

1 **Assessing the virulence of *Cryptococcus neoformans* causing meningitis in HIV infected and**  
2 **uninfected patients in Vietnam**

3 Lam Tuan Thanh<sup>1</sup>, Dena L. Toffaletti<sup>2</sup>, Jennifer L. Tenor<sup>2</sup>, Charles Giamberardino<sup>2</sup>, Gregory D.  
4 Sempowski<sup>3</sup>, Yohannes Asfaw<sup>4</sup>, Hai Trieu Phan<sup>1</sup>, Anh Van Duong<sup>1</sup>, Trinh Mai Nguyen<sup>1</sup>, Guy E.  
5 Thwaites<sup>1,5</sup>, Philip M. Ashton<sup>1,5</sup>, Chau Van Vinh Nguyen<sup>6</sup>, Stephen G. Baker<sup>7</sup>, John R. Perfect<sup>2</sup>,  
6 and Jeremy N. Day<sup>1,5</sup>

7 <sup>1</sup>Oxford University Clinical Research Unit, Wellcome Trust Asia Africa Programme, Ho Chi Minh  
8 City, Vietnam

9 <sup>2</sup>Division of Infectious Diseases, Department of Medicine and Department of Molecular  
10 Genetics and Microbiology, Duke University, North Carolina, USA

11 <sup>3</sup>Duke Human Vaccine Institute and Regional Biocontainment Laboratory, Duke University,  
12 North Carolina, USA

13 <sup>4</sup>Division of Laboratory Animal Resources, Duke University, North Carolina, USA

14 <sup>5</sup>Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University  
15 of Oxford, Oxford, UK

16 <sup>6</sup>Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam

17 <sup>7</sup>Cambridge Institute of Therapeutic immunology and Infectious Disease, Department of  
18 Medicine, University of Cambridge, Cambridge, UK

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20 **Correspondence:** Jeremy Day: [jday@oucru.org](mailto:jday@oucru.org) Tel : (+84 8) 39237954 Fax : (+84 8) 39238904

21

22 **Short title:** Characterizing *Cryptococcus neoformans* from HIV uninfected patients

23

24 **Keywords**

25 Cryptococcal meningitis, *Cryptococcus neoformans*, immunocompetent, HIV, virulence

26 **Abstract**

27 We previously observed a substantial burden of cryptococcal meningitis in Vietnam atypically  
28 arising in HIV-uninfected individuals. This disease was associated with a single genotype of  
29 *Cryptococcus neoformans* (Sequence Type (ST)5), which was significantly less common in HIV-  
30 infected individuals. Aiming to compare the phenotypic characteristics of ST5 and non-ST5 *C.*  
31 *neoformans* we selected 30 representative Vietnamese isolates, compared their *in vitro*  
32 pathogenic potential and *in vivo* virulence. ST5 and non-ST5 organisms exhibited comparable  
33 characteristics with respect to *in vitro* virulence markers including melanin production,  
34 replication at 37°C, and growth in cerebrospinal fluid. However, the ST5 isolates had  
35 significantly increased variability in cellular and capsular sizing compared with non-ST5  
36 organisms ( $p < 0.001$ ). Counter-intuitively, mice infected with ST5 isolates had significantly  
37 longer survival with lower fungal burdens at day 7 than non-ST5 isolates. Notably, ST5 isolates  
38 induced significantly greater initial inflammatory responses than non-ST5 strains, measured by  
39 TNF- $\alpha$  concentrations ( $p < 0.001$ ). Despite being generally less virulent in the mouse model, we  
40 hypothesize that the significant within strain variation seen in ST5 isolates in the tested  
41 phenotypes may represent an evolutionary advantage enabling adaptation to novel niches  
42 including apparently immunocompetent human hosts.

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## 47 Introduction

48 Cryptococcal meningitis (CM) is a life-threatening fungal infection caused principally by  
49 *Cryptococcus neoformans* and *Cryptococcus gattii*<sup>1-3</sup>. Despite CM being more common in  
50 immunocompromised patients, CM can also arise in apparently immunocompetent patients.  
51 Recent figures estimate an annual global CM incidence of 223,100 cases in HIV infected  
52 patients, resulting in 181,100 deaths<sup>4</sup>.  
53 Generally, *C. gattii* is the most common cause of CM in immunocompetent patients, while *C.*  
54 *neoformans* is primarily responsible for disease in immunocompromised patients<sup>5</sup>. When CM  
55 associated with *C. neoformans* var. *grubii* occurs in HIV-uninfected individuals, reports often  
56 describe patients with an increased susceptibility due to other underlying immunosuppressive  
57 conditions<sup>6,7</sup>. In Vietnam, *C. neoformans* var. *grubii* CM in HIV-uninfected patients accounts for  
58 approximately 20% of all CM cases admitted to our hospital in Ho Chi Minh City (HCMC)<sup>8</sup>. We  
59 previously reported that the majority of HIV-uninfected CM patients had no identified cause or  
60 other medical history suggestive of immunosuppression, and that >80% of HIV-uninfected CM  
61 patients were infected by a single genotype of *C. neoformans* var. *grubii*<sup>9</sup>, which we later  
62 confirmed to be Sequence Type 5 (ST5).

63  
64 Our clinical observations have been replicated in China, where >70% of CM cases from  
65 apparently immunocompetent individuals were infected with *C. neoformans*<sup>10,11</sup>. Again these  
66 organisms were latterly identified as genotype ST5<sup>12,13</sup>. Comparably, ST5 accounted for >80% of  
67 HIV-uninfected CM patients in South Korea, although some patients from this location had  
68 potentially immunosuppressive conditions<sup>14</sup>. This association between a ST and host immune

69 phenotype could be explained by lineage-specific increases in pathogenic potential, fitness in  
70 the human host, an unidentified host immune deficit, or a combination of these three factors.  
71 Recent high-resolution genomic investigation of the population structure of clinical *C.*  
72 *neoformans* var. *grubii* isolates in Vietnam has verified that ST5 and ST4 are genetically distinct  
73 lineages of *C. neoformans* var. *grubii*, namely VNla-5 and VNla-4<sup>15</sup>.

74  
75 Here, in order to explore the hypothesis that *C. neoformans* var. *grubii* ST5 have an increased  
76 pathogenic potential in comparison to non-ST5 organisms, we compared their *in vitro* virulence  
77 phenotypes and exploited an established cryptococcosis mouse model to compare their relative  
78 pathogenicity and ability to induce systemic inflammation.

79

## 80 **Materials and methods**

### 81 *C. neoformans* isolates and culture conditions

82 We used clinical isolates obtained at the point of diagnosis, prior to antifungal therapy, from  
83 the cerebrospinal fluid (CSF) of patients enrolled in a prospective descriptive study of HIV-  
84 uninfected patients with central nervous system (CNS) infections, and a randomized controlled  
85 trial of antifungal therapy in HIV-infected patients<sup>8,16</sup>. We randomly selected 15 isolates from  
86 HIV uninfected CM patients and 15 from HIV-infected CM patients. MLST profiles (CAP59,  
87 GPD1, IGS1, LAC1, PLB1, SOD1, URA5) for all isolates were previously determined<sup>17</sup>. *C.*  
88 *neoformans* yeasts were propagated using Yeast Peptone Dextrose (YPD) broth and incubated  
89 overnight at 30°C with agitation. Isolates and clinical information from corresponding patients  
90 are summarized in Table 1.

91  
92 *Growth at high temperature, in ex vivo human CSF and melanin production*  
93 Growth at high temperature and in *ex vivo* human CSF were tested as previously described<sup>18</sup>  
94 with modifications for quantitative assessment. To assess fungal growth at different  
95 temperatures, the inoculum was adjusted to 10<sup>8</sup> cells/ml, serially diluted and spot-inoculated in  
96 duplicate on YPD agar in 5µl aliquots and incubated at 30°C or 37°C for 48 hours. After 48  
97 hours, colony forming units (CFU) were counted and recorded in CFU/ml.

98  
99 For the *ex vivo* CSF growth assay, baseline pre-antifungal treatment CSF supernatant from  
100 random de-identified HIV-infected patients enrolled into an antifungal therapy trial was pooled,  
101 filtered, and stored at -80°C until use. 10µl of 10<sup>8</sup> cells/ml yeast suspension was inoculated into  
102 90µl of pooled CSF and incubated at 37°C with 5%CO<sub>2</sub>. Inoculated CSF was serially diluted and  
103 spotted on YPD agar at days 1 and 3 post-inoculation. All experiments were repeated in  
104 triplicate. The H99-derived mutant *Δena1*, which lacks a cation-ATPase-transporter resulting in  
105 decreased viability in human CSF and macrophages, was used as a negative control for the *ex*  
106 *vivo* CSF assay<sup>19</sup>. H99 was included as a reference in all experiments. Data were standardized  
107 by expressing the results as a ratio of the CFU/ml of the test isolate to the CFU/ml of H99.  
108 Melanin production was assessed by plating 5µl of 10<sup>6</sup> cells/mL cell suspension on L-DOPA agar  
109 containing 1g/L L-asparagine, 1g/L glucose, 3g/L KH<sub>2</sub>PO<sub>4</sub>, 250mg/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 1mg/L  
110 Thiamine HCl, 5µg/L Biotin, 100mg/L L-DOPA, 20g/L Bacto Agar<sup>20,21</sup>. Plates were incubated in  
111 the dark at 30°C or 37°C for 3 days. Differences in colony melanization were compared visually

112 with reference to H99 and an H99-derived mutant with diminished melanization in L-DOPA  
113 agar from the Perfect lab.

