Efficient phagocytosis and laccase activity affect the outcome of HIV-associated cryptococcosis

Wilber Sabiiti,1,2,3 Emma Robertson,1 Mathew A. Beale,1,4 Simon A. Johnston,5,6 Annemarie E. Brouwer,7 Angela Loyse,1 Joseph N. Jarvis,8 Andrew S. Gilbert,2 Matthew C. Fisher,4 Thomas S. Harrison,1 Robin C. May,2,9 and Tihana Bicanic1

1Institute of Infection and Immunity, St. George’s University of London, London, United Kingdom. 2Institute of Microbiology and Infection and the School of Biosciences, University of Birmingham, Birmingham, United Kingdom. 3School of Medicine University of St. Andrews, St. Andrews, United Kingdom. 4Department of Infectious Disease Epidemiology, Imperial College School of Public Health, London, United Kingdom. 5Department of Infection and Immunity, Medical School, and 6MRC Centre for Developmental Biology and Biomedical Genetics, Department of Biomedical Sciences, University of Sheffield, Sheffield, United Kingdom. 7St. Elisabeth Hospital, Tilburg, The Netherlands. 8Department of Clinical Research, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom. 9NIHR Surgical Reconstruction and Microbiology Research Centre, University Hospitals of Birmingham NHS Foundation Trust, Queen Elizabeth Hospital Birmingham, Birmingham, United Kingdom.

Background. Cryptococcal meningitis (CM) is a leading cause of HIV-associated mortality globally. High fungal burden in cerebrospinal fluid (CSF) at diagnosis and poor fungal clearance during treatment are recognized adverse prognostic markers; however, the underlying pathogenic factors that drive these clinical manifestations are incompletely understood. We profiled a large set of clinical isolates for established cryptococcal virulence traits to evaluate the contribution of C. neoformans phenotypic diversity to clinical presentation and outcome in human cryptococcosis.

Methods. Sixty-five C. neoformans isolates from clinical trial patients with matched clinical data were assayed in vitro to determine murine macrophage uptake, intracellular proliferation rate (IPR), capsule induction, and laccase activity. Analysis of the correlation between prognostic clinical and host immune parameters and fungal phenotypes was performed using Spearman’s r, while the fungal-dependent impact on long-term survival was determined by Cox regression analysis.

Results. High levels of fungal uptake by macrophages in vitro, but not the IPR, were associated with CSF fungal burden (r = 0.38, P = 0.002) and long-term patient survival (hazard ratio [HR] 2.6, 95% CI 1.2–5.5, P = 0.012). High-uptake strains were hypocapsular (r = –0.28, P = 0.05) and exhibited enhanced laccase activity (r = 0.36, P = 0.003). Fungal isolates with greater laccase activity exhibited heightened survival ex vivo in purified CSF (r = 0.49, P < 0.0001) and resistance to clearance following patient antifungal treatment (r = 0.39, P = 0.003).

Conclusion. These findings underscore the contribution of cryptococcal-phagocyte interactions and laccase-dependent melanin pathways to human clinical presentation and outcome. Furthermore, characterization of fungal-specific pathways that drive clinical manifestation provide potential targets for the development of therapeutics and the management of CM.

Funding. This work was made possible by funding from the Wellcome Trust (WT088148MF), the Medical Research Council (MR/J008176/1), the NIHR Surgical Reconstruction and Microbiology Research Centre and the Lister Institute for Preventive Medicine (to R.C. May), and a Wellcome Trust Intermediate fellowship (089966, to T. Bicanic). The C. neoformans isolates were collected within clinical trials funded by the British Infection Society (fellowship to T. Bicanic), the Wellcome Trust (research training fellowships WT069991, to A.E. Brouwer and WT081794, to J.N. Jarvis), and the Medical Research Council, United Kingdom (76201). The funding sources had no role in the design or conduct of this study, nor in preparation of the manuscript.

Introduction
Cryptococcal meningoencephalitis (CM), caused by the fungus C. neoformans, is a leading cause of mortality in HIV-infected individuals globally (1). Sub-Saharan Africa, with the highest HIV burden, is the most affected region, accounting for 80% of all CM-associated mortality, despite access to antiretroviral therapy (ART) (2, 3).

