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The cause and effect of *Cryptococcus* interactions with the host

Elizabeth R Ballou$^1$ and Simon A Johnston$^{2,3,*}$

1. Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Birmingham, B15 2TT, UK.

2. Department of Infection, Immunity and Cardiovascular disease, Medical School, University of Sheffield, UK.

3. Bateson Centre, University of Sheffield, Sheffield, UK

*These authors contributed equally to this work.
Upon *Cryptococcus neoformans* infection of the host lung, the fungus enters a nutrient poor environment and must adapt to a variety of host-specific stress conditions (temperature, nutrient limitation, pH, CO$_2$). Fungal spores enter this milieu with limited nutritional reserves, germinate, and begin proliferating by budding as yeast. Although relatively little is known about the initial stages of infection, recent work has characterized changes that occur upon germination[1, 2]. This program and subsequent yeast-phase proliferation progress in a dynamic environment as host nutrient immunity responds to the infection via toxic accumulation or sequestration of essential micronutrients (reviewed in[3]) and innate immune cells are recruited to the site of infection[4]. Adaptation to the host environment and evasion of the immune response through pathogenicity factor expression allows proliferation and dissemination to multiple sites throughout the body, including, most significantly for human disease, the central nervous system. Here we will discuss recent insights into mechanisms underlying *Cryptococcus neoformans* interactions with the host during infection.

*Initiation of infection by cryptococcal spores*

*C. neoformans* infection occurs via inhalation of desiccated yeast or spores, which germinate in the host lung (reviewed in [5-7]). Two recent studies from the Hull lab have significantly improved overall understanding of this transition[1, 2]. Barkal et al developed a high throughput, computational method for *in vitro* spore germination and analysis[2]. They show that *C. neoformans* germination, defined as a switch from ovoid spores to round yeast, is a synchronous process characterized by change in overall shape followed by isotropic growth. Germination requires microtubule reorganization, chromatin remodelling, and protein turn-over, and is slow relative to the typical yeast cell cycle, occurring over 12 hours[2]. Germination is triggered by nutrient availability rather than surface contact or temperature shift, and although human body temperature (37°C) modestly reduces spore viability, sensitivity appears to be strain dependent[2, 8]. Importantly, germination is a conversion from a metabolically inactive to active state, similar to *Saccharomyces cerevisiae* germination (reviewed in [9]). Consistent with this, whole proteome analysis of spore vs. yeast-enriched proteins identified proteins involved in carbohydrate metabolism, mating, and sporulation, rather than a specific germination
program[1], and germination was delayed in the absence of micronutrients[2]. Spore germination in the host lung therefore depends on nutrient acquisition, including carbon, nitrogen, and micronutrients (iron, copper, zinc). This is particularly relevant given recent insight into host nutritional immunity mechanisms for the sequestration of copper in the host lung[3, 10].

Cell cycle control in yeast phase virulence
While the importance of capsule and melanin for host evasion, immunomodulation, and stress resistance is well established, an emerging theme is the role of the cell cycle in regulating pathogenicity factor expression. The fundamental observation that, unlike model yeast, C. neoformans bud emergence can be uncoupled from DNA synthesis during host stress or nutrient limiting conditions (summarized in [11]) has clear morphological consequences. For example, Fu et al showed that G2 arrested cells (which accumulate in response to 37°C or during nocodazole treatment) are primed for hyphal growth and monokaryotic fruiting[12]. Data from the Casadevall lab suggest yeast-phase relevance of cell cycle in the host with the observation that capsule size is proportional to cell size[13]. Moreover, the cryptococcal G1/S cyclin CLN1 influences capsule and melanin production[14, 15], and the dramatic conversion via endoreduplication of proliferating haploid yeast to large, highly polyploid Titan cells is an in vivo-specific phenomenon that, like capsule and melanin, is regulated via cAMP/PKA signal transduction[16]. These observations are now supported by genetic and genomic analyses that reveal specific cell cycle regulation of pathogenicity factors via cAMP-dependent and -independent pathways.

