

Cryptococcus Strains with Different Pathogenic Potentials Have Diverse Protein Secretomes

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Secreted proteins are the frontline between the host and pathogen. In mammalian hosts, secreted proteins enable invasive infection and can modulate the host immune response. Cryptococcosis, caused by pathogenic *Cryptococcus* species, begins when inhaled infectious propagules establish to produce pulmonary infection, which, if not resolved, can disseminate to the central nervous system to cause meningoencephalitis. Strains of *Cryptococcus* species differ in their capacity to cause disease, and the mechanisms underlying this are not well understood. To investigate the role of secreted proteins in disease, we determined the secretome for three genome strains of *Cryptococcus* species, including a hypovirulent and a hypervirulent strain of *C. gattii* and a virulent strain of *C. neoformans*. Sixty-seven unique proteins were identified, with different numbers and types of proteins secreted by each strain. The secretomes of the virulent strains were largely limited to proteolytic and hydrolytic enzymes, while the hypovirulent strain had a diverse secretome, including non-conventionally secreted canonical cytosolic and immunogenic proteins that have been implicated in virulence. The hypovirulent strain cannot establish pulmonary infection in a mouse model, but strains of this genotype have caused human meningitis. To directly test brain infection, we used intracranial inoculation and found that the hypovirulent strain was substantially more invasive than its hypervirulent counterpart. We suggest that immunogenic proteins secreted by this strain invoke a host response that limits pulmonary infection but that there can be invasive growth and damage if infection reaches the brain. Given their known role in virulence, it is possible that non-conventionally secreted proteins mediate this process.

Protein secretion is an essential process for all cells. Secretion has roles in various aspects of cell physiology and lifestyle, including nutrient acquisition, cell wall remodeling, signaling, quorum sensing, and defense against other organisms (1). For pathogenic organisms, the secretion of specific proteins can be key to disease progression, allowing the pathogen both to invade and obtain nutrients and to directly modulate the host organism's immune response.

The ability to secrete proteins is of particular importance to fungal organisms. Fungi use exodigestion and absorptive nutrition to acquire nutrients and have evolved a complex suite of secreted proteins to degrade the diverse biopolymers encountered in the host or abiotic environment. Comparisons of characterized fungal secretomes suggest that their basal secretome consists of a core set of degradation enzymes. These include polysaccharide-active enzymes, including glycoside hydrolases, carbohydrate esterases, and polysaccharide lyases, which can degrade the major components of plant cell walls such as cellulose and pectin (2). Other degradative enzymes include proteases, for example, secreted aspartyl proteases (SAPs), which have been associated with pathogenicity in the human commensal yeast *Candida albicans* (3, 4).

Many proteins destined for secretion are tagged with a signal peptide that can be used to predict secretion via "classical" pathways (5). However, extracellular proteins that lack any known signal peptide have frequently been identified and are generally referred to as non-classically or unconventionally secreted proteins (6). A number of unconventional protein secretion mechanisms are known, and these include both vesicle-dependent and vesicle-independent pathways (7). Many proteins secreted via nonclassical pathways have been described as "moonlighting" proteins, which, in contrast to those proteins characterized as hav-

ing a single primary core function, are multifunctional, often with secondary roles that are unrelated to their core function (8–11).

Species of *Cryptococcus* are environmental fungi capable of causing disease in immunocompromised and immunocompetent individuals. The two predominant pathogenic species, *Cryptococcus neoformans* and *Cryptococcus gattii*, can cause cryptococcosis in animals and humans, with outcomes ranging from an asymptomatic state to severe, fatal meningitis (12). Notable differences between *C. gattii* and *C. neoformans* include the preferred environmental niche, basidiospore morphology, drug susceptibility, epidemiology, the clinical manifestations of associated disease, and host susceptibility (13). Additional differences have been observed within *Cryptococcus* species. In *C. gattii*, a hypervirulent subgenotype designated VGIIa caused a recent significant outbreak of cryptococcosis on Vancouver Island in British Columbia, Canada, and in the Pacific Northwest of the United States. *C. gattii* VGIIa occurs sympatrically with subgenotype VGIIb, which is

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globally distributed and is considered to be hypovirulent (14). These differences in virulence and epidemiology between *Cryptococcus* species and subgenotypes provide an opportunity for understanding pathogenicity and disease progression by what are otherwise very genetically similar fungal organisms.

The current study was designed to analyze the secretomes of three *Cryptococcus* strains that differ in virulence. Previous analyses of the protein cohort secreted by *Cryptococcus* spp. have used acapsular mutant strains or nutrient-replete culture media to suppress capsule formation (15–17). However, this may induce a secretion profile different from that seen under the nutrient-limited conditions encountered in the host (6, 15). In the current study, we set out to analyze secretion by encapsulated, wild-type *Cryptococcus* strains under conditions designed to be as similar as possible to those encountered within the host. As low-nutrient conditions provoke excess production and shedding of extracellular polysaccharide capsule (18), we developed a novel method of capturing secreted proteins using ProteoMiner (Bio-Rad) beads. We report here that different strains of *Cryptococcus* secrete distinctly different sets of proteins, with the *C. gattii* VGIIb isolate secreting many more proteins predicted to be non-classically secreted and immunogenic than the hypervirulent *C. gattii* VGIIa and *C. neoformans* strains. Infection of mice by intracerebral inoculation revealed that the VGIIb isolate produced rapidly fatal meningitis with substantially more growth than the *C. gattii* VGIIa strain. Given their role in infection and immunity, it is possible that non-classically secreted and nonimmunogenic proteins play a role in modulating cryptococcosis, on the one hand enhancing immune clearance from the lungs but on the other promoting disease following passage across the blood-brain barrier (BBB).

MATERIALS AND METHODS

Strains and culture conditions. *Cryptococcus* strains included *C. gattii* R265 (genotype VGIIa) and *C. gattii* R272 (genotype VGIIb) and *C. neoformans* KN99 α (secretome analysis) and H99 (congenic with KN99 α ; infection model, genotype VNI). Strains were retrieved from storage at -80°C and streaked onto yeast extract-peptone-dextrose (YPD) plates, which were incubated at 30°C for 2 days. Single yeast colonies were inoculated into a starter culture containing RPMI minimal media (Invitrogen), supplemented with $1\times$ minimal essential medium (MEM) complete amino acid mix (Sigma-Aldrich), 2.5 M MOPS (morpholinepropanesulfonic acid) buffer (Sigma-Aldrich) (pH 7.2), 0.2% glucose, and penicillin (100 U/ml)-streptomycin (100 $\mu\text{g/ml}$) (Life Technologies). Cultures were grown for 2 days at 37°C in an incubator with orbital shaking at 120 rpm. Cells were counted using an improved Bright-Line hemocytometer (Neubauer). Approximately 10^8 cells from the starter culture were inoculated into each of two 1-liter flasks containing the same medium as that described above. Cells were grown for a further 7 days at 37°C with shaking at 120 rpm. On day 7, all cultures were assayed for cell viability. A 10-ml aliquot was collected from each culture, and the cells were pelleted, resuspended in 500 μl phosphate-buffered saline, and stained with trypan blue, which is excluded from live but not dead cells. The level of viability was assessed by microscopic examination.

