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Know Your Enemy: How to Build and Vanquish a Global Fungal Scourge

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Abstract

The 8th International Conference on *Cryptococcus* and Cryptococcosis, chaired by Maurizio Del Poeta (Medical University of South Carolina), and organized together with June Kwon-Chung (National Institute of Allergy and Infectious Diseases), Stuart Levitz (University of Massachusetts Medical School), and John Perfect (Duke University), occurred in May 2011. This meeting brought together the world's leading researchers on *Cryptococcus* and cryptococcosis, including basic scientists, epidemiologists, and clinicians, to discuss new developments in *Cryptococcus* biology. With more than 60 oral presentations and 180 posters, this meeting enhanced our understanding of pathogenicity of *Cryptococcus* and served as a robust forum that facilitated cross-disciplinary discussions, research, and clinical collaborations. Due to space constraints, this brief overview highlights only a few of the topics discussed in this meeting, focusing on the evolution of virulence, host and pathogen interactions, fungal and host signaling, new advances of genomics studies on *Cryptococcus*, and the current status of the outbreak caused by *C. gattii*. The 8th International Conference on *Cryptococcus* and Cryptococcosis brought together scientists from across the globe in the beautiful historical downtown setting of Charleston to share their latest findings and highlight advances in *Cryptococcus* research. With more than 250 participants, this meeting was the largest gathering of the *Cryptococcus* international community in the 24-year history. Here, we review the advances presented and the current state of knowledge in the field.

Keywords

Cryptococcus; Cryptococcosis; ICCG; Meeting report

Evolution of Virulence

Spores generated through the mating of *Cryptococcus neoformans* have long been hypothesized to represent infectious propagules. However, due to the difficulties of purifying large quantities of spores, this hypothesis was hard to test. In a study by Michael Botts from Christina Hull's laboratory at the University of Wisconsin, a new protocol was developed to efficiently isolate large numbers of spores using high-speed centrifugation, thus enabling the authors to test this long-standing hypothesis for the first time. Specifically, they crossed two strains (B3501 and B3502) that are both attenuated and isolated large quantities of meiotic spores. The collected spore samples were then split into two: one half was maintained as spores, and the other half was germinated to yeast cells. They then used

the spores and yeast cells as inocula to test virulence in a murine model. What they found is that the spores show a significantly higher level of virulence compared to the two parents, as well as to the yeast cells germinated from the spores, supporting the hypothesis that the spores are primary infectious propagules. Furthermore, they found that while the yeast strains are rapidly cleared from the lung, infection with the spores results in massive dissemination to the central nervous system, likely due to spore-specific interactions with innate immune cells within the lung. This study provides the first direct evidence that spores have the competitive edge in causing disease compared to yeast cells.

How did *C. neoformans* evolve to be a human pathogen? Although interactions with the host may have contributed, little is currently known about how the host–microbe interaction has shaped the evolutionary trajectory of *C. neoformans* pathogenesis. In a study by Anastasia Litvintseva from Duke University Medical Center, this question was investigated by two different strategies: (1) analyzing naturally occurring isolates and (2) analyzing populations generated through experimental evolution. In the first approach, *C. neoformans* VNI isolates were assessed for their virulence profile both by using a murine model and by measuring the intra-macrophage proliferation rate. Not surprisingly, compared to environmental isolates, clinical isolates showed higher levels of virulence overall. What was interesting is that when environmental and clinical isolates with indistinguishable genotypes were compared, the clinical isolates again showed higher virulence. Furthermore, when grown in alveolar murine macrophage-like cells, environmental and clinical isolates with indistinguishable genotypes exhibit distinct gene expression profiles, although within environmental isolates or clinical isolates, a similar expression profile was observed. Specifically, the virulent strains displayed significant up-regulation of transporters and genes involved in protecting cells from oxidative stress and DNA damage. These results suggest that clinical *C. neoformans* isolates have evolved to adapt to the host environment by altering the expression of genes critical for survival within the host. It would be interesting to look in more detail at the nucleotide polymorphisms in these genes among natural isolates. In the second approach, two non-virulent environmental isolates with different genotypes were cultured in an environment that mimics host-like conditions of starvation and oxidative stress. After 200 generations, the experimentally evolved clones showed an enhanced growth rate when compared to the progenitor. Additionally, the evolved clones also showed greater proliferation in macrophage-like cells, consistent with the hypothesis that interacting with a host environment can enhance *C. neoformans* virulence.