114

#### 115 *Extracellular urease and phospholipase activity*

116 Extra-cellular urease production was semi-quantified using Christensen's agar.  $10\mu\text{l}$  of  $10^8$   
117 cells/ml yeast suspension was spotted on Christensen's agar and incubated at room  
118 temperature. The time to complete plate colouration was determined using a GoPro Hero 6  
119 camera (Gopro, USA) using the time-lapse setting set with a 1-minute interval. *C. neoformans*  
120 H99 was used as a positive control and *Candida albicans* as a negative control. Extracellular  
121 phospholipase activity was screened on egg yolk medium as previously described, with minor  
122 modifications<sup>22</sup>. The egg yolk medium contained Sabouraud agar with 1M sodium chloride,  
123 0.005M calcium chloride and 8% sterile egg yolk enrichment (Merck, USA). A  $5\mu\text{l}$  aliquot of *C.*  
124 *neoformans* yeast suspension ( $10^8$  cells/ml) was spotted on egg yolk agar and incubated at  $30^\circ\text{C}$   
125 for 72 hours. The diameters of the precipitation zone (D) formed around the colonies and of the  
126 respective colonies (d) were recorded after 72 hours incubation. The D/d ratio for each isolate  
127 was calculated. H99 was included for reference in each experimental batch. The final result for  
128 each isolate was expressed as the ratio between the test isolate's D/d ratio and that of H99. All  
129 isolates were tested in triplicate for each phenotype.

130

#### 131 *In vitro capsule and cell size measurement*

132 To measure *in vitro* cryptococcal capsule thickness, all isolates were streaked onto capsule-  
133 inducing agar containing powdered Dulbecco Modified Eagle Medium (DMEM) [supplemented

134 with 4.5g/L glucose, L-glutamine, sodium pyruvate], NaHCO<sub>3</sub> 250mM, NaMOPS 1M, Neomycin  
135 200mg/ml, Cefotaxime 100 mg/ml<sup>23</sup>. Plates were incubated at 37°C in 5% CO<sub>2</sub> until single  
136 colonies were visible. Unless otherwise specified, all reagents were purchased from Sigma-  
137 Aldrich. India ink smears from a single yeast colony were prepared on a glass slide and  
138 visualized at 100X magnification using a CX41 microscope (Olympus, Japan). Images of single  
139 microscopic yeast cells were captured using a DP71 Camera system with DP Controller software  
140 (Olympus, Japan) and processed using ImageJ ([rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)). Capsular thickness was  
141 calculated by subtracting yeast cell body diameter (D<sub>CD</sub>, no capsule) from whole cell diameter  
142 (D<sub>WC</sub>, including capsule). At least 30 individual microscopic yeast cells were assessed for each  
143 isolate.

144

#### 145 *Mouse inhalation infection model of cryptococcosis*

146 All mouse infection experiments were conducted as previously described according to Duke  
147 University's Institutional Animal Care and Use Committee guidelines and approvals<sup>24</sup>. Six-week  
148 old female A/J mice were sedated with isoflurane and inoculated intranasally with the selected  
149 *C. neoformans* var. *grubii* isolate by dropping 25µl of yeast suspension containing 5x10<sup>4</sup> cells  
150 into the nares. Eight isolates were randomly selected from the 30 for murine experiments. The  
151 isolates were five ST5 (BK147 and BK44 from HIV infected patients and BMD700, BMD1338 and  
152 BMD1646 from HIV uninfected patients) and three non-ST5 strains (BMD1415 (ST4) and  
153 BMD1367 (ST306) from HIV uninfected patients and BK80 (ST4) from an HIV infected patient).  
154 Animals were monitored daily and euthanized by CO<sub>2</sub> inhalation at indicated time points (fungal

155 burden and *in vivo* responses) or until weight loss  $\geq$  15% body weight was observed (virulence  
156 assay).

157

#### 158 *Determining in vivo fungal burden*

159 Five mice were infected with each isolate in two independent experiments for assessment of  
160 fungal burden at 7- or 14-days post-infection. All animals in each experiment set were  
161 euthanized by CO<sub>2</sub> inhalation either on day 7 or day 14 post-infection. Fungal burden at each  
162 time point was assessed by excising the left superior lobe of the lung and brain and  
163 homogenizing the tissue by bead beating. Tissue homogenate was serially diluted and plated  
164 onto YPD agar supplemented with 100mg/ml ampicillin. The plates were incubated at 30°C for  
165 48 hours and the number of *C. neoformans* colony forming units (CFU) recorded. Fungal  
166 burdens were expressed as CFU per gram of tissue (CFU/g). At each time point, additional lung  
167 lobes were also collected for determining *in vivo* histopathology and cytokine response, as  
168 described below. In addition, fungal burdens were separately determined at the point of death  
169 in animals from the survival assays described below.

170

#### 171 *Determining in vivo histopathology*

172 At specific time points (7 or 14 days post-infection, as described above), the right superior lung  
173 lobe from each mouse was excised and immersed in 10% formalin (replaced with 70% ethanol  
174 after 24 hours) for fixation. Fixed, uninflated lung specimens were stored at 4°C until further  
175 processing. After paraffin embedding, sliced sections were stained using the periodic acid-Schiff  
176 (PAS) or mucicarmine stains. Histopathological examination was performed by an independent



177 pathologist blinded to infecting strain. Tissue damage was scored from 0 (no changes) to 10  
178 (severe changes), corresponding to the severity of pathology in 4 different categories: necrosis,  
179 hemorrhage, edema and inflammation, as per the Duke Veterinary Diagnostic Laboratory  
180 (Division of Laboratory Animal Resources).

181

### 182 *Determining in vivo cytokine response*

183 To assess the severity of the inflammatory responses at specific time points (day 7 and day 14  
184 post-infection), the middle lobe from the right lung of each infected mouse was excised and  
185 homogenized by bead beating in 1ml sterile PBS/Protease inhibitor. 500µl of lung homogenate  
186 was used for cytokine profiling. Cytokines representing T-helper type 1 (Th1) (IL-12p70, TNF-α,  
187 IFN-γ), T-helper type 17 (IL-17) and T-helper type 2 (Th2) (IL-4, IL-5, IL-10) responses were  
188 measured using a customized Bio-Plex Pro™ Mouse Cytokine Th1/Th2 Assay kit (Biorad, USA)  
189 with the BioPlex 200 platform according to the manufacturer's guidelines. Data were retrieved  
190 using BioPlex Manager Software. The upper and lower limits of quantification (ULOQ and LLOQ)  
191 were based on a standard curve. All values falling below the LLOQ were replaced with the  
192 midpoint between zero and the LLOQ. Data were standardized by lung weight and presented as  
193 picogram of cytokines per gram lung tissue (pg/g).

194

### 195 *In vivo virulence assay*

196 The virulence assay was conducted independently from the day 7/day 14 experiment. Each of  
197 the 8 selected isolates was inoculated intranasally into 10 A/J mice. Mice were monitored daily

198 until death, or loss of more than 15% body weight (impending death), at which point they were  
199 euthanized by CO<sub>2</sub> inhalation, necropsied and had fungal burden in lung and brain determined.

200

### 201 *Statistical analysis*

202 GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego California USA;  
203 [www.graphpad.com](http://www.graphpad.com)) was used for data visualization and statistical analyses of fungal loads,  
204 cytokine profiling, capsular/cell size, and survival proportions. The Mann-Whitney U-test was  
205 used for comparing fungal load and cytokine concentrations. Kaplan-Meier survival curves and  
206 the log-rank test were used for survival analysis. Capsule/cell size was compared using Welch's  
207 t-test. The Fligner-Killeen test of variance homogeneity for analyzing variation in capsule/cell  
208 size and Fisher's exact test were performed using R software, version 3.2.4 ([http://www.r-](http://www.r-project.org)  
209 [project.org](http://www.r-project.org)). One way ANOVA with post hoc multiple comparison tests (Dunnett or Bonferroni)  
210 was used to compare cytokine concentrations between individual isolates.

211

## 212 **Results**

### 213 *C. neoformans in vitro virulence*

214 We compared the capsule size, extracellular urease, phospholipase production, melanin  
215 production, growth at high temperature, and growth in pooled human CSF between the 15 ST5  
216 and the 15 non-ST5 *C. neoformans* isolates. We observed no significant genotype-specific  
217 differences between growth at 30°C ( $p=0.10$ ), growth at 37°C ( $p=0.23$ ), *ex vivo* survival in CSF  
218 after 1-day ( $p=0.72$ ), *ex vivo* survival in CSF after 3-days of exposure ( $p=0.77$ ), extracellular  
219 urease activity, or phospholipase activity (Figure S1). ST5 *C. neoformans* cells developed

220 significantly thicker capsules during *in vitro* culture than non-ST5 isolates (Table 2). Individual  
221 ST5 *C. neoformans* cells were also significantly larger than non-ST5 cells (Figure 1), and we  
222 observed significantly greater variation in capsule size and cell diameter within ST5 cells than  
223 non-ST5 cells (Figure 1;  $p < 0.0001$ , Fligner-Killeen test). Notably, a single organism (BMD1646),  
224 was clearly larger and with thicker capsules than other isolates. However, the difference in  
225 capsule thickness and cell diameter between STs remained statistically significant even when  
226 this isolate was removed from analysis.

227

#### 228 *In vivo* mouse infections with ST5 and non-ST5 isolates

229 We hypothesized that a higher prevalence of ST5 infections in apparently healthy hosts was  
230 associated with higher virulence. Therefore, we challenged A/J with equivalent doses of  
231 representative isolates (5 ST5 and 3 non-ST5; the individual *in vitro* virulence phenotyping  
232 results for these 8 isolates are presented in supplementary Figures S2-S4). Contrary to our  
233 hypothesis, we found that mice infected with ST5 organisms had significantly longer survival  
234 times than mice infected with non-ST5 organisms ( $p < 0.0001$ , Figure 2A). However, there was  
235 significant variability in the effect of individual organisms of the same ST on survival (Figure 2B).  
236 Specifically, two ST5 organisms (BK147 and BMD1646) were substantially attenuated in  
237 comparison to other ST5 organisms; the mice infected with these organisms survived up to 40  
238 days. However, differences in survival times between mice infected with ST5 and non-ST5  
239 organisms remained significant when these two isolates were removed from the analysis  
240 ( $p = 0.003$ ; log-rank test).