Isolation of secreted protein. For each *Cryptococcus* strain, the two 1-liter cultures produced as described above were pooled and centrifuged at $4,500\times g$ for 10 min to pellet yeast cells, and the supernatant was decanted and saved. For KN99 α , the culture was centrifuged twice due to the presence of residual cells in the medium after the first spin. Secreted proteins were then isolated from the cell-free supernatant using ProteoMiner beads (Bio-Rad Laboratories). The beads were prepared according to the instructions of the manufacturers before being transferred to a 9-cm-long Poly-Prep gravity flow chromatography column (Bio-Rad Laboratories) connected to a system of sterile silicon tubing. This tubing

started and terminated in a flask containing the culture supernatant, which was circulated through the tubing via the action of a peristaltic pump (LongerPump; BT100-2J). This apparatus allowed the supernatant to be continually recirculated across the ProteoMiner beads at a flow rate of 12 ml/min for 2 days. The entire process was carried out at 4°C to minimize proteolysis. Captured proteins were eluted by transferring the beads back into the original column and adding 100 μl of $2\times$ sample buffer (20% glycerol, 200 mM Tris-HCl [pH 6.8], 4% SDS, 5% beta-mercaptoethanol, bromophenol blue) before the column was placed at 100°C for 10 min. This was repeated 4 times to ensure removal of all protein from the beads. A 10- μl aliquot of the eluted protein sample was electrophoresed in a 4%-to-12% XT Bis-Tris Criterion gel (Bio-Rad Laboratories) and quantified by comparison with a dilution series of bovine serum albumin standards. The remaining protein samples were stored at -20°C .

Preparation of protein for liquid chromatography-tandem mass spectrometry (LC-MS/MS). The entire remaining protein sample was reduced to approximately 45 μl by vacuum centrifugation, boiled for 10 min, and electrophoresed in a 4%-to-12% Bis-Tris Criterion gel (Bio-Rad Laboratories) at 160 V for 10 min. Gels were then fixed for 30 min in 40% methanol plus 10% acetic acid and stained with Bio-Safe Coomassie stain (Bio-Rad Laboratories). Lanes containing proteins were excised from the stained gels, finely diced into approximately 1-mm-by-1-mm pieces, and then subjected to trypsin in-gel digestion for mass spectrometry, as previously described (19).

LC-MS/MS analysis. Peptides were separated using liquid chromatography (LC) and analyzed via tandem mass spectrometry (MS/MS). Briefly, an Eksigent AS-1 autosampler was used to load the peptide samples onto a Tempo nanoLC system (Eksigent, USA) at a flow rate of 20 $\mu\text{l}/\text{min}$ with MS loading solvent (2% acetonitrile–0.2% trifluoroacetic acid) onto a C_8 trap column (CapTrap; Michrom Biosciences, USA). After washing for 3 min was performed, the peptides were washed off the C_8 trap at a flow rate of 300 nl/min with MS solvent A (2% acetonitrile–0.2% formic acid) and collected onto a PicoFrit column (75 μm by 150 mm) packed with Magic C18AQ resin (Michrom Biosciences). The LC-separated peptides were eluted from the column and into the source of a QStar Elite hybrid quadrupole time of flight mass spectrometer (Applied Biosystems/MDS Sciex) using the following program: 5% to 50% MS solvent B (98% acetonitrile–0.2% formic acid) over 60 min, 50% to 80% MS solvent B over 5 min, 80% MS solvent B for 2 min, and, finally, 80% to 5% MS solvent B for 3 min. The eluted peptides were ionized from the PicoFrit column at 2,300 V. An intelligent data acquisition (IDA) experiment was performed, with a mass range of 375 to 1,500 Da continuously scanned for peptides of charge state $2+$ to $5+$ and with an intensity of more than 30 counts/s. Selected peptides were fragmented, and the product ion fragment masses were measured over a mass range of 100 to 1,500 Da. The mass of the precursor peptide was then excluded for 1 min.

Generation of LC-MS/MS data. The MS/MS data files produced by a QStar Elite hybrid quadrupole time of flight mass spectrometer were searched using Mascot Daemon (version 2.2.2, provided by the Walter and Eliza Hall Institute) (20) and the LudwigNR database (comprised of the UniProt, plasmoDB, and Ensembl databases). The searched parameters were as follows: variable modifications of propionamide, oxidized methionine, and deamidated asparagine and a semi-trypsin digest, with a maximum of three missed cleavages, namely, a peptide mass tolerance of 100 ppm, a MS/MS mass tolerance of 0.2 Da, and charge states of $2+$, $3+$, and $4+$. The results of the search were then filtered by including only protein hits with at least one unique peptide and a Mascot score of >60 . Single peptide identifications were further validated by manual inspection of the MS/MS spectra for the peptide to ensure that the b-ion and y-ion series were sufficiently extensive for an accurate identification, with the y1 ion identified as either arginine or lysine, as the samples were trypsin digested. The list was filtered again by keeping only those proteins that were identified in three or more biological replicates and were identified as predicted proteins of *Cryptococcus* spp. via the LudwigNR database.

Bioinformatic analysis of identified proteins. Biological functions for protein identifications were assigned using the UniProtKB and InterPro databases (21, 22). UniProtKB uses the International Nucleotide Sequence Database Collaboration, which encompasses all sequences submitted to the EMBL-Bank/GenBank/DBJ databases. These resources were used to assign putative functions to uncharacterized proteins. All identified proteins were analyzed for secretory signals using the FunSecKB2 database (23). This resource combines multiple search algorithms, including SignalP, WolfPsort, Phobius, and FragAnchor, to interrogate all fungal protein data in the NCBIRefSeq database to predict secreted fungal proteins. The online tool SecretomeP was further used to predict protein secretion by both classical and nonclassical mechanisms. This program produces *ab initio* predictions, i.e., those generated without reference to previous secretion predictions, of nonclassical protein secretion. It does this by compiling information from feature prediction servers on posttranslational and localization aspects of a protein and using this information to produce a secretion prediction. A protein with a SecretomeP score of >0.5 is predicted to be secreted via a nonclassical pathway (24).