Host–Pathogen Interactions and Morphogenesis

Environmental fungal pathogens frequently exhibit morphological changes upon infection of mammalian hosts. Whether morphogenesis promotes fungal virulence is a central question. Hyphae formation is a common morphological transition among dimorphic fungi required for pathogenicity [1, 2]. *C. neoformans* is predominantly found in host tissues as yeast cells and thus is not considered a dimorphic fungus. However, Kirsten Nielsen (University of Minnesota) and Arturo Casadevall (Albert Einstein College of Medicine) reported a specific morphological change associated with cryptococcal infection that involves cell enlargement, with sizes up to 50–100 μm in diameter. This morphological transition to giant or titan cells is associated with a significant increase in capsule size and DNA content [3, 4]. This new paradigm for fungal morphological change affects pathogenicity by reducing phagocytosis [4] and decreasing dissemination to the central nervous system [3], thus suggesting a novel strategy for *C. neoformans* to evade the immune response by enlarging cell size. Features of the enlarged cells such as polyploidy suggest that alterations in cell growth and mitosis occur, leading to DNA replication without cell division.

Work presented by Kirsten Nielsen revealed a signal transduction pathway leading to titan/giant cell formation. Titan cell production is regulated via G-protein-coupled receptors (GPCR) and the cAMP-dependent PKA pathway. GPCRs involved in cell enlargement have been identified, including Ste3 in mating type A cells and two additional GPCRs not associated with the mating type locus, Gpr4 and Gpr5 [5]. Rim101, a transcriptional factor downstream of this pathway, was identified as a key element promoting titan cell formation. *rim101* mutant strains did not form titan cells in vivo. The Casadevall laboratory found that incubation of *C. neoformans* with phospholipids, in particular phosphatidylcholine, and derived molecules led to the formation of giant cells [6]. These phospholipids are the first identified host-derived stimulus (possibly functioning as ligands of GPCRs) that is sensed by *C. neoformans* cells and triggers capsule enlargement and the formation of giant cells. Interestingly, the Nielsen laboratory also found an unknown mutation (*mutX*) that can lock *Cryptococcus* cells in the titan cell form, in contrast to wild-type titan cells that bud normal-sized daughter cells. Work is in progress to identify the *mutX* gene. Analysis of mutants that either over-produce titan cells or fail to produce titan cells will provide insights into how titan cell formation modulates the host immune response. In addition, this cell enlargement phenomenon also occurs in other fungal pathogens. A novel example of size dimorphism linked to virulence of *Mucor circinelloides* was recently reported in which larger sporangiospores are more virulent than small spores [7]. Thus, it is possible that cell enlargement is a general mechanism promoting fungal persistence in the host that allows survival of a subset of the microbe population during the early stages of infection.

