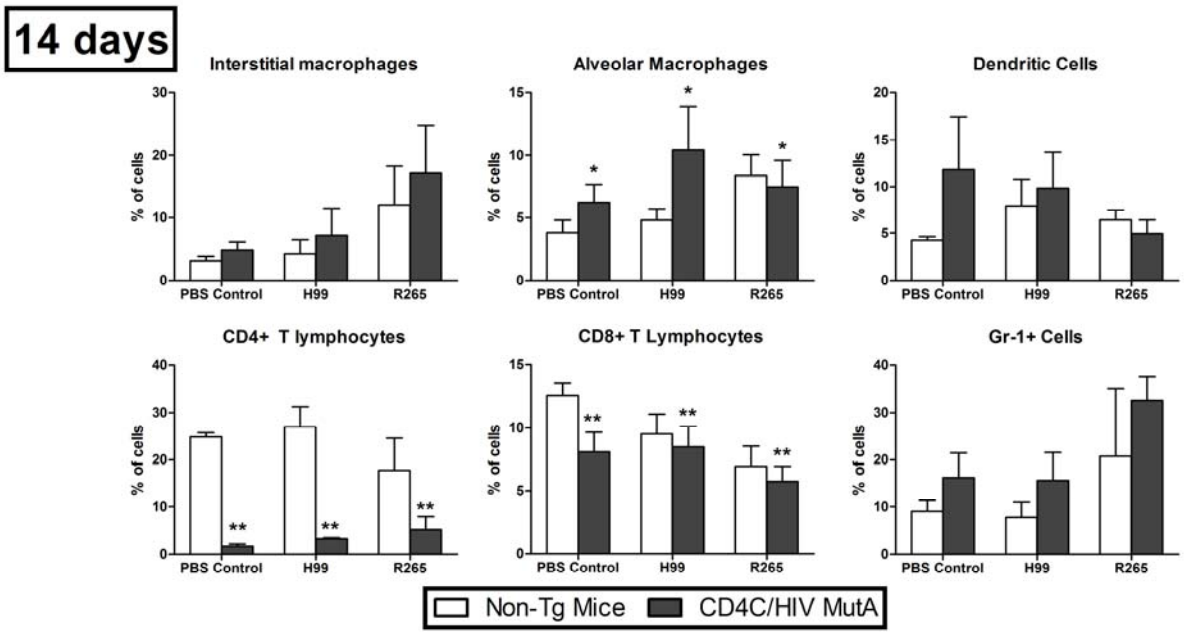

Figure 7. Percentages of lung immune cell populations, quantified by flow cytometry, in CD4C/HIV^{MutA} Tg and non-Tg mice 14 days after inoculation with PBS control, *C. neoformans* H99, or *C. gattii* R265. Data are presented as percentages of CD45^{+} cells and represent the means ± standard error of the means (SEM). * Tg > non-Tg mice ($P < 0.05$) and ** Tg < non-Tg mice ($P < 0.001$).

Expression of the HIV-1 transgene caused a prominent decrease in the percentages of CD4+ and CD8+ T lymphocytes ($P<0.001$), independently of infection with *C. neoformans* and *C. gattii*. The percentage of alveolar macrophages recruited in Tg mice was also significantly higher ($P<0.05$) compared to non-Tg mice, independently of cryptococcal infection. The percentages of Gr-1+ cells also showed a trend towards enhanced percentages in Tg mice ($P=0.075$). R265 infection displayed a higher percentage of interstitial macrophages compared to the PBS control or H99 infection in both Tg and non-Tg mice ($P=0.024$). No significant differences were observed between percentages of dendritic cells in Tg and non-Tg mice ($P>0.05$).

Figure 8 represents the number of extracted pulmonary cells in Tg and non-Tg mice 14 days after inoculation with PBS control, H99, or R265. Numbers of extracted cells, determined by hemacytometer count of cell suspensions after collagenase treatment, were significantly lower in Tg mice compared to non-Tg mice ($P=0.02$).

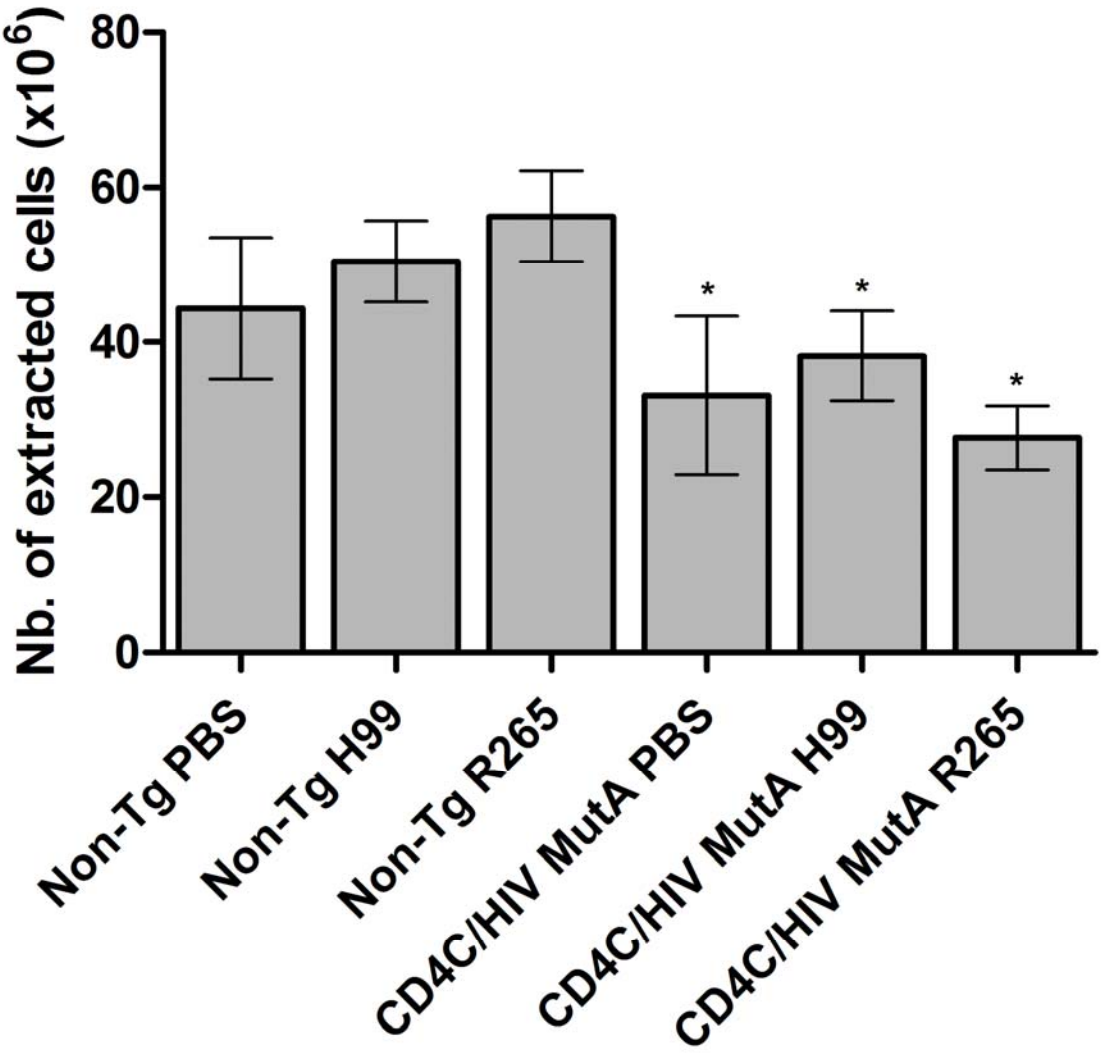

Figure 8. Total number of extracted pulmonary cells 14 days after inoculation of CD4C/HIV^{MutA} Tg and non-Tg mice with PBS control, *C. neoformans* H99, or *C. gattii* R265. Cells were counted using a hemacytometer after treatment of disrupted lung tissue with collagenase and red blood cell lysing solution. Results represent the means ± standard error of the means (SEM). * Tg < non-Tg (P<0.02).

Altered pulmonary cytokine response to *Cryptococcus* in Tg and non-Tg mice

To determine if the previously observed reduced inflammatory response in the lungs of Tg mice (Leongson K. M.Sc. thesis, Université de Montréal, 2011) was associated with defective cytokine production, cytokines from the lungs of CD4C/HIV^{MutA} Tg and non-Tg mice were quantified 7 and 14 days after inoculation with PBS control, *C. neoformans* H99, or *C. gattii* R265 (Figure 9).

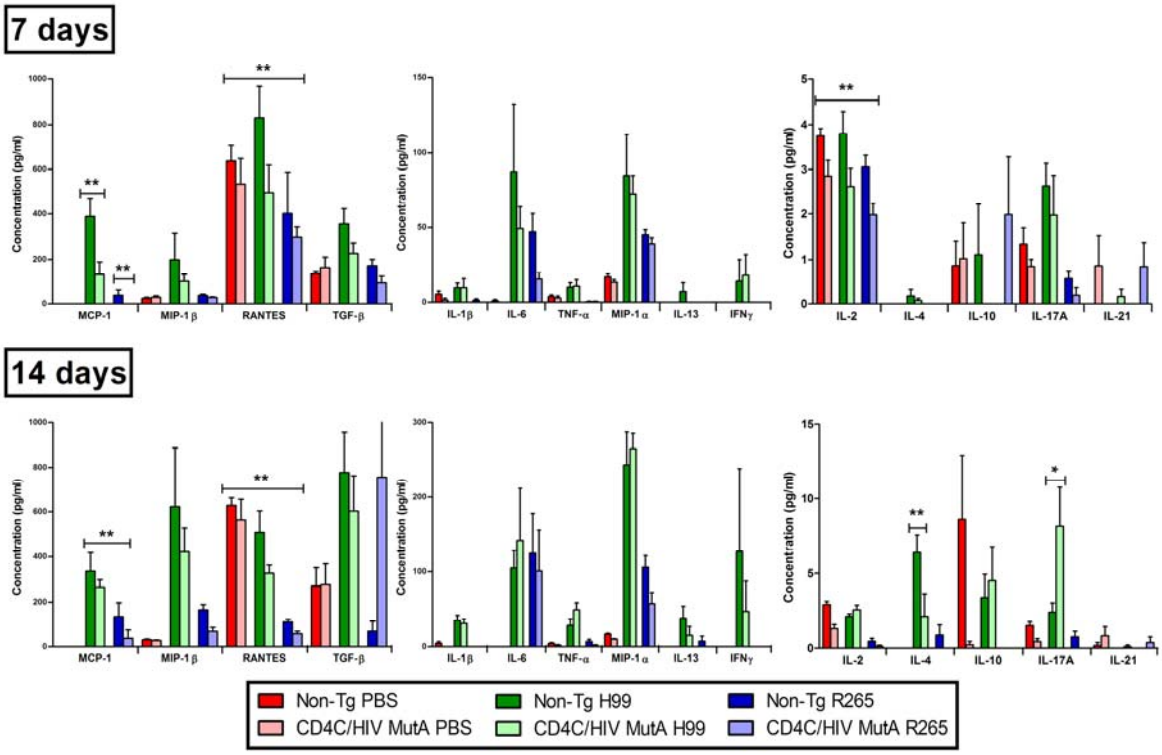

Figure 9. Cytokine production in the lungs of CD4C/HIV^{MutA} Tg mice and non-Tg mice 7 and 14 days after inoculation with PBS control, *C. neoformans* H99, and *C. gattii* R265. IL-12p70, not shown, was undetectable in all mice. Data are the means ± standard error of the means (SEM) of the results from six mice. * Tg > non-Tg mice ($P < 0.05$) and ** Tg < non-Tg mice ($P < 0.05$).

The production of RANTES by Tg mice was reduced under all three conditions after both 7 and 14 days ($P<0.01$). IL-2 production in Tg mice was also significantly reduced ($P<0.03$) at 7 days after inoculation. This same effect can be seen with the diminished production of MCP-1 in Tg mice both 7 and 14 days after infection with *C. neoformans* or *C. gattii* ($P<0.01$). 14 days after *C. neoformans* infection, the production of IL-4 was decreased and IL-17A production was increased ($P<0.001$) in Tg compared to non-Tg mice. The production of cytokines was also affected by *C. neoformans* and *C. gattii* infections independently of transgene expression. The production of MCP-1 and RANTES, 7 and 14 days after infection, was significantly reduced ($P<0.001$) in mice infected with *C. gattii* compared to *C. neoformans*. Fourteen days after infection, higher concentrations of MIP-1α, MIP-1β, TNF-α, TGF-β, IL-13, IL-2, and IL-4 were detected in mice infected with *C. neoformans* compared to *C. gattii* ($P<0.001$). The production of MIP-1β, TGF-β, IL-1β, TNF-α, IL-13, and IL-4 increased significantly from day 7 to day 14 after infection with *C. neoformans* ($P<0.001$), but not *C. gattii* ($P>0.05$). At both 7 and 14 days after infection, *C. neoformans* triggered production of IFN-γ, while this cytokine was undetectable in mice infected with *C. gattii*. The concentration of IL-6 increased significantly ($P<0.05$) from day 7 to day 14 after infection with either *C. neoformans* or *C. gattii*. Production of IL-12p70 was undetectable under all conditions in both Tg and non-Tg mice.