Intracranial cryptococcal meningitis infection model. This study was conducted in conformity with the Australian Code for the Care and Use of Animals for Scientific Purposes and with the review and approval of the University of Sydney Animal Ethics Committee (project number L02/4-2013/1/5887). *Cryptococcus* infection in the brain was studied in mice using an intracranial infection model (25). Six-week-old male C57BL/6 mice ($n = 3$) and SCID mice ($n = 3$) (Animal Resources Centre, Western Australia) were infected with each *Cryptococcus* strain. Animals were anesthetized, and 1,000 yeast cells were injected directly into the brain in a volume of 0.02 ml (5×10^4 cells/ml inoculum) using a 26-gauge needle inserted through the midline of the dorsal surface of the skull. Mice were monitored daily for weight loss and symptoms of illness, including excessive lethargy and ruffled fur. Once weight loss exceeded 15% of the weight of the animal at the time of infection, the mouse was euthanized. All surviving animals were euthanized at 14 days postinfection. At that time, the brain was harvested and immediately fixed in 4% paraformaldehyde. Statistical differences between survival curves were assessed by the Mann-Whitney U test.

Histochemistry and immunohistochemistry of cryptococcal brain infection. Longitudinal sections were prepared from fixed brain tissue for each of the infecting cryptococcal strains and mouse strains. Hematoxylin and eosin stains were performed and counterstained with alcian blue to visualize cryptococcal cells. Alcian blue stains mucin and reacts strongly with cryptococcal capsular material. Immunohistochemistry was performed on unstained slides using an anti-gial fibrillary acidic protein (anti-GFAP) antibody (DAKO Cytoation) for visualization of astrocytes and anti-Iba1 (Wako) to visualize microglial cells. Briefly, paraformaldehyde-fixed tissue sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Prior to antibody hybridization, for antigen retrieval, slides were treated either with Tris-HCl buffer (25 mM; pH 8.5) containing 0.05% SDS (wt/vol) and EDTA (1 mM) or with sodium citrate buffer (10 mM; pH 8.5) for 40 min at 97°C. Endogenous peroxidases were then blocked by incubation of slides for 5 min in 1% hydrogen peroxide and then blocked with 5% goat serum–Tris-buffered saline–0.1% Tween 20 (TBS-T). Slides treated with Tris-SDS-EDTA were incubated overnight at 4°C with anti-Iba1 antibody (diluted 1:1,000), while slides treated with sodium citrate buffer were incubated under similar conditions with anti-GFAP antibody (diluted 1:1,000). Following the primary antibody incubation, all slides were incubated with biotinylated secondary antibodies (Vector Laboratories) (diluted 1:200; 45 min at room temperature [RT]) and then with horseradish peroxidase (HRP)-coupled streptavidin (Vector Laboratories) (diluted 1:200; 45 min at RT). Visualization of the immunohistochemistry was performed using diaminobenzidine peroxidase substrate (Vector Laboratories). Finally, sections were counterstained with hematoxylin (Sigma-Aldrich) for 3 min before being dehydrated in graded ethanol and xylene. Stained sections

were examined under a DM4000B bright-field microscope (Leica, Wetzlar, Germany), and images were captured using a Spot Flex camera and Spot V4.5 software (Diagnostic Instruments, USA).

RESULTS

Proteins secreted by *Cryptococcus* strains of high (*C. neoformans* and *C. gattii* VGIIa) and low (*C. gattii* VGIIb) virulence were identified using a novel method of protein capture, followed by liquid chromatography-mass spectroscopy. FunSecKB:v2 and SecretomeP were used to determine if proteins that were present in the fungal secretomes were likely to be classically or nonclassically secreted. It was hypothesized that differences in the secretomes might contribute to differences in host response and pathology, and this was tested in a murine intracranial infection model.

ProteoMiner beads allow isolation of proteins secreted from *Cryptococcus* species under low-nutrient conditions designed to approximate those in the mammalian host lung. Species of *Cryptococcus* produce and shed large quantities of extracellular polysaccharide into culture medium, particularly when the availability of nutrients is limited. This complicates the isolation and analysis of secreted *Cryptococcus* proteins when culture conditions are designed to be similar to those encountered within the mammalian host. To overcome this, we supplemented protein-free RPMI tissue-culture media with amino acids, which we have found to limit the production of excessive shed capsule (D. A. Carter, unpublished data). To maximize the capture of secreted proteins from the culture supernatant, we developed a novel method using ProteoMiner beads (Bio-Rad Laboratories, USA). These beads contain randomly generated hexapeptide ligands designed to capture proteins following direct contact. This process yielded 50 to 60 µg of protein from each 2-liter biological replicate culture of *C. neoformans* and 40 to 50 µg from the 2-liter biological replicate cultures of the *C. gattii* strains.

To ensure that proteins collected from the supernatants had not been released as a result of cell death and lysis, cultures were assayed throughout the experimental process using trypan blue viability staining (see Fig. S1 in the supplemental material). All strains appeared similar, and there was no appreciable cell death or debris observed. This was further confirmed by subsequent mass spectroscopy, as ribosomal proteins, which would be present as a result of significant cell lysis, were not identified in any of the secretomes (see Table S1).

Different *Cryptococcus* strains secrete different numbers and types of proteins with limited overlap among strains. After manual validation of the LC-MS/MS protein identifications, a total of 67 cryptococcal proteins were identified. Of these, 17 had characterized functions and 24 had putative characterizations according to the UniProt KnowledgeBase. The remaining 26 proteins were annotated as “putative uncharacterized protein” ($n = 25$) or as “expressed protein” ($n = 1$) and were assigned functional roles according to predicted domains using InterPro and by gene ontology using UniProtKB. Using these approaches, putative functions were assigned to all but seven of the uncharacterized proteins (Table 1; see also Table S1 in the supplemental material).