HIV-associated CM is characterized by a paucity of inflammation and a large fungal burden in the cerebrospinal fluid (CSF) at diagnosis (4, 5). In addition to high baseline fungal burden and a poor proinflammatory immune response, altered mental status and a slow rate of fungal clearance upon treatment are associated with acute mortality (6–8). Understanding the factors that link these prognostic markers with cryptococcal virulence is crucial for the development of effective anticytococcal therapy.

Cryptococcal infection is acquired through inhalation of cryptococcal spores or desiccated yeast cells into the lung, where the
Clinical medicine

To date, most studies providing mechanistic links between cryptococcal virulence and outcome have been conducted in rodent models using laboratory reference strains. It remains unclear how these data relate to human infections. We report here for the first time, to our knowledge, a large-scale analysis of the relationship between the cryptococcal virulence factors (growth rate at 37°C, capsule expression, laccase activity, phagocytic uptake and intracellular proliferation in macrophages) and patient clinical parameters. We show that CNS fungal burden and consequent patient death are associated with high cryptococcal uptake by macrophages. Thus, effective phagocytosis counterintuitively predisposes to poor outcome upon cryptococcal infection. Finally, we show that laccase activity is associated with cryptococcal survival in human CSF ex vivo and poor in vivo CSF fungal clearance over a 2-week period of antifungal treatment.

### Results

**High cryptococcal uptake by macrophages is associated with high patient CSF fungal burden.** In *Cryptoccus gattii* infections of immunocompetent hosts, intracellular proliferation within phagocytes correlates with virulence in a murine model of cryptococcosis (16). Using a large patient-matched set of clinical isolates and the established murine macrophage–like cell line J774 (see Table 1 for a summary of patient baseline characteristics, isolate genotype, and in vitro phenotype), we tested whether a similar relationship exists for *C. neoformans*, but found no significant correlation between the intracellular proliferation rate (IPR) and patient fungal burden upon presentation (Spearman’s rank correlation $r = −0.2$, $P = 0.22$). However, we found a positive correlation between fungal burden in the CNS and phagocytic uptake of opsonized cryptococci ($r = 0.38$, $P = 0.002$; Figure 1A). We divided isolates into high- and low-uptake groups, based on median uptake (378 cryptococci/μl lysate), which confirmed that fungal burden in the high-uptake group of isolates was significantly higher than in the low-uptake isolates ($P < 0.001$, unpaired $t$ test; Figure 1B).

**Macrophage uptake, not intracellular proliferation, drives intracellular fungal load in *C. neoformans*.** To dissect this relationship, we analyzed the relationship between uptake and intracellular proliferation. This analysis showed that uptake and IPR are inversely correlated in this set of isolates ($r = −0.37$, $P = 0.003$), implying that highly phagocytosed isolates have lower intracellular proliferation rates. However, high-uptake isolates had a larger quantity of intracellular cryptococci at 18 hours than the low-uptake counterparts, exhibiting a strong positive correlation between uptake at 2 hours and intracellular cryptococcal load (ICL) at 18 hours of incubation ($r = 0.90$, $P < 0.0001$). High phagocytosis coupled with minimal proliferation was enough to drive intracellular fungal burden in the high-uptake isolates, suggesting a high retention and survival of the initially phagocytosed cryptococci inside the macrophage. Thus, for *C. neoformans*, the intracellular burden of cryptococci within macrophages appeared to be driven primarily by the rate at which the pathogen was engulfed and not by subsequent proliferation within macrophages (Figure 2).

To test whether the uptake phenotype is host specific, we repeated the phagocytosis assay using a selection of high-uptake and low-uptake isolates (5 strains from each group) and human primary monocyte–derived macrophages. This analysis showed a significant ($r^2 = 0.8$, $P < 0.001$, linear regression) correlation with uptake by J774 macrophages, suggesting that the traits mediating cryptococcal uptake by macrophages were conserved in these isolates and could be recognized by both murine and human macrophages (Figure 3).

