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Review article

What makes *Cryptococcus gattii* a pathogen?

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Abstract

Cryptococcosis is an invasive fungal infection of humans and other animals, typically caused by the species *Cryptococcus neoformans* in patients with impaired immunity. However, there is growing recognition of the importance of the related species *C. gattii* in causing infections in apparently immunocompetent individuals. In particular, an ongoing outbreak of cryptococcal disease in the Pacific Northwest region, which started in 1999, has driven an intense research effort into this previously neglected pathogen. Here, we discuss some of the recent discoveries in this organism from the Pacific Northwest region and highlight areas for future investigation.

Introduction

Cryptococcus gattii is a fungal pathogen of humans and other animals that can be found both as an opportunistic infection (Hagen *et al.*, 2012) and as a primary pathogen (Kwon-Chung & Varma, 2006). *C. gattii* is a haploid, encapsulated basidiomycete yeast that is widespread in soil, trees and tree hollows (reviewed in (Springer & Chaturvedi, 2010, Harris *et al.*, 2012)).

Cryptococcosis is thought to commence upon inhalation of airborne infectious propagules, such as spores or dried yeast cells, allowing the pathogen to settle in the lungs, where it can survive and proliferate within alveolar

macrophages (Fig. 1). Typical symptoms that are associated with cryptococcosis are fever, weight loss, fatigue, night sweats, cough, chest pain, headache, vomiting and neck stiffness (Phillips *et al.*, 2015). If the pathogen reaches the central nervous system, this can lead to meningoencephalitis, the most severe form of cryptococcosis, which is always lethal without rapid treatment. Interestingly, there is a predilection of *C. neoformans* for central nervous system infection and *C. gattii* for lung infection. On the other hand pulmonary (and cerebral) cryptococcomas (large inflammatory masses) are formed during infection with *C. gattii*, but not with *C. neoformans* (Mitchell *et al.*, 1995, Chen *et al.*, 2000, Galanis *et al.*, 2010, Byrnes & Marr, 2011); the latter leading instead mainly to small pulmonary lesions (Speed & Dunt, 1995, Chen *et al.*, 2000). This might be due to higher transmigration of *C. neoformans* through brain blood barrier via the Trojan horse mechanism according to *in vitro* studies (Sorrell *et al.*, 2015).

Early reports describing patients suffering from cryptococcosis highlighted the prevalence of men over women (Chen *et al.*, 2000) which was thought due to the exposure of males to environmental sources. Recent data indicate that in fact presence of testosterone in men, but not β -estradiol in women, may influence capsule growth and reduce phagocytosis of yeast by macrophages (McClelland *et al.*, 2013, Costa *et al.*, 2015).

Interestingly, while *C. neoformans* mainly infects immunosuppressed patients, with HIV/AIDS being the most common underlying condition, *C. gattii* is considered as a primary pathogen, since it frequently infects immunocompetent and apparently healthy individuals (Speed & Dunt, 1995, Sorrell, 2001), although, recent studies suggest several factors such as smoking, oral corticosteroids usage and older age may increase the risk of infection by this species (reviewed in (MacDougall *et al.*, 2011)). Interestingly, anti-cryptococcal antibody levels are higher during *C. gattii* than *C. neoformans* infections in immunocompetent patients (Speed *et al.*, 1996) and in cats (Malik *et al.*, 1999), and thus it is possible that undiagnosed antibody deficiencies may predispose to *C. gattii* infections (Marr *et al.*, 2012). Similarly, high concentrations of

granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibodies can be found in the plasma of otherwise healthy HIV-negative individuals suffering from cryptococcal meningitis (Rosen *et al.*, 2013) and, interestingly, are a significant risk factor for CNS infection by *C. gattii* but not *C. neoformans* (Saijo *et al.*, 2014).

In comparison to cryptococcosis caused by *C. neoformans* which kills 650,000 immunocompromised people suffering from HIV/AIDS every year worldwide (Park *et al.*, 2009) as well as a significant additional number of organ transplant recipients (Pappas, 2013), *C. gattii* infections are rather rare, although recent studies indicate that they may be mis- or under-diagnosed (Iverson *et al.*, 2012, Tintelnot *et al.*, 2015). *C. gattii* meningitis can be cured completely in the early stages of disease (Chen *et al.*, 2012), but often the disease is misdiagnosed as tuberculosis or other bacterial/viral pulmonary infections at this stage. Antifungal treatment is mainly based on amphotericin B in combination with 5-flucytosine and/or fluconazole (Chen *et al.*, 2013) and at later stages of the disease surgery and corticosteroids may be required (Sorrell & Chen, 2010).

There are many methods to differentiate between *Cryptococcus* species. Only *C. gattii* will produce blue colonies if grown on CGB (L-canavanine, glycine and bromthymol blue) agar (Kwon-Chung *et al.*, 1982, Min & Kwon-Chung, 1986). Similarly, capsular agglutination reactions can discriminate between *C. neoformans*, which exhibits serotypes A (*C. neoformans* var. *grubii*), D (*C. neoformans* var. *neoformans*) and AD) and *C. gattii* (which is comprised of serotypes B and C (Kwon-Chung *et al.*, 1982, Franzot *et al.*, 1999, Boekhout *et al.*, 2001)). However the most reliable methods for differentiating between *Cryptococcus* species are sequence based (McTaggart *et al.*, 2011, Kwon-Chung *et al.*, 2014, Hagen *et al.*, 2015) and rely on amplified fragment length polymorphism (AFLP; (Boekhout *et al.*, 2001)), PCR and multiplex PCR fingerprinting (Meyer *et al.*, 1999, Meyer *et al.*, 2003, Ito-Kuwa *et al.*, 2007) or sequencing of intergenic spacers (IGS; (Diaz *et al.*, 2000, Diaz *et al.*, 2005)).

For most of the time that cryptococcosis has been recognized, research efforts have focused on *C. neoformans* as the dominant pathogenic species. However, in 1999 an outbreak of cryptococcosis started on Vancouver Island (British Columbia, Canada) that was later identified as being caused by *C. gattii*. This outbreak subsequently spread to mainland Canada and the northwestern part of the USA (Oregon and Washington (MacDougall *et al.*, 2007)). Although *C. gattii* was previously known to be prevalent in tropical regions (Kwon-Chung & Bennett, 1984), its abrupt appearance in the moderate climate of the Pacific Northwest (PNW) region led to disease not only in otherwise healthy humans but also domestic, terrestrial and sea animals including dolphins (Stephen *et al.*, 2002, Kidd *et al.*, 2004, MacDougall *et al.*, 2007, Upton *et al.*, 2007). During 1999–2007 the outbreak affected 218 people (5.8 people per million in the region per year) and 19 died (8.7% associated deaths; (Galanis *et al.*, 2010, Phillips *et al.*, 2015)). In addition, during 2004-2011 the outbreak affected approximately 100 people, with a 33% mortality rate, in the US Pacific Northwest (Harris *et al.*, 2011). Most people suffered from respiratory illness (76.6%) or lung cryptococcomas (75.4%; (Galanis *et al.*, 2010)), with a third of those patients also showing central nervous system infection (Phillips *et al.*, 2015).

97% of documented cryptococcosis cases from the Vancouver Island outbreak were comprised of molecular genotype VGII (VG=variety *gattii*; (Boekhout *et al.*, 2001, Kidd *et al.*, 2004)), with subtype VGIIa being responsible for 86.3% of cases in BC (Galanis *et al.*, 2010) and 81% in Washington/Oregon (Harris *et al.*, 2011). The remaining cases were caused by a closely related lineage, VGIIb (also termed the minor lineage; (Kidd *et al.*, 2004)), while a third lineage, VGIIc (novel) has been reported mainly from Oregon State (Harris *et al.*, 2011). VGIIa exhibits higher fertility than other *C. gattii* strains (Ngamskulrunroj *et al.*, 2008) and the molecular type is now considered as the most virulent within this species (Lizarazo *et al.*, 2014).

In comparison to non-outbreak isolates from Australia, where *C. gattii* is endemic, the majority of the Vancouver Island outbreak isolates were highly fertile (70% in BC versus 10% in Australia; (Fraser *et al.*, 2003)), hypervirulent

(Fraser *et al.*, 2005) and showed low susceptibility to anti-fungal drugs (Trilles *et al.*, 2012). Although detailed analysis of many *C. gattii* isolates has been performed, no individual cryptococcal pathogenicity factors have yet been found (Ma *et al.*, 2009), leading to the suggestion that virulence is a complex, multifactorial phenotype (Garcia-Solache *et al.*, 2013, Firacative *et al.*, 2014). Below we discuss some of the features of this pathogen which may contribute to this multifactorial pathogenicity.