Light Sensing Pathway

Sunlight, specifically UV radiation, is a significant source of stress for unicellular organisms. UV light can jeopardize the integrity of genetic material and thus compromise survival. Hence, the protection of DNA is crucial for *C. neoformans*, an opportunistic pathogen abundant in the environment. In previous studies by Alex Idnurm, the White Collar proteins of *C. neoformans* (Bwc1 and Bwc2) have been implicated in protection against UV stress [8]. However, the targets of this pathway were largely unknown. In this pioneering study, Alex Idnurm's group (University of Missouri, Kansas City) conducted an *Agrobacterium tumefaciens* T-DNA insertional mutagenesis screen and identified an apurinic/apyrimidic (AP) endonuclease homologous to *Schizosaccharomyces pombe* Uve1, which when mutated causes a UV-hypersensitive phenotype. The protein is a component of the UV-dependent excision repair pathway, and the *C. neoformans* *UVE1* gene can complement *uve1* mutants of *S. pombe*. Idnurm's group found that Uve1 is a downstream target of the Bwc1-Bwc2 complex, as over-expression of Uve1 suppresses the UV-sensitive phenotype of *bwc1* mutants. Interestingly, transcriptional regulation of *UVE1* is differentially controlled in light and the dark. In darkness, a short transcript produces a protein that is equally distributed throughout the cell, while during illumination, a longer transcript generates a protein localized to the mitochondria. These findings reveal that *UVE1* is a downstream, light-regulated target of the Bwc1-Bwc2 complex and is possibly involved in mitochondrial DNA protection against UV light.

Involvement of TLR9 in Early Immune Responses to Cryptococcal Infection

While the incidence of cryptococcosis in both immunocompetent and immunocompromised patients is increasing, many key aspects of the protective host response to *Cryptococcus* infection remain unknown. Innate and adaptive immunity are both required for the development of optimal resistance. *C. neoformans* introduced to the lungs of the host faces the innate immune response and also generates a robust adaptive immune response in the host that involves the induction of cytokines, activation of macrophages, and clearance of

the pathogen. Toll-like receptors play a key role in the innate recognition of pathogens and mediate the activation of innate immunity, promoting adaptive responses.

The group of Michal Olszewski (University of Michigan) found that Toll-like receptor 9 (TLR9) is required for the development of protective immunity to *C. neoformans*. This study, presented by Stuart M. Zeltzer, demonstrated that TLR9 promotes early induction of IFN-gamma, monocyte chemokine proteins 3 and 5 (MCP3 and MCP5), and the development of a robust innate immune response. The induction of MCP3 triggered by TLR9 provides a likely pathway through which TLR9 impacts the induction of the adaptive response to *C. neoformans*. Olszewski's group compared wild-type (TLR9^{+/+}) and TLR9 knockout (TLR9^{-/-}) BALB/c mice infected with *C. neoformans* and characterized leukocyte populations present in the lungs post-infection. Cytokine/chemokine induction and macrophage activation during both innate and adaptive phases of the immune response were evaluated. They noted significant differences in early/innate responses, despite equivalent "early" growth of *C. neoformans* in the lungs of both TLR9^{-/-} and TLR9^{+/+} mice; however, TLR9 deletion resulted in the absence of *C. neoformans* clearance observed in TLR9^{+/+} mice during the adaptive phase of the immune response (from week 3 post-infection and onwards).

The impaired clearance in TLR9^{-/-} mice was accompanied by diminished recruitment and evidence of alternative activation of pulmonary exudate macrophages, contrasting with classical activation of macrophages in TLR9^{+/+} mice. TLR9^{-/-} mice also showed diminished recruitment of CD4⁺ and CD8⁺ T cells and changes in cytokine polarizations. Early reconstitution of MCP3 in TLR9^{-/-} mice restored the recruitment of leukocytes and increased the production of IFN-gamma. Furthermore, early MCP3 reconstitution restored CD4⁺ and CD8⁺ cells and the number of macrophages in pulmonary exudates. In addition, the clearance of *C. neoformans* reappeared after week 3 post-infection when MCP3 was reconstituted. These data indicate that MCP3 induction is an important mechanism downstream of TLR9 signaling, which leads to the development of protective anticytotoxic immunity and provides insight into the early molecular events crucial for anticytotoxic host defenses.

Genomics and Population Genetics

C. neoformans is regarded as a haploid organism with 14 chromosomes. Previous studies have identified chromosome disomy in several natural strains of *C. neoformans* [9]. Researchers in James Kronstad's laboratory at the University of British Columbia further investigated genome variation of *C. neoformans* and its association with virulence. They found that melanin production is correlated with Chr 1 monosomy, and disomic variants were less melanized and attenuated for virulence in mice. They also screened a number of environmental and clinical isolates and identified numerous variations in genome organization. These studies suggest that *C. neoformans* has a highly diverse genome organization that may be an important mechanism implicated in *C. neoformans* virulence.