We next tested whether the high-/low-uptake effect was opsonin dependent. Using the same set of 10 strains (tested in both J774 and human primary macrophages), we examined uptake following

### Table 1

<table>
<thead>
<tr>
<th>Patient baseline characteristics, isolate genotype, and phenotype</th>
<th>Value $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient baseline characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>32 (19–55)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>28 (43%)</td>
</tr>
<tr>
<td>CD4 count</td>
<td>26 (2–242)</td>
</tr>
<tr>
<td>Altered mental status (%)</td>
<td>19 (29%)</td>
</tr>
<tr>
<td><strong>CSF</strong> $^b$</td>
<td></td>
</tr>
<tr>
<td>Protein (g/l)</td>
<td>0.9 (0.13–5)</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>2.2 (0.1–4.8)</td>
</tr>
<tr>
<td>White cell count (cells/μl)</td>
<td>13 (0–1215)</td>
</tr>
<tr>
<td>IFN-γ (log₁₀ pg/ml)</td>
<td>1.7 (0–2.7)</td>
</tr>
<tr>
<td>TNF-α (log₁₀ pg/ml)</td>
<td>1.0 (−0.1–2.1)</td>
</tr>
<tr>
<td>IL-6 (log₁₀ pg/ml)</td>
<td>2.8 (−0.14–3.2)</td>
</tr>
<tr>
<td>Opening pressure (cm H₂O)</td>
<td>25 (5–80)</td>
</tr>
<tr>
<td>Fungal burden (log₁₀ CFU/ml)</td>
<td>5.8 (2.5–6.9)</td>
</tr>
<tr>
<td><strong>Deaths (%)</strong></td>
<td></td>
</tr>
<tr>
<td>2 weeks</td>
<td>10 (15%)</td>
</tr>
<tr>
<td>10 weeks</td>
<td>22 (34%)</td>
</tr>
<tr>
<td>52 weeks</td>
<td>32 (49%)</td>
</tr>
</tbody>
</table>

$^a$ Values are for 65 isolates derived from 65 patients and are the median (range), unless otherwise stated. $^b$ CSF inflammation is represented by total white blood cell count and the level of proinflammatory cytokines IFN-γ, TNF-α, and IL-6. $^c$ Rate of CSF fungal clearance (mean decrease in the number of viable cryptococci per day).

---

Clinical medicine
Clinical medicine

2 hours of macrophage infection in the absence of any opsonin (i.e., in serum-free media without the addition of anticapsule mAb 18B7). High-uptake strains again showed better engulfment than low-uptake strains (median 438 vs. 60 cryptococci/μl lysate after a 2-hour exposure to macrophages, \( P = 0.02 \), Mann-Whitney U test), suggesting that this phenotype is an intrinsic feature of the pathogen and not a reflection of variable opsonin deposition (Figure 4).

Cryptococcal uptake by macrophages and CSF IFN-γ levels are independently associated with fungal burden. To gain insight into other host factors that could influence fungal burden, we performed univariable analysis of patient CSF immune parameters, including total CSF white blood cell count and CSF cytokines and chemokines (RANTES, VEGF, IFN-α, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, GM-CSF, MCP1, MIP1α, CSF), and not a reflection of variable opsonin deposition (Figure 4). Cryptococcal uptake by macrophages and CSF IFN-γ levels are independently associated with fungal burden. To gain insight into other host factors that could influence fungal burden, we performed univariable analysis of patient CSF immune parameters, including total CSF white blood cell count and CSF cytokines and chemokines (RANTES, VEGF, IFN-α, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, GM-CSF, MCP1, MIP1α, CSF), and not a reflection of variable opsonin deposition (Figure 4).

Figure 1
Association of cryptococcal uptake by macrophages with patient fungal burden. (A) Positive correlation between cryptococcal uptake by J774 macrophages and patient CSF fungal burden. (B) The average log CSF fungal burden was significantly higher in the high-uptake than in the low-uptake group of isolates. Mean ± SEM, 5.856 ± 0.1457 and 4.922 ± 0.2188, respectively, \( P < 0.001 \), \( n = 32 \) in each group of isolates.

link between macrophage cryptococcal uptake and patient survival is likely partly mediated through fungal burden. Adjusting for CSF fungal burden rendered the 10-week uptake-survival association nonsignificant (HR 2.0, 95% CI 0.8–5.3, \( P = 0.14 \)).