Cytokine production by alveolar macrophages of Tg and non-Tg mice 24 and 48 hours after agonist exposure

Figure 10 represents the production of cytokines by alveolar macrophages of CD4C/HIV^{MutA} Tg and non-Tg mice 24 and 48 hours after exposure to KRPG control, heat-killed *C.*

neoformans H99 and *C. gattii* R265, viable H99 and R265, lipoteichoic acid (LTA) from *S. aureus*, and lipopolysaccharide (LPS) from *E. coli*.

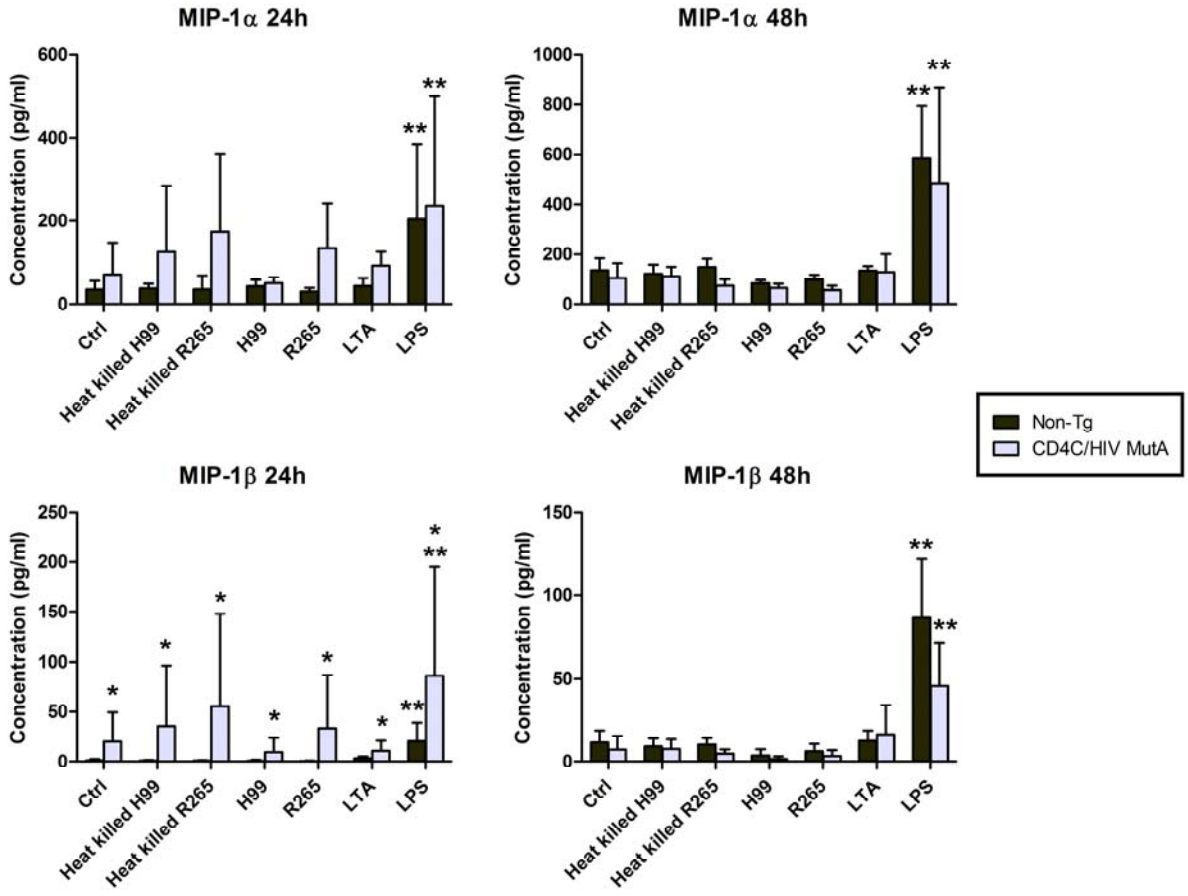

Figure 10. Cytokine production by alveolar macrophages of CD4C/HIV^{MutA} Tg and non-Tg mice 24 or 48 hours after exposure to KRPG control, heat-killed *C. neoformans* H99 and *C. gattii* R265, live H99 and R265, lipoteichoic acid, or lipopolysaccharide. MCP-1 and RANTES, not shown, were undetectable in the supernatants. Data are the means ± standard error of the means (SEM) of the results. *Tg > non-Tg ($P = 0.03$) and ** LPS > agonists and control ($P < 0.05$).

Both MCP-1 and RANTES were undetectable at both 24 and 48 hours after exposure in both Tg and non-Tg mice independently of the agonist present. At 24 hours, alveolar macrophages from Tg mice had a higher production of MIP-1β than non-Tg macrophages independently of the agonist present ($P = 0.03$). In contrast, at 48 hours after agonist exposure, the concentrations of both MIP-1α and MIP-1β in Tg and non-Tg alveolar macrophages were not significantly different ($P>0.05$). The concentration of MIP-1α did not increase significantly between 24 and 48 hours under all conditions, while MIP-1β concentrations from Tg mice were significantly higher at 24 hours compared to 48 hours ($P = 0.025$). MIP-1α and MIP-1β production by alveolar macrophages from Tg and non-Tg mice exposed to LPS was significantly higher compared to other agonists and control at both 24 and 48 hours ($P < 0.05$).

CHAPTER 7- DISCUSSION

Histopathological examination of the lungs at 14 days postinfection with *C. neoformans* or *C. gattii*, previously conducted by Kassandre Leongson, showed an increased inflammatory cell response compared to 7 days postinfection (Leongson et al. 2013). Flow cytometry analysis of pulmonary immune cell populations 7 days postinfection (Annex 2), previously performed by Kassandre Leongson, showed reduced percentages of CD4+ and CD8+ T lymphocytes, and increased percentages of alveolar macrophages and Gr-1+ cells, in Tg mice infected with *C. neoformans* or *C. gattii* (Leongson et al. 2013). Therefore, flow cytometry analysis of immune cell populations 14 days after infection with *C. neoformans* H99 or *C. gattii* R265 was conducted to establish a quantitative assessment of the progression of immune cell recruitment to the lungs of CD4C/HIV^{MutA} Tg and non-Tg mice, from day 7 to day 14 after inoculation (Figure 7).

The expression of the HIV-1 transgene, independently of cryptococcal infection, resulted in an increased percentage of alveolar macrophages. The HIV-1 nef protein, expressed in CD4C/HIV^{MutA} Tg mice, interacts with the macrophage CD40 ligand signaling pathway, enabling the recruitment and subsequent infection by HIV-1 of T lymphocytes (Swingler et al. 1999; Swingler et al. 2003; de Repentigny et al. 2004; Mangino et al. 2007; Goupil et al. 2009). *Nef* is also responsible for modulating signal transduction pathways including apoptosis, MAPK, STAT1, STAT3, JNK, and ERK1/2 in macrophages (Federico et al. 2001; Mangino et al. 2007). The increased percentage of macrophages in Tg mice is consistent with the enhanced survival of HIV-1-infected macrophages, which act as long-term reservoirs for the virus (Vazquez et al. 2005). Infection with either *C. neoformans* or *C. gattii* had no significant effect on the percentage of alveolar macrophages; the same result was shown on

total lung macrophages in C57BL/6 mice (Cheng et al. 2009). Interestingly, interstitial macrophages displayed a significant increase in percentage (P=0.043) during infection with *C. gattii* compared to *C. neoformans*. The percentages of both alveolar and interstitial macrophages significantly increased ($P \leq 0.001$) 14 days after infection, compared to the percentages previously observed at 7 days by Kassandre Leongson. This could be a result of macrophage recruitment to the lungs caused by the increased production of MCP-1, RANTES, MIP-1α, and MIP-1β (Huffnagle et al. 1995; Cook 1996; Murooka et al. 2008).

Interestingly, transgene expression had no significant effect (P=0.371) on the percentage of dendritic cells (DC) in the lungs 14 days after infection (Figure 7). This result is congruent with the results obtained 7 days postinfection, but is surprising because CD4C/HIV^{MutA} Tg mice showed depletion of mature DCs (de Repentigny et al. 2004). Moreover, the percentage of DCs was significantly increased (P=0.02) at 14 days after infection compared to day 7. Since we did not differentiate between mature and immature DCs during the present experiments, further work will be needed to investigate the possible depletion of mature DCs which has been shown in Tg mice aged between 3 and 5 months (Poudrier et al. 2003).

Cheng and colleagues showed a significant decrease in the percentage of DCs when C57BL/6 mice were infected with *C. gattii* compared to *C. neoformans*, an effect also seen, but not significantly so, in Tg and non-Tg mice (Cheng et al. 2009). This could be caused by the differential susceptibility of mouse genetic lineages; C57BL/6 mice have been shown to be more susceptible to *Cryptococcus* infections, while C3H mice are more resistant (Rhodes et al. 1980; Huffnagle et al. 1998). It must also be noted that the flow cytometry method used by Cheng et al. to arbitrarily differentiate DCs and macrophages by low (DCs) and high

(macrophages) autofluorescence is not very precise and prone to experimental and user bias which could affect the results (Cheng et al. 2009).

The expression of the transgene significantly decreased ($P<0.001$) the percentages of both CD4+ and CD8+ T lymphocytes (Figure 7). These results are consistent with the well-characterized reduction of these populations in the oral mucosa, secondary lymphoid organs, and peripheral blood of these Tg mice (Lewandowski et al. 2006), and correlate with the reduced number of CD4+ T lymphocytes in human cases of HIV-1 infection. *Cryptococcus* infection had no significant effect on the recruitment of CD4+ or CD8+ T lymphocytes. There was also no significant increase in the percentage of CD4+ T lymphocytes when comparing results 7 and 14 days after infection. Interestingly, the percentage of CD8+ T lymphocytes was significantly decreased ($P=0.001$) 14 days after infection compared to 7 days. The reduction of CD8+ T lymphocytes could be caused by the upregulation of TNF-α and FasL production by DCs, and the activation of caspase 8 by *nef*, resulting in CD8+ T lymphocyte apoptosis (Quaranta et al. 2004).

Transgene expression did not significantly increase ($P=0.75$) the percentage of Gr-1+ cells at day 14 after inoculation. Gr-1+ cells include not only PMNs, but also plasmacytoid dendritic cells and inflammatory monocytes that express Ly6C but not CD3. The increase in Gr-1+ cells previously showed at day 7 after infection could result from the constitutive activation of macrophages and their enhanced production of cytokines such as TNF-α, IL-1β, and IL-8, which recruit PMNs (Wesselius et al. 1997; de Repentigny et al. 2004). Moreover, it has been shown that CD4C/HIV^{MutA} Tg mice show elevated percentages of neutrophils in peripheral blood (Lewandowski et al. 2006). *C. gattii* infection increased the percentage of Gr-1+ cells compared to *C. neoformans* infection, but the difference was not significant ($P=0.09$). This

result was surprising because Cheng et al. (2009) observed a significant reduction of pulmonary neutrophils in *C. gattii* infections compared to *C. neoformans* infection (Cheng et al. 2009). The differences could be attributed to the fact that Gr-1+ cell populations may include, in addition to PMNs, plasmacytoid DCs and inflammatory monocytes recruited to the lungs. Moreover, Cheng et al. (2009) manually counted pulmonary cell suspensions and calculated Gr-1+ cells as a percentage of total leukocytes present in the sample; in addition, the different susceptibilities to cryptococcosis of the mouse genetic lineages employed may be associated with differing host immune responses (Rhodes et al. 1980; Huffnagle et al. 1998; Cheng et al. 2009). Nevertheless, percentages of Gr-1+ cells were significantly increased ($P<0.001$) between 7 and 14 days after infection, which could be the result of Gr-1+ cell recruitment to the site of infection.