Environmental niche of *C. gattii*

While *C. neoformans* spores can be found in birds' droppings, the environmental presence of *C. gattii* is strictly associated with plants. To date 54 species of trees growing globally (Hagen & Boekhout, 2010, Springer & Chaturvedi, 2010) have been found to host *C. gattii*, with Australian eucalyptus (Ellis & Pfeiffer, 1990), almond trees (Callejas *et al.*, 1998) in tropical and semi-tropical regions and, quite recently, *Pseudotsuga menziesii* (Oregon pine) in regions with a more moderate climate (Springer & Chaturvedi, 2010) acting as dominant host species. *C. gattii* can proliferate and mate on plant surfaces rich in myo-inositol (Xue *et al.*, 2007, Springer *et al.*, 2010) and there is some evidence that *C. gattii* may persist longer in the environment in the presence of plant tissue (Huerfano *et al.*, 2001).

The origin of the Pacific Northwest Outbreak

Early investigations proposed several sources for the VGII outbreak strain, but more recent work points to an origin in South America (VGIIa; (Hagen *et al.*, 2013, Billmyre *et al.*, 2014, Engelthaler *et al.*, 2014)) and Australia (VGIIb; (Fraser *et al.*, 2005, Billmyre *et al.*, 2014)). Separation of the VGI and VGII *C. gattii* strains occurred around 12.4 million years ago (D'Souza *et al.*, 2011), and this extended period of genetic isolation has contributed to recent suggestions to raise these lineages to species level (Hagen *et al.*, 2015). Although the molecular genotype of the VGII population differs significantly from other strains of *C. gattii*, due to mutations and recombinations, within the VGII group, the four subpopulations VGIIa, VGIIb, VGIIc and VGIIx are highly clonal and not very

diverse (Billmyre *et al.*, 2014, Engelthaler *et al.*, 2014, Farrer *et al.*, 2015). A comparison between genomes from VGIIa isolates suggests that these groups diverged less than 100 years ago from a less virulent strain in which a frameshift mutation in a DNA repair gene *msh2* was found (Billmyre *et al.*, 2014). VGIIa genomes from the outbreak do not contain the frameshift mutation and it has been hypothesized that its genome reverted via mitotic microevolution (Billmyre *et al.*, 2014). If this is true, there is a possibility that the reversion occurred after gaining adaptation for higher virulence. Since the other non-pathogenic VGIIa-like isolates have retained this hypermutator mutation, it remains possible that such an event may recur leading to the emergence of novel outbreak strains (Billmyre *et al.*, 2014).

In addition, several studies have highlighted the potential for gene transfer (introgression) occurring between *C. neoformans* var. *grubii* and *C. gattii* (Engelthaler *et al.*, 2014) as well as between different *C. gattii* clades (Billmyre *et al.*, 2014) through sexual reproduction (bisexual or unisexual). Support for the latter model comes from evidence for genomic islands of high polymorphism within a VGIIa genome, which were potentially introduced from two distinct VGII clades (Billmyre *et al.*, 2014).

To date, all clonal *C. gattii* VGII isolates identified from the PNW have been mating type MAT α (Lockhart *et al.*, 2013). Unlike MAT α strains, MAT α strains are capable of same-sex mating (Wiesner *et al.*, 2012) and this has been proposed as a potential origin for the PNW outbreak (Fraser *et al.*, 2005). In *C. neoformans*, MAT α strains are also associated with higher virulence (Kwon-Chung *et al.*, 1992, Barchiesi *et al.*, 2005, Nielsen *et al.*, 2005), but this appears not to be the case with *C. gattii* (Zhu *et al.*, 2013).

Genomic differences

Separation between the VGI and VGII *C. gattii* strains has resulted in significant genome differences including chromosomal rearrangements and higher than expected overall nucleotide sequence divergence (D'Souza *et al.*, 2011). Recently a whole genome analysis was performed for all lineages of *C.*

gattii (Farrer *et al.*, 2015), where the authors compared nuclear and mitochondrial DNAs between lineages. Interestingly, they found that the PNW outbreak VGII lost 146 genes (three times more than number of genes lost in VGI-III-IV lineages combined) that are still present in the three other lineages, including a mitochondrial cytochrome c peroxidase gene and several other genes that are typically thought of as being essential for nuclear and mitochondrial genome maintenance. At the same time, the VGII lineage has gained several unique genes encoding proteins with COX6B, HSP70 and iron-binding domains and proteins possibly involved in membrane trafficking.

Lack of RNA interference machinery

One of the most remarkable discoveries to emerge from the extensive genome sequencing effort in this species is the VGII-specific absence of genes encoding Argonaute, Ago1 and Ago2 (D'Souza *et al.*, 2011), which are critical components of the RNA interference (RNAi) machinery in other fungi including *C. neoformans* (Janbon *et al.*, 2010). This lack of *ago* genes was found in all VGII isolates, including those from beyond the PNW region (Farrer *et al.*, 2015). Thus the *C. gattii* VGII lineage is lacking an RNAi-mediated genome defense during both the sexual cycle (Wang *et al.*, 2010, Dumesic *et al.*, 2013) and vegetative growth (Wang *et al.*, 2012). This loss of silencing machinery appears to have independently occurred in several pathogens (reviewed in (Nicolas *et al.*, 2013)), a finding which remains enigmatic. However, this loss of RNAi typically leads to transposon reactivation, which may accelerate genome evolution and potentially help in developing novel anti-host mechanisms (Oliver & Greene, 2009, Biemont, 2010, Wang *et al.*, 2010). Alternatively, the loss of RNAi may be a protective response to pathogens that can otherwise hijack this pathway. Cross-kingdom hijacking of RNAi silencing is known for other pathogens, such as the plant fungal pathogen *Botrytis cinerea* which is able to hijack *Arabidopsis* and tomato RNAi machineries by binding to host AGO1, leading to the silencing of host immunity genes and facilitating infection (Weiberg *et al.*, 2013). To date, pathogens of Cryptococci have not been identified, but such a hypothesis remains at least a theoretical possibility.

Fertility

Although Cryptococci can reproduce sexually, where two opposite mating types, MAT α and MAT α , mate and produce spores, a predominance of α mating type in the environment means that alternative reproduction strategies are common. In particular, same-sex mating between two α mating-type parents (Lin *et al.*, 2005), or spontaneous generation of spores by haploid strains (known as monokaryotic fruiting) can both produce infectious propagules. It has been suggested that the rapid expansion of the PNW outbreak has been driven primarily by clonal reproduction (Fraser *et al.*, 2005) and it is therefore enigmatic that the majority of the PNW outbreak isolates are highly fertile (Fraser *et al.*, 2003, Ngamskulrunroj *et al.*, 2008). Despite this, whole genome analysis has revealed very limited nuclear genetic exchange between *C. gattii* lineages (Farrer *et al.*, 2015), although interestingly several instances of recombination within the mitochondrial genome (Voelz *et al.*, 2013, Farrer *et al.*, 2015).

Inflammation and the cytokine response

In contrast to *C. neoformans*, *C. gattii* is able to infect immunocompetent individuals, suggesting that the latter uses different or additional methods to inhibit immune responses. Somewhat counterintuitively, in human peripheral blood mononuclear cells *C. gattii* induces higher concentrations of cytokines such as pro-inflammatory interleukin IL-1 β , TNF- α and IL-6 and the T-cell cytokines IL-17 and IL-22 than *C. neoformans* (Schoffelen *et al.*, 2013). Interestingly, however, the authors found that Toll-like receptor (TLR) 4 and TLR9 were involved in the recognition of the pathogen, but not TLR2, unlike *C. neoformans* (Vecchiarelli, 2005). These results suggested that a different innate cytokine response of the host might be related to different pathogen-activated molecular pattern (PAMPS) molecules localized on the *C. gattii* surface in comparison to *C. neoformans*. TLR2 is known to recognize chitin (Da Silva *et al.*, 2008). Chitin-like structures in *Cryptococci* are only exposed in the limited parts of the cell surface under the capsule (Rodrigues *et al.*, 2008), which may be the reason why they are not normally recognized by TLR2. Thus it is possible that

differences in the organization and localization of chitin-derived structures between *C. neoformans* and PNW *C. gattii* strains might explain different preferences in organ colonization, since *C. gattii* preferentially targets the lungs, whilst the brain is the primary target organ for *C. neoformans* (Ngamskulrunroj *et al.*, 2012, Sorrell *et al.*, 2015).