High CSF fungal burden is most likely a reflection of meningeal or brain parenchymal infection, not replication in situ. At the time of CM diagnosis, there is often a high organism load in the CSF, an indication either of cryptococcal “immigration” from other body sites, or an ability to proliferate within the CNS. We tested the latter possibility through ex vivo exposure of the strains to normal human CSF. None of the isolates grew in CSF, and in most cases, fungal burden dropped significantly during incubation over a 96-hour period (mean rate of decrease of \(-1.1 \log_{10} \text{CFU/ml per day of incubation}\), suggesting that high fungal burden in the CNS may be due to replication in the brain parenchyma rather than in the CSF, which may just reflect “spillover” from the brain and meninges (Figure 6A). As controls, isolates were grown in nutrient-rich Saboraud dextrose broth (SDB) nutrient–deficient PBS and in 1% saline at pH 5.6, 7.4, 8.5, and 10. Isolates grew efficiently in SDB (mean ± SD rate of increase 0.47 ± 0.11 CFU/ml SDB/day) and had comparatively better survival in PBS (0.012 ± 0.067) and saline (–0.001 ± 0.033) than in CSF, indicating that cryptococcal failure to grow in CSF was neither nutrient nor pH dependent (Figure 6B).

High-uptake strains are hypocapsular. High-uptake and low-uptake strains showed similar in vitro growth rates in SDB at 37°C (mean ± SD doubling time of 4.1 ± 0.16 hours and 4.9 ± 0.36 hours, respectively, \( P = 0.1 \), unpaired t test), which correlated neither with uptake nor with patient CSF fungal burden. Following capsule induction for all strains, the average capsule diameter was inversely correlated with macrophage uptake \((r = -0.28, P = 0.05)\); mean ± SD diameter 1.9 ± 0.7 μm for high-uptake and 2.2 ± 0.5 μm for low-uptake strains). Thus, as previously reported, the capsule represents a major inhibitor of phagocytic uptake in both opsonic and nonopsonic conditions (17). We did not find any
**Discussion**

In this study of the virulence phenotype of clinical *C. neoformans* isolates, we have shown an association of two cryptococcal virulence traits, macrophage uptake and laccase activity, with fungal burden and rate of clearance of infection, two in vivo adverse prognostic markers in CM, and mortality. Infection with cryptococcal strains exhibiting high-uptake and high laccase activity was correlated with higher pretreatment CSF fungal burden and poor cryptococcal clearance from CSF on antifungal therapy, significantly increasing the likelihood of death from CM. We have demonstrated, to our knowledge, the first direct link between these pathogen virulence factors and poor clinical outcome in HIV-associated CM. Importantly, by drawing on isolates from five clinical trials, four of which were conducted at different times and in different regions of South Africa and the fourth conducted in Thailand (4, 22–25), our analysis includes a diverse set of human hosts, pathogen genotypes, and commonly used antifungal treatment regimens. Our findings are thus likely to reflect common virulence traits associated with clinical phenotypes in patients across a wide spectrum of cryptococcal disease.

The key, and perhaps surprising, observation arising from this study is that strains showing high engulfment by phagocytes in vitro were associated with higher fungal burden in patients. Interestingly, this correlation does not reflect higher replication rates either extracellularly in nutrient-rich medium or intracellularly, as measured by doubling time at 37°C, or higher IPRs; nor did we see evidence of growth using an *ex vivo* CSF model. Instead, we
hypothesize that strains that are more easily phagocytosed have a higher chance of intracellular survival within phagocytes and thus of more efficient dissemination to the brain, resulting ultimately in a higher CNS fungal burden and poorer patient outcome. In this context, it is interesting to note that Rohartgi et al. very recently reported that a higher-affinity variant of the phagocytic receptor FcγRIIIA is a risk factor for HIV-associated cryptococcal disease (26). Thus, both host factors (26) and pathogen factors (this study) that enhance phagocytic uptake appear to increase the risk of (severe) cryptococcal disease.