241

242 The tissue-specific fungal burden data are shown in Table 3. All organisms established lung  
243 infection and disseminated brain infection as early as 7 days post infection. Non-ST5 infections  
244 resulted in higher fungal burdens in lung than ST5 infections at all time-points (day 7,  $p<0.001$ ;  
245 day 14,  $p<0.0001$ ; and end of the experiment,  $p<0.0001$  (Figure 3). Again, these data were not  
246 driven by the two apparently attenuated ST5 isolates as the fungal loads associated with the  
247 ST5 infections were significantly lower even when excluded from the analysis. The majority of  
248 animals infected with the attenuated ST5 organisms (BMD1646 (4/5 mice) and BK147 (5/5  
249 mice)) had low fungal burdens in the lungs up to day 14 (Figure 3). Fungal burdens in the brain  
250 were higher in non-ST5 infected animals at all time points; however, this difference was only  
251 statistically significant at the end of the experiment ( $p=0.054$ ,  $p=0.36$ , and  $p=0.01$ ; Mann-  
252 Whitney test at days 7, 14 and point of impending death (time when 15% body weight loss is  
253 observed).

254

#### 255 *ST5 C. neoformans isolates induce of TNF- $\alpha$ in the lungs of infected mice*

256 We next measured the cytokine concentrations in lung homogenates at days 7 and 14 post-  
257 infection (Figure 4). The concentrations of TNF- $\alpha$  were significantly higher in lung homogenates  
258 from mice infected with ST5 than non-ST5 infections ( $p=0.01$ ). Of note, the highest day 7 lung  
259 TNF- $\alpha$  concentrations were associated with the ST5 organisms that were most attenuated in  
260 the mouse survival model (BK147 and BMD1646). Correspondingly, the lowest TNF- $\alpha$   
261 concentrations were measured in the most virulent ST5 isolate in the mouse model (BMD1338).  
262

263 By day 14, the mean TNF- $\alpha$  concentrations in lung homogenates associated with the ST5  
264 infections had declined from 3933.71 pg/g lung to 2802.36 pg/g lung ( $p < 0.001$ ). Other Th-1  
265 cytokines including IL-12, IL-17, and IFN- $\gamma$  also decreased in ST5-infected mice between days 7  
266 and 14 ( $p < 0.001$ ,  $p < 0.01$  and  $p = 0.02$ , respectively). This contrasted with the non-ST5 infections,  
267 in which the mean TNF- $\alpha$  levels had increased from 3256.25 pg/g to 6378.76 pg/g ( $p = 0.02$ ) over  
268 this same period. The high TNF- $\alpha$  concentration associated with non-ST5 infections at day 14  
269 was driven by a single isolate - BMD1415. The TNF- $\alpha$  concentrations in lung homogenates  
270 from mice infected with this isolate were significantly higher than for infections with any other  
271 isolate of any lineage ( $p < 0.001$ ). There was no statistically significant differences in TNF- $\alpha$   
272 concentrations at day 14 between lineages when this isolate was excluded from the analysis.  
273 Similarly, the concentrations of these two cytokines in BMD1415-infected mice was significantly  
274 higher than in mice infected with any other isolate ( $p < 0.001$ ). However, the IL-12  
275 concentrations declined in non-ST5-infected mice, including BMD1415, between day 7 and 14  
276 (4 fold decrease,  $p < 0.0001$ ) (Figure 5).

277  
278 We measured the TNF- $\alpha$ :IL-10 ratio as a proxy for the relationship between a Th-1 and a Th-2  
279 response<sup>25</sup>. From day 7 to day 14 days post-infection, the TNF- $\alpha$ :IL-10 ratio decreased by a  
280 factor of 0.78 in mice infected with ST5 isolates, while those infected with non-ST5 strains  
281 exhibited an increase in the ratio of 1.25-fold. However, when BMD1415 (ST4) was excluded  
282 from the analysis the TNF- $\alpha$ /IL-10 ratio in non-ST5 infected mice decreased by a factor of 0.67.  
283 By day 14 we detected elevated concentrations of IL-17 and IFN- $\gamma$  in non-ST5 infected mice (5-  
284 fold and 17-fold increments, respectively (Table 4), but not in ST5 infected mice. Again, this

285 effect was largely associated with BMD1415, since the concentrations of IL-17 and IFN- $\gamma$   
286 induced by this isolate at day 14 were significantly higher than for all others ( $p < 0.001$ ).

287

### 288 *Histopathological examination*

289 Histological examination of infected lung tissue revealed evidence of inflammation,  
290 hemorrhage, edema and necrosis in most cases. These changes were generally greater by day  
291 14 in comparison to day 7. There were no clear differences in histological scores between ST,  
292 other than with BMD1646 (ST5) which generated only mild inflammation with no evidence of  
293 necrosis or hemorrhage (Figure S5). PAS staining revealed extensive perivascular infiltration of  
294 leukocytes in mice tissue associated with both infecting genotypes (BMD1338-ST5 and  
295 BMD1415-ST4, Figure 6). Extensive *in vivo* encapsulation of *C. neoformans* var. *grubii* in mouse  
296 lung was visualized using Mucicarmine staining (Figure 7). However, it was unknown whether  
297 the bigger capsule and cell size associated with ST5 organisms *in vitro*, as described earlier, was  
298 also evident *in vivo* since we were unable to perform this measurement.

299

### 300 **Discussion**

301 Most cases of cryptococcal meningitis in HIV uninfected, apparently immunocompetent,  
302 patients in Vietnam and East Asia are due to *C. neoformans* strains of multi-locus sequence type  
303 5 (ST5)<sup>9,10,12,17,26–28</sup>. This phenomenon could be explained either by ST5 strains being  
304 intrinsically more pathogenic, or due to unidentified lineage-specific host immune defects or  
305 exposures. It is unlikely that the high prevalence of ST5 infection observed in HIV-uninfected  
306 patients is explained by a significantly greater prevalence of the lineage in the environment

307 since it causes only 35% of cases in HIV-infected patients within the same geographical area <sup>9</sup>.  
308 Furthermore, data from China suggest that ST5 strains are significantly less prevalent in the  
309 environment, making up only 5% of isolates recovered in a recent study <sup>29</sup>. We investigated the  
310 first hypothesis by comparing previously identified *in vitro* virulence-associated phenotypes,  
311 along with murine *in vivo* virulence and immune responses, between lineages. All isolates were  
312 derived from HIV infected or uninfected Vietnamese patients with cryptococcal meningitis. The  
313 comparison by MLST defined lineage is appropriate because ST5 is a coherent and distinct  
314 group, whole genome sequence data revealing that there are few intra-lineage genomic  
315 variations between ST5 strains <sup>17</sup>. More recent phylogenetic analysis using whole genome  
316 sequencing of a larger collection of clinical isolates from Vietnam, including the eight strains  
317 tested in the mouse model, has established that the MLST ST5 and ST4/ST6/306 groups are  
318 genetically distinct lineages (VN1a-5 and VN1a-4, respectively) <sup>15</sup>. It is unlikely that any  
319 phenotypic variations among ST5 isolates are primarily attributable to *subtle* intra-lineage  
320 genomic variations since phenotypic and genotypic diversity are not tightly coupled in *C.*  
321 *neoformans* var. *grubii* <sup>30</sup>. Due to the fact that ST5 has been shown to be consistently associated  
322 with the clinical phenotype of interest (infection in HIV-uninfected patients), and that strains  
323 from HIV uninfected patients are dispersed throughout the VN1a-5 cluster, we believe the  
324 ability to infect apparently immunocompetent hosts is common to all ST5 isolates. All  
325 comparisons of ST versus non-ST5 in this study were thus essentially VN1a-5 versus VN1a-4.  
326 We found that isolates from all STs were able to grow in *ex vivo* human CSF and at 37°C -  
327 essential characteristics for establishing human CNS infection. While these qualities would be  
328 needed for disease in both HIV infected and immunocompetent patients, it might have been

329 expected that ST5 strains would grow more rapidly in these conditions. The lack of ST-specific  
330 differences in these phenotypes suggests that the ability to establish disease in HIV-  
331 uninfected/immunocompetent patients is not driven by simple adaptations to these conditions.  
332 Noticeably, ST5 cells were significantly larger than non-ST5 cells, had thicker capsules *in vitro*,  
333 and had more within lineage variation in these characteristics. Capsule size and composition are  
334 known to vary during infection and under specific stress conditions<sup>31,32</sup>, influencing  
335 macrophage phagocytosis and modulating host immune response<sup>32-35</sup>, and in human disease,  
336 *ex vivo* capsule size has been associated with higher intracranial pressures, slower yeast  
337 clearance and attenuated inflammation<sup>34</sup>. While we did not formally measure yeast cell or  
338 capsule size in our *in vivo* experiments, mucicarmine staining was suggestive that capsules were  
339 indeed larger during mouse infection. It is possible that the ability of ST5 strains to cause  
340 infections in immunocompetent patients is a function of increased responsiveness to capsule-  
341 inducing conditions. Further investigation of genotype-specific characteristics of *in vitro* and *in*  
342 *vivo* capsular polysaccharide production, composition, and morphology, may elucidate a  
343 specific role in ST5-associated pathogenesis. Of note, cryptococcal virulence factors frequently  
344 have additional metabolic functions; the increased cell and capsule size seen in ST5 isolates  
345 may be a side effect of other processes involving capsular-biosynthesis genes (for example  
346 carbon source sensing, sugar transport and spore formation)<sup>36,37</sup>.