Although in vitro blood infections by *C. gattii* result in potent inflammatory signalling, in pulmonary tissue Hoang *et al.* found only minimal inflammatory responses to *C. gattii* (Hoang *et al.*, 2004). This may be accounted for by the ability of *C. gattii* to weaken pulmonary Th1 and Th17 responses (at least in mice) via altered dendritic cell (DC) function through down-regulation of pulmonary chemokine expression (Angkasekwinai *et al.*, 2014). This restricted DC function is related to reduced levels of TNF- α , and indeed addition of recombinant TNF- α fully restores DC maturation and thus T cell responses (Huston *et al.*, 2013).

Thus acute introduction of *C. gattii* may induce rapid inflammation, but longer-lasting systemic inflammation is dampened by poor dendritic cell activation. This biphasic response may also explain otherwise contradictory findings, such as the relatively slower growth of *C. gattii* than *C. neoformans* in blood (Ngamskulrunroj *et al.*, 2012) (suggesting strong induction of defense) and yet reduced neutrophil infiltration to sites of infection (Cheng *et al.*, 2009).

Virulence strategies

Human fungal pathogens often use a huge repertoire of virulence strategies in order to survive inside the host. In cryptococci, the polysaccharide capsule, chitin and melanin within the cell wall, phospholipases, urease, laccase and the ability to growth at 37°C are the most studied virulence factors involved in pathogenesis. However, although these features are shared by all pathogenic Cryptococci, there are some crucial differences among them that might play a role in the hypervirulence of PNW *C. gattii* isolates.

Growth at 37°C

The ability to survive at elevated temperature is crucial for human pathogens. In Cryptococci this is regulated by Calcineurin, a Ca²⁺/calmodulin-activated serine/threonine-specific phosphatase (Liu *et al.*, 1991). Typically, mutants lacking calcineurin gene, such as *cna1Δ*, are avirulent in both *C. neoformans* and *C. gattii* isolates. However, PNW VGIIa strains lacking calcineurin function are still viable at elevated temperature (Chen *et al.*, 2013), suggesting there may be as-yet unidentified differences in temperature tolerance in this lineage.

Capsule

Cryptococcal capsule is a highly hydrated and a negatively charged mesh of polysaccharides surrounding the yeast cell (Fig. 2), mainly composed of glucuronoxylomannan (GXM; composed of mannose, xylose and glucuronic acid), and glucuronoxylomannogalactan (GXMGal) plus mannoproteins (Vartivarian *et al.*, 1989), and its growth is activated during host infection. Most studies to date have focused on *C. neoformans* capsule, which is considered a major virulence factor (McClelland *et al.*, 2006) and has antiphagocytic properties in macrophages (Kozel & Mastroianni, 1976). This is correlated with a reduction of systemic inflammation (reviewed in (Vecchiarelli *et al.*, 2013)) mainly due to a suppression of T lymphocyte proliferation (Syme *et al.*, 1999), induced secretion of the anti-inflammatory cytokine IL-10 (Vecchiarelli *et al.*, 1996) and inhibited secretion of TNF- α and IL-1 β (Vecchiarelli *et al.*, 1995) by human monocytes.

GXM is a large molecule (around 4,600,000 Daltons in serotype B strain I23) and has different structures (McFadden *et al.*, 2006) which correlate with differences in antibody reactivity (Fonseca *et al.*, 2010). Capsule size depends on environmental conditions (summarized in (Zaragoza & Casadevall, 2004, Gupta & Fries, 2010)) and capsule enlargement is usually observed during infection (Garcia-Hermoso *et al.*, 2004). This is linked to the presence of mammalian serum (Zaragoza *et al.*, 2003), higher CO₂ concentration (Granger *et al.*, 1985) and tissue-specific conditions such as iron deficiency in the lungs (Vartivarian *et al.*, 1993) (Rivera *et al.*, 1998) or the high concentration of urea in cerebrospinal fluid (Frazzitta *et al.*, 2013). In addition, capsular size can change during the cell

cycle and its enlargement is mainly observed during the G₁ phase when no budding occurs (Garcia-Rodas *et al.*, 2014).

Depending on the *C. gattii* strain, age of the cells, temperature, conditions and methodology used for studies, capsule thickness can differ dramatically. For instance, relative to the canonical *C. neoformans* strain H99 (McFadden *et al.*, 2006), *C. gattii* capsules can be very similar (NIH191 and NIH198 (Frases *et al.*, 2009)), much smaller (strains CN23/10.993 and the PNW strain R265 (Cheng *et al.*, 2009, Fonseca *et al.*, 2010) or significantly larger (strain I23 and R265; (Frazzitta *et al.*, 2013)).

Although the major capsular polysaccharide GXM is generally immunosuppressive, fractions with molecular masses below 10,000 Daltons isolated from *C. gattii* strains were effective in stimulating nitric oxide (NO) production by host macrophages and in activation of TLRs (TLR2/1 and TLR2/6) and NF- κ B (Fonseca *et al.*, 2010). Increased production of NO has also been observed after incorporation of extracellular vesicles (EVs) by murine macrophages, but it was diminished after adding fractions of GXM (Oliveira *et al.*, 2010), suggesting that these two components may act in concert to reduce host inflammatory responses.

Interestingly, the cryptococcal capsule is also likely to play an important role in the environment. On plant surfaces, some strains of *C. gattii* form 40–100 nm length extracellular fibrils which then allow yeast cells to escape from human neutrophils *in vivo*, potentially by inhibiting the production of neutrophil extracellular traps (Rocha *et al.*, 2015). Consequently, infection of mice with yeast cells grown on leaf agar was more severe and showed higher proliferation in the lung and brain than when yeast cells grown on YPD agar were used (Springer *et al.*, 2010).

Extracellular vesicles

GXM, the main polysaccharide component of the cryptococcal capsule, is synthesized intracellularly and transferred from the Golgi apparatus (Hu *et al.*, 2007) to the outside of the cell wall via EVs (Yoneda & Doering, 2006). The

bilayered membrane-EVs serve not only as transporting ports for capsule components, but also are used by cryptococci as 20 to 400 nm diameter “virulence bags” (Rodrigues *et al.*, 2007, Rodrigues *et al.*, 2008). Studies performed so far on *C. neoformans* revealed that EVs contain ribosomal proteins as well as proteins related to virulence and anti-oxidant defense, including laccase (melanin synthesis), urease, superoxide dismutase and heat shock proteins ((Rodrigues *et al.*, 2008) and reviewed in (Rodrigues *et al.*, 2014)). Interestingly, a *C. neoformans sec6* RNAi mutant, which is impaired in EV secretion, was attenuated in virulence in mice, although growth at 37°C, capsule formation and phospholipase activity were not affected (Panepinto *et al.*, 2009). Recent studies on EVs from different fungi including *C. neoformans* revealed that these vesicles are packed with a spectrum of short non-coding mRNAs, which are thought to play a role in cell communication and pathogenesis (Peres da Silva *et al.*, 2015). Unfortunately there is no data regarding function and content of EVs isolated from *C. gattii* to date.

On the other hand, cryptococcal EVs can enhance host antimicrobial activity after incorporation by murine macrophages where increased levels of NO and cytokines (extracellular TNF- α , IL-10, and transforming growth factor (TGF- β)) were observed (Oliveira *et al.*, 2010). Similar results were obtained after treatment of macrophages with EVs isolated from *Candida albicans* (Vargas *et al.*, 2015) suggesting that EVs can serve as a platform of secreted virulence for pathogenic fungi.

At first glance, enhancing host phagocytosis in this way seems like a disadvantageous step for a pathogen. However, since *C. gattii* can happily survive within the phagosome and, at the same time, be protected from other immune cells as well as extracellular antifungal molecules such as complement, it may be that EV-induced boosting of phagocytosis offers survival advantages to pathogens such as *C. neoformans* and *C. albicans* by facilitating their entry into an intracellular niche (Oliveira *et al.*, 2010, Vargas *et al.*, 2015).

Survival within macrophages

Although Cryptococci are phagocytosed by macrophages, in most cases they can then survive and proliferate inside these host cells. Cryptococci have developed an amazing repertoire of anti-phagocytic strategies (reviewed in (Johnston & May, 2013)), most probably as a result of prolonged selective pressure from environmental predators such as amoebae (Steenbergen *et al.*, 2001). As a result, virulence and defense mechanisms against phagocytic cells could be acquired and selected during the evolution of fungus-amoebal interactions in the environment. In support of this model, transcriptional profiles show strong similarities between genes upregulated by yeast internalized by amoebae and murine macrophages (Derengowski Lda *et al.*, 2013). Interestingly, however, relative to *C. neoformans*, *C. gattii* is rarely phagocytosed by the model amoeba *A. castellanii*, perhaps reflecting differences in their capsule structure (Malliaris *et al.*, 2004).