A global transcriptional analysis of elutriated C. neoformans cells revealed that 40 virulence-related genes have periodic expression profiles linked to cell cycle control [17]. This omics-level observation is validated in several detailed analyses connecting C. neoformans stress response, capsule and melanin regulation to cell cycle events [11, 14, 15, 18]. The sole C. neoformans G1 cyclin Cln1 is not essential, however the cln1Δ mutant exhibits delayed DNA synthesis and bud emergence[11]. This delay may be exacerbated under stress conditions: the Cncln1Δ mutant is temperature sensitive at 37°C, but
Thermotolerance is rescued by sorbitol [15]. Consistent with correlation between cell and capsule size, large, G2 accumulated $\text{cln1} \Delta$ cells have more capsule than $\text{CLN1}$ cells [14] and exhibited cell wall organization defects [15]. Loss of $\text{CLN1}$ also abrogates $\text{LAC1}$ expression [15]. In a separate study, Gish et al. characterised the cell-cycle regulated transcription factor Usv101, a paradoxical negative regulator of capsule that, in response to capsule inducing conditions, specifically represses the $\text{GAT201}$ transcription factor and the $\text{UXS1}$ UDP-xylose synthase, both required for capsule. Usv101 also induces the $\text{CTR1}$ copper transporter. Ctr1 protein degradation is adaptive to the high copper environment of the host lung [19]. Despite the apparently contradictory roles of Usv101 in pathogenicity factor elaboration and host adaptation, loss of $\text{USV101}$ reduces pathogenicity, preventing proliferation in the host lung [18]. Network analyses suggest that Usv101 is itself regulated by the cell-cycle transcription factor Swi6/Mbs2 (CNAG_01438) independent of cAMP/PKA regulation [18, 20]. All together, these data suggest that the temporal regulation of pathogenicity factor expression during proliferation is essential to host adaptation and pathogenicity.

**Host condition dependent changes in C. neoformans**

The question of how pathogenicity factor expression became integrated into cell cycle control remains unexplored, however the conserved role of Usv101 in regulating cell wall synthesis genes ($\text{AGS1}$ ($\alpha$-glucan), $\text{CHS5}$ (chitin), and $\text{SKO1}$ ($\beta$-glucan)) in C. neoformans and the distantly related, non-periodic S. cerevisiae Usv1 suggest that Usv101 is a cell wall transcription factor that has co-opted pathogenicity factor expression pathways and been incorporated into cell cycle regulation. This link may facilitate changes associated with pathogenesis. For example, Usv101 itself regulates Rim101 [18], which also influences capsule, the cell wall, and tetanisation [16] (Ost 2017). In fact, $\text{USV101}$ was originally identified in a microarray-based screen for regulators of cryptococcal capsule [21].

New insights have revealed the impact of capsule structure on host interaction. Proteomic analysis identified the enzyme lactonohydrolase (Lhc1) as a capsule component [22]. Loss resulted in only small changes capsule composition but
significantly increased capsule thickness. Intriguingly, cryoelectron microscopy of \textit{lhc1}Δ mutants suggest that larger capsules have a more open structure, and a corresponding increase in antibody mediated phagocytosis was observed\cite{22}.

The effect of a potentially more open capsule structure on pattern recognition receptor-mediated phagocytosis was not investigated, but the finding is reinforced by the observation that deletion of \textit{USV101} resulted in increased capsule thickness and enhanced phagocytosis by macrophage-like cells \textit{in vitro}\cite{18, 22}. Likewise, \textit{CLN1} deletion increased the amount of capsule containing vesicles and resulted in increased capsule thickness in liquid culture and decreased phagocytosis by macrophages\cite{14}.