347 Our data indicate that phenotypic heterogeneity may be a hallmark characteristic of the ST5  
348 lineage. Heterogeneity is a desirable trait for microbial populations under selection pressure  
349 allowing the exploitation of, and survival, in novel niches<sup>38</sup>. Chow *et al* (2008) has previously  
350 reported that ST5 *C. neoformans* var. *grubii* possesses unique genomic features which may



351 drive niche adaptation<sup>39</sup>. We speculate that the phenotypic heterogeneity associated with  
352 strains from the ST5 lineage is a strategy that facilitates successful colonization of novel  
353 environmental niches, including the exploitation of infrequent specific human immune deficits.  
354 Morphological variation including cell size, capsule size and cell shape has also been associated  
355 with different patient clinical symptoms, suggesting greater capacity for pathogenicity, immune  
356 evasion and pathogenesis<sup>40</sup>.

357 However, paradoxically, we found no evidence that ST5 isolates have greater virulence in the  
358 mouse model. There are several possible explanations. First, virulence in mice is variable  
359 depending on mouse breed and may not accurately reflect the immunological heterogeneity of  
360 the human population<sup>41</sup>. Second, yeasts with different pathogenic potentials, associated with  
361 their isolation from different sources (i.e. clinical versus environmental, immunocompromised  
362 versus immunocompetent patients) may have the same or paradoxical pathogenic potential in  
363 experimental animal models. An example is *Cryptococcus gattii* which is associated with  
364 infection in immunocompetent patients and therefore is considered to be more fit in the  
365 human host than *C. neoformans*. However, the hypervirulent *C. gattii* strain R265, responsible  
366 for the on-going Vancouver outbreak, has similar virulence in both C57BL/6 and A/J mice to the  
367 *C. neoformans* H99 strain, which was derived from a patient with Hodgkin's disease on  
368 chemotherapy<sup>42</sup>. Third, the A/J mouse breed is not immunologically intact; it may be an  
369 imperfect model of infection for immunocompetent hosts<sup>43</sup>. Rather, A/J mice may be a better  
370 model of disease in immunosuppressed patients, as they are highly susceptible to cryptococcal  
371 disease, and the patterns of cytokine expression in mice with disseminated cryptococcosis are  
372 similar to those seen in HIV-infected patients with CM<sup>44</sup>. Consistent with this, we could not

373 detect clinical differences in disease course or outcome between HIV patients infected with ST5  
374 versus other strains in Vietnam<sup>17</sup>. Models that better mimic infection in immunocompetent  
375 hosts are needed.

376 We did identify lineage specific differences in immune response in the mouse model. Previous  
377 research has suggested that a Th1 type immune response, defined by the TNF- $\alpha$ /IL-10 ratio, is  
378 protective, and a Th2 response is associated with poor outcomes<sup>45,46</sup>. We found no evidence of  
379 genotype-specific differences in TNF- $\alpha$ /IL-10 ratios by lineage in the murine infection model.  
380 Rather, we found higher initial (day 7) TNF- $\alpha$  concentrations in mice infected with ST5 isolates,  
381 suggesting this genotype elicits a more intense initial inflammatory response. Previous studies  
382 have suggested that capsule components, or cryptococcal cells themselves, have a dose-  
383 dependent ability to stimulate TNF- $\alpha$  production by various immune effector cells<sup>47,48</sup>. The  
384 more robust initial inflammatory response we observed may have been due to the ST5 capsular  
385 phenotype. Previously, it has been suggested that the ability of *C. gattii* to cause disease in  
386 apparently immunocompetent patients is because it induces a less severe inflammatory  
387 response compared with other cryptococcal species<sup>42</sup>. The robust initial inflammatory  
388 responses seen in our murine infection experiments are not consistent with this being the  
389 mechanism underlying the ability of ST5 *C. neoformans* var. *grubii* organisms to cause disease in  
390 the immunocompetent.

391 *In vivo* controlled infection studies in mice, including ours, commonly employ the classic  
392 definition of pathogenicity as the microbe's capability to cause disease in a susceptible host,  
393 whereas virulence corresponds to the severity of the ensuing pathology<sup>49</sup>. Using the same  
394 infective dose for all strains we failed to demonstrate that ST5 strains had greater virulence.

395 The difference we observe in prevalence of different lineages in immunocompetent and  
396 immunosuppressed humans may actually represent specific differences in pathogenicity - the  
397 ability of the organisms to colonize the host and establish infection. We could not assess this  
398 with our experimental system.

399 In summary, our cohort of ST5 *Cryptococcus* isolates displayed two notable phenotypes. First,  
400 despite their well-documented ability to cause disease in HIV uninfected humans, they  
401 appeared to be less virulent in a murine model than the other sequence types, as demonstrated  
402 by reduced fungal burdens in tissue and prolonged mouse survival. Second, ST5 strains had  
403 larger capsules and cell sizes than the other genotypes, and greater variability in this phenotype  
404 throughout the lineage. These data lead us to the following conclusions. First, clinical isolates,  
405 which have by their nature already undergone selection within the human host, can possess  
406 wide variability in the expression of virulence phenotypes within a single lineage. Secondly, the  
407 use of host risk factors and immune phenotypes to derive an understanding of the factors that  
408 drive the pathogenicity of *Cryptococcus neoformans* may be more complex than anticipated.

409 Associations may be difficult to make due to the relevance of the particular *in vitro* phenotypes,  
410 the animal models used, within strain heterogeneity, and population substructure. Moreover,  
411 there may be heterogeneity in the immune response of apparently immunocompetent patients  
412 which selects particular sub-cohorts of isolates of the same lineage. Laboratory phenotyping of  
413 larger numbers of clinical isolates is needed to define the lineage-specific differences that  
414 determine different human disease phenotypes.

415 Finally, it is possible that the categorization of strains into specific clades with limited genetic  
416 information such as MLST may lack precision to understand the relative fitness of specific

417 strains in the human host. It is likely that whole genome sequencing will provide better  
418 mapping of the relationships between strains and virulence.

419 In conclusion, in this study, we demonstrated genotype-specific differences in *in vitro* and *in*  
420 *vivo* virulence phenotypes between *C. neoformans* var. *grubii* strains isolated from host with  
421 different immune status. However, there was also significant variation among strains isolated  
422 from apparently immunocompetent patients in specific *in vitro* and *in vivo* phenotypes tested.  
423 This higher rate of phenotypic variation may represent an evolutionary strategy for *C.*  
424 *neoformans* var. *grubii* to take advantage of novel niches and contribute to their ability to infect  
425 apparently immunocompetent hosts, despite generally being less virulent in a mammalian  
426 animal model. Furthering the understanding of the pathogenesis of cryptococcal meningitis will  
427 require investigation of large numbers of strains with associated robust clinical information,  
428 and the development of high throughput laboratory phenotypic studies that have clinical  
429 relevance in humans.

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438

439 **Conflicts of Interest**

440 None

441

442 **References**

- 443 1. Fang W, Chen M, Liu J, et al. Cryptococcal meningitis in systemic lupus erythematosus  
444 patients: pooled analysis and systematic review. *Emerg Microbes Infect.* 2016;5(9):e95.  
445 doi:10.1038/emi.2016.93
- 446 2. Schmalzle SA, Buchwald UK, Gilliam BL, Riedel DJ. *Cryptococcus neoformans* infection in  
447 malignancy. *Mycoses.* 2016;59(9):542-552. doi:10.1111/myc.12496
- 448 3. Williamson PR, Jarvis JN, Panackal AA, et al. Cryptococcal meningitis: epidemiology,  
449 immunology, diagnosis and therapy. *Nat Rev Neurol.* 2016;13(1):13-24.  
450 doi:10.1038/nrneurol.2016.167
- 451 4. Rajasingham R, Smith RM, Park BJ, et al. Global burden of disease of HIV-associated  
452 cryptococcal meningitis: an updated analysis. *Lancet Infect Dis.* 2017;17(8):873-881.  
453 doi:10.1016/S1473-3099(17)30243-8
- 454 5. Kwon-Chung KJ, Fraser JA, Doering TL, et al. *Cryptococcus neoformans* and *Cryptococcus*  
455 *gattii*, the etiologic agents of cryptococcosis. *Cold Spring Harb Perspect Med.*  
456 2014;4(7):a019760. doi:10.1101/cshperspect.a019760
- 457 6. Pappas PG. Cryptococcal infections in non-HIV-infected patients. *Trans Am Clin Climatol*  
458 *Assoc.* 2013;124:61-79.
- 459 7. Shih CC, Chen YC, Chang SC, Luh KT, Hsieh WC. Cryptococcal meningitis in non-HIV-  
460 infected patients. *QJM.* 2000;93(4):245-251. doi:10.1093/qjmed/93.4.245