C. neoformans heteroresistance to fluconazole was first described in 1999 [10], but the underlying mechanism was not fully understood until recently when comparative genome hybridization and micro-array techniques were applied [11]. Duplication of *C. neoformans* Chr 1, on which genes (*AFR1* and *ERG11*) that confer drug resistance are located, contributes to heteroresistance to fluconazole at the lowest concentrations [11]. Disomy of other chromosomes (Chr 4, Chr 10, and Chr 14) was observed at higher drug concentrations. June Kwon-Chung and colleagues (NIAID, NIH) furthered our knowledge about the mechanisms of heteroresistance by studying genes on these chromosomes and through in vivo studies. Deletion of several genes involved in drug efflux on Chr 4 and Chr 7 reduced

the frequency of Chr 4 disomy. The researchers linked abnormal ER morphology with chromosomal disomy and heteroresistance to fluconazole. Similar genomic dynamics of *C. neoformans* isolates from the brain of infected mice have also been observed. These findings suggest that chromosome duplication and aneuploidy are important mechanisms that modulate *Cryptococcus* resistance to fluconazole and virulence, which may underlie therapy failure and relapses of infection.

Sexual reproduction in fungi generates progeny with genome-wide recombination. Joseph Heitman's group (Duke University) found that aneuploid progeny are generated at a high frequency from both opposite-sex and same-sex mating and that aneuploidy is associated with temperature sensitive growth, drug resistance, and melanin production. Their study suggests that sex, although costly, is an efficient way for *C. neoformans* to generate *de novo* genotypic and phenotypic variation and to facilitate rapid adaption to changing environments.

With rapid advances in genome sequencing technology, a large number of *Cryptococcus* isolates have been sequenced or are on the proposed list for whole-genome sequencing. Vishnu Chaturvedi (Wadsworth Center, New York State Department of Health) is sequencing 12 *C. gattii* genomes and cDNA for the four molecular types. This will improve molecular epidemiology studies of *C. gattii* and assist with the design of diagnostics, discovery of drug targets, and development of vaccines. The CDC has sequenced 54 *C. gattii* isolates (mostly VGII isolates), identifying more than 100 SNPs and constructing a robust phylogenetic tree of *C. gattii*. The genome-wide phylogenetic organization confirmed that the VGIIc genotype has recently emerged and is related to VGIIa. Genome comparison of the sequenced isolates will shed light on the origin and evolution of the VGII molecular type. With the rapid accumulation of fungal DNA sequences, especially genomic DNA sequences, sequence analysis and comparison are critically important to fungal research. Jason Stajich (University of California, Riverside) is constructing the FungiDB database (www.fungidb.org) to include the genomic sequences of most human and plant fungal pathogens and provide broad web services such as the extraction of DNA sequences from the genome, local BLAST, and genome comparisons.

Molecular Epidemiology of *C. gattii*

C. gattii, a sibling species of *C. neoformans*, has four defined molecular types: VGI, VGII, VGIII, and VGIV. While VGIII and VGIV are more commonly found in immunocompromised patients, VGII mostly causes infections in immunocompetent individuals. Due to the emergence of *C. gattii* VGII on Vancouver Island in 1999 [12], molecular identification and genotyping of *C. gattii* have been conducted extensively globally. Identification of the outbreak genotypes in the Pacific Northwest region documents the outbreak is expanding beyond Canada [13]. A majority of human and animal infections in this region are caused by the major genotype VGIIa that is extremely clonal [12]. Recently, a novel VGIIc genotype that is as virulent as the outbreak VGIIa genotype has been identified in Oregon [14]. However, the molecular mechanism of the emergence of the outbreak genotypes is still unclear. Wieland Meyer (University of Sydney) recently identified both mating types of *C. gattii* VGII molecular type in Australia and found that genetic recombination may occur [15]. In addition to same-sex mating [16], this may be another potential model of the emergence and evolution of *C. gattii* VGII molecular types. Teun Boekhout (CBS Fungal Biodiversity Center, the Netherlands) has identified *C. gattii* and *C. neoformans* in carob trees in the Mediterranean area. The carob tree may be an important source of infection for humans and animals in this region. In addition, two groups led by Joseph Heitman (Duke University) and Wieland Meyer (University of Sydney) are working on the population structure of *C. gattii* VGIII molecular type based on multilocus sequence typing (MLST).