The 67 secreted proteins were unevenly distributed across the three *Cryptococcus* strains, with significant differences with respect to both the number and type of proteins secreted (Table 1 and Fig. 1). A smaller number of less diverse proteins were identified in the secretomes of the high-virulence *C. neoformans* and

TABLE 1 Proteins identified from the *Cryptococcus* secretomes

Protein name	Strain(s) in which the protein was identified	Molecular function or predicted function ^a	Functional grouping	Protein accession no.
Classically secreted hydrolytic and proteolytic proteins				
Glycosyl hydrolase, putative	All strains	Hydrolase	Hydrolysis	E6QY56
Carboxypeptidase D, putative	<i>C. gattii</i> VGIIa & VGIIb	Serine-type carboxypeptidase activity	Proteolysis	E6RCV7
Peptidase, putative	<i>C. gattii</i> VGIIa & VGIIb	Serine-type endopeptidase activity	Proteolysis	E6R030
Chitin deacetylase	<i>C. neoformans</i> & <i>C. gattii</i> VGIIa	Chitin deacetylase activity	Hydrolysis	P82476
Putative uncharacterized protein	<i>C. neoformans</i> & <i>C. gattii</i> VGIIa	<i>Glycoside hydrolase</i>	Hydrolysis	Q55ZC4
Alpha-amylase AmyA, putative	<i>C. neoformans</i> & <i>C. gattii</i> VGIIb	Alpha-amylase activity	Hydrolysis	E6RCN1
Chitinase, putative	<i>C. gattii</i> VGIIa	Chitinase activity	Hydrolysis	E6RB39
Cellulase, putative	<i>C. gattii</i> VGIIa	Hydrolyzing O-glycosyl compounds	Hydrolysis	E6R762
Endopeptidase, putative	<i>C. gattii</i> VGIIb	Aspartyl protease	Proteolysis	E6QY25
Serine-type endopeptidase, putative	<i>C. gattii</i> VGIIb	Serine-type endopeptidase activity	Proteolysis	E6R505
Putative uncharacterized protein	<i>C. gattii</i> VGIIb	<i>Glycoside hydrolase</i>	Hydrolysis	E6QYQ1
Putative uncharacterized protein	<i>C. gattii</i> VGIIb	<i>Glycoside hydrolase</i>	Hydrolysis	E6R0L9
Putative uncharacterized protein	<i>C. neoformans</i>	<i>Glycoside hydrolase</i>	Hydrolysis	E6QZ12
Putative uncharacterized protein	<i>C. neoformans</i>	<i>Glycoside hydrolase</i>	Hydrolysis	E6R597
Putative uncharacterized protein	<i>C. neoformans</i>	<i>Alpha amylase</i>	Hydrolysis	F5HAT9
Putative uncharacterized protein	<i>C. neoformans</i>	<i>Peptidase</i>	Proteolysis	Q560V8
Putative uncharacterized protein	<i>C. neoformans</i>	<i>Glucanase</i>	Hydrolysis	Q5KA52
Putative uncharacterized protein	<i>C. neoformans</i>	<i>Carboxylesterase</i>	Hydrolysis	Q5KA58
Putative uncharacterized protein	<i>C. neoformans</i>	<i>Glycoside hydrolase</i>	Hydrolysis	Q5KN45
Putative uncharacterized protein	<i>C. neoformans</i>	<i>Glycoside hydrolase</i>	Hydrolysis	Q55S66
Putative uncharacterized protein	<i>C. neoformans</i>	<i>Glycoside hydrolase</i>	Hydrolysis	Q5KPL2
Classically secreted nondegradative proteins				
Transmembrane receptor, putative	<i>C. gattii</i> VGIIa & VGIIb	Receptor	Signaling	E6QYF2
Cytokine inducing-glycoprotein, putative	<i>C. gattii</i> VGIIa & VGIIb	<i>Cytokine inducing-glycoprotein</i>	Unknown	E6R316
Putative uncharacterized protein	<i>C. gattii</i> VGIIa & VGIIb	<i>Unknown/lyase activity</i>	Unknown	E6R9N5
Putative uncharacterized protein	<i>C. gattii</i> VGIIa & VGIIb	<i>Oxidoreductase</i>	Redox	E6RF45
Meiotic recombination-related protein	<i>C. neoformans</i> & <i>C. gattii</i> VGIIb	<i>Stress response nuclear envelope protein</i>	Stress response	E6R3P7
Putative uncharacterized protein	<i>C. gattii</i> VGIIa	<i>Beta-glucan synthesis</i>	Metabolism	Q5K715
Glycoprotein, putative	<i>C. gattii</i> VGIIa	<i>Unknown</i>	Unknown	Q5K852
Sterol-binding protein	<i>C. neoformans</i>	Sterol binding	Transport	E6RAN9
Expressed protein	<i>C. neoformans</i>	<i>Beta-glucan synthesis</i>	Metabolism	Q5KIN3
Transmembrane receptor	<i>C. neoformans</i>	Receptor	Signaling	Q5KNE4
Non-classically secreted proteins				
G protein beta subunit Gib2	<i>C. gattii</i> VGIIb	cAMP signaling	Signaling	A0AUJ0
Fructose-bisphosphate aldolase, putative	<i>C. gattii</i> VGIIb	Fructose-bisphosphate aldolase activity	Metabolism	E6R1G7
Aminotransferase, putative	<i>C. gattii</i> VGIIb	Transaminase activity	Metabolism	E6R1V5
Actin-binding protein Cofilin, putative	<i>C. gattii</i> VGIIb	Actin filament depolarization	Organelle organization	E6R286
Orotidine 5'-phosphate decarboxylase	<i>C. gattii</i> VGIIb	Decarboxylase activity	Metabolism	Q5K890
ATP synthase subunit beta	<i>C. gattii</i> VGIIb	ATP binding	Transport	E6R8N5
Phosphoglycerate kinase	<i>C. gattii</i> VGIIb	Phosphoglycerate kinase activity	Metabolism	E6R9I3
Phosphomannomutase, putative	<i>C. gattii</i> VGIIb	Phosphomannomutase activity	Metabolism	E6RA79
Guanosine-diphosphatase, putative	<i>C. gattii</i> VGIIb	Guanosine-diphosphatase activity	Metabolism	Q5KLL8
Oxidoreductase, putative	<i>C. gattii</i> VGIIb	Oxidoreductase activity	Redox	Q5KLM8
Dihydrolipoyl dehydrogenase	<i>C. gattii</i> VGIIb	Oxidoreductase	Redox	Q5Y229
Dihydrolipoyl dehydrogenase	<i>C. gattii</i> VGIIb	Oxidoreductase	Redox	Q8J0Z3
Putative uncharacterized protein	<i>C. gattii</i> VGIIb	<i>Unknown</i>	Unknown	E6RDV2
Putative uncharacterized protein	<i>C. gattii</i> VGIIb	TolB-like propeller	Unknown	F5HD57
Putative uncharacterized protein	<i>C. gattii</i> VGIIb	<i>Unknown</i>	Unknown	E6RET6
UDP-glucose 6-dehydrogenase ^b	<i>C. gattii</i> VGIIb	Oxidoreductase	Redox	E6R5A2
Thioredoxin peroxidase, putative ^b	<i>C. gattii</i> VGIIb	Peroxioredoxin activity	Redox	E6RE09
Citrate synthase ^b	<i>C. gattii</i> VGIIb	Citrate (Si)-synthase activity	Metabolism	Q5KQ45
Putative uncharacterized protein	<i>C. neoformans</i>	<i>Glutaredoxin/oxidoreductase activity</i>	Redox	E6QYZ1

(Continued on following page)

TABLE 1 (Continued)