The total number of extracted pulmonary cells was lower in Tg compared to non-Tg mice, 14 days after infection with *C. neoformans* H99 or *C. gattii* R265. Some technical issues need to be considered when interpreting these results. The lungs were manually disrupted with a mortar and pestle, and it is impossible to completely disrupt the lungs. Lungs from mice infected with *C. gattii* were rubbery and proved very difficult to disrupt. Since the lungs were not completely disrupted into a homogenized mixture, some portions of the lung would be lost when the mixture is filtered. These factors resulted in a loss of pulmonary cells. The expression of the transgene resulted in a significantly reduced number of extracted pulmonary cells. This result correlates with histopathological examination that shows reduced inflammation and cellular recruitment in Tg mice compared to non-Tg mice. There was also a significant increase ($P<0.001$) in the number of extracted pulmonary cells, in both Tg and non-Tg mice, 14 days after infection compared to 7 days, which is congruent with the increased

inflammation and cellular recruitment between day 7 and 14 postinfection observed on histopathology. *Cryptococcus* infection had no significant effect on the number of extracted pulmonary cells. To assess if the reduced inflammatory cell recruitment observed in the lungs of Tg mice infected with *Cryptococcus* was caused by an altered cytokine response, cytokines were quantified from the lungs of Tg and non-Tg mice 7 or 14 days after infection. MCP-1 and RANTES concentrations were significantly reduced ($P<0.05$) as a result of transgene expression, independently of *Cryptococcus* infection and the duration of infection. Specifically MCP-1 and RANTES play critical roles in the recruitment of leukocytes in response to *Cryptococcus* infection in the lungs (Huffnagle et al. 1995; Murooka et al. 2008). This effect can be seen in the significantly reduced recruitment of CD4+ and CD8+ T lymphocytes in the lungs of Tg mice 7 and 14 days after infection, which is likely a combination of both HIV-1 transgene-induced depletion and impaired recruitment to the lungs of these lymphocyte populations. However, production of MCP-1 has been shown to be increased in peritoneal macrophages of Tg mice (Goupil et al. 2009). There might be a site-specific difference between the pulmonary and peritoneal macrophage response, since the production of MCP-1 by pulmonary macrophages was consistently reduced after exposure to *C. neoformans* compared to LPS- exposure in a rat model of cryptococcosis (He et al. 2003). This reduced production of MCP-1 could be caused by the induction of TGF-β by cryptococcal extracellular vesicles inhibiting macrophage proliferation (Oliveira et al. 2010; Kubiczkova et al. 2012). It would be interesting to examine if the production of MCP-2 and MCP-3 are impaired due to the expression of the transgene, because the increased production of these cytokines could possibly compensate for the reduced production of MCP-1. The significant increase of IL-17 in Tg mice infected with *C. neoformans* is interesting because its

effects are associated with a protective antifungal response (Kleinschek et al. 2006). The presence of IL-17 along with IL-6 and TNF-α, which are markers of a Th17 response, in Tg mice indicates that transgene expression has no major effect on Th17 polarization and response in the lungs. The significant reduction of IL-4 in Tg mice infected with *C. neoformans* at day 14 after infection is also surprising because IL-4 is usually associated with a non-protective Th2 response that alternatively activates macrophages, reducing their anti-cryptococcal activity (Arora et al. 2011). CD4C/HIV^{MutA} Tg mice usually display a constitutive Th2-like cytokine pattern (Lewandowski et al. 2006).

The production of IL-10 was not significantly altered by the expression of the HIV-1 transgene, while some studies have shown that human monocytes and macrophages have an increased production of IL-10 in HIV-1 infection (Borghi et al. 1995; Leghmari et al. 2008). Since the HIV-1 Tat protein activates the classical and alternative NF-κB pathways, inducing the production of IL-10, this effect cannot be seen in CD4C/HIV^{MutA} Tg mice because these mice express *nef*, *env* and *rev* but not the *tat* gene (Hanna, Kay, Rebai, et al. 1998; Leghmari et al. 2008).

The lower cytokine response to *C. gattii* compared to *C. neoformans* infection correlates with the diminished immune cell response observed on histopathology. The production of MCP-1, RANTES, MIP-1α, MIP-1β, IL-1β, IL-2, IL-4, IL-13, TNF-α, IFN-γ, and TGF-β are all reduced, in comparison to *C. neoformans*, in Tg and non-Tg mice infected with *C. gattii*. The production of MCP-1, RANTES, MIP-1α, and TNF-α play important roles in pulmonary leukocyte recruitment in response to *Cryptococcus* infection (Huffnagle et al. 1996; Huffnagle et al. 2000; Traynor et al. 2002). This reduced cytokine response could potentially explain the slightly diminished percentage of CD4+ and CD8+ T lymphocytes 14 days after infection with

C. gattii. IFN-γ is a marker for a Th1 protective response and is responsible for classically activating macrophages, thus augmenting the anti-cryptococcal effects of the host (Goldsby and Goldsby 2003; Arora et al. 2011). The lack of IFN-γ production at both 7 and 14 days after *C. gattii* infection suggests a non-protective response. A reduced production of IFN-γ has been observed in HIV-infected patients infected with *C. gattii* compared to *C. neoformans* infection (Brouwer et al. 2007). The production of most cytokines increased between days 7 and 14, which correlates with the increased number of extracted pulmonary cells 14 days after infection compared to day 7.

The effect of transgene expression on the production of MIP-1α, MIP-1β, MCP-1, and RANTES by alveolar macrophages 24 and 48 hours after agonist exposure produced surprising results (Figure 10). In Tg mice, the production of MIP-1β was increased compared to non-Tg mice 24 hours after exposure to the agonists. The production in non-Tg mice is nearly nonexistent, and all Tg mice produced elevated concentrations of MIP-1β. Interestingly, this effect is no longer distinguishable 48 hours after exposure to the agonists; the concentrations of MIP-1β are relatively equal (Figure 10). The elevated production of MIP-1β by alveolar macrophages 24 hours after exposure could result from Tg mouse macrophages being constitutively activated (de Repentigny et al. 2004). The increased production of MIP-1β in Tg mice may also be caused by upregulation of NF-κB by *nef*, causing the macrophages from Tg mice to respond more quickly to agonist exposure (Olivetta et al. 2003). Surprisingly, the production of MCP-1 and RANTES by alveolar macrophages was undetectable 24 and 48 hours after exposure to different agonists. The theory for the lack of production of these cytokines is that alveolar macrophages need to be stimulated by both the agonist and IFN-γ in order to fully activate and produce MCP-1 and RANTES (Hu,

Chakravarty, et al. 2008). The use of IFN-γ, which inactivates feedback inhibitory mechanisms, to prime alveolar macrophages may result in the increased production of MCP-1 and RANTES after exposure to the same agonists (Hu, Chakravarty, et al. 2008; Goupil et al. 2009). Moreover, the production of MCP-1 has been shown to peak at 1-2 months after infection in both rats and CBA/J mice (Huffnagle et al. 1995; He et al. 2003). It would therefore be interesting to quantify the cytokine production of alveolar macrophages 7 days after exposure to the agonists.

PERSPECTIVES

The results obtained using this novel murine model to examine *Cryptococcus* infection creates a solid foundation from which to continue the study of the pathogenesis of cryptococcosis with regards to HIV-1 infection.

Since there was no production of MCP-1 and RANTES in alveolar macrophages after both 24 and 48 hours of exposure to agonists, it would be interesting to examine if priming the macrophages with IFN-γ to classically activate them would result in an increased production of these cytokines. IFN-γ priming of macrophages has previously been done in our laboratory to examine the production of H_2O_2 and NO in peritoneal macrophages (Goupil et al. 2009).

RNA from the alveolar macrophages, 24 and 48 hours after agonist exposure has already been extracted using RNeasy kit (Qiagen) and stored at -80°C. The same process could be used for IFN-γ primed alveolar macrophages. It would therefore be interesting to examine if there is an upregulation of certain genes following agonist exposure and IFN-γ priming. The genes that would be fascinating to assess would be for the cytokines analyzed (MIP-1α, MIP-1β, MCP-1, and RANTES); both TLR2 and TLR4; important proteins in the MyD88 pathway (MyD88, MAPK, PI3K, TRAF6, and IRAK-4); and transcription factors (NF-κB and AP-1) (Medzhitov et al. 1998; Suzuki et al. 2002; Biondo et al. 2005; Laird et al. 2009).

It has been shown that both alveolar macrophages and dendritic cells play an important role in the initial innate immune response following *C. neoformans* infection (Osterholzer, Milam, et al. 2009). DCs have the ability to produce MIP-1α, MIP-1β, and TGF-β; and the stimulation of immature murine DC with LPS has been shown to upregulate the expression of both TLR2 and TLR4 (Morelli et al. 2001; An et al. 2002; Maurer and von Stebut 2004). The production of cytokines by purified, flow-sorted, DCs following exposure to the same agonists that were

used with alveolar macrophages would be interesting to examine. Accounting for the fact that Tg mice have an elevated percentage of immature DCs, this would enable us to examine the altered response by DCs due to transgene expression (de Repentigny et al. 2004).

It would be fascinating to examine if the expression of the transgene modulates phosphorylation in the pathways involved in the production of MCP-1, RANTES, MIP-1α, and MIP-1β. This could be analyzed in alveolar macrophages cultured in a phosphate-free medium and the addition of orthophosphate (^{33}P) containing a radiolabel followed by an exposure to an agonist (Anderson and Roche 1998). This would allow the examination of the downstream effects that transgene expression could have in pathway signal transduction.

Better understanding the mechanisms involved in enhanced susceptibility of Tg mice to *C. neoformans* and *C. gattii* infections could enable the production of new and more efficient treatments for cryptococcosis in healthy and immunocompromised patients, as well as developing new strategies to control the outbreak on Vancouver Island and limit the expansion in the Pacific Northwest.

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ANNEX 1- CULTURE MEDIA

YPD (Agar)	
Yeast Extract	10g
Peptone	20g
Dextrose	20g
Agar	20g
H2O	1L

YPD (Broth)	
Yeast Extract	2.5g
Peptone	5g
Dextrose	5g
H20	250mL

Krebs Ringer Phosphate Glucose (KRPG) Medium	
NaCl	145mM
Sodium Phosphate	5.7mM
KCl	4.86mM
CaCl2	0.54mM
Glucose	5.5mM
Adjust pH to 7.35	

Modified Supplemented RPMI Medium	
RPMI 1640 without phenol red	
Penicillin/Streptomycin	100 U/mL
Amphotericin B	0.25 ug/mL
Gentamycin	50 ug/mL
L- glutamine	2 mM
HEPES	20 mM

ANNEX 2- ARTICLE

The article entitled "Altered immune response differentially enhances susceptibility to *Cryptococcus neoformans* and *Cryptococcus gattii* infection in mice expressing the HIV-1 transgene" published in the April 2013 issue of Infection and Immunity, of which I am a co-senior author with Kassandre Leongson, includes histopathological and 7 day postinfection pulmonary immune cell recruitment results that are used in the discussion. These results were produced by Kassandre Leongson. My contributions to this article are quantification and analysis of pulmonary immune cell recruitment at day 14 postinfection, and pulmonary cytokine production at days 7 and 14 after infection.

Infection and Immunity

Altered Immune Response Differentially Enhances Susceptibility to Cryptococcus neoformans and Cryptococcus gattii Infection in Mice Expressing the HIV-1 Transgene

Kassandre Leongson, Vincent Cousineau-Côté, Mathieu Goupil, Francine Aumont, Serge Sénéchal, Louis Gaboury, Paul Jolicoeur, James W. Kronstad and Louis de Repentigny

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Altered Immune Response Differentially Enhances Susceptibility to *Cryptococcus neoformans* and *Cryptococcus gattii* Infection in Mice Expressing the HIV-1 Transgene

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***Cryptococcus neoformans* var. *grubii* is the most frequent cause of AIDS-associated cryptococcosis worldwide, while *Cryptococcus gattii* usually infects immunocompetent people. To understand the mechanisms which cause differential susceptibility to these cryptococcal species in HIV infection, we established and characterized a model of cryptococcosis in CD4C/HIV^{MutA} transgenic (Tg) mice expressing gene products of HIV-1 and developing an AIDS-like disease. Tg mice infected intranasally with *C. neoformans* var. *grubii* strain H99 or C23 consistently displayed reduced survival compared to non-Tg mice at three graded inocula, while shortened survival of Tg mice infected with *C. gattii* strain R265 or R272 was restricted to a single high inoculum. HIV-1 transgene expression selectively augmented systemic dissemination to the liver and spleen for strains H99 and C23 but not strains R265 and R272. Histopathologic examination of lungs of Tg mice revealed large numbers of widely scattered H99 cells, with a minimal inflammatory cell response, while in the non-Tg mice H99 was almost completely embedded within extensive mixed inflammatory cell infiltrates. In contrast to H99, R265 was dispersed throughout the lung parenchyma and failed to induce a strong inflammatory response in both Tg and non-Tg mice. HIV-1 transgene expression reduced pulmonary production of CCL2 and CCL5 after infection with H99 or R265, and production of these two chemokines was lower after infection with R265. These results indicate that an altered immune response in these Tg mice markedly enhances *C. neoformans* but not *C. gattii* infection. This model therefore provides a powerful new tool to further investigate the immunopathogenesis of cryptococcosis.**

Cryptococcal meningitis is one of the most important HIV-related opportunistic infections worldwide, especially in sub-Saharan Africa (1). Globally, approximately 957,900 cases occur each year, resulting in 624,700 deaths among persons living with HIV/AIDS (1). Although cryptococcosis can occur in apparently healthy hosts, most infections are observed in HIV-infected patients, who are particularly susceptible to this life-threatening fungal infection (1). Inhalation of basidiospores or yeast cells of *Cryptococcus* from the environment results in pulmonary infection and preferential dissemination to the central nervous system, causing meningoencephalitis. *Cryptococcus neoformans* var. *grubii* (serotype A) is by far the most frequent cause of AIDS-associated cryptococcosis worldwide, with fewer cases caused by *Cryptococcus neoformans* var. *neoformans* (serotype D), *Cryptococcus gattii* (serotypes B and C) (2–7), or, exceptionally, a *C. neoformans* var. *grubii* serotype A × *C. gattii* serotype B hybrid (8, 9). In contrast to *C. neoformans* var. *grubii*, *C. gattii* usually infects immunocompetent people (10) and is only occasionally found in patients with HIV/AIDS (2–6). In a survey from South Africa, however, although only 2.4% of all *Cryptococcus* isolates were confirmed to be *C. gattii*, 24 of these cases occurred in HIV-infected patients, and only a single case involved an HIV-uninfected person (6). Accordingly, although HIV/AIDS may potentially augment susceptibility to *C. gattii* infection in specific circumstances combining both environmental exposure in an area of endemicity and limited access to antiretroviral therapy, most of the enhanced burden of cryptococcal infection in HIV/AIDS is caused by the ubiquitous *C. neoformans* var. *grubii* (6).