- 461 8. Chau TT, Mai NH, Phu NH, et al. A prospective descriptive study of cryptococcal  
462 meningitis in HIV uninfected patients in Vietnam - high prevalence of *Cryptococcus*  
463 *neoformans* var. *grubii* in the absence of underlying disease. *BMC Infect Dis.*  
464 2010;10(1):199. doi:10.1186/1471-2334-10-199
- 465 9. Day JN, Hoang TN, Duong A V, et al. Most cases of cryptococcal meningitis in HIV-  
466 uninfected patients in Vietnam are due to a distinct amplified fragment length  
467 polymorphism-defined cluster of *Cryptococcus neoformans* var. *grubii* VN1. *J Clin*  
468 *Microbiol.* 2011;49(2):658-664. doi:10.1128/JCM.01985-10
- 469 10. Chen J, Varma A, Diaz M. *Cryptococcus neoformans* strains and infection in apparently  
470 immunocompetent patients, China. *Emerg Infect Dis.* 2008;14(5).
- 471 11. Li Z, Liu Y, Cao H, Huang S, Long M. Epidemiology and clinical characteristics of  
472 cryptococcal meningitis in china ( 1981-2013 ): A review of the literature. *Med Mycol*  
473 *Open Access.* 2017;3(1):1-6. doi:10.4172/2471-8521.100022
- 474 12. Fan X, Xiao M, Chen S-L, et al. Predominance of *Cryptococcus neoformans* var. *grubii*  
475 multilocus sequence type 5 and emergence of isolates with non-wild-type minimum  
476 inhibitory concentrations to fluconazole: a multi-centre study in China. *Clin Microbiol*  
477 *Infect.* 2016;22(10):887.e1-887.e9. doi:10.1016/j.cmi.2016.07.008
- 478 13. Dou H-T, Xu Y-C, Wang H-Z, Li T-S. Molecular epidemiology of *Cryptococcus neoformans*  
479 and *Cryptococcus gattii* in China between 2007 and 2013 using multilocus sequence  
480 typing and the DiversiLab system. *Eur J Clin Microbiol Infect Dis.* 2015;34(4):753-762.  
481 doi:10.1007/s10096-014-2289-2
- 482 14. Choi YH, Ngamskulrungrroj P, Varma A, et al. Prevalence of the VN1c genotype of

- 483 *Cryptococcus neoformans* in non-HIV-associated cryptococcosis in the Republic of Korea.  
484 *FEMS Yeast Res.* 2010;10(6):769-778. doi:10.1111/j.1567-1364.2010.00648.x
- 485 15. Ashton PM, Thanh LT, Trieu PH, et al. Three phylogenetic groups have driven the recent  
486 population expansion of *Cryptococcus neoformans*. *Nat Commun.* 2019;10(1):1-10.  
487 doi:10.1038/s41467-019-10092-5
- 488 16. Day JN, Chau TTH, Wolbers M, et al. Combination antifungal therapy for cryptococcal  
489 meningitis. *N Engl J Med.* 2013;368(14):1291-1302. doi:10.1056/NEJMoa1110404
- 490 17. Day JN, Qihui S, Thanh LT, et al. Comparative genomics of *Cryptococcus neoformans* var.  
491 *grubii* associated with meningitis in HIV infected and uninfected patients in Vietnam.  
492 *PLoS Negl Trop Dis.* 2017;11(6):e0005628. doi:10.1371/journal.pntd.0005628
- 493 18. Lee A, Toffaletti DL, Tenor J, et al. Survival defects of *Cryptococcus neoformans* mutants  
494 exposed to human cerebrospinal fluid result in attenuated virulence in an experimental  
495 model of meningitis. *Infect Immun.* 2010;78(10):4213-4225. doi:10.1128/IAI.00551-10
- 496 19. Idnurm A, Walton FJ, Floyd A, Reedy JL, Heitman J. Identification of ENA1 as a virulence  
497 gene of the human pathogenic fungus *Cryptococcus neoformans* through signature-  
498 tagged insertional mutagenesis. *Eukaryot Cell.* 2009;8(3):315-326. doi:10.1128/EC.00375-  
499 08
- 500 20. Salas SD, Bennett JE, Kwon-Chung KJ, Perfect JR, Williamson PR. Effect of the laccase  
501 gene CNLAC1, on virulence of *Cryptococcus neoformans*. *J Exp Med.*  
502 1996;184(August):377-386. doi:10.1084/jem.184.2.377
- 503 21. Eisenman HC, Mues M, Weber SE, et al. *Cryptococcus neoformans* laccase catalyses  
504 melanin synthesis from both D- and L-DOPA. *Microbiology.* 2007;153(12):3954-3962.

- 505           doi:10.1099/mic.0.2007/011049-0
- 506   22.   Chen SC, Muller M, Zhou JZ, Wright LC, Sorrell TC. Phospholipase activity in *Cryptococcus*  
507       *neoformans*: a new virulence factor? *J Infect Dis.* 1997;175(2):414-420.  
508       doi:10.1093/infdis/175.2.414
- 509   23.   Zaragoza O, Casadevall A. Experimental modulation of capsule size in *Cryptococcus*  
510       *neoformans*. *Biol Proced Online.* 2004;6(1):10-15. doi:10.1251/bpo68
- 511   24.   Hu G, Cheng P-Y, Sham A, Perfect JR, Kronstad JW. Metabolic adaptation in *Cryptococcus*  
512       *neoformans* during early murine pulmonary infection. *Mol Microbiol.* 2008;69(6):1456-  
513       1475. doi:10.1111/j.1365-2958.2008.06374.x
- 514   25.   Levitz SM, Tabuni A, Nong SH, Golenbock DT. Effects of interleukin-10 on human  
515       peripheral blood mononuclear cell responses to *Cryptococcus neoformans*, *Candida*  
516       *albicans*, and lipopolysaccharide. *Infect Immun.* 1996;64(3):945-951.
- 517   26.   Fang W, Fa Z, Liao W. Epidemiology of *Cryptococcus* and cryptococcosis in China. *Fungal*  
518       *Genet Biol.* November 2014. doi:10.1016/j.fgb.2014.10.017
- 519   27.   Khayhan K, Hagen F, Pan W, et al. Geographically structured populations of *Cryptococcus*  
520       *neoformans* variety *grubii* in Asia correlate with HIV status and show a clonal population  
521       structure. Zaragoza O, ed. *PLoS One.* 2013;8(9):e72222.  
522       doi:10.1371/journal.pone.0072222
- 523   28.   Chen M, Xu Y, Hong N, et al. Epidemiology of fungal infections in China. *Front Med.*  
524       2018;12(1):58-75. doi:10.1007/s11684-017-0601-0
- 525   29.   Dou H, Wang H, Xie S, Chen X, Xu Z, Xu Y. Molecular characterization of *Cryptococcus*  
526       *neoformans* isolated from the environment in Beijing, China. *Med Mycol.* 2017;38:1-11.



- 527 doi:10.1093/mmy/myx026
- 528 30. Beale MA, Sabiiti W, Robertson EJ, et al. Genotypic diversity is associated with clinical  
529 outcome and phenotype in cryptococcal meningitis across Southern Africa. Vinetz JM,  
530 ed. *PLoS Negl Trop Dis*. 2015;9(6):e0003847. doi:10.1371/journal.pntd.0003847
- 531 31. Bojarczuk A, Miller KA, Hotham R, et al. *Cryptococcus neoformans* intracellular  
532 proliferation and capsule size determines early macrophage control of infection. *Sci Rep*.  
533 2016;6(October 2015):21489. doi:10.1038/srep21489
- 534 32. Zaragoza O, Chrisman CJ, Castelli MV, et al. Capsule enlargement in *Cryptococcus*  
535 *neoformans* confers resistance to oxidative stress suggesting a mechanism for  
536 intracellular survival. *Cell Microbiol*. 2008;10(10):2043-2057. doi:10.1111/j.1462-  
537 5822.2008.01186.x
- 538 33. García-Rodas R, Casadevall A, Rodríguez-Tudela JL, Cuenca-Estrella M, Zaragoza O.  
539 *Cryptococcus neoformans* capsular enlargement and cellular gigantism during *Galleria*  
540 *mellonella* infection. *PLoS One*. 2011;6(9):e24485. doi:10.1371/journal.pone.0024485
- 541 34. Robertson EJ, Najjuka G, Rolfes M a, et al. *Cryptococcus neoformans* ex vivo capsule size  
542 is associated with intracranial pressure and host immune response in HIV-associated  
543 cryptococcal meningitis. *J Infect Dis*. 2014;209(1):74-82. doi:10.1093/infdis/jit435
- 544 35. Vecchiarelli A, Pericolini E, Gabrielli E, et al. *Cryptococcus neoformans* galactoxylomannan  
545 is a potent negative immunomodulator, inspiring new approaches in anti-inflammatory  
546 immunotherapy. *Immunotherapy*. 2011;3:997-1005. doi:10.2217/imt.11.86
- 547 36. Kronstad J, Saikia S, Nielson ED, et al. Adaptation of *Cryptococcus neoformans* to  
548 mammalian hosts: integrated regulation of metabolism and virulence. *Eukaryot Cell*.