Protein name	Strain(s) in which the protein was identified	Molecular function or predicted function ^a	Functional grouping	Protein accession no.
Putative uncharacterized protein	<i>C. neoformans</i>	<i>Unknown</i>	Unknown	Q5KCM3
Putative uncharacterized protein	<i>C. neoformans</i>	<i>Unknown</i>	Unknown	Q5KD42
Nondegradative proteins secreted via unknown mechanisms				
3-Isopropylmalate dehydrogenase ^b	<i>C. gattii</i> VGIIb	Oxidoreductase	Redox	E6QXQ4
Enolase ^{b,c}	<i>C. gattii</i> VGIIb	Phosphopyruvate hydratase activity	Metabolism	Q55UX4
Glutamate dehydrogenase ^b	<i>C. gattii</i> VGIIb	Oxidoreductase	Redox	E6R2U4
Glyceraldehyde-3-phosphate dehydrogenase ^{b,c}	<i>C. gattii</i> VGIIb	NAD/P binding	Metabolism	E6R7Z5
6-Phosphogluconate dehydrogenase ^{b,c}	<i>C. gattii</i> VGIIb	NADP binding	Metabolism	E6RDR8
Heat shock protein, putative ^b	<i>C. gattii</i> VGIIb	ATP binding	Stress response	E6RFH1
Putative uncharacterized protein ^b	<i>C. gattii</i> VGIIb	HSP90-like	Stress response	F5HDC9
Putative uncharacterized protein ^b	<i>C. gattii</i> VGIIb	<i>14-3-3-Like</i>	Signaling	F5HI88
Superoxide dismutase (Cu-Zn) ^{b,c}	<i>C. neoformans</i>	Oxidoreductase activity	Redox	Q9C0S4
Eukaryotic ADP/ATP carrier	<i>C. gattii</i> VGIIb	Transporter activity	Transport	Q2XPZ3
Glucose-6-phosphate isomerase	<i>C. gattii</i> VGIIb	Isomerase	Metabolism	E6QZC4
Transaldolase	<i>C. gattii</i> VGIIb	Transaldolase activity	Metabolism	E6RD54
Putative uncharacterized protein	<i>C. gattii</i> VGIIb	<i>Unknown</i>	Unknown	E6R123
Putative uncharacterized protein	<i>C. gattii</i> VGIIb	AAA ⁺ ATPase	Transport	F5HD04
Cytokine-inducing glycoprotein	<i>C. neoformans</i>	<i>Unknown</i>	Unknown	Q96UH1

^a Italic entries represent predicted or unknown functions.

^b Reported in association with microvesicles (26).

^c Potentially immunogenic.

C. gattii VGIIa strains, with 22 and 13 identified secreted proteins, respectively, compared to those identified in the secretome of the lower-virulence *C. gattii* VGIIb strain with 44 identified proteins.

Eleven proteins were identified in the secretomes of two or more of the three strains. One protein, a putative glycosyl hydrolase (E6QY56) predicted to belong to the glycosyl hydrolase 61 family, was found in all three strains. The *C. gattii* strains had six proteins in common, including a putative transmembrane receptor (E6QYF2), a putative cytokine-inducing glycoprotein (E6R316), a putative peptidase (E6R030), and a putative carboxy-

peptidase D (E6RCV7). Two proteins were uncharacterized but were predicted to have lyase (E6R9N5) and oxidoreductase (E6RF45) activity. Four proteins were common to one of the *C. gattii* strains and the *C. neoformans* strain. *C. neoformans* and the high-virulence *C. gattii* VGIIa strain shared a chitin deacetylase (P82476) and a putative uncharacterized protein (Q55ZC4). *C. neoformans* and the low-virulence *C. gattii* VGIIb strain both secreted a putative meiotic recombination-related protein (E6R3P7) and an α -amylase (E6RCN1). The latter has been linked to virulence in some fungi, including *Aspergillus flavus* and *Histoplasma capsulatum* (27, 28).

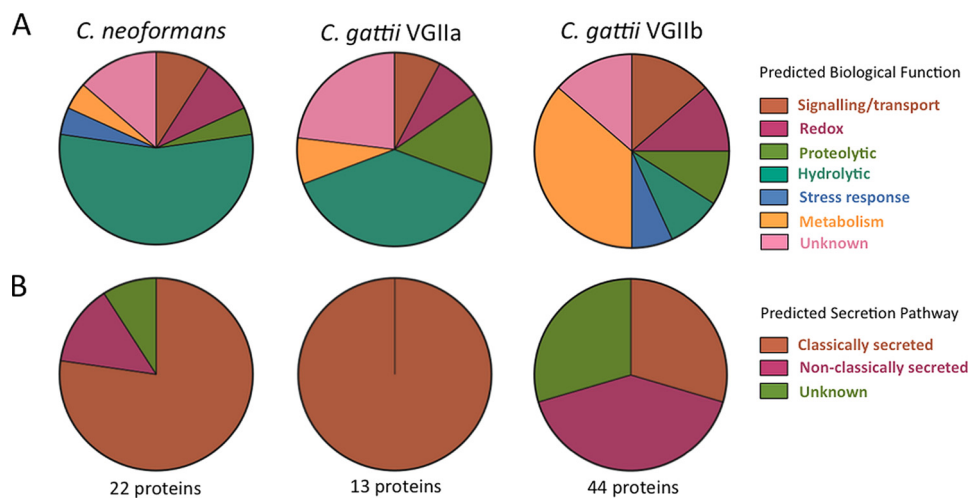


FIG 1 Predicted functional groups and secretory pathways for the secreted *Cryptococcus* proteins. (A) The secretomes of high-virulence *C. neoformans* and *C. gattii* VGIIa are dominated by hydrolytic and proteolytic proteins, while the secretome of *C. gattii* VGIIb contains a large number of proteins involved in metabolism. (B) *C. gattii* VGIIb has a much greater proportion of proteins secreted by nonclassical pathways than *C. neoformans* and *C. gattii* VGIIa.

All secretomes contained a core set of hydrolytic and proteolytic proteins with potential nutrient-scavenging roles. Approximately half of the proteins identified in the secretomes of the high-virulence *C. gattii* VGIIa and *C. neoformans* strains (7/13 and 13/22 proteins, respectively) are known or predicted to have hydrolytic or proteolytic functions (Fig. 1). These enzymes are secreted by a wide range of fungi and have a role in nutrient scavenging via exodigestion (2). In the *C. gattii* VGIIa secretome, these proteins included a putative cellulase (E6R762) and a chitinase (E6RB39), while those identified in the *C. neoformans* secretome were generally uncharacterized proteins with putative glycoside hydrolase, peptidase, or carboxyesterase activity (Table 1). The *C. gattii* VGIIb secretome contained a number of proteins with hydrolytic and proteolytic functions similar to those seen with the other two strains, but these comprised a substantially smaller fraction of the total proteins in its secretome (8/44 [18%]). These hydrolytic and proteolytic proteins may represent a “core set” of degradative enzymes, similar to those known to be secreted by many fungal species (2).