A major endemic outbreak of *C. gattii* infection that began on Vancouver Island in 1999 led to 239 reported cases and at least 19 deaths by the end of 2008 (10–12; www.BCCDC.ca), and it has now spread to mainland British Columbia and the Pacific Northwest in the United States (10, 13–15). Consistent with the epidemiology of *C. gattii* infections in Australia and New Zealand (7, 16), these infections in the British Columbia outbreak occurred mainly in immunocompetent people, and only 6.2% of confirmed *C. gattii*-infected patients were infected with HIV (12).

The mechanisms underlying the differential ability of *C. gattii* and *C. neoformans* var. *grubii* to cause disease in healthy persons or patients with HIV/AIDS are largely unknown. As a first step toward understanding the ability of *C. gattii* to cause disease in immunocompetent hosts, a previous study revealed reduced levels of neutrophil infiltration and reduced inflammatory cytokine production in the lungs of C57BL/6 mice infected with *C. gattii* com-

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pared to those of mice infected with *C. neoformans* var. *grubii* (17). However, a comprehensive analysis of virulence and host immune cell responses to these *Cryptococcus* species would be facilitated greatly by the availability of a relevant animal model of cryptococcosis in HIV infection. We previously devised a novel model of mucosal candidiasis in CD4C/HIV transgenic (Tg) mice expressing gene products of HIV-1 in immune cells and developing an AIDS-like disease (18). These CD4C/HIV Tg mice are immunodeficient and exhibit severe atrophy and fibrosis of lymphoid organs and a preferential depletion of $CD4^+$ T cells, with altered $CD4^+$ T-cell proliferation *in vitro*, loss of $CD4^+$ T-cell help, $CD4^+$ T-cell and B-cell activation, and impaired dendritic cell (DC) function (19–23). In addition, diseases of the lung (lymphocytic interstitial pneumonitis), heart (myocytolysis and myocarditis), and kidney (tubulointerstitial nephritis, segmental glomerulosclerosis, and microcystic dilatation) develop in these Tg mice (19, 24). Mucosal *Candida* infection in these Tg mice closely mimics the clinical and pathological features of candidal infection in human HIV infection (18, 25) and has allowed us to perform controlled studies on the immunopathogenesis of mucosal candidiasis in HIV infection (26–28).

With the recognition that a cause-and-effect analysis of the immunopathogenesis of cryptococcosis and the virulence of *Cryptococcus* species could potentially be achieved with these Tg mice, the present study was undertaken to establish and characterize a novel model of cryptococcosis in these animals and to examine the infections caused by *C. neoformans* var. *grubii* and *C. gattii*, using survival assays, organ fungal burdens, histopathology, and assessments of the host immune response during a time course of infection.

MATERIALS AND METHODS

Strains. *C. neoformans* var. *grubii* strains H99 and C23 and *C. gattii* strains R265 and R272 were used in this study. Clinical strains H99 and C23, both of molecular type VNI (29), were obtained from Joseph Heitman and Thomas Mitchell (Duke University Medical Center). R265 and R272 were both isolated in 2001 from the bronchial washings of immunocompetent patients infected during the outbreak on Vancouver Island and belong to the major VGIIa and less frequent VGIIb molecular types of *C. gattii* causing this outbreak, respectively (11).

Infection of Tg mice expressing HIV-1. CD4C/HIV^{MutA} Tg mice have been described elsewhere (19). CD4C/HIV^{MutA} mutant DNA harbors mouse CD4 enhancer and human CD4 promoter elements to drive expression of the *nef*, *env*, and *rev* genes of HIV-1 in $CD4^+$ $CD8^+$ and $CD4^+$ thymocytes, peripheral $CD4^+$ T cells, macrophages, and DCs. The founder mouse F21388 was bred on the C3H background. Animals from this line express moderate levels of the transgene, with 50% survival at 3 months (19). Several HIV-1 genes (*gag*, *pol*, *vif*, *vpr*, *tat*, and *vpu*) are mutated in the CD4C/HIV^{MutA} DNA, whereas *nef*, *env*, and *rev* are intact. Specific-pathogen-free male and female Tg mice and non-Tg littermates were housed in sterilized individual cages equipped with filter hoods, supplied with sterile water, and fed with sterile mouse chow. All animal experiments were approved by the animal care committee of the University of Montreal.

Cryptococcus strains were grown in yeast extract-peptone-dextrose (YPD) medium for 24 h at 30°C, washed twice with phosphate-buffered saline (PBS), counted in a hemacytometer, and resuspended in PBS at a density of 2.5×10^6 or 2.5×10^5 yeast cells/ml. Intranasal inoculation of the mice was performed as described previously (17). For the survival assay, animals reaching predetermined morbidity endpoints (>20% weight loss, immobile, no response when stimulated, or irregular/labored abdominal respiration) were designated premortem and euthanized with a lethal dose of ketamine and xylazine (18). For all other assays, mice were euthanized on the indicated days. Quantification of *Cryptococcus* in internal organs, histopathology, and determination of *Cryptococcus* cell body diameters and capsule thicknesses in mucicarmine-stained tissue sections were done using methods described elsewhere (17, 18, 30).

Flow cytometry analysis of lung immune cell populations. Groups of five CD4C/HIV^{MutA} Tg and non-Tg littermates (42 to 69 days old) were infected intranasally with 1.25×10^4 CFU of *C. neoformans* H99 or 1.25×10^5 CFU of *C. gattii* R265 and assessed at 7 and 14 days postinfection. Uninfected control mice received intranasal PBS alone. Independent experiments were conducted by pooling cells from all mice within each group. Mice were anesthetized with a mixture of ketamine and xylazine and then exsanguinated with 0.9% NaCl. Single-cell suspensions of lung tissue were prepared by mechanical disruption in a mortar containing 3 ml of PBS and incubation at 37°C for 1 h with 1% collagenase type IV (Sigma) in RPMI 1640 medium (Wisent Inc., St. Bruno, Canada) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Wisent), 100 U/ml penicillin-streptomycin, and 50 μg/ml gentamicin. Cells were filtered through a sterile nylon mesh (pore size, 80 μm) to obtain a homogeneous suspension. Cells were surface stained with anti-mouse anti-CD45, anti-CD11b, anti-CD11c, and anti-F4/80 fluorescence-labeled monoclonal antibodies and their respective isotype controls (all from BioLegend, San Diego, CA) for quantitation of interstitial ($CD45^+$ $CD11b^+$ $CD11c^-$ $F4/80^+$) and alveolar ($CD45^+$ $CD11b^+$ $CD11c^+$ $F4/80^+$) macrophages and dendritic cells ($CD45^+$ $CD11b^+$ $CD11c^+$ $F4/80^-$); with anti-CD45, anti-CD3, and anti-Gr-1 to quantitate $Gr\text{-}1^+$ cells ($CD45^+$ $CD3^-$ $Gr\text{-}1^+$); and with anti-CD45, anti-CD4, and anti-CD8 to quantitate $CD4^+$ ($CD45^+$ $CD4^+$ $CD8^-$) and $CD8^+$ ($CD45^+$ $CD4^-$ $CD8^+$) T-cell populations. Red blood cells were removed with FACS lysing solution (BD Biosciences), and the remaining total extracted cells were counted using a hemacytometer. Cell surface marker analysis was conducted on a FACSCalibur flow cytometer (BD Biosciences) equipped with CellQuest software. Data were acquired for 30,000 events by gating on $CD45^+$ cells. Results for each immune cell population were calculated as both the percentage of $CD45^+$ cells and the absolute number of cells extracted from the lungs of a single mouse.

Production of cytokines. To assay the production of cytokines, lungs were harvested from CD4C/HIV^{MutA} Tg mice and non-Tg littermates 7 or 14 days after intranasal infection with 1.25×10^4 CFU of *C. neoformans* H99 or 1.25×10^5 CFU of *C. gattii* R265. Uninfected control mice received intranasal PBS. Lungs were mechanically disrupted in a mortar containing 2 ml of PBS. Lung homogenates were centrifuged, and supernatants were stored at −80°C. Cytokines in supernatants were assayed using a BD Flex cytometric bead array set (BD Biosciences) according to the manufacturer's protocol on a FACSCalibur flow cytometer equipped with BD CellQuest software. Data analysis was performed using BD FCAP array software 3.0.

Statistical analysis. Kaplan-Meier modeling and a log rank (Mantel-Cox) test were used to compare survival of *C. neoformans* var. *grubii*- and *C. gattii*-infected Tg and non-Tg mice. Organ burdens of *Cryptococcus* were compared using the Kruskal-Wallis test, and significant interactions were further analyzed by use of the Mann-Whitney test. *Cryptococcus* cell body diameters and capsule thicknesses, lung immune cell populations, and cytokine production were analyzed with SPSS, version 19, software (SPSS, Chicago, IL), using analysis of variance. Differences were considered significant if the P value was <0.05.

RESULTS

Enhanced susceptibility to cryptococcosis in Tg mice. Tg and non-Tg mice were infected intranasally with three graded inocula of *C. neoformans* (strain H99 or C23) or *C. gattii* (strain R265 or R272) and then assessed for survival and organ burdens. Survival of both Tg and non-Tg mice was inversely correlated with the inoculum size of *C. neoformans* and *C. gattii*, with the single exception of Tg mice infected with strain R265 (Fig. 1A). Although *C. neoformans* strain C23 was less virulent than *C. neoformans*

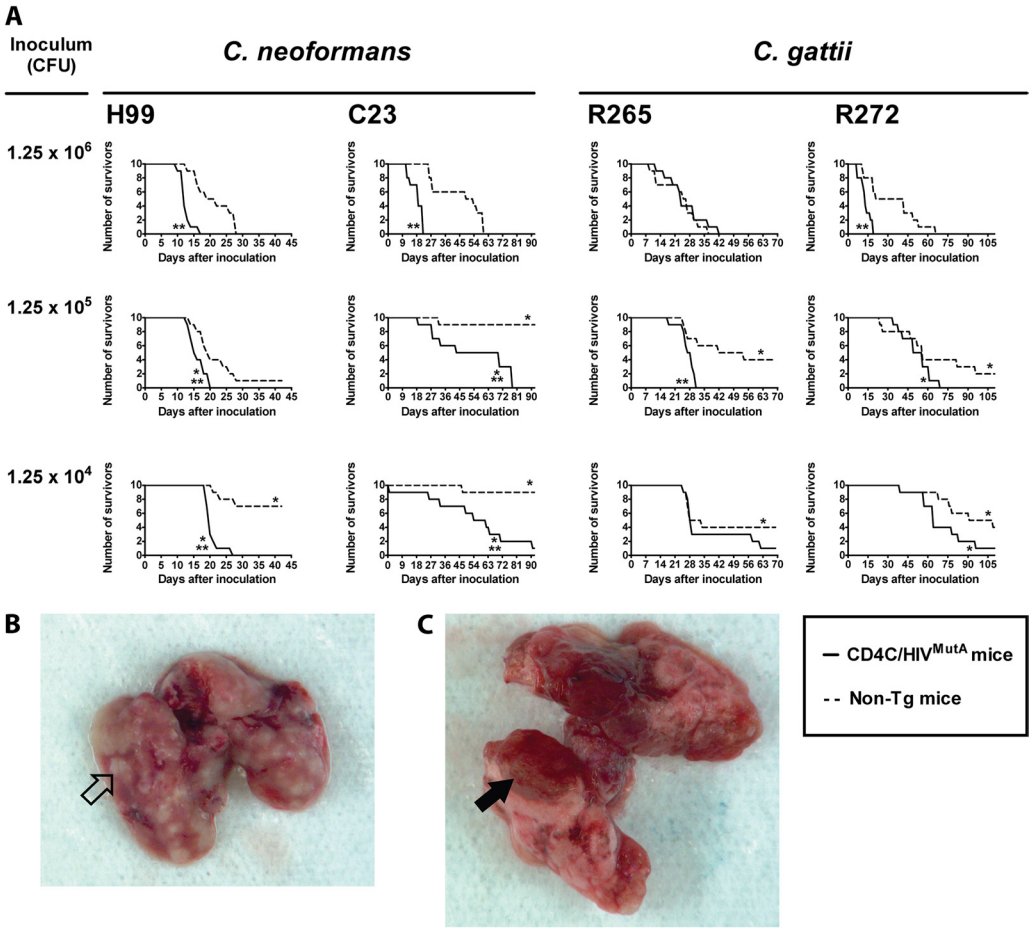

FIG 1 (A) Survival of Tg and non-Tg mice infected with *Cryptococcus neoformans* (strain H99 or C23) or *Cryptococcus gattii* (strain R265 or R272). Ten mice were infected intranasally at each of the indicated inocula. Significant differences are indicated as follows: *, $P < 0.01$ versus mice infected with the same strain at an inoculum of 1.25×10^6 CFU; and **, $P < 0.01$ for Tg versus non-Tg mice infected with identical inocula of the same strain. (B and C) Lungs harvested at necropsy from Tg and non-Tg mice infected with *C. neoformans* H99 or *C. gattii* R265 all showed multiple hemorrhagic (filled arrowhead) and abscess-like (open arrowhead) surface lesions. Representative examples are shown for a non-Tg mouse infected with *C. neoformans* H99 (B) and a Tg mouse infected with *C. gattii* R265 (C).

strain H99 in Tg and non-Tg mice at all three inocula ($P < 0.03$), Tg mice infected with these two *C. neoformans* strains consistently displayed reduced survival compared to non-Tg mice infected at the same three inocula. The enhanced susceptibility to cryptococcosis in the Tg mice was especially prominent in animals infected with the low inoculum of 1.25×10^4 CFU of *C. neoformans* H99, none of which survived, in comparison to the 70% survival of the non-Tg animals (Fig. 1A). Likewise, the mortality of Tg mice infected with *C. neoformans* C23 at this inoculum was 90%, compared to 10% for the non-Tg mice (Fig. 1A). In contrast to the *C. neoformans* infections, shortened survival of Tg mice infected with *C. gattii* strain R265 or R272 was restricted to a single higher inoculum (1.25×10^5 or 1.25×10^6, respectively) (Fig. 1A). Lungs harvested at necropsy from Tg and non-Tg mice infected with *C. neoformans* H99 or *C. gattii* R265 were macroscopically indistinguishable. All showed multiple hemorrhagic and abscess-like surface lesions (Fig. 1B and C). Taken together, the results of these survival studies clearly demonstrated that HIV-1 transgene expression markedly and consistently enhanced susceptibility to *C. neoformans*, independent of the inoculum, while this effect was discernible only at a single inoculum with *C. gattii*.