- 549 2012;11(2):109-118. doi:10.1128/EC.05273-11
- 550 37. Botts MR, Giles SS, Gates MA, Kozel TR, Hull CM. Isolation and characterization of  
551 *Cryptococcus neoformans* spores reveal a critical role for capsule biosynthesis genes in  
552 spore biogenesis. *Eukaryot Cell*. 2009;8(4):595-605. doi:10.1128/EC.00352-08
- 553 38. Bódi Z, Farkas Z, Nevozhay D, et al. Phenotypic heterogeneity promotes adaptive  
554 evolution. *PLoS Biol*. 2017;15(5):e2000644. doi:10.1371/journal.pbio.2000644
- 555 39. Chow EWL, Morrow CA, Djordjevic JT, Wood IA, Fraser JA. Microevolution of  
556 *Cryptococcus neoformans* driven by massive tandem gene amplification. *Mol Biol Evol*.  
557 2012;29(8):1987-2000. doi:10.1093/molbev/mss066
- 558 40. Fernandes KE, Brockway A, Haverkamp M, et al. Phenotypic variability correlates with  
559 clinical outcome in *Cryptococcus* isolates obtained from Botswanan HIV/AIDS Patients.  
560 *MBio*. 2018;9(5):e02016-18. doi:10.1128/mBio.02016-18
- 561 41. Zaragoza O, Alvarez M, Telzak A, Rivera J, Casadevall A. The relative susceptibility of  
562 mouse strains to pulmonary *Cryptococcus neoformans* infection is associated with  
563 pleiotropic differences in the immune response. *Infect Immun*. 2007;75(6):2729-2739.  
564 doi:10.1128/IAI.00094-07
- 565 42. Cheng P-Y, Sham A, Kronstad JW. *Cryptococcus gattii* isolates from the British Columbia  
566 cryptococcosis outbreak induce less protective inflammation in a murine model of  
567 infection than *Cryptococcus neoformans*. *Infect Immun*. 2009;77(10):4284-4294.  
568 doi:10.1128/IAI.00628-09
- 569 43. Sellers RS, Clifford CB, Treuting PM, Brayton C. Immunological variation between inbred  
570 laboratory mouse strains: points to consider in phenotyping genetically immunomodified

- 571 mice. *Vet Pathol.* 2012;49(1):32-43. doi:10.1177/0300985811429314
- 572 44. Lortholary O, Improvisi L, Rayhane N, et al. Cytokine profiles of AIDS patients are similar  
573 to those of mice with disseminated *Cryptococcus neoformans* infection. *Infect Immun.*  
574 1999;67(12):6314-6320.
- 575 45. Jain A V, Zhang Y, Fields WB, et al. Th2 but not Th1 immune bias results in altered lung  
576 functions in a murine model of pulmonary *Cryptococcus neoformans* infection. *Infect*  
577 *Immun.* 2009;77(12):5389-5399. doi:10.1128/IAI.00809-09
- 578 46. Koguchi Y, Kawakami K. Cryptococcal infection and Th1-Th2 cytokine balance. *Int Rev*  
579 *Immunol.* 2002;21(4-5):423-438. doi:10.1080/08830180190048109
- 580 47. Chaka W, Verheul AF, Vaishnav V V, et al. *Cryptococcus neoformans* and cryptococcal  
581 glucuronoxylomannan, galactoxylomannan, and mannoprotein induce different levels of  
582 tumor necrosis factor alpha in human peripheral blood mononuclear cells. *Infect Immun.*  
583 1997;65(1):272-278.
- 584 48. Levitz SM, Tabuni A, Kornfeld H, Reardon CC, Golenbock DT. Production of tumor  
585 necrosis factor alpha in human leukocytes stimulated by *Cryptococcus neoformans*. *Infect*  
586 *Immun.* 1994;62(5):1975-1981.
- 587 49. García-Rivera J, Casadevall A. Melanization of *Cryptococcus neoformans* reduces its  
588 susceptibility to the antimicrobial effects of silver nitrate. *Med Mycol.* 2001;39:353-357.  
589 doi:10.1080/mmy.39.4.353.357
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595

596 **FIGURE LEGENDS**

597 **Figure 1. *In vitro* induced capsule thickness and cell diameter of individual *Cryptococcus***

598 ***neoformans* strains from Vietnam:** Cells were grown on DMEM medium/5% CO<sub>2</sub> and visually

599 assessed by India ink staining. Images were taken for single cells measurement using ImageJ

600 software. Capsule thickness is obtained by subtracting cell body diameter from total cell

601 diameter. AFLP-VNI-γ/MLST-ST5 strains expressed higher degree of variation in both capsule

602 size and cell diameter *in vitro*, which remains significant even when the outlier BMD1646 was

603 removed from the analysis ( $p < 0.0001$  for both capsule and cell size, Fligner-Killeen test).

604 Scattered plot represents single cells from an individual strain. Data for individual strains are

605 presented as mean with error bars denoting standard deviation. Strains selected for experiment

606 in mice were indicated by asterisks.

607

608 **Figure 2. Kaplan-Meier survival curves for mice infected with either ST5 (n = 5) or non-ST5 (n=**

609 **3) *Cryptococcus neoformans* strains.** 10 A/J mice were infected per strain (five ST5 strains and

610 three non-ST5 strains). Mice were monitored daily until the point of more than 15% weight loss

611 or visible suffering and were then sacrificed by CO<sub>2</sub> inhalation. Mice infected with ST5 strains

612 had statistically significantly longer survival times than those infected with non-ST5 strains

613 ( $P < 0.0001$ , Mantel-Cox log rank test) (Panel A). Two ST5 strains, BK147 and BMD1646, were

614 attenuated, suggesting high degree of heterogeneity within the ST5 cluster. Mice infected with

615 BK147 and BMD1646 survived for as long as 42 days post-infection, at which point the  
616 experiment was terminated and all infected mice sacrificed (Panel B).

617

618 **Figure 3. Fungal burden in mouse lung and brain tissue at days 7, 14 and the point of**  
619 **impending death (mortality experiment) according to infecting genotype.** *In vivo* virulence  
620 (tissue fungal burden and mortality) was assessed in three independent mouse infection  
621 experiments. In the first two experiments, five A/J mice were infected with each of the eight  
622 test *C. neoformans* isolate (five ST5 isolates and 3 non-ST5 isolates, total N=40 mice in each  
623 experiment). All mice were sacrificed at day 7 post-infection in the first experiment and at day  
624 14 post-infection in the second experiment to assess *in vivo* fungal burden in mouse lung and  
625 brain. Fungal burden in the lung and brain at either day 7 or day 14 for each isolate is presented  
626 in panel A. In the last experiment, ten A/J mice were infected with each of the 8 test *C.*  
627 *neoformans* isolate (five ST5 isolates and 3 non-ST5 isolates, N=80 mice in total) and monitored  
628 daily until the point when 15% of body weight loss, a sign of distress and impending death, was  
629 evident. Fungal burden in lung and brain at point of death was again assessed as before. Here,  
630 for each strain, five mice were randomly selected from the ten test mice for fungal burden  
631 investigation. The pooled fungal burden in non-ST5 infections was higher than in ST5 infections  
632 in both lung tissue at all time points, and in brain tissue at the point of sacrifice (Panel B) (For P  
633 values see text). Boxplots (Tukey's method) describe the median and interquartile range, the  
634 whiskers demarcate the largest or smallest values that were not outliers (black dots); outliers  
635 are defined as more than 1.5 times the interquartile range from the nearest quartile.

636

637 **Figure 4. Genotype-specific cytokine concentrations from lung homogenate of A/J mice at 7**  
638 **and 14 days post infection with  $5 \times 10^4$  *C. neoformans* cells/mouse.** Five mice were infected  
639 with each strain from each genotype at each time points. ST5 strains induced significantly  
640 higher levels of TNF- $\alpha$  at day 7, suggesting an earlier and more profound initial inflammatory  
641 response in infected mice. By day 14 mice infected with non-ST5 strains have higher levels  
642 proinflammatory cytokines, probably a result of ST5 yeasts being cleared more rapidly from  
643 infected mice. The horizontal line within the box indicates the median; boundaries of the box  
644 indicate the 25<sup>th</sup> and 75<sup>th</sup> percentile and the whiskers indicate the highest and lowest values of  
645 the results; outliers are denoted as black dots (Tukey's method). Data are standardized as  
646 picograms of cytokine per gram lung tissue. Asterisks indicate statistically significant differences  
647 (Mann-Whitney test).

648

649 **Figure 5. Genotype-specific changes in cytokine concentrations from lung homogenate of A/J**  
650 **mice infected with *C. neoformans* between day 7 and day 14 post-infection.** Box and whisker  
651 plots (Tukey's method) compare levels of each cytokine between day 7 and day 14 for each  
652 genotype. Data are standardized as picograms of cytokine per gram lung tissue. Asterisks  
653 indicate statistically significant differences (Mann-Whitney test).

654

655 **Figure 6. Periodic Acid Schiff (PAS) staining of pulmonary tissue from mice infected with**  
656 **BMD1338 and BMD1415 on days 7 and 14.** The two strains represent ST5 and non-ST5,  
657 respectively. A/J mice were inoculated intranasally with  $5 \times 10^4$  yeast cells. Lung specimens were

658 harvested at days 7 and 14 for histopathological examination. Photomicrographs were obtained  
659 at 200X magnification; the scale bar represents 215  $\mu\text{m}$ ). (A-B): lung sections from mice infected  
660 with BMD1338 (VNI- $\gamma$ /ST5) at day 7 and day 14, respectively. (C-D): lung sections from mice  
661 infected with BMD1415 (VNI- $\delta$ /ST4) at day 7 and day 14, respectively. Perivascular infiltration  
662 (red arrows) and necrosis are more marked by day 14 for both strains. Encapsulated yeasts  
663 (yellow arrows), notable for the larger cell size and capsule thickness of BMD 1338 compared  
664 with BMD1415.

665

666 **Figure 7. Mucicarmine staining of capsular material in paraffin-embedded mice pulmonary**  
667 **tissue.**

668 Uninflated lung specimens were harvested from mice as described in the methods.

669 Mucicarmine staining was performed to visualize the cryptococcal capsule. Photomicrographs  
670 were captured at 400X magnification with scale bar indicating 55 $\mu\text{m}$ . Capsular polysaccharide is  
671 stained pink (indicated by blue arrows), demonstrating diffuse localization consistent with  
672 extensive capsule production by yeasts in the alveolar space.

673 **FIGURE S1. Phenotyping of *C. neoformans* var. *grubii* isolates from Vietnam (ST5: n=15 and**  
674 **non-ST5: n=15).** Data were obtained from 30 isolates (15 ST5 and 15 non-ST5) in 3 different  
675 experiment batches, with 3 technical replicates of each isolate per batch. Data are expressed as  
676 the ratio between measurements of the test isolates and that of H99. Boxplots (Tukey's  
677 method) describe the median and interquartile range (panel A-C). For assessment of urease  
678 activity (panel D), we used the time to complete color change of the agar plate as an indirect  
679 measure of extracellular urease activity since all *C. neoformans* var. *grubii* isolates in our

680 collection were positive for urease activity. Each isolate were tested in triplicate and monitored  
681 in real time by live-imaging. No significant difference in any virulence-associated phenotype  
682 were observed between the two MLST groups.