It is clear that interaction with the host is influenced by both capsule secretion and composition\cite{23-25}. Newly synthesized polysaccharide is added to the existing capsule via secretion of extracellular vesicles \cite{24, 25}. Deletion of the flippase Apt1 alters Golgi morphology (where capsule biosynthesis occurs) and limits growth of the capsule in the mouse lung but not liquid culture\cite{26}. Polysaccharide modification may contribute to the increased virulence of some \textit{C. gatti} strains\cite{25}: decreased O-acetylation of capsule in the \textit{C. gatti} VGIIc isolate JP02 was associated with lower pro-inflammatory cytokine production, a result recapitulated by deacetylation of \textit{C. neoformans} H99 capsule. \textit{C. gatti} capsule prevents dendritic cell maturation independent of internalisation, and this can be overcome by co-stimulation with tumour necrosis alpha\cite{27}. Thus, it may be reduced internalisation of the JP02 strain that causes reduced cytokine release by DCs.

\textit{Surviving the warm-blooded host}

Thermotolerance is essential to \textit{C. neoformans} virulence, and recent findings have emphasized the requirement for calcineurin-Crz1 signalling\cite{28}, the unfolded protein response\cite{29}, and amino acid permeases\cite{30}. Capsule synthesis induction by differences in temperature varies between \textit{C. neoformans} strains\cite{31}. Analysis of the signalling networks responsible for the increased thermotolerance of the \textit{SCH9} protein kinase mutant identified the heat shock transcription factor \textit{HSF1} to have both transcriptional repressing and activating activities with thermal stress\cite{32}. Interestingly,
C. gattii strains exhibit reduced thermotolerance in comparison to C. neoformans, apart from the VGII group containing the Vancouver Outbreak strains[33]. However, environmental VGII C. gattii growth in Vancouver Island was positively correlated with temperate conditions[34]. C. neoformans is associated with bird guano, and analysis of growth within bird macrophages identified that incubation at bird body temperature of 42°C, but not 37°C, was sufficient to suppress intracellular growth of cryptococci but not extracellular growth[35].

The immune response and interaction with cryptococci
How the different aspects of the host immune system protect against cryptococcal infection is highly complex, and our understanding of normal immunity, and the defects in immuno-compromise, are still incomplete (reviewed in [36]). A critical area of investigation is pro-inflammatory activation of macrophages. Recent clinical studies have highlighted the complexity of the immunology associated with cryptococcal meningitis in HIV positive patients (reviewed in [37]) and increased clarity on the vital requirements for protective pro-inflammatory responses[38]. While there is a clear role for Th2 responses to cryptococci in mouse models, how this corresponds to human disease is not known [37, 39]). Pro-inflammatory macrophage activation by interferon gamma requires STAT1 activity in macrophages [40, 41]. Ex vivo analysis demonstrated reduced macrophage iNOS expression and activity in the absence of STAT1. STAT1 deficiency was also associated with a higher intracellular burden of cryptococci in lung macrophages but the relative contribution of phagocytosis, intracellular proliferation, macrophage lysis and vomocytosis was not determined. Deletion of the cryptococcal inositol hexaphosphate kinase KCS1 resulted in a persistent weak infection in mice with reduced inflammatory responses in the lung, and perhaps due to a reduced capsular mannoprotein content[42].

Great progress has been made in understanding the different aspects of macrophage interactions with Cryptococcus. Direct in vivo imaging has demonstrated how phagocytosis of cryptococcal yeast very early in infection is critical for control of cryptococcosis prior to protective immunity [43]. However, the phagocytic receptors
required for uptake of yeast or spore cryptococcal cells are not known, despite extensive testing of the requirements for the known fungal receptors Dectin 1 and 2, Mincle, and mannose receptor[44, 45]. One possible explanation is that capsule masks the C. neoformans cell wall, leaving fungal PAMPs (β-glucan, mannans) largely inaccessible (reviewed in[4]). However, an investigation of the capsule-deficient rim101Δ mutant revealed condition-dependent cell wall remodelling[46]. The mutant elicited significantly more TNF-α than wildtype cells, and loss of capsule masking alone was insufficient to explain increased TNF-α, as the rim101Δ cap59Δ mutant was more immunostimulatory than cap59Δ alone[46]. Instead, Rim101 appears to play a condition-specific role in the organization of chito-oligomers in the cell wall during interaction with the host[46]. Relative to other fungi, the C. neoformans cell wall contains high levels of acetylated (chitin) and deacetylated (chitosan) N-acetylglucosamine polymers. Chitin has been identified as a possible determinant of the non-protective T\textsubscript{H}2 immune response elicited upon C. neoformans lung infection, and chitin content increases during endoreduplication and titanisation [39].