Organ burdens of euthanized mice premortem, determined as CFU/g (Tables 1 and 2), demonstrated a close correlation with survival of Tg and non-Tg mice infected with *C. neoformans* or *C. gattii*. In non-Tg mice, organ burdens in the liver and spleen increased significantly with the inoculum size of the two *C. neoformans* strains ($P \leq 0.001$), but not the two *C. gattii* strains ($P > 0.05$), but in Tg mice, inoculum size had no significant effect on organ burdens of either *C. neoformans* or *C. gattii* ($P > 0.05$). The two *C. neoformans* strains produced comparable burdens in the liver and spleen within the Tg and non-Tg groups of mice ($P > 0.05$), but both sets of burdens were greater than those produced by the two *C. gattii* strains ($P < 0.03$), which did not differ significantly from each other ($P > 0.05$). Interestingly, the reduced survival of Tg mice infected with *C. neoformans* compared to infected non-Tg animals was correlated with strikingly enhanced systemic dissemination to the liver and spleen of strains H99 and C23 at the two lowest inocula (1.25×10^4 and 1.25×10^5 CFU) ($P < 0.03$) (Table 1). In contrast, burdens of *C. gattii* strains R265 and R272 in these organs were comparable at all three inocula in Tg and non-Tg mice ($P > 0.05$) (Table 2), demonstrating that HIV-1 transgene expression selectively augments systemic dis-

TABLE 1 Viable CFU in organs of CD4C/HIV^{MutA} Tg mice inoculated intranasally with *Cryptococcus neoformans*

Strain and variable	Value[a]					
	Tg mice			Control non-Tg mice		
C. neoformans H99						
Inoculum (CFU)	1.25×10^6	1.25×10^5	1.25×10^4	1.25×10^6	1.25×10^5	1.25×10^4
No. of mice inoculated	10	10	10	10	10	10
Mean (range) age at inoculation (days)	59 (49–63)	54 (50–57)	60 (50–64)	49 (42–63)	53 (45–67)	61 (50–64)
Mean (range) age at assessment (days)	72 (61–80)[b]	70 (64–75)[b]	80 (70–84)[b]	70 (58–80)[b]	76 (65–93)[c]	96 (73–106)[c]
Variables for organs culture positive for *C. neoformans*						
Brain						
No. of mice	7	9	9	10	8	5
C. neoformans count (CFU/g)	8.8×10^7	2.5×10^7	7.1×10^7	6.0×10^7	5.3×10^7	6.2×10^7
Range of counts	7.4×10^3-3.8×10^8	1.4×10^4-1.0×10^8	2.7×10^4-2.8×10^8	3.3×10^5-1.6×10^8	2.7×10^4-1.3×10^8	7.9×10^4-2.0×10^8
Lungs						
No. of mice	9	10	9	10	9	6
C. neoformans count (CFU/g)	2.0×10^9	1.3×10^9	2.5×10^9	1.2×10^9	1.0×10^9	3.8×10^8
Range of counts	2.7×10^8-8.7×10^9	1.8×10^7-5.0×10^9	9.6×10^7-4.7×10^9	8.6×10^7-5.4×10^9	6.2×10^6-6.3×10^9	6.9×10^5-6.9×10^8
Liver						
No. of mice	9	10	9	10	9	4
C. neoformans count (CFU/g)	4.6×10^5	1.3×10^6	3.5×10^5	2.9×10^5	8.8×10^4	7.3×10^4
Range of counts	2.5×10^4-2.5×10^6	4.2×10^4-7.6×10^6	1.3×10^5-6.5×10^5	2.9×10^4-8.4×10^5	1.1×10^4-3.2×10^5	1.9×10^4-2.1×10^5
Spleen						
No. of mice	8	9	9	10	8	3
C. neoformans count (CFU/g)	3.6×10^6	1.5×10^6	1.5×10^6	4.0×10^5	2.2×10^5	6.0×10^4
Range of counts	1.1×10^5-2.2×10^7	2.3×10^5-2.8×10^6	3.6×10^5-4.0×10^6	5.4×10^4-1.1×10^6	1.8×10^4-6.5×10^5	9.1×10^3-1.2×10^5
C. neoformans C23						
Inoculum (CFU)	1.25×10^6	1.25×10^5	1.25×10^4	1.25×10^6	1.25×10^5	1.25×10^4
No. of inoculated mice	10	10	10	10	10	10
Mean (range) age at inoculation (days)	53 (50–56)	53 (46–60)	46 (43–52)	45 (43–57)	49 (49–50)	52 (50–54)
Mean (range) age at assessment (days)	71 (62–77)[b]	105 (65–134)[b]	100 (53–135)[d]	90 (69–113)[b]	135 (81–142)[d]	140 (99–146)[d]
Variables for organs culture positive for *C. neoformans*						
Brain						
No. of mice	8	7	6	6	0	0
C. neoformans count (CFU/g)	2.2×10^7	4.4×10^6	8.1×10^6	1.2×10^7	NA	NA
Range of counts	3.6×10^4-7.1×10^7	5.9×10^3-2.0×10^7	9.5×10^3-1.4×10^7	8.2×10^5-4.3×10^7	NA	NA
Lungs						
No. of mice	10	7	4	8	1	0
C. neoformans count (CFU/g)	4.2×10^8	1.3×10^8	2.2×10^8	5.7×10^8	4.4×10^6	NA
Range of counts	1.2×10^8-9.4×10^8	7.7×10^5-3.2×10^8	2.1×10^6-3.9×10^8	1.7×10^7-1.5×10^9	NA	NA
Liver						
No. of mice	10	7	4	7	1	0
C. neoformans count (CFU/g)	6.1×10^5	6.0×10^5	2.2×10^6	3.4×10^6	4.4×10^3	NA
Range of counts	3.9×10^4-2.5×10^6	1.5×10^3-2.1×10^6	1.4×10^4-4.3×10^6	9.6×10^3-8.6×10^6	NA	NA
Spleen						
No. of mice	10	5	4	5	0	0
C. neoformans count (CFU/g)	2.8×10^6	2.8×10^6	3.1×10^7	4.1×10^6	NA	NA
Range of counts	1.8×10^5-1.7×10^7	2.0×10^5-7.7×10^6	1.0×10^5-7.4×10^7	1.6×10^5-8.5×10^6	NA	NA

[a] Mice studied included Tg and control non-Tg offspring derived from the founder mouse F21388. NA, not applicable.

[b] Assessment was done on the day of euthanization because of severe illness.

[c] Assessment was done on the day of euthanization because of severe illness; survivors were euthanized 42 days after inoculation with *C. neoformans*.

[d] Assessment was done on the day of euthanization because of severe illness; survivors were euthanized 92 days after inoculation with *C. neoformans*.

TABLE 2 Viable CFU in organs of CD4C/HIV^{MutA} Tg mice inoculated intranasally with *Cryptococcus gattii*

Strain and variable	Value[a]					
	Tg mice			Control non-Tg mice		
C. gattii R265						
Inoculum (CFU)	1.25×10^6	1.25×10^5	1.25×10^4	1.25×10^6	1.25×10^5	1.25×10^4
No. of inoculated mice	10	10	10	10	10	10
Mean (range) age at inoculation (days)	57 (55–62)	57 (54–61)	53 (49–62)	56 (52–62)	59 (57–61)	53 (49–62)
Mean (range) age at assessment (days)	83 (70–100)[b]	84 (72–91)[b]	91 (74–131)[c]	79 (61–92)[b]	107 (83–130)[c]	98 (74–131)[c]
Variables for organs culture positive for *C. gattii*						
Brain						
No. of mice	6	6	0	4	3	0
C. gattii count (CFU/g)	5.7×10^4	1.4×10^5	NA	8.3×10^4	3.9×10^4	NA
Range of counts	1.4×10^4-2.2×10^5	6.1×10^3-7.1×10^5	NA	7.4×10^4-2.9×10^5	6.1×10^3-9.0×10^4	NA
Lungs						
No. of mice	10	10	7	9	7	7
C. gattii count (CFU/g)	1.5×10^8	1.3×10^8	8.3×10^7	1.3×10^8	4.2×10^7	6.4×10^7
Range of counts	4.6×10^7-3.8×10^8	7.7×10^7-1.9×10^8	2.3×10^7-1.9×10^8	5.9×10^7-3.2×10^8	1.1×10^7-9.2×10^7	7.5×10^6-1.2×10^8
Liver						
No. of mice	4	4	1	3	2	3
C. gattii count (CFU/g)	1.3×10^6	1.5×10^6	2.4×10^3	3.3×10^4	6.2×10^3	2.8×10^5
Range of counts	1.8×10^3-3.9×10^6	8.8×10^4-4.3×10^6	NA	1.4×10^4-6.8×10^4	2.0×10^3-1.0×10^4	3.6×10^4-7.4×10^5
Spleen						
No. of mice	1	0	0	1	0	0
C. gattii count (CFU/g)	1.8×10^6	NA	NA	3.1×10^5	NA	NA
Range of counts	NA	NA	NA	NA	NA	NA
C. gattii R272						
Inoculum (CFU)	1.25×10^6	1.25×10^5	1.25×10^4	1.25×10^6	1.25×10^5	1.25×10^4
No. of inoculated mice	10	10	10	10	10	10
Mean (range) age at inoculation (days)	53 (47–59)	64 (63–69)	49 (44–53)	56 (46–59)	63 (60–64)	59 (57–61)
Mean (range) age at assessment (days)	66 (58–75)[b]	115 (98–133)[b]	120 (84–162)[d]	89 (62–125)[b]	129 (84–173)[d]	149 (96–170)[d]
Variables for organs culture positive for *C. gattii*						
Brain						
No. of mice	1	5	1	5	1	5
C. gattii count (CFU/g)	5.9×10^3	2.4×10^4	6.4×10^6	9.6×10^4	NA	5.9×10^7
Range of counts	NA	5.6×10^3-4.2×10^4	NA	1.8×10^4-2.4×10^5	NA	1.2×10^7-1.7×10^8
Lungs						
No. of mice	8	9	6	8	6	8
C. gattii count (CFU/g)	7.2×10^7	5.9×10^7	3.8×10^7	1.0×10^8	4.9×10^7	1.5×10^7
Range of counts	5.0×10^7-1.1×10^8	2.1×10^7-1.5×10^8	1.7×10^7-6.2×10^7	2.0×10^8-3.5×10^8	8.3×10^6-1.7×10^8	3.6×10^6-3.4×10^7
Liver						
No. of mice	1	3	3	2	1	3
C. gattii count (CFU/g)	1.9×10^4	1.2×10^5	7.7×10^4	2.7×10^4	8.7×10^5	1.3×10^4
Range of counts	NA	4.6×10^3-2.5×10^5	3.9×10^3-2.0×10^5	2.1×10^4-3.2×10^4	NA	1.2×10^3-2.1×10^4
Spleen						
No. of mice	0	0	0	0	0	0
C. gattii count (CFU/g)	NA	NA	NA	NA	NA	NA
Range of counts	NA	NA	NA	NA	NA	NA

[a] Mice studied included Tg and control non-Tg offspring derived from the founder mouse F21388. NA, not applicable.

[b] Assessment was done on the day of euthanization because of severe illness.

[c] Assessment was done on the day of euthanization because of severe illness; survivors were euthanized 69 days after inoculation with *C. gattii*.

[d] Assessment was done on the day of euthanization because of severe illness; survivors were euthanized 110 days after inoculation with *C. gattii*.

TABLE 3 Viable CFU in organs of CD4C/HIV^{MutA} Tg mice inoculated intranasally with *Cryptococcus* spp.