Extracellular *Cryptococcus* proteins are secreted by both classical and nonclassical mechanisms. FunSecKB:v2 and SecretomeP were used to predict if proteins identified in the cryptococcal secretomes were secreted via classical or nonclassical mechanisms. Of the 67 unique proteins identified across the three secretomes, 32 were predicted as being classically secreted. These included the majority of the proteins identified in the secretomes of the virulent strains, with 13/13 of the *C. gattii* VGIIa and 18/22 of the *C. neoformans* proteins predicted to be secreted via classical mechanisms. Four of these classically secreted proteins were predicted to have glycosylphosphatidylinositol (GPI)-anchor signal sequences, suggesting an interaction with the fungal cell wall (Fig. 1; see also Table S1 in the supplemental material). In contrast, less than a third (13/44) of the proteins identified in the *C. gattii* VGIIb secretome were predicted to be classically secreted. All of the hydrolytic and proteolytic proteins across the three secretomes, which potentially belong to a core suite of nutrient-scavenging enzymes, were predicted to be classically secreted, consistent with a role in fungal exodigestion.

In the *C. gattii* VGIIb and the *C. neoformans* secretomes, 18 and 3 proteins, respectively, were predicted as being secreted via alternative or nonclassical mechanisms (Table 1). These proteins included dihydrolipoyl dehydrogenase (Q5Y229 and Q8J0Z3) and a putative thioredoxin peroxidase (E6RE09), both involved in redox homeostasis within the cell, the G-protein Gib2 beta subunit (A0AUJ0) involved in cyclic AMP (cAMP) signaling, and metabolic proteins, including a putative fructose-bisphosphate aldolase (E6R1G7), a putative aminotransferase (E6R1V5), and a UDP-glucose 6-dehydrogenase (E6R5A2).

Fifteen proteins were not predicted by FunSecKB or SecretomeP to be secreted by either classical or nonclassical mechanisms. *Cryptococcus* species are known to secrete microvesicles, which are analogous to mammalian exosomes and are described as an alternative, nonclassical secretion mechanism (29). Of the 67 proteins identified as secreted in this study, 12 have been reported as contained within *Cryptococcus* microvesicles, and nine of these were among the 15 proteins with no predicted secretion mechanism, with the remaining three predicted to be nonclassically secreted. These microvesicle-associated proteins included the common intracellular proteins enolase (Q55UX4), glyceraldehyde-3-phosphate dehydrogenase (E6R7D5), 6-phos-

phogluconate dehydrogenase (E6RDR8), and Cu-Zn superoxide dismutase (Q9C0S4) as well as a putative uncharacterized protein (F5HI88) with a predicted 14-3-3 regulatory-protein-like function.

The *C. gattii* VGIIb secretome contains a unique set of secreted proteins, some of which may have “moonlighting” or immunogenic functions. Thirty-five of the 67 proteins identified across the three cryptococcal secretomes analyzed in this study were unique to the secretome of the low-virulence *C. gattii* VGIIb strain (Table 1). These proteins are primarily involved in metabolism, signaling/transport, glycolysis, and redox processes and are canonical intracellular proteins. A number of these have orthologues that have previously been identified in the extracellular milieu of various cell types from other organisms (30–33). Their presence in this alternative environment, where interacting protein partners or substrates are typically absent, suggests they may have alternative or “moonlighting” functions when secreted. In addition, some of the proteins secreted by the *C. gattii* VGIIb strain have orthologues that can initiate an immune response in the host. These included the glycolytic proteins glyceraldehyde-3-phosphate dehydrogenase (E6R7D5), enolase (Q55UX4), and 6-phosphogluconate dehydrogenase (E6RDR8) and the stress response protein Cu-Zn superoxide dismutase (Q9C0S4) (Table 1).

***C. gattii* high-virulence and low-virulence strains have reversed pathogenicity characteristics in an intracranial infection model of cryptococcal meningitis.** Past epidemiological data and studies using a murine inhalation model of virulence have suggested that the *C. gattii* VGIIa genotype is more virulent than the VGIIb genotype (14, 34). However, the results of a recent epidemiological study of the Vancouver Island cryptococcal outbreak indicated that, while a much smaller number of patients had been infected with the VGIIb genotype, a greater proportion of these progressed to cerebral sequelae resulting in death. In contrast, *C. gattii* VGIIa infections were more likely to be pulmonary (35). The difference in the levels of immunogenicity of the proteins secreted by the VGIIa and VGIIb strains noted here suggested that differences between these genotypes with respect to virulence and pathology might be mediated in part by the fungal secretome, which on the one hand might trigger a more rapid immune clearance of *C. gattii* VGIIb strains but on the other might result in more-severe symptoms if the immune system were breached. To test this, we bypassed the lung, using an intracranial infection model with direct injection of 1,000 CFU of *Cryptococcus* yeast cells into the mouse brain. *C. neoformans* strain H99, which is widely used as a model for virulent cryptococcosis, was included for comparison. Two different strains of mice were used for intracerebral infection, inbred wild-type C57BL/6 mice and immunocompromised SCID mice, which are T cell deficient and are often used as an animal model for AIDS. The survival curve following infection is shown in Fig. 2. In contrast to the survival profile seen following inhalation, where mice infected with *C. neoformans* H99 and *C. gattii* VGIIa strains are rapidly killed while those infected with *C. gattii* VGIIb survive (14), infection by *C. gattii* VGIIb caused significantly more rapid death than infection by *C. gattii* VGIIa ($P < 0.05$).