Of interest is the recent investigation of the binding of cryptococcal spores, where, despite the presence of antibody-accessible β-1-3 glucan on the spore coat surface, very limited evidence for recognition by such fungal receptors was found[44]. How cryptococcal spores are recognised remains unclear: recognition through an unknown receptor and the impact of steric factors (distribution of ligands on spore surface, in vivo constraints on spores), are two possible mechanisms. The scavenger receptor MARCO has been shown to influence cryptococcal phagocytosis and subsequent progression of the immune response via monocytic cell recruitment and proinflammatory cytokine expression[47]. Inactivating mutations in the inhibitory Fc gamma receptor IIb, which are associated with Systemic Lupus Erythaematosus [48], were correlated with C. neoformans dissemination in a mouse model, and macrophages showed increased phagocytosis in vitro. The in vivo effect could be partially reversed by depletion of macrophages following infection[48]. Different phagocytosis rates of clinical isolates in vitro associated high uptake with higher fungal burden in the cerebral spinal fluid of patients[49]. In addition, laccase expression, independent of melanin production, was also positively correlated with fungal burden, perhaps indicating a role for fungal eicosanoids in virulence of C. neoformans[50].
Deletion of all three *C. neoformans* high-affinity phosphate transporters (*PHO84*, *PHO840*, and *PHO89*) reduced proliferation within macrophages, although whether this is due to a nutritional defect or reduced capsule and melanin production is unknown[51]. *C. neoformans* phospholipase B is also required for survival and growth within macrophages, and the deletion mutant forms a greater number of titan cells *in vivo*[52]. Intracellular proliferation appears critical to the virulence of the Vancouver Island Outbreak (VIO) *C. gattii* strains, but through a ‘division of labour’ mechanism between proliferating and non-proliferating yeasts within macrophages. VIO strains exhibit a high proportion of tubular mitochondria within macrophages, fungal cells with tubular mitochondria do not proliferate themselves, but seem to promote a permissive environment for their non-tubular, proliferating neighbours, a phenomenon that is dependent on host reactive oxygen species production[53].

The mechanism of non-lytic exocytosis/vomocytosis is unknown[54]. Annexin A2 is a calcium responsive phospholipid binding protein that is associated with diverse cellular processes including pro-inflammatory cytokine release and inflammasome activation in macrophages [55, 56]. Deletion of Annexin A2 reduced vomocytosis from mouse macrophages and increased host cell lysis, but how these two effects are related and if macrophage inflammatory cytokines are involved was not determined[57]. The atypical kinase Erk5 was identified in a compound screen to be a negative regulator of vomocytosis[58]. Specific inhibition of Erk5 increased rates of vomocytosis and this was associated with reduced macrophage actin dynamics and increased inflammatory markers.