Strain (inoculum) and variable	Value[a]			
	Tg mice		Control non-Tg mice	
Cryptococcus neoformans H99 (1.25×10^4 CFU)				
Days after inoculation	7	14	7	14
No. of inoculated mice	6	6	6	6
Variables for organs culture positive for *C. neoformans*				
Brain				
No. of mice	0	4	0	2
C. neoformans count (CFU/g)	NA	2.5×10^6	NA	6.9×10^6
Range of counts	NA	4.4×10^4-8.5×10^6	NA	3.8×10^5-1.4×10^7
Lungs				
No. of mice	6	6	6	6
C. neoformans count (CFU/g)	1.1×10^8	6.1×10^8	6.7×10^7	2.0×10^8
Range of counts	3.3×10^7-3.9×10^8	4.6×10^7-1.1×10^9	3.6×10^7-1.0×10^8	1.0×10^8-3.1×10^8
Liver				
No. of mice	0	4	2	1
C. neoformans count (CFU/g)	NA	4.1×10^4	9.6×10^3	6.5×10^3
Range of counts	NA	2.3×10^4-5.8×10^4	7.9×10^3-1.1×10^4	NA
Spleen				
No. of mice	0	4	0	1
C. neoformans count (CFU/g)	NA	2.7×10^5	NA	1.8×10^4
Range of counts	NA	1.6×10^5-4.2×10^5	NA	NA
Cryptococcus gattii R265 (1.25×10^5 CFU)				
Days after inoculation	7	14	7	14
No. of inoculated mice	6	6	6	6
Variables for organs culture positive for *C. gattii*				
Brain				
No. of mice	0	1	4	0
C. gattii count (CFU/g)	NA	3.5×10^5	2.4×10^4	NA
Range of counts	NA	NA	1.3×10^4-5.9×10^4	NA
Lungs				
No. of mice	6	6	6	6
C. gattii count (CFU/g)	1.7×10^8	1.9×10^8	1.6×10^8	2.5×10^8
Range of counts	3.9×10^7-2.4×10^8	1.0×10^8-3.6×10^8	4.0×10^7-2.6×10^8	1.8×10^8-3.3×10^8
Liver				
No. of mice	0	2	1	1
C. gattii count (CFU/g)	NA	6.0×10^3	3.7×10^3	5.7×10^4
Range of counts	NA	4.2×10^3-7.8×10^3	NA	NA
Spleen				
No. of mice	0	0	0	0
C. gattii count (CFU/g)	NA	NA	NA	NA
Range of counts	NA	NA	NA	NA

[a] Mice studied included Tg and control non-Tg offspring derived from the founder mouse F21388. NA, not applicable.

semination to the liver and spleen for *C. neoformans* but not *C. gattii*. However, enhanced burdens in brains of Tg compared to non-Tg mice were observed at the two lowest inocula with *C. neoformans* strain C23 only ($P \leq 0.002$), not strain H99 ($P > 0.05$) or the two *C. gattii* strains ($P > 0.05$) (Tables 1 and 2), showing that HIV-1 transgene-mediated augmentation of *C. neoformans* dissemination to the brain may be strain dependent.

Enhanced cryptococcal burdens and more frequent dissemination to the liver and spleen were also found in Tg compared to non-Tg mice euthanized at the fixed time of 14 days after infection with the lowest inoculum (1.25×10^4 CFU) of *C. neoformans* H99 ($P < 0.05$) (Table 3). Seven days after infection, however, no systemic dissemination had yet occurred, and pulmonary burdens were comparatively lower than those at day 14 ($P < 0.02$) and were not significantly different ($P > 0.05$) in Tg and non-Tg mice (Table 3). In contrast to the case with *C. neoformans* H99, however, lung burdens were comparable at days 7 and 14 ($P > 0.05$), the frequency of systemic dissemination remained low, and cryptococcal burdens in the liver and spleen were comparable in Tg and non-Tg mice 14 days after infection with an intermediate inoculum (1.25×10^5 CFU) of *C. gattii* strain R265 ($P > 0.05$) (Table 3).

Defective inflammatory cell response to *Cryptococcus* in Tg mice. Histopathologic examination of lungs was conducted on days 7 and 14 after infection and premortem to identify the nature, location, and extent of the inflammatory cell response to *C. neoformans* strain H99 and *C. gattii* strain R265 (Fig. 2; see Fig. S1 to S3 in the supplemental material). The pulmonary inflammatory responses to *C. neoformans* were strikingly and consistently different in Tg and non-Tg mice. Seven days after infection of the Tg mice, numerous *C. neoformans* cells were located in the bronchioles and formed cysts or were individually dispersed throughout the lung parenchyma, with a minimal scattered mononuclear

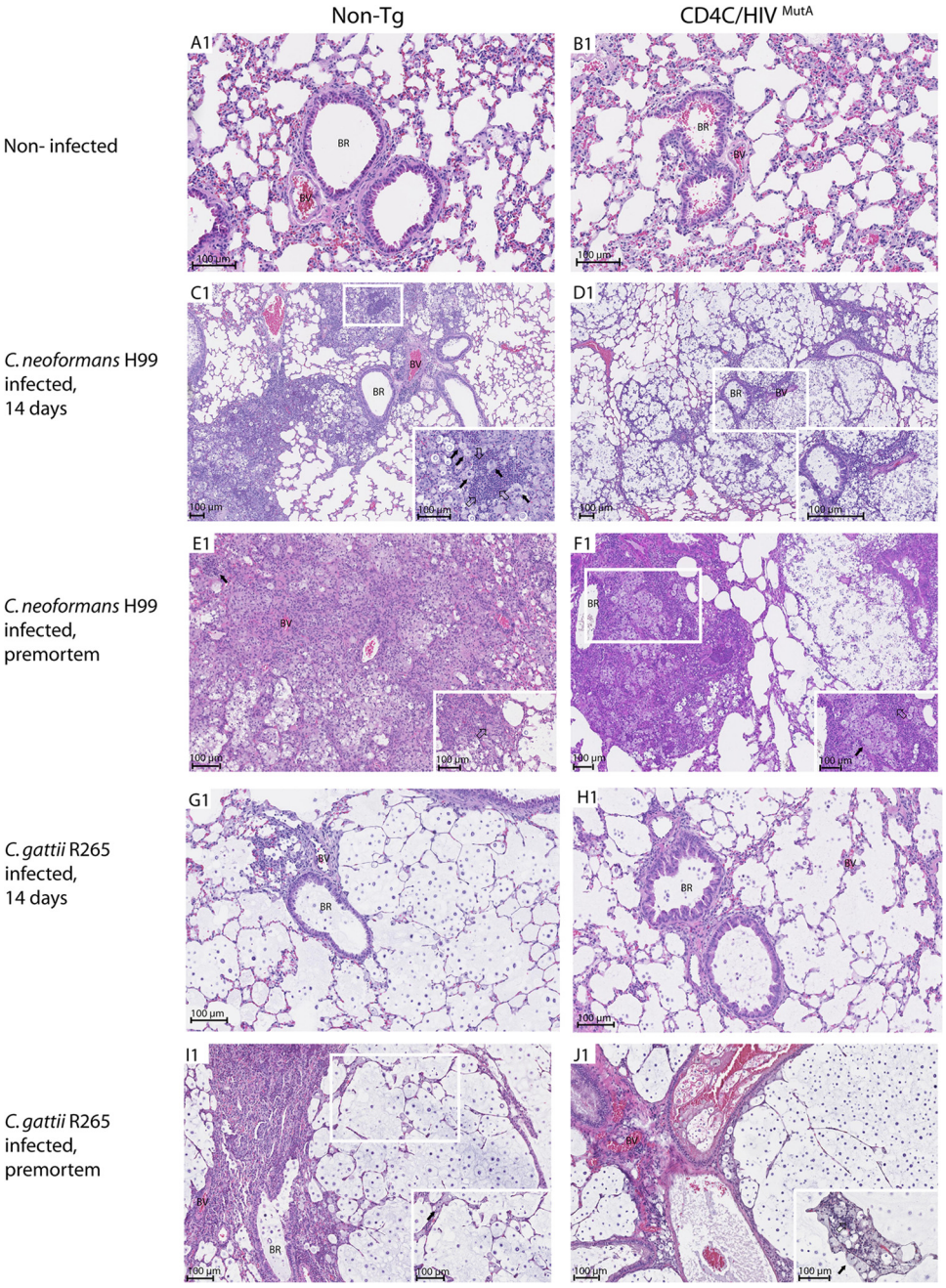

FIG 2 Histopathology of lungs from Tg and non-Tg mice, either uninfected or assessed on day 14 or premortem after intranasal infection with 1.25×10^4 CFU of *Cryptococcus neoformans* H99 or 1.25×10^5 CFU of *Cryptococcus gattii* R265. Tissues were stained with hematoxylin phloxine saffron (HPS). Fourteen days after infection of non-Tg mice (C1), *C. neoformans* was present in great numbers and was almost entirely embedded within extensive mixed inflammatory infiltrates comprised of macrophages (filled arrowheads) and polymorphonuclear leukocytes (open arrowheads) (C1, enlarged inset) and only rarely observed in the remaining lung parenchyma, in marked contrast to the case with Tg mice, which displayed predominantly innumerable and widely scattered *C. neoformans* cells, with a minimal inflammatory cell response (D1) and only rarely enclosed within discrete bronchovascular infiltrates (enlarged inset). Premortem non-Tg mice displayed necrotizing granulomas containing epithelioid cells (filled arrowhead) and Langhans-type giant cells (open arrowhead) (E1), in contrast to Tg mice, which showed no granulomas but numerous macrophages (filled arrowhead) and polymorphonuclear leukocytes (open arrowhead) and wide areas of lung parenchyma containing numerous *C. neoformans* cells but no inflammatory response (F1). *C. gattii* was widely dispersed throughout the lung tissue and induced only a modest and localized inflammatory response comprised of macrophages (filled arrowheads) and polymorphonuclear leukocytes (open arrowhead) in Tg and non-Tg mice (G1 to J1). BR, bronchiole; BV, blood vessel. Images are representative of 2 (A1 and B1) or 6 (C1 to J1) mice per group, with consistent results.

cell infiltrate (see Fig. S2L1 and S3L2). In contrast, the non-Tg mice displayed dense bronchovascular infiltrates containing mononuclear cells and polymorphonuclear leukocytes (PMNs) completely enclosing *C. neoformans* (see Fig. S2K1). Fourteen days after infection of the Tg mice, much larger numbers of *C. neoformans* cells were widely scattered in the lung tissue, with a minimal inflammatory cell response, and were rarely observed within discrete bronchovascular infiltrates containing mostly

PMNs and a few mononuclear cells (Fig. 2D1; see Fig. S1D2). In striking contrast, in non-Tg mice, *C. neoformans* cells were almost entirely embedded within far more extensive mixed inflammatory infiltrates comprised of PMNs and macrophages and were seldom observed in the remaining lung parenchyma, which was devoid of inflammatory cells (Fig. 2C1). Finally, premortem non-Tg mice again displayed a widespread inflammatory response, with the added appearance at this late time point of necrotizing granulomas containing epithelioid cells and Langhans-type giant cells (Fig. 2E1). This was in contrast to the Tg mice, which displayed more limited inflammatory foci containing abundant macrophages and PMNs but no granulomas, as well as broad areas of lung parenchyma containing numerous *C. neoformans* cells but no inflammatory response (Fig. 2F1).

In sharp contrast to the case for infection with *C. neoformans*, numerous *C. gattii* cells were widely dispersed throughout the lung tissue and induced only a sparse inflammatory response on days 7 and 14 after infection in both Tg and non-Tg mice (Fig. 2G1 and H1; see Fig. S1 to S3 in the supplemental material). A modest and circumscribed inflammatory response comprised of macrophages and PMNs appeared only in premortem animals and was independent of HIV-1 transgene expression (Fig. 2I1 and J1).

Interestingly, macrophages in lung tissue sections from Tg and non-Tg mice infected with *C. neoformans* or *C. gattii* often displayed the distinctive appearance of "hueco" cells filled with vesicles containing capsular polysaccharide (31, 32). These cells were observed beginning on day 14 after infection and became more abundant in mice assessed premortem.

Histopathologic examination of the brains of Tg and non-Tg mice on day 7 after infection with *C. neoformans* showed that the brains were entirely normal, in accordance with the absence of systemic dissemination to this organ at this early time point (Table 3). On day 14 after infection, however, histopathology revealed *C. neoformans* in the brain parenchyma of a single non-Tg mouse which displayed culture evidence of dissemination to this organ, but not in the other animals, which were either culture positive or negative (Table 3). Taken together with the organ burdens, these results indicated that the onset of dissemination to the brain for *C. neoformans* was detectable more than 7 days after infection in both Tg and non-Tg mice and did not occur earlier in the Tg mice, despite their enhanced frequency of systemic dissemination (Tables 1 and 2). Examination of the brains of Tg and non-Tg mice 7 and 14 days after infection with *C. gattii* did not show histopathologic evidence of the fungus, in accordance with lower burdens of *C. gattii* than of *C. neoformans* in this organ (Table 3).