Cryptococcal nonlytic escape and spread from cell to cell has been described in in vitro cultures of both murine and human macrophages (27–29) and has been recently confirmed to occur in vivo in a murine model of cryptococcosis (30). Moreover, cryptococci-laden macrophages have been observed in the brain capillaries and leptomeninges of a mouse with severe cryptococcal meningitis (31). The ability of monocytes to systemically disseminate the fungus to the brain has further been demonstrated in a mouse model of cryptococcosis (32). These observations point to the potential of phagocytes to drive host fungal burden through the uptake of fungal cells, providing a niche for cryptococcal survival in the host and spreading it from the site of infection to the brain in a “Trojan horse” model.

The cryptococcal capsule is well described for its role in resisting phagocytosis (17), and thus possession of a smaller capsule may partly explain why high-uptake isolates were easily engulfed by macrophages. In the environment, such a phenotype is likely to be deleterious, since fungi will be effectively engulfed and killed by soil amoebae and other predators. Thus, cryptococcal strains causing human CM are most likely a selected subset of a more diverse environmental population and happen to exhibit a combination of rapid engulfment but good intracellular survival.

We also observed that strains exhibiting high phagocytic uptake also exhibited higher laccase activity. Laccase is a critical enzyme for the synthesis of melanin, a pigment that is protective against both macrophage antimicrobial activities (such as the reactive oxygen burst) and amphotericin B, a drug that is the basis of the induction of CM therapy, including in our patients (33–35). Acid extraction of a small subset of these strains showed no significant correlation between phagocytic uptake and cell wall melanin. This may have been due to the small sample size (n = 10); alternatively, the link between laccase activity and phagocytic uptake in this group of isolates may reflect one of the other functions of this enzyme. In particular, it is tempting to speculate that the production of prostaglandins by cryptococci, which is a laccase-
clonal medicine

We also suggest that high laccase activity, which results in unchecked proliferation, is important in appropriate macrophage activation and in which the ability to orchestrate an effective IFN-γ–mediated response and proinflammatory cytokines, in particular IFN-γ, is determined using multilocus sequence typing (MLST) according to the ISHAM (International Society for Human and Animal Mycology) consensus typing scheme (40). All isolates included were from patients who were ART naïve at enrollment and treated for a first episode of CM with amphotericin B–based therapies (sometimes in combination with fluconazole, flucytosine, or adjuvant IFN-γ). Patient baseline characteristics including mental status, CSF white cell count, protein levels, cytokine levels, opening pressure, and fungal burden were determined before therapeutic intervention.

Cryptococcal culture

The reference serotype A C. neoformans var. grubii strain H99 (obtained from R.C. May’s laboratory at the University of Birmingham) was used as the control strain in all assays. Prior to assay, clinical isolates and H99 were propagated either in yeast peptone dextrose (YPD) broth (1% yeast extract, 1% peptone, and 2% glucose; Sigma-Aldrich) or on SDA (Thermo Fisher Scientific) at 30°C. Infection inocula were generated by amphotericin B (39).

In summary, our study has shown that the relationship between the macrophage-cryptococcal interaction and poor clinical outcome is mediated through increased patient fungal burden. Building on a recent study linking macrophage-cryptococcal interaction to high mortality at 1 year of follow-up in HIV-associated CM (14), we have shown an association with mortality at 10 weeks in our cohort, a time when most mortality is attributed to CM as the major cause of death. Furthermore, we are the first to demonstrate an association of laccase activity and poor CSF fungal clearance during amphotericin-based treatment of human CM, which may either be mediated through an effect on melanization in vivo or through an alternative immunomodulatory effect of laccase. These findings underscore the importance of cryptococcal-phagocyte interactions and the laccase-dependent melanin pathway and their relevance to human clinical outcome and identify these as important targets for future therapeutics and management of CM.