They identified two subgroups of distinct virulence in the VGIII molecular type, VGIIIa and VGIIIb [17]. This is the first comprehensive genetic study of *C. gattii* VGIII, which causes infections in immunocompromised individuals. Given the previously unappreciated high prevalence of *C. gattii* VGIII infection in AIDS patients, these studies are important not only to the epidemiological survey but also to the clinical study of the infection. Current molecular epidemiology studies in the USA, Canada, South America, Australia, and Asia will reveal a clearer view of the population structure of *C. gattii* VGII and other molecular types.

Coupled mRNA Synthesis and Degradation at Host Temperature

Survival in the host environment is challenging, and thus pathogens develop diverse strategies to infect the hosts. How has the human pathogenic fungus *C. neoformans* adapted to the stressful host condition of high temperature? John Panepinto (University at Buffalo) presented a clue to begin to answer this fundamental question.

Disruption of the *CCR4* and *RPB4* genes, which encode an mRNA deadenylase and RNA polymerase subunit, respectively, led to a failure of *Cryptococcus* to grow at host temperature. Both proteins are involved in mRNA destabilization, especially of ribosomal protein transcripts. Accordingly, during a temperature shift to 37°C, ribosomal protein mRNA levels decreased. Concomitant ER stress response is manifested by an up-regulation of stress responsive genes. By showing ER stress gene expression and stability, Panepinto and his colleagues connected two events, mRNA synthesis and decay during high-temperature challenge, which have to be in balance. Kar2 is a protein chaperone localized to the ER that is responsive to stress conditions. Panepinto showed that the *KAR2* gene is highly expressed but the half-life of the transcript is decreased during temperature shift; however, in the *ccr4Δ* mutant, the half-life of the transcript is longer than in wild type suggesting an increased stability of the mRNA. Other ER stress responsive protein genes, including *OST2* and *ALG7*, which encode an ER oligosaccharyl transferase subunit and a protein involved in aspartate-linked glycosylation in the ER, are regulated similarly. The findings by Panepinto's group clearly demonstrate that mRNA synthesis and decay are coupled to ER stress responsive genes, thereby allowing *C. neoformans* to adapt to host temperature stress conditions.

Conclusions

In summary, the 8th ICCG witnessed another successful and stimulating gathering of the *Cryptococcus* community and clear advances for the field since the 7th ICCG held in Nagasaki, Japan, 2008. With rapid advances in complete genome sequencing and more *Cryptococcus* isolates genomes currently being assembled, multiple genome comparisons will be made and the impact of genomics will continue to advance. From the studies presented in the conference, there was great progress in understanding the nature of the infectious propagule for *Cryptococcus*, and the elucidation of the host–pathogen interface is still a work in progress. This conference also served as an ideal forum to discuss the possibilities and strategies to implement new assays for early diagnosis and treatment for cryptococcosis. Exciting data were presented on a lateral flow immunoassay dipstick for the detection of cryptococcal glucuronoxylomannan by Tom Kozel (University of Nevada) and colleagues, and this assay represents an inexpensive and facile diagnostic test of cryptococcosis for the developing world. This assay will soon be deployed in South Africa, Kenya, Uganda, and other regions of the developing world. The 9th ICCG will take place in 2014 in Amsterdam, where we will next take stock of progress in this rapidly advancing area.

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