Cell body diameters and capsule thicknesses of 100 randomly selected *C. neoformans* or *C. gattii* cells were determined in lung tissue sections from Tg and non-Tg mice 7 or 14 days after infection. For both *C. neoformans* and *C. gattii*, cell body diameters and capsule thicknesses increased significantly from day 7 to day 14 after infection of non-Tg mice ($P < 0.001$) but not Tg mice ($P > 0.05$), and both measurements were greater in non-Tg than in Tg mice on day 14 after infection with these two species ($P < 0.001$) (Table 4). However, cell body diameters and capsule thicknesses of *C. neoformans* H99 were markedly greater than those of *C. gattii* R265 both 7 and 14 days after infection of both Tg and non-Tg mice ($P < 0.001$), showing that the dimensions of these two species consistently differ *in vivo*, irrespective of time after infection or HIV-1 transgene expression (Table 4). Interestingly, using a cell body diameter threshold of 15 μm, 22 to 53% of *C. neoformans* H99 cells comprised giant (titan) cells (33), but these cells were seen less frequently (3 to 12% of cells) in tissue sections from mice infected with *C. gattii* 265.

TABLE 4 Cell body diameters and capsule thicknesses of *C. neoformans* H99 and *C. gattii* R265 in mucicarmine-stained lung tissue sections 7 or 14 days after infection of CD4C/HIVMutA Tg or non-Tg mice

Measurement and strain	Value after infection[a]			
	Tg mice		Non-Tg mice	
	7 days	14 days	7 days	14 days
Cell body diameter (μm)				
C. neoformans H99	11.8 ± 4.2[b]	11.4 ± 4.5[b]	12.4 ± 3.6[b]	15.4 ± 3.5[b,c,d]
C. gattii R265	10.3 ± 2.8	9.8 ± 2.7	9.5 ± 2.9	12.0 ± 2.8[c,d]
Capsule thickness (μm)				
C. neoformans H99	5.0 ± 1.9[b]	5.6 ± 2.5[b]	6.3 ± 1.8[b]	8.2 ± 2.0[b,c,d]
C. gattii R265	4.9 ± 1.4	4.7 ± 2.3	3.5 ± 1.3	6.2 ± 2.0[c,d]

[a] Data are means ± standard deviations for 100 randomly selected cells.
[b] $P < 0.001$ compared to *C. gattii* R265.
[c] $P < 0.001$ compared to non-Tg mice at day 7.
[d] $P < 0.001$ compared to Tg mice at day 14.

Altered lung immune cell populations in response to *Cryptococcus* in Tg mice. To quantitatively assess the impact of HIV-1 transgene expression on lung immune cell populations, multiparametric flow cytometry analysis was conducted on CD4C/HIV^{MutA} Tg mice and non-Tg littermates 7 and 14 days after infection or no infection with *C. neoformans* H99 or *C. gattii* R265. On both days, transgene expression independently caused striking reductions in the percentages of CD4$^+$ and CD8$^+$ T cells ($P < 0.001$) (Fig. 3). Furthermore, on day 14, total numbers of extracted pulmonary cells were significantly lower in Tg mice than in non-Tg mice ($P = 0.002$), correlating with the defective inflammatory cell response to *Cryptococcus* observed on histopathology. Independent of cryptococcal infection, percentages of alveolar macrophages were significantly increased ($P < 0.05$) in Tg compared to non-Tg mice on days 7 and 14 (Fig. 3). Similar findings were observed with Gr-1$^+$ cells, but they reached statistical significance only on day 7 (Fig. 3). In addition, from day 7 to day 14, in both Tg and non-Tg mice, the percentages of dendritic cells, alveolar macrophages, and Gr-1$^+$ cells were significantly increased in animals infected with either *C. neoformans* or *C. gattii* ($P \leq 0.02$), while a similar increase in interstitial macrophages during the same interval was restricted to *C. gattii* ($P < 0.001$). We cannot formally exclude the possibility that in addition to PMNs, plasmacytoid dendritic cells and inflammatory monocytes, expressing Ly6C but not CD3, may have been recognized by the anti-Gr-1 antibody. Finally, absolute numbers of CD4$^+$ and CD8$^+$ cells, but not the other cell populations, were significantly diminished ($P < 0.05$) in the Tg compared to non-Tg mice on days 7 and 14 after infection or no infection with *C. neoformans* or *C. gattii* (data not shown).

Altered production of pulmonary cytokines in response to *Cryptococcus* in Tg mice. To determine if the reduced pulmonary inflammatory response to *Cryptococcus* observed in the Tg mice was associated with defective production of cytokines, Tg and non-Tg mice were assessed 7 or 14 days after infection or no infection with *C. neoformans* H99 or *C. gattii* R265. In comparison to the levels in non-Tg mice, HIV-1 transgene expres-

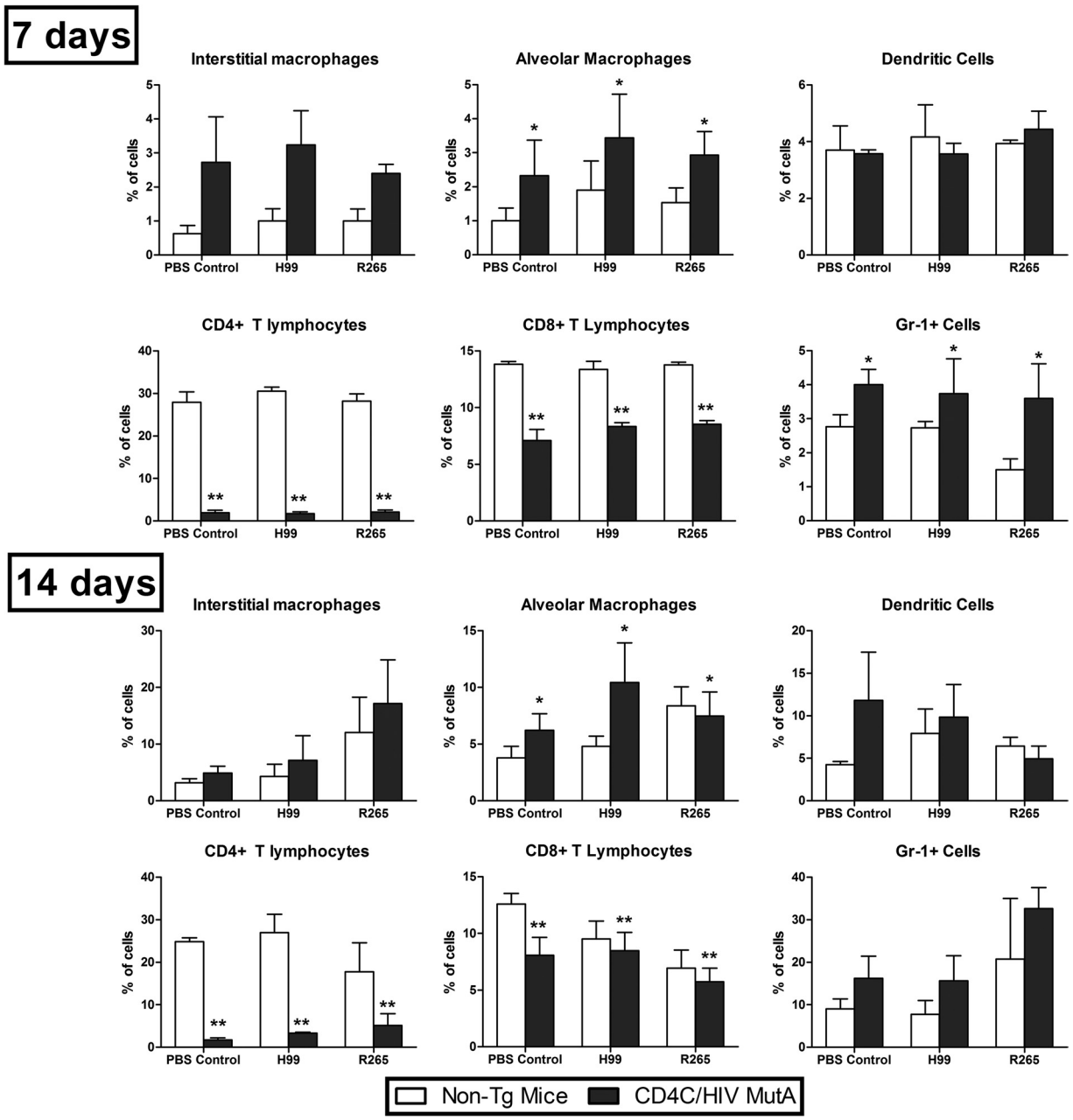

FIG 3 Flow cytometry analysis of lung immune cell populations in CD4C/HIV^{MutA} Tg and non-Tg mice 7 and 14 days after infection or no infection with *C. neoformans* H99 or *C. gattii* R265. Data are presented as percentages of CD45$^+$ cells and are the means ± standard errors of the means (SEM) of results from three or four independent experiments. Significant differences are indicated as follows: *, Tg > non-Tg mice ($P < 0.05$); and **, Tg < non-Tg mice ($P < 0.001$).

sion consistently reduced pulmonary production of the CC chemokines monocyte chemoattractant protein 1 (MCP-1; CCL2) and RANTES (CCL5) both 7 and 14 days after infection with *C. neoformans* or *C. gattii* ($P \leq 0.01$) (Fig. 4), suggesting that defective production of these chemotactic cytokines may contribute to the defective inflammatory response to *Cryptococcus* in Tg mice. In addition, in comparison to the case with *C. neoformans*, production of these two chemokines was significantly lower after infection with *C. gattii* ($P < 0.001$), which may partially explain the markedly reduced pulmonary inflammatory response to *C. gattii* in comparison to that to *C. neoformans* in the non-Tg mice (Fig. 2). Indeed, a wide array of cytokines (interleukin-1β [IL-1β], tumor necrosis factor alpha [TNF-α], macrophage inflammatory protein 1β [MIP-1β], IL-13, transforming growth factor beta [TGF-β], and IL-4) increased significantly from day 7 to day 14 after infection with *C. neoformans* ($P < 0.001$) but not *C. gattii* ($P > 0.05$), and higher concentrations of TNF-α, MIP-1α, MIP-1β, IL-13, TGF-β, IL-2, and IL-4 were also found on day 14 after infection with *C. neoformans* compared to *C. gattii* ($P < 0.001$), independent of transgene expression. This differential production of cytokines after infection by the two species was especially prominent in

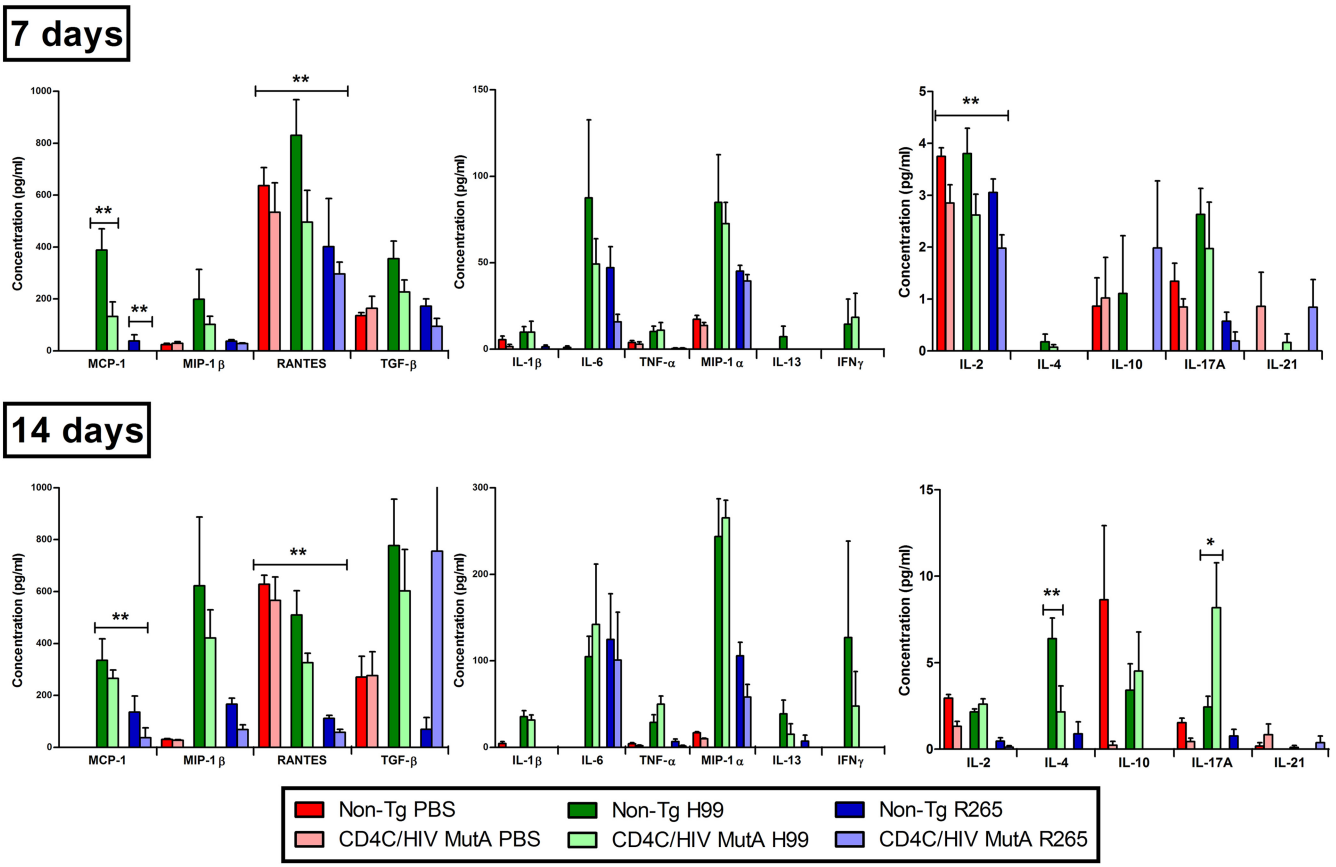

FIG 4 Cytokine production in lungs of CD4C/HIV^{MutA} Tg and non-Tg mice 7 and 14 days after infection or no infection with *C. neoformans* H99 or *C. gattii* R265. IL-12p70 (not shown) was undetectable in all mice. Data are the means ± SEM of results from six mice. Significant differences are indicated as follows: *, Tg > non-Tg mice ($P < 0.05$); and **, Tg < non-Tg mice ($P < 0.05$).

the case of gamma interferon (IFN-γ), which was produced exclusively in response to infection with *C. neoformans*.