Methods

Patients and isolates

A total of 65 cryptococcal isolates were assayed from a cohort of more than 300 clinically characterized HIV-associated CM patients from five completed clinical trials in Thailand and South Africa (4, 22–25). Participants provided written informed consent, and all trials were approved by the local research ethics committees as well as the ethics committee of Wandsworth (London, United Kingdom). Isolates for this study were selected to reflect the range of clinical trials, geographic locations, and isolate genotypes of serotype A C. neoformans var. grubii. Genotypes were determined using multilocus sequence typing (MLST) according to the ISHAM (International Society for Human and Animal Mycology) consensus typing scheme (40). All isolates included were from patients who were ART naïve at enrollment and treated for a first episode of CM with amphotericin B–based therapies (sometimes in combination with fluconazole, flucytosine, or adjuvant IFN-γ). Patient baseline characteristics including mental status, CSF white cell count, protein levels, cytokine levels, opening pressure, and fungal burden were determined before therapeutic intervention.

Cryptococcal culture

The reference serotype A C. neoformans var. grubii strain H99 (obtained from R.C. May’s laboratory at the University of Birmingham) was used as the control strain in all assays. Prior to assay, clinical isolates and H99 were propagated either in yeast peptone dextrose (YPD) broth (1% yeast extract, 1% peptone, and 2% glucose; Sigma-Aldrich) or on SDA (Thermo Fisher Scientific) at 30°C. Infection inocula were made from 24-hour starter cultures in YPD broth or SDB (Thermo Fisher Scientific) at 30°C with rotation.

The Journal of Clinical Investigation http://www.jci.org Volume 124 Number 5 May 2014
Macrophage culture

The murine-derived J774 macrophage cell line and human monocyte-derived macrophages were used for the study. Prior to use, 1.0 × 10^6 J774 macrophages were grown in 24-well tissue culture plates (Greiner) containing DMEM supplemented with 10% FBS, 1 mM L-glutamine, and 1% penicillin-streptomycin (Sigma-Aldrich) for 24 hours at 37°C with 5% CO₂. The macrophage batches used were kept within the range of three passages to limit passage-to-passage variations.

Primary human macrophages were derived from peripheral blood monocytes from healthy donor buffy coats (National Blood Service, United Kingdom). PBMCs were isolated by ficoll (Sigma-Aldrich) gradient centrifugation, and monocytes were purified from the PBMC population by adherence to plastic following overnight culture in tissue culture flasks (T75 Greiner) in RPMI 1640 medium with L-glutamine (Gibco), supplemented with either 10% AB male human serum (First Link) or 10% FBS (Sigma-Aldrich) plus 1% penicillin-streptomycin, in the presence of 1,000 U/ml GM-CSF. Monocytes were detached and counted by hemocytometry to achieve a final cell density of 5 × 10^5 cells per well in a 24-well tissue culture plate (Corning). Cells were maintained for 7 to 14 days in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin for differentiation into macrophages. The medium was changed every 3 days.

Phagocytosis and intracellular proliferation assay

Isolates were tested for the rate at which they were phagocytosed by (uptake) — and proliferated inside (IPR) — macrophages. Prior to infection, J774 macrophages were activated with 15 μg/ml PMA for 30 to 40 minutes in serum-free medium (DMEM with L-glutamine and 1% penicillin-streptomycin). Cryptococci were harvested from the 24-hour starter culture by centrifugation, washed twice in PBS, and opsonized with 1 μg/ml of the anticapsule mAb 18B7 (provided by Arturo Casadevall, Albert Einstein College of Medicine, New York, New York, USA) for 30 to 60 minutes at room temperature. Macrophages were then exposed to the opsonized cryptococci at a 1:10 ratio and incubated for 2 hours at 37°C with 5% CO₂ to promote phagocytosis. After 2 hours, the medium was removed, and macrophages were extensively washed (4–5 times) with sterile PBS to remove extracellular cryptococci. Part of the culture (time point 0) was lysed with 200 μl of sterile water for 20 minutes at 37°C to release intracellular cryptococci, which were counted using a hemocytometer. The remaining culture was maintained in serum-free medium at 37°C, and cells were lysed and intracellular cryptococci were counted at 18, 24, and 48 hours after infection. The rate of phagocytosis (uptake) was determined as the number of cryptococci engulfed by macrophages 2 hours after infection. The IPR was determined as the ratio of the number of intracellular cryptococci at 18 hours to the number of intracellular cryptococci at 2 hours. Results were expressed as the mean of 5 to 7 experimental repeats. Ten isolates were selected from the J774 macrophage uptake profile, representing the top 5 (high-uptake) and bottom 5 (low-uptake) strains and then tested in primary human macrophages. The human monocyte-derived macrophages were stimulated with 1,000 U/ml human recombinant IFN-γ (Immunoools) and 10 μg/ml LPS (Sigma-Aldrich) 24 hours prior to infection. The rate of cryptococcal uptake and the IPR were determined as described above with J774 macrophages. To evaluate opsonin influence on cryptococcal uptake, the experiment was repeated with nonopsonized cryptococci, and the rate of uptake was determined after 2 hours of incubation at 37°C with 5% CO₂.