683 **FIGURE S2. Growth at different temperature of 8 *C. neoformans* (five ST5 and three non-ST5)**  
684 **isolates from Vietnamese patients used in the murine infection experiments.** All strains  
685 expressed similar growth at 30°C. Two ST5 isolates (BMD1646 and BK147) displayed diminished  
686 growth at 37°C compared to other test isolates.

687 **FIGURE S3. *Ex vivo* growth in human cerebrospinal fluid (CSF) of 8 clinical *C. neoformans***  
688 **isolates from Vietnamese patients that were tested for virulence in mice, representing both**  
689 **ST5 (n = 5) and non-ST5 (n = 3) genotypes.** The same inoculum of yeasts were inoculated in  
690 pooled human CSF and incubated at 37°C. The inoculated CSF was serially diluted and plated on  
691 YPD agar at day 1 and day 3 post-inoculation. The wild-type H99 strain and the H99-derived  
692 mutant *Δena1*, lacking a cation ATPase transporter which results in decreased viability in  
693 human CSF and within macrophages, were included as controls. An apparent difference in  
694 growth between ST5 and non-ST5 isolates could be observed at day 3.

695 **FIGURE S4. Melanin production on L-DOPA medium of eight clinical *C. neoformans* isolates**  
696 **from Vietnamese patients representing both ST5 (n =5) and non-ST5 (n = 3) genotypes that**  
697 **were tested for virulence in mice.** For each isolates, 10μl of 10<sup>6</sup> cell suspension was inoculated  
698 on L-DOPA agar and incubated in the dark at 30°C and 37°C. No clear genotype-specific patterns  
699 of melanization were observed at either 30°C or 37°C. The five ST5 strains displayed marked



700 variation in the degree of pigment production, from mildly melanized (BK147) to highly  
701 melanized (BK44 and BMD1646).

702 **FIGURE S5: Histopathological scores across 4 categories of tissue damage (Inflammation,**  
703 **necrosis, hemorrhage and edema).** Thin sections of paraffin-embedded lung specimens from  
704 infected mice were stained using the Periodic Acid Schiff (PAS) method. Specimens were  
705 assessed by an independent pathologist who was blind to infecting isolate and in randomized  
706 order. Scores ranged from 0 (no changes) to 10 (severe changes), corresponding to the severity  
707 of pathology in in each category as per the Duke Veterinary Diagnostic Laboratory protocol  
708 (Division of Laboratory Animal Resources).

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721 **TABLES**

722 **TABLE 1** *Cryptococcus neoformans* isolate clinical source and typing (n=30).

Isolate data			Patient data		
Order	Strain name	WGS	MLST	Underlying Disease	Sex
		cluster	sequence type		
1	BK14	VNIa-4	4	HIV	M
2	BK163	VNIa-4	4	HIV	M
4	BK225	VNIa-4	4	HIV	M
6	BK48	VNIa-4	4	HIV	M
7	BK59	VNIa-4	4	HIV	F
8	BK69	VNIa-4	4	HIV	M
9	BK74	VNIa-4	4	HIV	M
10	BK80*	VNIa-4	4	HIV	M
11	BK87	VNIa-4	4	HIV	M
12	BK88	VNIa-4	4	HIV	M
13	BK89	VNIa-4	4	HIV	M
15	BMD1415*	VNIa-4	4	Lupus	M
3	BK218	VNIa-4	6	HIV	M
5	BK234	VNIa-4	6	HIV	F
14	BMD1367*	VNIa-4	306	Gastric cancer	F

16	BK147*	VNIa-5	5	HIV	M
17	BK44*	VNIa-5	5	HIV	M
18	BMD101	VNIa-5	5	None known	M
19	BMD1228	VNIa-5	5	None known	F
20	BMD1291	VNIa-5	5	None known	F
21	BMD1338*	VNIa-5	5	None known	M
22	BMD1353	VNIa-5	5	None known	M
23	BMD1452	VNIa-5	5	None known	F
24	BMD1646*	VNIa-5	5	None known	M
25	BMD1716	VNIa-5	5	None known	F
26	BMD367	VNIa-5	5	None known	M
27	BMD673	VNIa-5	5	None known	F
28	BMD700*	VNIa-5	5	None known	M
29	BMD854	VNIa-5	5	None known	F
30	BMD899	VNIa-5	5	None known	F

*WGS = whole genome sequencing*

*MLST = multi locus sequence typing*

*M = Male, F = Female*

*\* indicates isolates selected for mouse experiment*

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725 **TABLE 2** Variability in *in vitro* capsule thickness and cell diameter of *Cryptococcus neoformans*  
 726 strains by Sequence Type (ST)

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Variable	MLST Group	
	ST5	Non-ST5
<b>Capsule Thickness (<math>\mu\text{m}</math>)</b>		
Mean*	2.64	2.01
95% CI	2.49 - 2.79	1.94 - 2.07
Range	0.01 - 9.56	0.02 - 6.16
Coefficient of Variation <sup>§</sup>		
(%)	0.63	0.37
<b>Cell diameter (<math>\mu\text{m}</math>)</b>		
Mean*	11.38	9.69
95% CI of Mean	10.96 - 11.79	9.52 - 9.87
Range	4.74 - 27.22	5.25 - 19.89
Coefficient of Variation <sup>§</sup>		
(%)	0.41	0.20

728 \*  $P < 0.0001$ , *t*-test with Welch's correction.

729 <sup>§</sup>  $P < 0.0001$ , Fligner-Killeen test of homogeneity of variance.

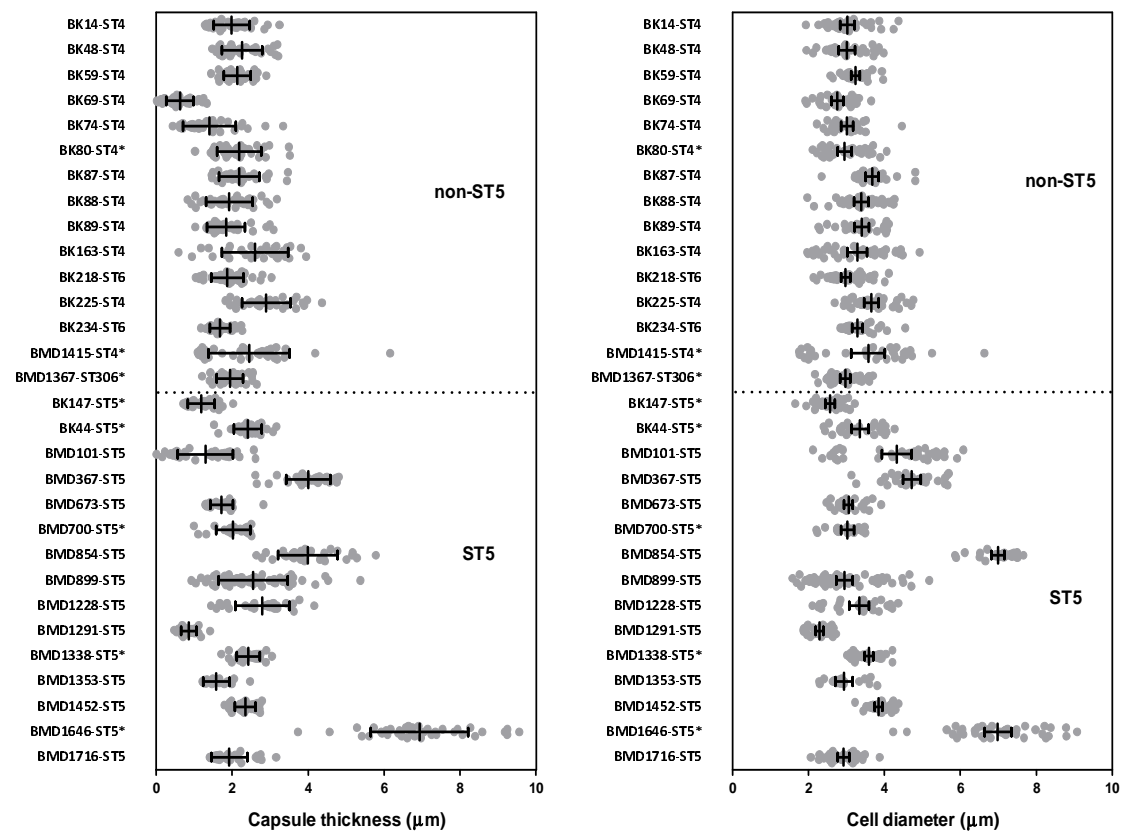
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732 **TABLE 3** Tissue fungal burden in lung and brains of mice at days 7, 14 and at time of death by  
 733 infecting MLST Sequence Type (ST)

<b>Fungal burden</b>			
(Mean log <sub>10</sub> colony forming units per gram of tissue)			
[95%CI]			
Tissue	ST5	Non-ST5	P Value
Lung (Day 7)	4.96 [3.86 - 6.05]	7.07 [6.85 - 7.29]*	<0.001
Brain (Day 7)	0.81 [0.38 - 1.24]	1.59 [0.87 - 2.30]	0.054
Lung (Day 14)	5.48 [4.34 - 6.62]	8.00 [7.77 - 8.23]*	<0.0001
Brain (Day 14)	1.56 [0.81 - 2.31]	1.91 [1.12 - 2.69]	0.36
Lung (Death)	3.81 [3.05 - 4.57]	6.53 [6.30 - 6.76]*	<0.0001
Brain (Death)	2.90 [2.23 - 3.56]	4.29 [3.90 - 4.68]*	0.01

\*Mann-Whitney test.