Although the severely immunocompromised SCID mice succumbed slightly earlier than the immunocompetent mice, the results were not significantly different for any of the infecting *Cryptococcus* strains ($P > 0.05$), suggesting that the host immune status was having little influence on cryptococcal infection once this had

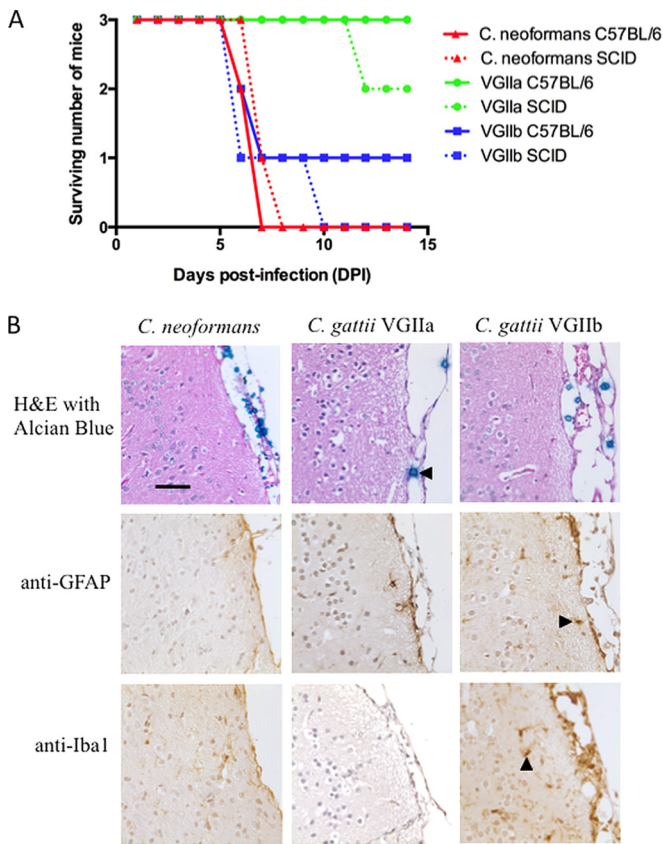


FIG 2 Survival curve, histochemistry, and immunohistochemistry for C57BL/6 and SCID mice infected intracranially with *Cryptococcus* strains. (A) Intracranial infection results in rapid death of mice infected with *C. neoformans* and *C. gattii* strain R272, which is considered hypovirulent in a pulmonary infection model. In contrast, most mice survive infection with the “hypervirulent” *C. gattii* VGIIa strain. (B) Histochemistry and immunohistochemistry for representative infected C57BL/6 mice. (Top panel) alcian blue counterstaining showing cryptococcal cells in the mice meninges (left-pointing arrowhead). The fungal burden is considerably lower in mice infected with *C. gattii* VGIIa (see also Fig. S2 in the supplemental material for a zoomed-out field of view). (Middle panel) Anti-GFAP antibody staining showing astrocytes (right-pointing arrowhead). (Bottom panel) Anti-Iba1 visualizing microglial cells (upward-pointing arrowhead). The immune response is extremely limited, particularly for *C. neoformans* infection, despite the significant fungal burden. There was a greater microglial response in mice infected with *C. gattii* VGIIb than in those infected with *C. neoformans* and *C. gattii* VGIIa. Bar, 50 μ m. H&E, hematoxylin and eosin.

established in the brain. To assess pathology, we examined embedded sections of brain tissue from infected C57BL/6 mice by histochemistry and immunohistochemistry. Brains infected with the *C. gattii* VGIIb and *C. neoformans* strains contained a large number of *Cryptococcus* cells located predominantly in the meninges, with evidence of extensive polysaccharide capsule production. In contrast, the brain sections from mice infected with the *C. gattii* VGIIa strain had very few visible *Cryptococcus* cells (Fig. 2; see also Fig. S2 in the supplemental material). Interestingly, while the *C. gattii* cells visible in the meninges were relatively large (~10 to 20 μ m) and uniform in size, the *C. neoformans* cells were very heterogeneous, with a large number of very small (<5- μ m-diameter) cells. An observable effect on the meninges was apparent only with the VGIIb strain, where a slight to moderate thickening was apparent. Immunohistochemistry was performed on tissue sec-

tions from the C57BL/6 mice to determine the presence and activation state of astrocytes, which respond rapidly to tissue damage in the brain, and of microglia, the immune cells resident in the central nervous system (CNS). Activation of some astrocyte and microglial cells was observed adjacent to the meninges in the VGIIb-infected tissue; however, compared to the results seen with other intracerebral infection models, this response was very weak (36, 37). There was no apparent response to infection seen in brain tissue from mice infected with the *C. gattii* VGIIa or the *C. neoformans* strain.

DISCUSSION

In our study, we compared individual proteins secreted from *Cryptococcus* strains with different virulence attributes. Our aims were to identify proteins likely to be secreted by the *Cryptococcus* strains during mammalian infection and to determine if these differed between strains known to have different degrees of pathogenicity. Our results suggest that the secretomes of the strains differ and may mediate a complex interplay between host recognition and clearance on the one hand and invasion and pathogenic disease on the other.

The most striking finding in this study was that the proteins secreted by *C. gattii* VGIIa and VGIIb and *C. neoformans* were very different, with little overlap among strains. However, in terms of number and function of proteins, there was substantially greater similarity between the secretomes of the two physiologically related high-virulence strains than between the two genetically related *C. gattii* strains. We took a very conservative approach to calling protein hits, with a requirement of identification in at least three independent biological replicates; in most cases, however, proteins were either present in the majority of samples or entirely absent, and few appeared in only one or two samples (see Table S1 in the supplemental material). At the outset of this study, we hypothesized that the higher-virulence strains would have distinctive, highly active cell-damaging enzymes that might promote host invasion. While we did identify secreted enzymes with predicted hydrolytic or proteolytic functions, these were present in all three cryptococcal secretomes and were not restricted to the virulent strains. Similar enzymes have been described in a range of fungi, and they may be a fundamental component of fungal secretomes, regardless of ecological niche or lifestyle (2). Although their primary function is to degrade polymers in the environment for nutrition, these enzymes can have a crossover function in fungal pathogenesis, where they can promote invasion and cause cell damage (3, 4, 27, 28).

A second striking difference was that the secretome of the “hypovirulent” VGIIb strain included a suite of canonical intracellular proteins, many with known immunogenic properties, which were absent from the secretomes of the virulent strains. These types of proteins have been observed in the secretomes of many different organisms; however, they have been noted as a particular feature of the secretomes of pathogenic fungal species compared with nonpathogenic fungi (2). Further, in fungal pathogens, these proteins have been associated with secretion during growth within the host. For example, the plant pathogen *Fusarium graminearum* secretes a large number of canonical intracellular proteins when grown *in planta* that are not secreted when the fungus is cultured *in vitro* (38).

Orthologues of many of the canonical intracellular proteins secreted by the VGIIb strain are known to have alternative extra-

cellular “moonlighting” functions that appear unrelated to their intracellular roles (8). These proteins do not contain secretory signal motifs and hence are generally not predicted to be extracellular. The moonlighting roles of similar proteins in different organisms differ greatly and appear to be organism specific, making it difficult to extrapolate their functions among even closely related species (8, 30). It has been noted, however, that certain functional groups are prominent among known moonlighting proteins. Glycolytic proteins are one such group, with a large number of the enzymes involved in glycolysis and gluconeogenesis found to have moonlighting roles, including enolase and GAPDH (glyceraldehyde 3-phosphate dehydrogenase), which were both present in the VGIIb secretome (31).