In sharp contrast, however, production of IL-6 and IL-10 increased significantly from day 7 to day 14 after infection with either of the two species ($P < 0.05$), suggesting that the lower pulmonary inflammatory response observed after infection with *C. gattii* than after infection with *C. neoformans* was not associated with a differing production of these two cytokines.

In addition to MCP-1 and RANTES, HIV-1 transgene expression resulted in decreased production of IL-2 on day 7 after infection ($P \leq 0.03$), a defect previously associated with enhanced susceptibility to cryptococcosis at this early time point of infection (34). However, in comparison to non-Tg controls on day 14, Tg mice infected with *C. neoformans* unexpectedly had reduced production of IL-4 ($P \leq 0.001$) and increased production of IL-17A ($P \leq 0.001$), both of which are associated with a protective rather than nonprotective anticryptococcal host response (35–37).

DISCUSSION

The model which we established recapitulates the hallmark histopathological features of human pulmonary *C. neoformans* (38, 39) and *C. gattii* (40) infections, including a minimal inflammatory cell infiltrate in transgenic mice infected with *C. neoformans* that reproduces the pathological findings in AIDS patients (38). The present results also clearly establish, for the first time in an animal model, using controlled conditions with two strains each and three inocula of *C. neoformans* and *C. gattii*, that HIV-1 expression consistently augments susceptibility to *C. neoformans* but not that to *C. gattii*. This finding provides experimental evidence to support the results of epidemiological studies of cryptococcosis, which demonstrate that *C. neoformans* causes the overwhelming majority of infections in the setting of HIV infection, while *C. gattii* infections occur mostly in immunocompetent persons (12, 16). The lack of a significant transgene effect on mortality at the lowest inoculum of *C. gattii* (1.25×10^4 CFU), in contrast to an inoculum of 1.25×10^5 CFU, may have resulted from differing levels of the inflammatory response to *C. gattii* at these two inocula.

Assessments of organ burdens, lung histopathology, immune cell populations, and cytokine production were conducted at the fixed time points of 7 and 14 days after infection with 1.25×10^4 CFU of *C. neoformans* H99 or 1.25×10^5 CFU of *C. gattii* R265. These inocula were selected on the basis of the results of the survival study, which showed the greatest transgene effect on mortality at these inocula (Fig. 1), and therefore they were most likely to reveal differences in organ burdens at the fixed time points. Immune response parameters were assessed for the same inocula to allow a meaningful correlation with organ burden data.

In comparison to *C. neoformans*, infection of immunocompetent non-Tg C3H mice with *C. gattii* elicited a markedly reduced pulmonary inflammatory cell response, as reported previously for

C57BL/6 and A/JCr mice infected with identical inocula of the two species (17, 41). It is therefore unlikely that the less robust pulmonary inflammatory cell response to *C. gattii* than that to *C. neoformans* which we found in the non-Tg mice was caused by the higher inoculum.

The lower pulmonary inflammatory cell response to *C. gattii* was closely correlated with diminished production of several cytokines and chemokines, including MCP-1, RANTES, MIP-1α, MIP-1β, IL-1β, IL-2, IL-4, IL-13, TNF-α, IFN-γ, and TGF-β. Among these, MCP-1, MIP-1α, TNF-α, and IFN-γ all play a role in leukocyte recruitment to the lungs in response to *C. neoformans* infection (42–52). Accordingly, reduced production of these four cytokines may explain, at least in part, the strikingly sparse inflammatory cell response to *C. gattii* compared to that to *C. neoformans* in the non-Tg C3H mice. Interestingly, we found greater capsule thicknesses of *C. neoformans* than *C. gattii*, and it has been reported that increasing capsule thicknesses of *C. neoformans* augment the magnitudes of IL-1β and TNF-α release by human PMNs (53). It would be relevant in future work to examine infection by *C. neoformans* 145A, which like *C. gattii* R265 induces a limited pulmonary inflammatory response (54), to determine if it behaves similarly to *C. gattii* in HIV-1-expressing Tg mice.

Despite these strikingly dissimilar host immune responses to *C. neoformans* and *C. gattii*, comparable lung burdens of both cryptococcal species were found on days 7 and 14 after infection and premortem. This seemingly paradoxical finding could possibly be explained by the antiphagocytic properties of the cryptococcal capsule (55) and the reduced phagocytosis of cryptococcal giant (titan) cells (33, 56–58), which would allow *C. neoformans* to proliferate at a rate comparable to that of *C. gattii* despite the enhanced inflammatory cell response. However, in a recent report (41), *C. gattii* R265 produced higher lung burdens than those of *C. neoformans* H99 after infection of C57BL/6 and BALB/c mice, suggesting that the protective pulmonary immune responses to *Cryptococcus* of these two mouse strains may differ qualitatively or quantitatively from those of non-Tg C3H mice. Nevertheless, in the non-Tg C3H mice, dissemination of *C. neoformans* to the liver and spleen at the time of euthanasia largely exceeded that of *C. gattii*, demonstrating a greater capacity of *C. neoformans* for systemic dissemination in the immunocompetent host (41). The greater capsule thickness of *C. neoformans* than that of *C. gattii*, providing protection against reactive oxygen and nitrogen species within phagocytes (55), may have facilitated dissemination by a "Trojan horse" mechanism (59). Despite this enhanced dissemination, however, the survival of non-Tg C3H mice infected with the *C. neoformans* and *C. gattii* strains did not differ significantly, suggesting that the variable virulence of strains within each species outweighs any potentially consistent difference in virulence between these two cryptococcal species. In fact, previous studies comparing the virulence of *C. neoformans* H99 and *C. gattii* R265 in C57BL/6 and BALB/c mice produced inconsistent results (17, 41), indicating that the virulence of *C. neoformans* and *C. gattii* is likely comparable in many, if not most, strains of immunocompetent mice. This interpretation is supported by the balanced upregulation in production of protective (IFN-γ) and nonprotective (IL-4 and IL-13) cytokines (36, 51, 52, 60, 61) in non-Tg C3H mice infected with *C. neoformans* compared to those infected with *C. gattii*. Taken together, the results of our survival studies demonstrate that HIV-1 transgene expression alters the course of cryptococcal infection to a far larger degree than any intrinsic differences in virulence, systemic dissemination, or host immune responses between *C. neoformans* and *C. gattii*.

Enhanced susceptibility to *C. neoformans* infection in the Tg mice was associated with a sharply reduced pulmonary inflammatory cell response and decreased production of the CC chemokines MCP-1 (CCL2) and RANTES (CCL5). The striking depletion of pulmonary $CD4^+$ and $CD8^+$ T cells in infected or uninfected Tg mice is congruent with the quantitative reductions of these cell populations in the oral mucosa, secondary lymphoid organs, and peripheral blood of these Tg mice (18, 23). The present results therefore suggest that the defective pulmonary $CD4^+$ and $CD8^+$ T-cell response to *C. neoformans* infection in Tg mice resulted from the primary depletion of these cell populations as a consequence of HIV-1 transgene expression, combined with a failure of their recruitment as a result of reduced production of the chemokines MCP-1 and RANTES, which attract activated T cells, monocytes, and dendritic cells. During pulmonary *C. neoformans* infection, upregulation of MCP-1 and MCP-3 (CCL7) production is required for CCR2-mediated recruitment of T cells, dendritic cells, and macrophages, formation of bronchovascular cell infiltrates, and development of protective Th1 immunity (42–48). Furthermore, SJL/J mice, which are resistant to *C. neoformans* infection, show enhanced MCP-1 mRNA expression compared to susceptible C57BL/6 mice (62). Potential cellular sources of MCP-1 in the lungs include epithelial cells, endothelial cells, fibroblasts, and macrophages (42). Of these specific cell populations, only macrophages express the HIV-1 transgene (19) and would thus be susceptible primarily to altered cytokine expression. In this regard, we have previously shown that $F4/80^+$ macrophages recruited to the gastric submucosa and oral mucosa of HIV-1-expressing Tg mice in response to *Candida albicans* infection express the mannose receptor (CD206) almost uniformly, but MCP-1 only very infrequently (26), consistent with an alternatively activated (M2) phenotype known to be associated with susceptibility to cryptococcosis (36, 52). Furthermore, because it has been shown that experimental depletion of $CD4^+$ and $CD8^+$ T cells independently abrogates the appearance of a protective inflammatory response to pulmonary *C. neoformans* infection and augments systemic dissemination (63–65), it is likely that the depletion of these T-cell populations in the Tg mice contributed to the reduced pulmonary inflammatory cell response to *C. neoformans* and the augmented systemic dissemination to the liver and spleen. Despite the defective pulmonary inflammatory cell response to *C. neoformans* in the Tg mice, pulmonary fungal burdens were remarkably comparable to those in non-Tg mice, suggesting that reduced survival of the Tg mice was caused primarily by enhanced systemic dissemination rather than increased proliferation of *C. neoformans* in the lungs (66). Surprisingly, augmented susceptibility of the Tg mice to *C. neoformans* infection was associated with diminished pulmonary production of IL-4 and increased production of IL-17A, which result in an alteration of the Th1-Th2-Th17 balance associated with a protective rather than a nonprotective host response to *C. neoformans* (35–37, 55). The augmented dissemination of *C. neoformans* to the liver and spleen in Tg mice, also previously observed in IL-23p19$^{-/-}$ mice with impaired production of IL-17 (35), was therefore likely caused by perturbations other than a defective Th17 response.

Capsule thicknesses of *C. neoformans* and *C. gattii* in the lungs increased significantly during the course of infection of non-Tg mice (30) but not Tg mice. The mechanisms responsible for dif-

ferences in capsule thickness *in vivo* are unknown (30) but could potentially include variations in iron, CO_2, and nutrient concentrations in host tissues (30, 67). Interestingly, CD4C/HIV^{Nef} transgenic mice display increased circulating ferritin levels due to Nef-dependent release of ferritin from macrophages, and plasma ferritin levels are correlated with viral RNA in HIV-1-infected patients (68). *C. neoformans* can acquire iron bound to the major carrier transferrin by a reductive iron uptake pathway (69). Because growth of *C. neoformans* at high iron concentrations results in cells with thinner capsules (30) and lower expression of the CAP60 gene that is required for capsule production (70), increased availability of iron from the ferritin carrier may have contributed to the lack of capsule thickening during the course of cryptococcal infection in the Tg mice. However, despite the absence of capsule thickening during infection by both species, the capsule thickness of *C. neoformans* remained greater than that of *C. gattii* in the Tg mice and may have contributed to its enhanced systemic dissemination to the liver and spleen, which was also observed in the non-Tg mice.

The percentages of pulmonary dendritic cells, alveolar macrophages, and Gr-1$^+$ cells increased from day 7 to day 14 after infection of Tg and non-Tg mice with *C. neoformans*, and absolute numbers of these cell populations extracted from the lungs were not significantly diminished in the Tg mice. Dendritic cells in CD4C/HIV^{MutA} Tg mice have an immature phenotype, with low expression of major histocompatibility complex (MHC) class II and costimulatory molecules and a decreased capacity to present antigen *in vitro* (20, 27). In view of the defective production of MCP-1 in the Tg mice, dendritic cells could potentially have failed to accumulate in the lungs in response to *C. neoformans* infection because of defective CCR2-mediated recruitment and differentiation of monocytes (46). Preserved production of other CCR2 agonists, such as MCP-2 and MCP-3, may have compensated for the defective production of MCP-1. Because dendritic cells and alveolar macrophages play a critical role in the early innate protective host response against *C. neoformans* (71) and are associated with natural resistance to progressive infection (62), it is likely that functional defects of these cell populations also contributed to the increased susceptibility of the Tg mice to *C. neoformans* infection. Blood monocytes and alveolar macrophages from HIV-infected patients have impaired fungistatic activity against *C. neoformans* (72–76).

In summary, the present findings clearly demonstrate that HIV-1 transgene expression consistently augments susceptibility to *C. neoformans* but not *C. gattii* infection, and it reduces the pulmonary inflammatory cell response by both depletion of immune cells and diminished production of chemokines. In the absence of this protective host response in Tg mice, the greater capsule thickness of *C. neoformans* than that of *C. gattii in vivo* may become a primary determinant of the host-pathogen interaction and result in selectively enhanced virulence of *C. neoformans*, considering that both species qualitatively share all of the known major *C. neoformans* virulence traits (7, 77).

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