Laccase activity

Isolates were grown for 12 hours in SDB at 37°C with shaking at 200 rpm. The growth medium was removed, and the yeast cells were washed twice in PBS, counted by hemocytometry, and adjusted to achieve an inoculum of 4 × 10^5 cells/ml. Cryptococci were then incubated in L-DOPA medium (0.1% glucose anhydrous, 0.1% L-asparagine, 0.3% KH₂PO₄, 0.025% MgSO₄·7H₂O, and 0.01% L-DOPA, pH 5.56) for 16 hours at 37°C and then 24 hours at 25°C, with shaking at 250 rpm to induce melanin production. After incubation, the supernatants were harvested at 4,000 g for 5 minutes, and the amount of pigment produced was determined spectrophotometrically at a 475-nm wavelength and converted into laccase units (U) by a factor of 0.001 OD = 1 U (41). Assays were repeated three to four times. To adjust for interexperimental variation, laccase activity of the clinical isolates was expressed as a ratio to H99 (positive control), and results were reported as the median of the repeats.

Cell wall melanin quantification

Acid-insoluble melanin production was quantified following the protocol of Wang and colleagues (21). Ten strains (5 with low laccase activity: IFN16, RCT7, RCT33, CM50, and CM36, and 5 with high laccase activity: CPT2, CPT3, CPT10, CPT13, and CPT30) were subcultured on YPD agar and then cultured in 3 ml of YPD broth with rotation (20 rpm) at 25°C for 24 hours. 1.0 × 10^6 yeast cells were then transferred into 100 ml of defined minimal media (41) and incubated in the dark on a shaking incubator at 200 rpm at 30°C, for 10 days.

Yeast cells were then pelleted, washed once with 1.0 M sorbitol in 0.1 M sodium citrate (pH 5.0), and resuspended in 5 ml of this solution. The number of cells present in this resuspension was counted by hemocytometry before lysis in a Precellys cell homogenizer (two 45-second periods of lysis, separated by 1 minute on ice). The cell debris was collected by centrifugation and resuspended in 4.0 M guanidinium isothiocyanate, a protein denaturant, for 30 minutes at room temperature. Cell debris was pellets by centrifugation and resuspended in 6.0 M HCl at 100°C for 30 minutes. This treatment dissolved cells completely, leaving a black/brown pellet of insoluble melanin. This pellet was washed in water and left to dry completely before being weighed and the mass per yeast cell calculated.

Capsule induction

To induce capsule production in vitro, C. neoformans isolates were inoculated into SDB and grown overnight at 37°C with shaking. The following day, 20 μl of culture was inoculated into 5-ml capsule-inducing medium (DMEM with 1% NCTC-109 medium and 10% heat-inactivated FBS; Sigma-Aldrich) and incubated for 48 hours at 37°C with 10% CO₂. Cells were subsequently harvested by centrifugation at 941 g for 5 minutes and observed by counterstaining with India ink, using a ×40 bright-field objective. Cells were measured using ImageJ software, version 1.440 (NIH), and the capsule diameter was calculated as the average of: (total cell diameter – cell body diameter) / 2 for 50 to 100 cells.

CSF survival

Human CSF was obtained from patients undergoing therapeutic lumbar punctures for benign intracranial hypertension at the Neurosciences day unit of St. George’s Hospital NHS Trust in London. CSF parameters in these patients, including white cell count and protein and glucose levels, were within the normal range for human CSF and did not contain antifungal drugs.