Fungal persistence and reactivation

C. neoformans is classically thought of as a long-term latent pathogen that reactivates upon immunocompromisation, but this picture is more complex for *C. gattii*. Recent multilocus sequence typing between European and worldwide isolates has revealed that dormant *C. gattii* infections can be reactivated many years after the initial infection (Hagen *et al.*, 2012), for instance following treatment with corticosteroids (Hagen *et al.*, 2010). However, unlike *C. neoformans* it appears that many *C. gattii* infections represent de novo acquisition of the organism from the environment, rather than (re)activation of latent disease (MacDougall & Fyfe, 2006).

Proliferation inside macrophages and “Division of Labour”

All pathogenic Cryptococci appear capable of survival and proliferation within macrophages. However, outbreak *C. gattii* isolates are capable of intracellular proliferation rates that exceed those of all other isolates and which correlate with virulence (Ma *et al.*, 2009). In contrast, *C. neoformans* virulence is associated to macrophage uptake and laccase activity, but not to intracellular proliferation rate (IPR; (Sabiiti *et al.*, 2014)). This difference offers a potential explanation for their varying host profiles; *C. gattii* infections in otherwise

healthy individuals can only proceed if intracellular proliferation is rapid enough to overwhelm the host immune system. In contrast, *C. neoformans* infections in immunocompromised hosts instead rely on “stealth”, in which rapid proliferation is not necessarily beneficial but an intracellular niche is critical for survival.

In the case of *C. gattii* outbreak isolates, rapid intracellular proliferation is associated with changes in mitochondrial morphology (Ma *et al.*, 2009, Voelz *et al.*, 2014). It was initially proposed that this change in mitochondrial morphology could protect the pathogen against the intracellular environment of the phagocytic cells (Ma *et al.*, 2009, Ma & May, 2010). However, more recent analyses of this group have indicated a more complex and intriguing model. Upon entry into host phagocytes PNW outbreak strains of *C. gattii* trigger a “Division of Labour” mechanism in which some cells adopt this mitochondrial morphology and cease division, but in doing so they facilitate extremely rapid proliferation of neighboring Cryptococci, thus driving amplification of the population as a whole (Fig. 3; (Voelz *et al.*, 2014)).

Surprisingly, a comparison of mitochondrial genomes between *C. gattii* and *C. neoformans* revealed similar gene content (D'Souza *et al.*, 2011). Likewise, a very recent whole genome analysis did not identify any single gene that is characteristic of the PNW strains (Farrer *et al.*, 2015). However, several studies have highlighted unusual patterns of mitochondrial inheritance and recombination in this lineage (Bovers *et al.*, 2009, Xu *et al.*, 2009, Voelz *et al.*, 2013), suggesting that unusual combinations of nuclear and mitochondrial alleles may contribute to the virulence of this group.

Interestingly, the inheritance patterns of mitochondria in *C. gattii* can be influenced by several environmental variables including UV exposure and higher temperatures (Wang *et al.*, 2015), suggesting an intriguing link between environmental conditions and the evolution of novel genotypes in this group.

Escaping

In addition to intracellular proliferation, *Cryptococci* can also escape from host cells in a poorly understood process called vomocytosis (Alvarez & Casadevall, 2006, Ma *et al.*, 2006). Interestingly, the frequency of this non-lytic expulsion process *in vivo* seems to be higher than the rates obtained *in vitro* (Nicola *et al.*, 2011). There is considerable interest in the contribution that vomocytosis may make to tissue dissemination by allowing infected phagocytes to “deposit” *Cryptococci* at distant sites; the so-called “Trojan Horse” model. Charlier and colleagues have previously provided evidence for this mechanism of entry across the blood-brain barrier (Charlier *et al.*, 2009), although recent work using *C. neoformans* mutants with reduced phagocytosis by macrophages showed no difference in rates of CNS entry (Tseng *et al.*, 2012). Both *C. neoformans* and *C. gattii* undergo vomocytosis *in vitro*, and rates appear similar, at least between *C. neoformans* H99 and *C. gattii* R265 (Voelz *et al.*, 2009) suggesting that differential vomocytosis is unlikely to be a major factor in PNW virulence. Rather, it appears that the slower growth of PNW *C. gattii* isolates in blood (10-100 times slower than *C. neoformans*) coupled with their exceptionally fast replication within host cells means that *C. gattii* infections frequently present as pulmonary infections rather than disseminated CNS disease (Ngamskulrunroj *et al.*, 2012, Sorrell *et al.*, 2015).

Cell gigantism

Enlargement of the cryptococcal capsule has been documented as a mechanism of protection against phagocytosis and the phagocytic oxidative burst for many years (Zaragoza *et al.*, 2008). However, recently an additional role for cell size increase has become apparent. During *in vivo* infections, giant or ‘titan’ cells (50-100 μm in diameter) form and represent about 20% of the cryptococcal population during pulmonary infection (Okagaki *et al.*, 2010, Zaragoza *et al.*, 2010). Intriguingly, the presence of titan cells in the cryptococcal population reduces overall phagocytosis (not just of the titan cells themselves) by macrophages (Okagaki & Nielsen, 2012). Recent studies using the moth larvae *G. mellonella* showed that during *C. gattii* infections, both the capsule and the cell sizes of VGII cells underwent significant enlargements up to 75 μm , but this was not observed in a very virulent PNW isolate R265 (Firacative *et al.*, 2014). These

observations are consistent with a suggestion that cell ‘titanisation’ provides an additional defense mechanism of the isolates attenuated in virulence (Evans *et al.*, 2015) and/or that this strategy is critical for long-term latent infections, but perhaps less vital for highly virulent acute infections caused by PNW strains.

Conclusions

Recent epidemiological data indicate that the Pacific Northwest outbreak of *C. gattii* infection is receding, although the fungus now appears endemic to Vancouver Island (Espinel-Ingroff & Kidd, 2015, Kwon-Chung & Saijo, 2015), However, understanding the apparently recent and dramatic evolutionary history of virulent VGIIa isolates is of profound importance both for improving our understanding of fungal pathogenesis (Fig. 4) in general and for determining the likelihood of other such outbreaks in the near future.

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Figure Legends

Fig. 1. A schematic illustration of an infection process of *C. gattii* (left) and *C. neoformans* (right). An infection starts upon inhalation of airborne infectious propagules, which may allow the pathogen to settle in the lungs. If the fungus reaches the central nervous system, this can lead to a brain infection, which can be lethal. Note the differences between *C. neoformans* and *C. gattii* environmental origin, the immune condition of the hosts and organ preference between pathogens.

Fig. 2. Diagram representing the role of cryptococcal polysaccharide capsule and its involvement in several immune responses. Chitin-like structures (not shown) composed of β -1,4-N-acetylglucosamine oligomers link

the capsule to the cell wall (Rodrigues *et al.*, 2008). GXMGal molecules (shown in red) are mainly found in growing capsules of budding daughter cell (De Jesus *et al.*, 2009) and also in the capsules of mature cells but only transiently due to secretion (De Jesus *et al.*, 2010). GXM, glucuronoxylomannan; GXMGal, glucuronoxylomannogalactan; EVs, extracellular vesicles.

Fig. 3. Diagram representing a scheme of 'Division of Labour' during engulfment of cryptococcal cell by a macrophage. A) Receptor-mediated phagocytosis allows recognition of the fungal cell **B)** Phagocytosis of the pathogen cell by an alveolar macrophage **C)** One of the first steps of host defense is an oxidative burst during when macrophage releases reactive oxygen species **D)** A subpopulation of guardian cells sacrifice their proliferation and tubularize their mitochondria which is accompanied with reduction of host ROS **E)** This allows proliferation of neighboring cryptococcal cells **F)** and following escape from the macrophage.

Fig. 4. A cartoon representing a repertoire of cryptococcal pathogenic activities.