Enolase is one of the most abundant cytosolic proteins, with a key function in the pentose phosphate pathway. This protein is known to be multifunctional and has been identified in the secretomes of many different organisms and cell types (32). In the fungal pathogens *Candida albicans*, *Pneumocystis jirovecii*, and *Aspergillus fumigatus*, cell wall-associated enolase has been shown to be immunogenic, to bind to and activate host plasminogen, and to promote invasive disease (39–42). *C. neoformans* enolase has been implicated in plasminogen activation, allowing *Cryptococcus* strains to traverse endothelial cells and potentially cross the blood-brain barrier (BBB) (43, 44). Extracellular GAPDH appears to have different roles in the progression of infection by a wide range of pathogens, including plasminogen binding in parasites (45), adherence to host cells by pathogenic *Escherichia coli*, *Neisseria meningitidis*, *Bacillus anthracis*, *Paracoccidioides brasiliensis*, and *Candida albicans* (33), and complement and fibronectin binding in helminths (46, 47) and as an immunogen in *C. albicans* (26). Given these diverse roles, cryptococcal GAPDH secreted by the *C. gattii* VGIIb strain has the potential to trigger a host response leading to clearance from the lung or to aid disease progression, leading to cryptococcal meningitis.

Intracellular vesicles are increasingly implicated in nonclassical secretion by pathogens, with microvesicles, secretory lysosomes, and autophagosomes involved in this process (7, 48–51). A key role for microvesicles in the host-pathogen interface was recently described for the malaria parasite *Plasmodium falciparum* in a study in which infected host red blood cells produced microvesicles containing both host and parasite proteins, facilitating communication within the parasite population and between the parasite and the host immune system (52). *Cryptococcus* species are known to produce microvesicles, and these have been shown to be immunologically active, stimulating macrophages to produce cytokines and other antimicrobial compounds (29, 53). Microvesicles have also been observed during *in vivo* infection, and proteins derived from them are immunoreactive to sera from cryptococcosis patients (29, 54). Microvesicles appear to facilitate the traversal of *Cryptococcus* cells across the BBB and to aggregate at lesion sites during brain infection (55). There is considerable overlap between some of the non-classically secreted proteins released by the VGIIb strain shown here and a number of proteins previously identified from cryptococcal microvesicles; in particular, a 14-3-3 protein is considered a marker of microvesicle presence (Table 1) (29). Thus, although indirectly, our secretome data suggest that microvesicles may be associated with VGIIb infection, and this could be a further factor provoking more-severe CNS infection by this genotype (35).

Many non-classically secreted proteins, in addition to their

moonlighting roles, are known to be immunogenic. In addition to the results seen with enolase and GAPDH described above, the *C. gattii* VGIIb strain secreted glucose-6-phosphate isomerase, which has been linked to the development of rheumatoid arthritis following *Aspergillus* infection (56). Further, some non-classically secreted proteins identified in this study, including fungal serine-type endopeptidases (57) and transaldolase, an immunodominant protein in *C. neoformans* (58–60), have been found to bind IgE. The presence of immunogenic proteins in the VGIIb secretome might be expected to provoke rapid induction of the innate immune response, preventing the initiation of disease. In contrast, high-virulence strains that lack these secreted immune-stimulating proteins may bypass the host response and establish lung infection.

As our data suggested that a significant factor contributing to the differences in virulence seen between *Cryptococcus* strains may be the presence of immunogenic and invasion-promoting secreted proteins that on the one hand prevent low-virulence strains from establishing infection in the lung but on the other might provoke more-serious disease if the pulmonary immune response were to be bypassed, we performed intracranial infections in a mouse model (25). In agreement with our hypothesis, the hypovirulent *C. gattii* VGIIb strain was able to establish infection and was in fact considerably more pathogenic than the VGIIa strain, where all infected immunocompetent mice survived (Fig. 2). This is consistent with epidemiological evidence that VGIIb infections, while much more infrequent, are significantly more likely to progress to cryptococcal meningitis than VGIIa infections, where severe pulmonary disease is the most common cause of death (35). Analysis of the brains of infected mice revealed substantial colonization of the meninges in mice infected with *C. gattii* VGIIb and *C. neoformans*, while VGIIa cells were almost completely cleared (Fig. 2; see also Fig. S2 in the supplemental material). There was a surprising lack of immune response or associated cellular activation in the meninges and adjacent tissue in all mice regardless of which *Cryptococcus* strain was used for infection, although the anti-Iba1 stain indicated greater activation of microglia following VGIIb infection (Fig. 2). *C. neoformans* H99 was an effective pathogen in both the pulmonary and meningitis models of infection, consistent with its ability to cause fatal lung and CNS infection.

There are a number of limitations to this study that may be addressed with further work. First, only three *Cryptococcus* strains were analyzed, and follow-up work that includes more strains of the different species and genotypes is required to confirm our observations. Second, while we attempted to simulate the host lung using RPMI tissue culture medium, such attempts cannot recapitulate the complexities of the host environment. *In vivo* proteomics in a mammalian host is extremely challenging; however, the use of antibodies or new mass spectroscopy-based methods such as selected reaction monitoring (SRM), which is designed to detect individual proteins from a heterogeneous mixture, may be able to validate whether selected proteins are secreted during infection. Third, the use of ProteoMiner beads limited us to a qualitative analysis, and we had no way of assessing the relative abundances of the various secreted proteins, which may also be very important in determining the virulence of a strain. Finally, although our analysis found interesting correlations between the nature of the secretome and the ability to produce pulmonary and

CNS infection, we cannot assume causation, and for now, this link remains an interesting but untested hypothesis.

In conclusion, the three *Cryptococcus* secretomes contained a core set of hydrolytic and proteolytic functions with various proteins that are predicted to have roles in nutrient scavenging. The secretome of the VGIIb strain, which is hypovirulent in a lung infection model, included additional, non-classically secreted proteins, with many reported previously to be associated with microvesicles. As a number of these are immunogenic, their absence from the *C. neoformans* and *C. gattii* VGIIa secretomes suggests that these strains may evade host immune detection, allowing cryptococcosis to be initiated in the lung. However, microvesicles and their associated proteins may also facilitate dissemination to the CNS, consistent with the ability of the VGIIb genotype to cause cerebral infection with a poor outcome, despite a lower incidence of disease. We propose that the activity of secreted cryptococcal proteins and their interactions with the host immune system are likely to play an important role in cryptococcal disease progression. If particular secreted proteins can be associated with disease outcome, these could be useful as prognostic markers for disease progression. Secreted proteins may also be useful targets for diagnostics, vaccines, or antifungal therapy, which remain underdeveloped for fungal diseases.

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