CSF pH was determined before filter sterilization with a 0.2-μm filter and preserved at -80°C until use. Isolates were initially grown to the stationary phase in SDB at 37°C, with shaking at 150 rpm. Cultures were diluted in PBS and then inoculated into CSF at a concentration of 1 to 2 × 10^6 cells/ml. Cultures were then incubated at 37°C for 96 hours with shaking at 150 rpm. Aliquots were collected at time point 0 and subsequently at 12, 24, 36, 72, and 96 hours after inoculation and plated on SDA for CFU counts (42). In parallel, 1% saline at a different pH, PBS, and SDB

Clinical Medicine
were inoculated and monitored as pH, nutrient-deplete and nutrient-rich growth media controls, respectively. The survival slope was calculated as the mean rate of increase or decrease in CFU cryptococcal counts per day of incubation, derived by averaging the slope of the linear regression of log_{10} CFU/ml over time for each isolate.

**Growth curves**

Isolates were grown in SDB for 48 hours to reach the stationary phase. Cultures were then reincubated into fresh SDB in 24-well flat-bottomed, transparent polystyrol plates (Corning) to achieve an initial OD of 0.2 at a 600-nm wavelength. The plate was then incubated at 37°C with a combination of orbital and linear shaking (3-mm amplitude) for 48 hours (Tecan i-control 1.7.1.12, Infinite 200 PRO Machine). OD was measured every 30 minutes, and the growth curves were plotted in Microsoft Excel, version 2010, after subtraction of the blank. Doubling time was then determined using the formula: doubling time = cell concentration at time 0 \times \frac{\text{growth rate}}{\text{time}} (Roth V. 2006, http://www.doubling-time.com/compute.php).

**Statistics**

Analyses were performed using Prism, version 6.0b (GraphPad Software) and Stata, version 11 (StataCorp). Associations were tested between the isolate virulence factors and patient clinical variables and outcome using linear regression for continuous variables and logistic regression for categorical variables. P values less than or equal to 0.05 was considered significant. Fungal burden and CSF cytokines were log_{10} transformed before analysis. Spearman’s rank correlation was used to determine the correlation between in vitro phenotypes and in vivo (patient) characteristics. Linear regression was used to determine the relationship between cryptococcal uptake by the murine macrophage-like cell line J774 and human primary monocyte-derived macrophages. Differences between groups were determined using the Mann-Whitney U test for non-normally distributed data and an unpaired t test (2-tailed) for normally distributed (log-transformed) data. To elucidate the relationship between different and potentially confounding variables, factors found to be significantly associated (P ≤ 0.05) with CSF fungal burden on univariable analysis were entered into a multivariable regression model, with fungal burden as the dependent variable. Cox regression was used to assess the risk of death for patients infected with high-uptake cryptococcal strains.

**Study approval**

Clinical cryptococcal isolates. Isolates used in this study were obtained from patients enrolled in five published clinical trials in Thailand and South Africa (4, 22–25). All trials received approval from the ethics committee of Wandsworth, London, as well as from the local research ethics committees in the host countries. Patients gave written informed consent, including for the storage and use of their clinical isolates for future research.

**Human CSF.** We consulted the UK National Research Ethics Service as well as the Research and Development Office at St. George’s University of London regarding the requirement for ethical review. Given that the lumbar punctures were being performed for a clinical indication, that the CSF used would otherwise have been discarded, that no clinical data were being collected, and that CSF samples were anonymized, rendered acellular by filtration, and pooled for experimental use, ethical approval was deemed to be unnecessary.

**Acknowledgments**

We gratefully acknowledge the support of the May and Bicanc groups in making this study possible.

Received for publication August 29, 2013, and accepted in revised form February 6, 2014.

**Address correspondence to:** Robin C. May, Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Birmingham, B15 2TT, United Kingdom. Phone: 44.0.121.4145418; Fax: 44.0.1214145925; E-mail: r.c.may@bham.ac.uk. Or to: Tihana Bicanc, Institute of Infection and Immunity, St. George’s University of London, London, SW17 ORE, United Kingdom. Phone: 44.0.2087252911; Fax: 44.0.2087253487; E-mail: tbicanc@sgul.ac.uk.

---