References

- Alvarez M & Casadevall A (2006) Phagosome extrusion and host-cell survival after *Cryptococcus neoformans* phagocytosis by macrophages. *Current biology : CB* **16**: 2161-2165.
- Angkasekwinai P, Sringkarin N, Supasorn O, Fungkrajai M, Wang YH, Chayakulkeeree M, Ngamskulrunroj P, Angkasekwinai N & Pattanapanyasat K (2014) *Cryptococcus gattii* infection dampens Th1 and Th17 responses by attenuating dendritic cell function and pulmonary chemokine expression in the immunocompetent hosts. *Infection and immunity* **82**: 3880-3890.
- Barchiesi F, Cogliati M, Esposito MC, Spreghini E, Schimizzi AM, Wickes BL, Scalise G & Viviani MA (2005) Comparative analysis of pathogenicity of *Cryptococcus neoformans* serotypes A, D and AD in murine cryptococcosis. *The Journal of infection* **51**: 10-16.
- Biemont C (2010) A brief history of the status of transposable elements: from junk DNA to major players in evolution. *Genetics* **186**: 1085-1093.

Billmyre RB, Croll D, Li W, Mieczkowski P, Carter DA, Cuomo CA, Kronstad JW & Heitman J (2014) Highly recombinant VGII *Cryptococcus gattii* population develops clonal outbreak clusters through both sexual macroevolution and asexual microevolution. *mBio* **5**: e01494-01414.

Boekhout T, Theelen B, Diaz M, Fell JW, Hop WC, Abeln EC, Dromer F & Meyer W (2001) Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiology* **147**: 891-907.

Bovers M, Hagen F, Kuramae EE & Boekhout T (2009) Promiscuous mitochondria in *Cryptococcus gattii*. *FEMS yeast research* **9**: 489-503.

Byrnes EJ, 3rd & Marr KA (2011) The Outbreak of *Cryptococcus gattii* in Western North America: Epidemiology and Clinical Issues. *Current infectious disease reports* **13**: 256-261.

Callejas A, Ordonez N, Rodriguez MC & Castaneda E (1998) First isolation of *Cryptococcus neoformans* var. *gattii*, serotype C, from the environment in Colombia. *Medical mycology* **36**: 341-344.

Charlier C, Nielsen K, Daou S, Brigitte M, Chretien F & Dromer F (2009) Evidence of a role for monocytes in dissemination and brain invasion by *Cryptococcus neoformans*. *Infection and immunity* **77**: 120-127.

Chen S, Sorrell T, Nimmo G, Speed B, Currie B, Ellis D, Marriott D, Pfeiffer T, Parr D & Byth K (2000) Epidemiology and host- and variety-dependent characteristics of infection due to *Cryptococcus neoformans* in Australia and New Zealand. Australasian Cryptococcal Study Group. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **31**: 499-508.

Chen SC, Slavin MA, Heath CH, *et al.* (2012) Clinical manifestations of *Cryptococcus gattii* infection: determinants of neurological sequelae and death. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **55**: 789-798.

Chen SC, Korman TM, Slavin MA, *et al.* (2013) Antifungal therapy and management of complications of cryptococcosis due to *Cryptococcus gattii*. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **57**: 543-551.

Chen YL, Lehman VN, Lewit Y, Averette AF & Heitman J (2013) Calcineurin governs thermotolerance and virulence of *Cryptococcus gattii*. *G3* **3**: 527-539.

Cheng PY, Sham A & Kronstad JW (2009) *Cryptococcus gattii* isolates from the British Columbia cryptococcosis outbreak induce less protective inflammation in a murine model of infection than *Cryptococcus neoformans*. *Infection and immunity* **77**: 4284-4294.

Costa MC, Fernandes HB, Silveira RR, Freitas GJC, Oliveira LVN, Silva AM & Santos DA (2015) Gender influence on the macrophages interaction with *Cryptococcus gattii*. *XXVIII Congresso Brasileiro Microbiologia CBM 2015*.

D'Souza CA, Kronstad JW, Taylor G, *et al.* (2011) Genome variation in *Cryptococcus gattii*, an emerging pathogen of immunocompetent hosts. *mBio* **2**: e00342-00310.

Da Silva CA, Hartl D, Liu W, Lee CG & Elias JA (2008) TLR-2 and IL-17A in chitin-induced macrophage activation and acute inflammation. *Journal of immunology* **181**: 4279-4286.

De Jesus M, Nicola AM, Rodrigues ML, Janbon G & Casadevall A (2009) Capsular localization of the *Cryptococcus neoformans* polysaccharide component galactoxylomannan. *Eukaryotic cell* **8**: 96-103.

De Jesus M, Chow SK, Cordero RJ, Frases S & Casadevall A (2010) Galactoxylomannans from *Cryptococcus neoformans* varieties *neoformans* and *grubii* are structurally and antigenically variable. *Eukaryotic cell* **9**: 1018-1028.

Derengowski Lda S, Paes HC, Albuquerque P, Tavares AH, Fernandes L, Silva-Pereira I & Casadevall A (2013) The transcriptional response of *Cryptococcus neoformans* to ingestion by *Acanthamoeba castellanii* and macrophages provides insights into the evolutionary adaptation to the mammalian host. *Eukaryotic cell* **12**: 761-774.

Diaz MR, Boekhout T, Theelen B & Fell JW (2000) Molecular sequence analyses of the intergenic spacer (IGS) associated with rDNA of the two varieties of the pathogenic yeast, *Cryptococcus neoformans*. *Systematic and applied microbiology* **23**: 535-545.

Diaz MR, Boekhout T, Kiesling T & Fell JW (2005) Comparative analysis of the intergenic spacer regions and population structure of the species complex of the pathogenic yeast *Cryptococcus neoformans*. *FEMS yeast research* **5**: 1129-1140.

Dumesic PA, Natarajan P, Chen C, Drinnenberg IA, Schiller BJ, Thompson J, Moresco JJ, Yates JR, 3rd, Bartel DP & Madhani HD (2013) Stalled spliceosomes are a signal for RNAi-mediated genome defense. *Cell* **152**: 957-968.

Ellis DH & Pfeiffer TJ (1990) Natural habitat of *Cryptococcus neoformans* var. *gattii*. *Journal of clinical microbiology* **28**: 1642-1644.

Engelthaler DM, Hicks ND, Gillece JD, *et al.* (2014) *Cryptococcus gattii* in North American Pacific Northwest: whole-population genome analysis provides insights into species evolution and dispersal. *mBio* **5**: e01464-01414.

Espinel-Ingroff A & Kidd SE (2015) Current trends in the prevalence of *Cryptococcus gattii* in the United States and Canada. *Infection and drug resistance* **8**: 89-97.

Evans RJ, Li Z, Hughes WS, Djordjevic JT, Nielsen K & May RC (2015) Cryptococcal phospholipase B1 is required for intracellular proliferation and control of titan cell morphology during macrophage infection. *Infection and immunity* **83**: 1296-1304.

Farrer RA, Desjardins CA, Sakthikumar S, *et al.* (2015) Genome Evolution and Innovation across the Four Major Lineages of *Cryptococcus gattii*. *mBio* **6**.

Firacative C, Duan S & Meyer W (2014) *Galleria mellonella* model identifies highly virulent strains among all major molecular types of *Cryptococcus gattii*. *PLoS one* **9**: e105076.

Fonseca FL, Nohara LL, Cordero RJ, Frases S, Casadevall A, Almeida IC, Nimrichter L & Rodrigues ML (2010) Immunomodulatory effects of serotype B glucuronoxylomannan from *Cryptococcus gattii* correlate with polysaccharide diameter. *Infection and immunity* **78**: 3861-3870.

Franzot SP, Salkin IF & Casadevall A (1999) *Cryptococcus neoformans* var. *grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates. *Journal of clinical microbiology* **37**: 838-840.

Fraser JA, Subaran RL, Nichols CB & Heitman J (2003) Recapitulation of the sexual cycle of the primary fungal pathogen *Cryptococcus neoformans* var. *gattii*: implications for an outbreak on Vancouver Island, Canada. *Eukaryotic cell* **2**: 1036-1045.

Fraser JA, Giles SS, Wenink EC, *et al.* (2005) Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. *Nature* **437**: 1360-1364.

Frases S, Pontes B, Nimrichter L, Viana NB, Rodrigues ML & Casadevall A (2009) Capsule of *Cryptococcus neoformans* grows by enlargement of polysaccharide molecules. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 1228-1233.

Frazzitta AE, Vora H, Price MS, Tenor JL, Betancourt-Quiroz M, Toffaletti DL, Cheng N & Perfect JR (2013) Nitrogen source-dependent capsule induction in human-pathogenic *Cryptococcus* species. *Eukaryotic cell* **12**: 1439-1450.

Galanis E, Macdougall L, Kidd S, Morshed M & British Columbia *Cryptococcus gattii* Working G (2010) Epidemiology of *Cryptococcus gattii*, British Columbia, Canada, 1999-2007. *Emerging infectious diseases* **16**: 251-257.