*Initiation of cryptococcal meningitis*

Cryptococcal meningitis is the life-threatening form of cryptococcosis and requires the invasion of the central nervous system (CNS) by cryptococcal yeast. Three different mechanisms for CNS invasion have been identified: 1) disruption of the blood vessel endothelium integrity, 2) uptake and expulsion by blood vessel cells and 3) Trojan horse invasion via host macrophages. The cryptococcal matrixmetalloprotease *MPR1* has
been implicated in CNS invasion and adherence to endothelia in vitro, but the mechanism is currently unknown. Host inositol increases brain invasion in vivo, and free fungal cell transfer, but not macrophage mediated crossing of endothelia, in vitro[59, 60]. Identification of Erk5 as a regulator of vomocytosis provided the first opportunity to identify the relationship between vomocytosis and dissemination. In vivo analysis of infection progression in the presence of an Erk5 inhibitor increased rates vomocytosis and reduced dissemination, via reduced opportunities for Trojan horse mediated dissemination[58]. The role of proinflammatory cytokines is difficult to dissect in initiation of cryptococcal meningitis; inflammatory cytokines increased the traversal on endothelia in vitro but are clinically associated with better outcomes in cryptococcal meningitis and associated with lower dissemination following Erk5 inhibition [38, 58, 60].

The diverse studies of cryptococcal and host biology by the field has led to great progress in recent years in understanding the interactions of Cryptococcus with the host. We now possess a good working knowledge of the immune response and cryptococcal factors involved, as well as a growing understanding of fungal behaviour upon interaction with the host. There is much to be learnt in investigating very early interactions during infection and the interactions of late disease during cryptococcal meningitis. Out of necessity many experimental models are initiated with high fungal inocula, but this does not recapitulate cryptococcosis as we understand it and the study cryptococcal meningitis remains challenging. Progress in genetic tools, genomic and RNA sequencing, in vivo imaging and using the widest diversity of experimental models will be vital in the next phase of cryptococcal research.

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*Of special interest

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**Computational and experimental analysis identifying Usv101 as a master regulator of capsule, melanin, and pathogenesis.


*Of special interest


**A vital experimental medicine study on the immunology of cryptococcal meningitis that provides detailed data on the cytokine profiles associated with the different outcomes of cryptococcal infection.


*Of special interest


Figure 1. Host and pathogen determinants of cryptococcal interactions with the host. **A.** Cryptococcal spores are inhaled and germination is initiated via nutrient signals. **B.** Following germination cryptococci are able to proliferate in the host. Phagocytosis of cryptococci (D) is associated with preventing early high fungal burden, although cryptococci are able to proliferate intracellularly in macrophages (F). The requirements and timing of extracellular growth in the host are still unclear. **C.** In addition to proliferation in the host, cryptococcal cells can exhibit a number of phenotypes that modulate their virulence e.g., titan cell formation, melanisation and increased polysaccharide capsule thickness. Recent work has provided further evidence for the importance of protein kinase A and cAMP in these phenotypes, as well as roles for G1/S cyclin, USV101 and RIM101. **D.** Cryptococcal capsule is associated with reduced phagocytosis (dashed arrow). Deletion of the G1/S cyclin increased capsule thickness in in vitro culture and reduced phagocytosis by macrophages. The importance of capsule structure has been highlighted by the identification of two cryptococcal mutants (DLHC1 and DUSV1) that had increased capsule thickness but with a potentially more open structure and an associated increase in phagocytosis (solid arrow). **E.** The intracellular environment of the macrophage exhibits many potential stresses including temperature, nutrient limitation, pH and CO2 stress. **F.** Despite the stresses of the intracellular environment cryptococcal cells can proliferate in macrophages, an ability dependent on phosphate transport (PHO84, 840 and 89) and the activity of phospholipase B (PLB1). **G.** Vomocytosis is the ability of cryptococci to escape non-lytically from macrophages. Annexin A2 is a potential regulator of vomocytosis as its deletion reduces the rate of expulsion. In contrast, Erk5 is a negative regulator of vomocytosis. **H.** Classical activation of macrophages is required for protective immunity to cryptococcal infection. Cryptococcal chitin content stimulates T-helper cell Th2 polarisation of macrophages and alternative activation of macrophages. **I.** Trojan horse mediated dissemination of *Cryptococcus* relies on infected macrophages crossing the epithelial and endothelial barriers. Stimulating vomocytosis by inhibition of
Erk5 reduced dissemination. J. Cryptococci can cross tissue barriers directly, and binding of cryptococci is modulated by MPR1 and inositol.