Garcia-Hermoso D, Dromer F & Janbon G (2004) *Cryptococcus neoformans* capsule structure evolution in vitro and during murine infection. *Infection and immunity* **72**: 3359-3365.

Garcia-Rodas R, Cordero RJ, Trevijano-Contador N, Janbon G, Moyrand F, Casadevall A & Zaragoza O (2014) Capsule growth in *Cryptococcus neoformans* is coordinated with cell cycle progression. *mBio* **5**: e00945-00914.

Garcia-Solache MA, Izquierdo-Garcia D, Smith C, Bergman A & Casadevall A (2013) Fungal virulence in a lepidopteran model is an emergent property with deterministic features. *mBio* **4**: e00100-00113.

Granger DL, Perfect JR & Durack DT (1985) Virulence of *Cryptococcus neoformans*. Regulation of capsule synthesis by carbon dioxide. *The Journal of clinical investigation* **76**: 508-516.

Gupta G & Fries BC (2010) Variability of phenotypic traits in *Cryptococcus* varieties and species and the resulting implications for pathogenesis. *Future microbiology* **5**: 775-787.

Hagen F & Boekhout T (2010) The search for the natural habitat of *Cryptococcus gattii*. *Mycopathologia* **170**: 209-211.

Hagen F, van Assen S, Luijckx GJ, Boekhout T & Kampinga GA (2010) Activated dormant *Cryptococcus gattii* infection in a Dutch tourist who visited Vancouver Island (Canada): a molecular epidemiological approach. *Medical mycology* **48**: 528-531.

Hagen F, Khayhan K, Theelen B, Kolecka A, Polacheck I, Sionov E, Falk R, Parnmen S, Lumbsch HT & Boekhout T (2015) Recognition of seven species in the *Cryptococcus gattii*/*Cryptococcus neoformans* species complex. *Fungal genetics and biology : FG & B*.

Hagen F, Colom MF, Swinne D, *et al.* (2012) Autochthonous and dormant *Cryptococcus gattii* infections in Europe. *Emerging infectious diseases* **18**: 1618-1624.

Hagen F, Ceresini PC, Polacheck I, *et al.* (2013) Ancient dispersal of the human fungal pathogen *Cryptococcus gattii* from the Amazon rainforest. *PloS one* **8**: e71148.

Harris J, Lockhart S & Chiller T (2012) *Cryptococcus gattii*: where do we go from here? *Medical mycology* **50**: 113-129.

Harris JR, Lockhart SR, Debess E, Marsden-Haug N, Goldoft M, Wohrle R, Lee S, Smelser C, Park B & Chiller T (2011) *Cryptococcus gattii* in the United States: clinical aspects of infection with an emerging pathogen. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **53**: 1188-1195.

Hoang LM, Maguire JA, Doyle P, Fyfe M & Roscoe DL (2004) *Cryptococcus neoformans* infections at Vancouver Hospital and Health Sciences Centre (1997-

2002): epidemiology, microbiology and histopathology. *Journal of medical microbiology* **53**: 935-940.

Hu G, Steen BR, Lian T, Sham AP, Tam N, Tangen KL & Kronstad JW (2007) Transcriptional regulation by protein kinase A in *Cryptococcus neoformans*. *PLoS pathogens* **3**: e42.

Huerfano S, Castaneda A & Castaneda E (2001) Experimental infection of almond trees seedlings (*Terminalia catappa*) with an environmental isolate of *Cryptococcus neoformans* var. *gattii*, serotype C. *Revista iberoamericana de micología* **18**: 131-132.

Huston SM, Li SS, Stack D, Timm-McCann M, Jones GJ, Islam A, Berenger BM, Xiang RF, Colarusso P & Mody CH (2013) *Cryptococcus gattii* is killed by dendritic cells, but evades adaptive immunity by failing to induce dendritic cell maturation. *Journal of immunology* **191**: 249-261.

Ito-Kuwa S, Nakamura K, Aoki S & Vidotto V (2007) Serotype identification of *Cryptococcus neoformans* by multiplex PCR. *Mycoses* **50**: 277-281.

Iverson SA, Chiller T, Beekmann S, Polgreen PM & Harris J (2012) Recognition and diagnosis of *Cryptococcus gattii* infections in the United States. *Emerging infectious diseases* **18**: 1012-1015.

Janbon G, Maeng S, Yang DH, Ko YJ, Jung KW, Moyrand F, Floyd A, Heitman J & Bahn YS (2010) Characterizing the role of RNA silencing components in *Cryptococcus neoformans*. *Fungal genetics and biology : FG & B* **47**: 1070-1080.

Johnston SA & May RC (2013) *Cryptococcus* interactions with macrophages: evasion and manipulation of the phagosome by a fungal pathogen. *Cellular microbiology* **15**: 403-411.

Kidd SE, Hagen F, Tschärke RL, Huynh M, Bartlett KH, Fyfe M, Macdougall L, Boekhout T, Kwon-Chung KJ & Meyer W (2004) A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). *Proceedings of the National Academy of Sciences of the United States of America* **101**: 17258-17263.

Kozel TR & Mastroianni RP (1976) Inhibition of phagocytosis by cryptococcal polysaccharide: dissociation of the attachment and ingestion phases of phagocytosis. *Infection and immunity* **14**: 62-67.

Kwon-Chung KJ & Bennett JE (1984) High prevalence of *Cryptococcus neoformans* var. *gattii* in tropical and subtropical regions. *Zentralblatt für Bakteriologie, Mikrobiologie, und Hygiene Series A, Medical microbiology, infectious diseases, virology, parasitology* **257**: 213-218.

Kwon-Chung KJ & Varma A (2006) Do major species concepts support one, two or more species within *Cryptococcus neoformans*? *FEMS yeast research* **6**: 574-587.

Kwon-Chung KJ & Saijo T (2015) Is *Cryptococcus gattii* a Primary Pathogen? *J Fungi* **1**: 154-167.

Kwon-Chung KJ, Polacheck I & Bennett JE (1982) Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). *Journal of clinical microbiology* **15**: 535-537.

Kwon-Chung KJ, Edman JC & Wickes BL (1992) Genetic association of mating types and virulence in *Cryptococcus neoformans*. *Infection and immunity* **60**: 602-605.

Kwon-Chung KJ, Fraser JA, Doering TL, Wang Z, Janbon G, Idnurm A & Bahn YS (2014) Cryptococcus neoformans and Cryptococcus gattii, the etiologic agents of cryptococcosis. *Cold Spring Harbor perspectives in medicine* **4**: a019760.

Lin X, Hull CM & Heitman J (2005) Sexual reproduction between partners of the same mating type in Cryptococcus neoformans. *Nature* **434**: 1017-1021.

Liu J, Farmer JD, Jr., Lane WS, Friedman J, Weissman I & Schreiber SL (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**: 807-815.

Lizarazo J, Escandon P, Agudelo CI, Firacative C, Meyer W & Castaneda E (2014) Retrospective study of the epidemiology and clinical manifestations of Cryptococcus gattii infections in Colombia from 1997-2011. *PLoS neglected tropical diseases* **8**: e3272.

Lockhart SR, Iqbal N, Harris JR, Grossman NT, DeBess E, Wohrle R, Marsden-Haug N & Vugia DJ (2013) Cryptococcus gattii in the United States: genotypic diversity of human and veterinary isolates. *PLoS one* **8**: e74737.

Ma H & May RC (2010) Mitochondria and the regulation of hypervirulence in the fatal fungal outbreak on Vancouver Island. *Virulence* **1**: 197-201.

Ma H, Croudace JE, Lammas DA & May RC (2006) Expulsion of live pathogenic yeast by macrophages. *Current biology : CB* **16**: 2156-2160.

Ma H, Hagen F, Stekel DJ, Johnston SA, Sionov E, Falk R, Polacheck I, Boekhout T & May RC (2009) The fatal fungal outbreak on Vancouver Island is characterized by enhanced intracellular parasitism driven by mitochondrial regulation. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 12980-12985.

MacDougall L & Fyfe M (2006) Emergence of Cryptococcus gattii in a novel environment provides clues to its incubation period. *Journal of clinical microbiology* **44**: 1851-1852.

MacDougall L, Fyfe M, Romney M, Starr M & Galanis E (2011) Risk factors for Cryptococcus gattii infection, British Columbia, Canada. *Emerging infectious diseases* **17**: 193-199.

MacDougall L, Kidd SE, Galanis E, Mak S, Leslie MJ, Cieslak PR, Kronstad JW, Morshed MG & Bartlett KH (2007) Spread of Cryptococcus gattii in British Columbia, Canada, and detection in the Pacific Northwest, USA. *Emerging infectious diseases* **13**: 42-50.

Malik R, Speed BR, Kaldor J, Cairns B, Pegorer M, Wigney DI & Love DN (1999) Serum antibody response to Cryptococcus neoformans in cats, dogs and koalas with and without active infection. *Medical mycology* **37**: 43-51.

Malliaris SD, Steenbergen JN & Casadevall A (2004) Cryptococcus neoformans var. gattii can exploit Acanthamoeba castellanii for growth. *Medical mycology* **42**: 149-158.

Marr KA, Datta K, Pirofski LA & Barnes R (2012) Cryptococcus gattii infection in healthy hosts: a sentinel for subclinical immunodeficiency? *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **54**: 153-154.

McClelland EE, Bernhardt P & Casadevall A (2006) Estimating the relative contributions of virulence factors for pathogenic microbes. *Infection and immunity* **74**: 1500-1504.

McClelland EE, Hobbs LM, Rivera J, Casadevall A, Potts WK, Smith JM & Ory JJ (2013) The role of host gender in the pathogenesis of *Cryptococcus neoformans* infections. *PloS one* **8**: e63632.

McFadden DC, De Jesus M & Casadevall A (2006) The physical properties of the capsular polysaccharides from *Cryptococcus neoformans* suggest features for capsule construction. *The Journal of biological chemistry* **281**: 1868-1875.

McTaggart L, Richardson SE, Seah C, Hoang L, Fothergill A & Zhang SX (2011) Rapid identification of *Cryptococcus neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii* by use of rapid biochemical tests, differential media, and DNA sequencing. *Journal of clinical microbiology* **49**: 2522-2527.

Meyer W, Castaneda A, Jackson S, Huynh M, Castaneda E & IberoAmerican Cryptococcal Study G (2003) Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. *Emerging infectious diseases* **9**: 189-195.

Meyer W, Marszewska K, Amirmostofian M, *et al.* (1999) Molecular typing of global isolates of *Cryptococcus neoformans* var. *neoformans* by polymerase chain reaction fingerprinting and randomly amplified polymorphic DNA—a pilot study to standardize techniques on which to base a detailed epidemiological survey. *Electrophoresis* **20**: 1790-1799.

Min KH & Kwon-Chung KJ (1986) The biochemical basis for the distinction between the two *Cryptococcus neoformans* varieties with CGB medium. *Zentralblatt für Bakteriologie, Mikrobiologie, und Hygiene Series A, Medical microbiology, infectious diseases, virology, parasitology* **261**: 471-480.

Mitchell DH, Sorrell TC, Allworth AM, Heath CH, McGregor AR, Papanoum K, Richards MJ & Gottlieb T (1995) Cryptococcal disease of the CNS in immunocompetent hosts: influence of cryptococcal variety on clinical manifestations and outcome. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **20**: 611-616.

Ngamskulrungraj P, Chang Y, Sionov E & Kwon-Chung KJ (2012) The primary target organ of *Cryptococcus gattii* is different from that of *Cryptococcus neoformans* in a murine model. *mBio* **3**.

Ngamskulrungraj P, Sorrell TC, Chindamporn A, Chaiprasert A, Poonwan N & Meyer W (2008) Association between fertility and molecular sub-type of global isolates of *Cryptococcus gattii* molecular type VGII. *Medical mycology* **46**: 665-673.

Nicola AM, Robertson EJ, Albuquerque P, Derengowski Lda S & Casadevall A (2011) Nonlytic exocytosis of *Cryptococcus neoformans* from macrophages occurs in vivo and is influenced by phagosomal pH. *mBio* **2**.

Nicolas FE, Torres-Martinez S & Ruiz-Vazquez RM (2013) Loss and retention of RNA interference in fungi and parasites. *PLoS pathogens* **9**: e1003089.

Nielsen K, Marra RE, Hagen F, Boekhout T, Mitchell TG, Cox GM & Heitman J (2005) Interaction between genetic background and the mating-type locus in *Cryptococcus neoformans* virulence potential. *Genetics* **171**: 975-983.

Okagaki LH & Nielsen K (2012) Titan cells confer protection from phagocytosis in *Cryptococcus neoformans* infections. *Eukaryotic cell* **11**: 820-826.

Okagaki LH, Strain AK, Nielsen JN, Charlier C, Baltes NJ, Chretien F, Heitman J, Dromer F & Nielsen K (2010) Cryptococcal cell morphology affects host cell interactions and pathogenicity. *PLoS pathogens* **6**: e1000953.

Oliveira DL, Freire-de-Lima CG, Nosanchuk JD, Casadevall A, Rodrigues ML & Nimrichter L (2010) Extracellular vesicles from *Cryptococcus neoformans* modulate macrophage functions. *Infection and immunity* **78**: 1601-1609.

Oliver KR & Greene WK (2009) Transposable elements: powerful facilitators of evolution. *BioEssays : news and reviews in molecular, cellular and developmental biology* **31**: 703-714.

Panepinto J, Komperda K, Frases S, Park YD, Djordjevic JT, Casadevall A & Williamson PR (2009) Sec6-dependent sorting of fungal extracellular exosomes and laccase of *Cryptococcus neoformans*. *Molecular microbiology* **71**: 1165-1176.

Pappas PG (2013) Cryptococcal infections in non-HIV-infected patients. *Transactions of the American Clinical and Climatological Association* **124**: 61-79.

Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG & Chiller TM (2009) Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *Aids* **23**: 525-530.

Peres da Silva R, Puccia R, Rodrigues ML, Oliveira DL, Joffe LS, Cesar GV, Nimrichter L, Goldenberg S & Alves LR (2015) Extracellular vesicle-mediated export of fungal RNA. *Scientific reports* **5**: 7763.

Phillips P, Galanis E, MacDougall L, *et al.* (2015) Longitudinal Clinical Findings and Outcome Among Patients With *Cryptococcus gattii* Infection in British Columbia. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **60**: 1368-1376.

Rivera J, Feldmesser M, Cammer M & Casadevall A (1998) Organ-dependent variation of capsule thickness in *Cryptococcus neoformans* during experimental murine infection. *Infection and immunity* **66**: 5027-5030.

Rocha JD, Nascimento MT, Decote-Ricardo D, *et al.* (2015) Capsular polysaccharides from *Cryptococcus neoformans* modulate production of neutrophil extracellular traps (NETs) by human neutrophils. *Scientific reports* **5**: 8008.

Rodrigues ML, Alvarez M, Fonseca FL & Casadevall A (2008) Binding of the wheat germ lectin to *Cryptococcus neoformans* suggests an association of chitinlike structures with yeast budding and capsular glucuronoxylomannan. *Eukaryotic cell* **7**: 602-609.

Rodrigues ML, Nakayasu ES, Almeida IC & Nimrichter L (2014) The impact of proteomics on the understanding of functions and biogenesis of fungal extracellular vesicles. *Journal of proteomics* **97**: 177-186.

Rodrigues ML, Nakayasu ES, Oliveira DL, Nimrichter L, Nosanchuk JD, Almeida IC & Casadevall A (2008) Extracellular vesicles produced by *Cryptococcus neoformans* contain protein components associated with virulence. *Eukaryotic cell* **7**: 58-67.

Rodrigues ML, Nimrichter L, Oliveira DL, Frases S, Miranda K, Zaragoza O, Alvarez M, Nakouzi A, Feldmesser M & Casadevall A (2007) Vesicular polysaccharide export in *Cryptococcus neoformans* is a eukaryotic solution to the problem of fungal trans-cell wall transport. *Eukaryotic cell* **6**: 48-59.

Rosen LB, Freeman AF, Yang LM, *et al.* (2013) Anti-GM-CSF autoantibodies in patients with cryptococcal meningitis. *Journal of immunology* **190**: 3959-3966.

Sabiiti W, Robertson E, Beale MA, *et al.* (2014) Efficient phagocytosis and laccase activity affect the outcome of HIV-associated cryptococcosis. *The Journal of clinical investigation* **124**: 2000-2008.

Saijo T, Chen J, Chen SC, Rosen LB, Yi J, Sorrell TC, Bennett JE, Holland SM, Browne SK & Kwon-Chung KJ (2014) Anti-granulocyte-macrophage colony-stimulating factor autoantibodies are a risk factor for central nervous system infection by *Cryptococcus gattii* in otherwise immunocompetent patients. *mBio* **5**: e00912-00914.

Schoffelen T, Illnait-Zaragozi MT, Joosten LA, Netea MG, Boekhout T, Meis JF & Sprong T (2013) *Cryptococcus gattii* induces a cytokine pattern that is distinct from other cryptococcal species. *PLoS one* **8**: e55579.

Sorrell TC (2001) *Cryptococcus neoformans* variety *gattii*. *Medical mycology* **39**: 155-168.

Sorrell TC & Chen SC (2010) Recent advances in management of cryptococcal meningitis: commentary. *F1000 medicine reports* **2**: 82.

Sorrell TC, Juillard PG, Djordjevic JT, Kaufman-Francis K, Dietmann A, Milonig A, Combes V & Grau GE (2015) Cryptococcal transmigration across a model brain blood-barrier: evidence of the Trojan horse mechanism and differences between *Cryptococcus neoformans* var. *grubii* strain H99 and *Cryptococcus gattii* strain R265. *Microbes and infection / Institut Pasteur*.

Speed B & Dunt D (1995) Clinical and host differences between infections with the two varieties of *Cryptococcus neoformans*. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **21**: 28-34; discussion 35-26.

Speed BR, Kaldor J, Cairns B & Pegorer M (1996) Serum antibody response to active infection with *Cryptococcus neoformans* and its varieties in immunocompetent subjects. *Journal of medical and veterinary mycology : bi-monthly publication of the International Society for Human and Animal Mycology* **34**: 187-193.

Springer DJ & Chaturvedi V (2010) Projecting global occurrence of *Cryptococcus gattii*. *Emerging infectious diseases* **16**: 14-20.

Springer DJ, Ren P, Raina R, Dong Y, Behr MJ, McEwen BF, Bowser SS, Samsonoff WA, Chaturvedi S & Chaturvedi V (2010) Extracellular fibrils of pathogenic yeast *Cryptococcus gattii* are important for ecological niche, murine virulence and human neutrophil interactions. *PLoS one* **5**: e10978.

Steenbergen JN, Shuman HA & Casadevall A (2001) *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 15245-15250.

Stephen C, Lester S, Black W, Fyfe M & Raverty S (2002) Multispecies outbreak of cryptococcosis on southern Vancouver Island, British Columbia. *The Canadian veterinary journal La revue veterinaire canadienne* **43**: 792-794.

Syme RM, Bruno TF, Kozel TR & Mody CH (1999) The capsule of *Cryptococcus neoformans* reduces T-lymphocyte proliferation by reducing phagocytosis, which can be restored with anticapsular antibody. *Infection and immunity* **67**: 4620-4627.

Tintelnot K, Hagen F, Han CO, Seibold M, Rickerts V & Boekhout T (2015) Pitfalls in Serological Diagnosis of *Cryptococcus gattii* Infections. *Medical mycology*.

Trilles L, Meyer W, Wanke B, Guarro J & Lazera M (2012) Correlation of antifungal susceptibility and molecular type within the *Cryptococcus neoformans/C. gattii* species complex. *Medical mycology* **50**: 328-332.

Tseng HK, Liu CP, Price MS, Jong AY, Chang JC, Toffaletti DL, Betancourt-Quiroz M, Frazzitta AE, Cho WL & Perfect JR (2012) Identification of genes from the fungal

pathogen *Cryptococcus neoformans* related to transmigration into the central nervous system. *PloS one* **7**: e45083.

Upton A, Fraser JA, Kidd SE, Bretz C, Bartlett KH, Heitman J & Marr KA (2007) First contemporary case of human infection with *Cryptococcus gattii* in Puget Sound: evidence for spread of the Vancouver Island outbreak. *Journal of clinical microbiology* **45**: 3086-3088.

Vargas G, Rocha JD, Oliveira DL, *et al.* (2015) Compositional and immunobiological analyses of extracellular vesicles released by *Candida albicans*. *Cellular microbiology* **17**: 389-407.

Vartivarian SE, Anaissie EJ, Cowart RE, Sprigg HA, Tingler MJ & Jacobson ES (1993) Regulation of cryptococcal capsular polysaccharide by iron. *The Journal of infectious diseases* **167**: 186-190.

Vartivarian SE, Reyes GH, Jacobson ES, James PG, Cherniak R, Mumaw VR & Tingler MJ (1989) Localization of mannoprotein in *Cryptococcus neoformans*. *Journal of bacteriology* **171**: 6850-6852.

Vecchiarelli A (2005) The cellular responses induced by the capsular polysaccharide of *Cryptococcus neoformans* differ depending on the presence or absence of specific protective antibodies. *Current molecular medicine* **5**: 413-420.

Vecchiarelli A, Retini C, Monari C, Tascini C, Bistoni F & Kozel TR (1996) Purified capsular polysaccharide of *Cryptococcus neoformans* induces interleukin-10 secretion by human monocytes. *Infection and immunity* **64**: 2846-2849.

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

Vecchiarelli A, Pericolini E, Gabrielli E, Kenno S, Perito S, Cenci E & Monari C (2013) Elucidating the immunological function of the *Cryptococcus neoformans* capsule. *Future microbiology* **8**: 1107-1116.

Voelz K, Lammas DA & May RC (2009) Cytokine signaling regulates the outcome of intracellular macrophage parasitism by *Cryptococcus neoformans*. *Infection and immunity* **77**: 3450-3457.

Voelz K, Johnston SA, Smith LM, Hall RA, Idnurm A & May RC (2014) 'Division of labour' in response to host oxidative burst drives a fatal *Cryptococcus gattii* outbreak. *Nature communications* **5**: 5194.

Voelz K, Ma H, Phadke S, *et al.* (2013) Transmission of Hypervirulence traits via sexual reproduction within and between lineages of the human fungal pathogen *cryptococcus gattii*. *PLoS genetics* **9**: e1003771.

Wang X, Hsueh YP, Li W, Floyd A, Skalsky R & Heitman J (2010) Sex-induced silencing defends the genome of *Cryptococcus neoformans* via RNAi. *Genes & development* **24**: 2566-2582.

Wang X, Wang P, Sun S, Darwiche S, Idnurm A & Heitman J (2012) Transgene induced co-suppression during vegetative growth in *Cryptococcus neoformans*. *PLoS genetics* **8**: e1002885.

Wang Z, Wilson A & Xu J (2015) Mitochondrial DNA inheritance in the human fungal pathogen *Cryptococcus gattii*. *Fungal genetics and biology : FG & B* **75**: 1-10.

- Weiberg A, Wang M, Lin FM, Zhao H, Zhang Z, Kaloshian I, Huang HD & Jin H (2013) Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. *Science* **342**: 118-123.
- Wiesner DL, Moskalenko O, Corcoran JM, *et al.* (2012) Cryptococcal genotype influences immunologic response and human clinical outcome after meningitis. *mBio* **3**.
- Xu J, Yan Z & Guo H (2009) Divergence, hybridization, and recombination in the mitochondrial genome of the human pathogenic yeast *Cryptococcus gattii*. *Molecular ecology* **18**: 2628-2642.
- Xue C, Tada Y, Dong X & Heitman J (2007) The human fungal pathogen *Cryptococcus* can complete its sexual cycle during a pathogenic association with plants. *Cell host & microbe* **1**: 263-273.
- Yoneda A & Doering TL (2006) A eukaryotic capsular polysaccharide is synthesized intracellularly and secreted via exocytosis. *Molecular biology of the cell* **17**: 5131-5140.
- Zaragoza O & Casadevall A (2004) Experimental modulation of capsule size in *Cryptococcus neoformans*. *Biol Proced Online* **6**: 10-15.
- Zaragoza O, Fries BC & Casadevall A (2003) Induction of capsule growth in *Cryptococcus neoformans* by mammalian serum and CO₂. *Infection and immunity* **71**: 6155-6164.
- Zaragoza O, Garcia-Rodas R, Nosanchuk JD, Cuenca-Estrella M, Rodriguez-Tudela JL & Casadevall A (2010) Fungal cell gigantism during mammalian infection. *PLoS pathogens* **6**: e1000945.
- Zaragoza O, Chrisman CJ, Castelli MV, Frases S, Cuenca-Estrella M, Rodriguez-Tudela JL & Casadevall A (2008) Capsule enlargement in *Cryptococcus neoformans* confers resistance to oxidative stress suggesting a mechanism for intracellular survival. *Cellular microbiology* **10**: 2043-2057.
- Zhu P, Zhai B, Lin X & Idnurm A (2013) Congenic strains for genetic analysis of virulence traits in *Cryptococcus gattii*. *Infection and immunity* **81**: 2616-2625.