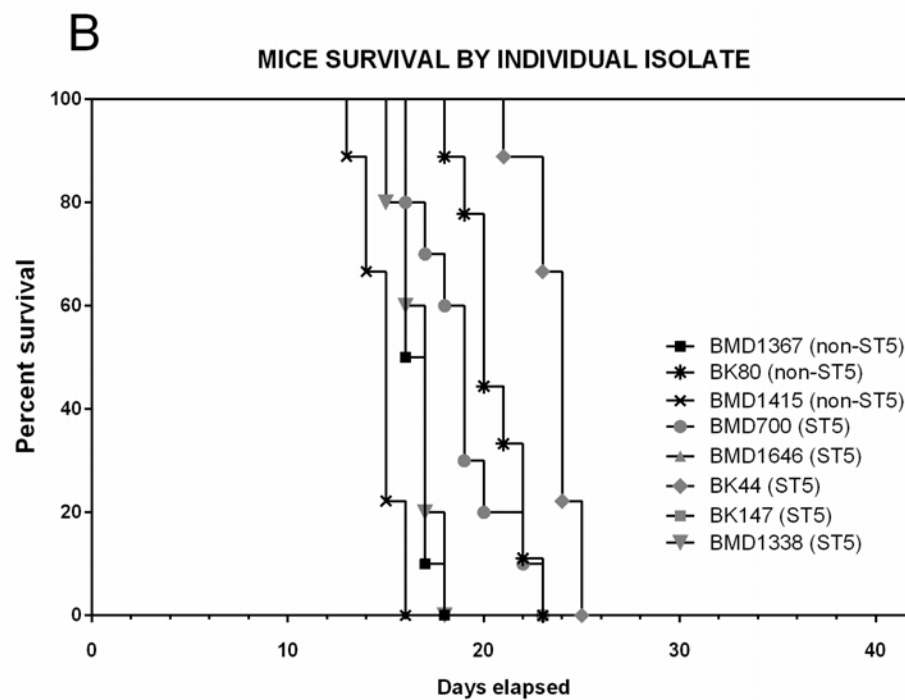
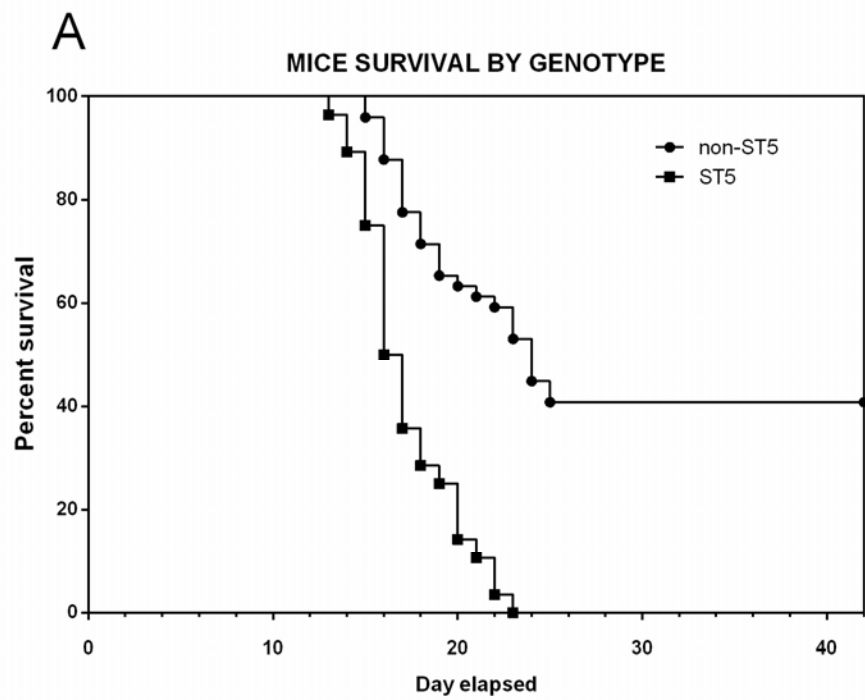
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735 **Figure 2. *In vitro* induced capsule thickness and cell diameter of individual *Cryptococcus neoformans* strains from Vietnam:** Cells were grown

736 on DMEM medium/5% CO<sub>2</sub> and visually assessed by India ink staining. Images were taken for single cells measurement using ImageJ software.

737 Capsule thickness is obtained by subtracting cell body diameter from total cell diameter. AFLP-VNI- $\gamma$ /MLST-ST5 strains expressed higher degree  
738 of variation in both capsule size and cell diameter *in vitro*, which remains significant even when the outlier BMD1646 was removed from the  
739 analysis ( $p < 0.0001$  for both capsule and cell size, Fligner-Killeen test). Scattered plot represents single cells from an individual strain. Data for  
740 individual strains are presented as mean with error bars denoting standard deviation. Strains selected for experiment in mice were indicated by  
741 asterisks.

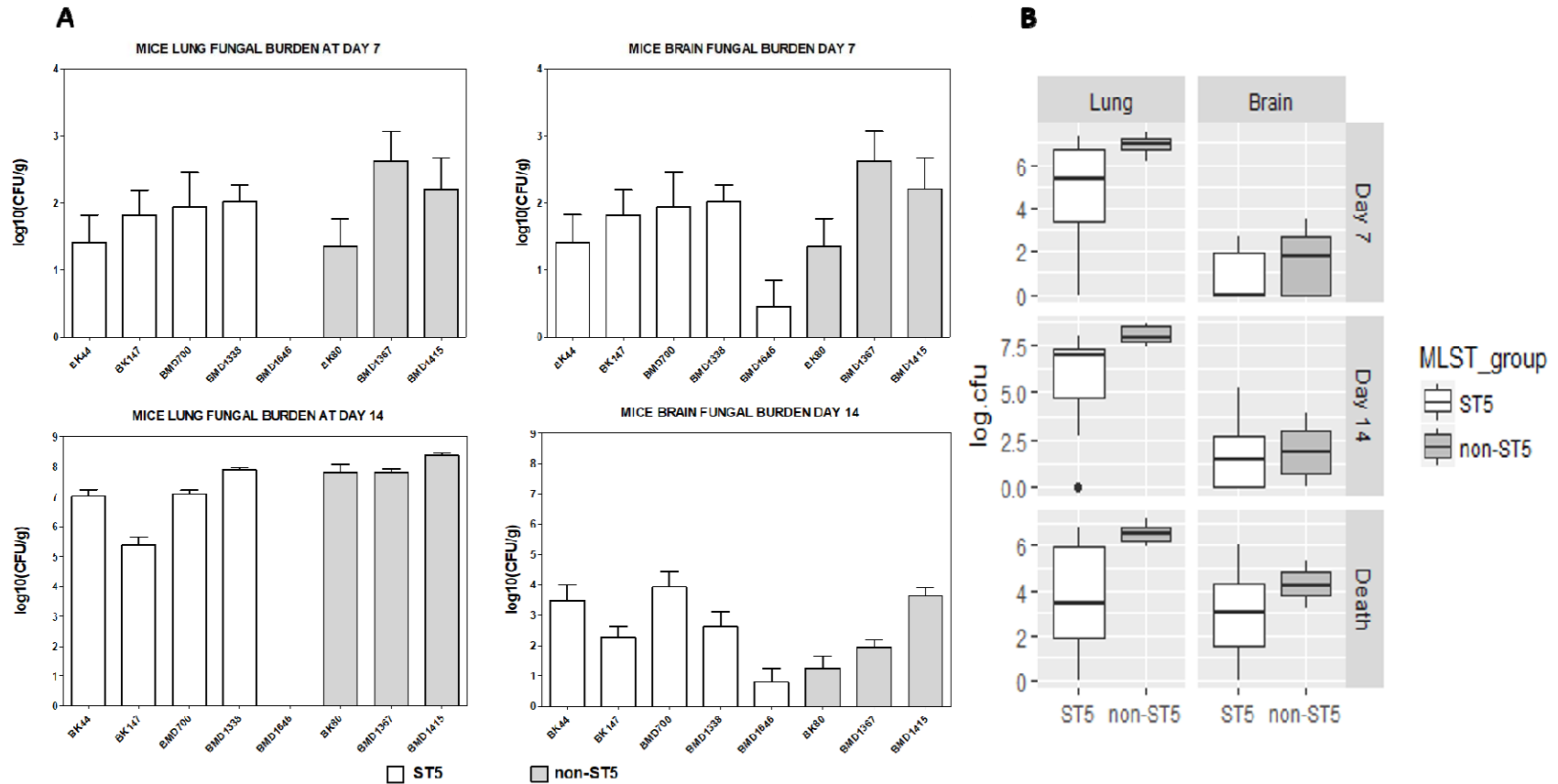
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744 **Figure 2. Kaplan-Meier survival curves for mice infected with either ST5 (n = 5) or non-ST5 (n= 3) *Cryptococcus neoformans* strains.** 10  
745 A/J mice were infected per strain (five ST5 strains and three non-ST5 strains). Mice were monitored daily until the point of more than 15%  
746 weight loss or visible suffering and were then sacrificed by CO<sub>2</sub> inhalation. Mice infected with ST5 strains had statistically significantly longer  
747 survival times than those infected with non-ST5 strains (P<0.0001, Mantel-Cox log rank test) (Panel A). Two ST5 strains, BK147 and  
748 BMD1646, were attenuated, suggesting high degree of heterogeneity within the ST5 cluster. Mice infected with BK147 and BMD1646 survived  
749 for as long as 42 days post-infection, at which point the experiment was terminated and all infected mice sacrificed (Panel B).  
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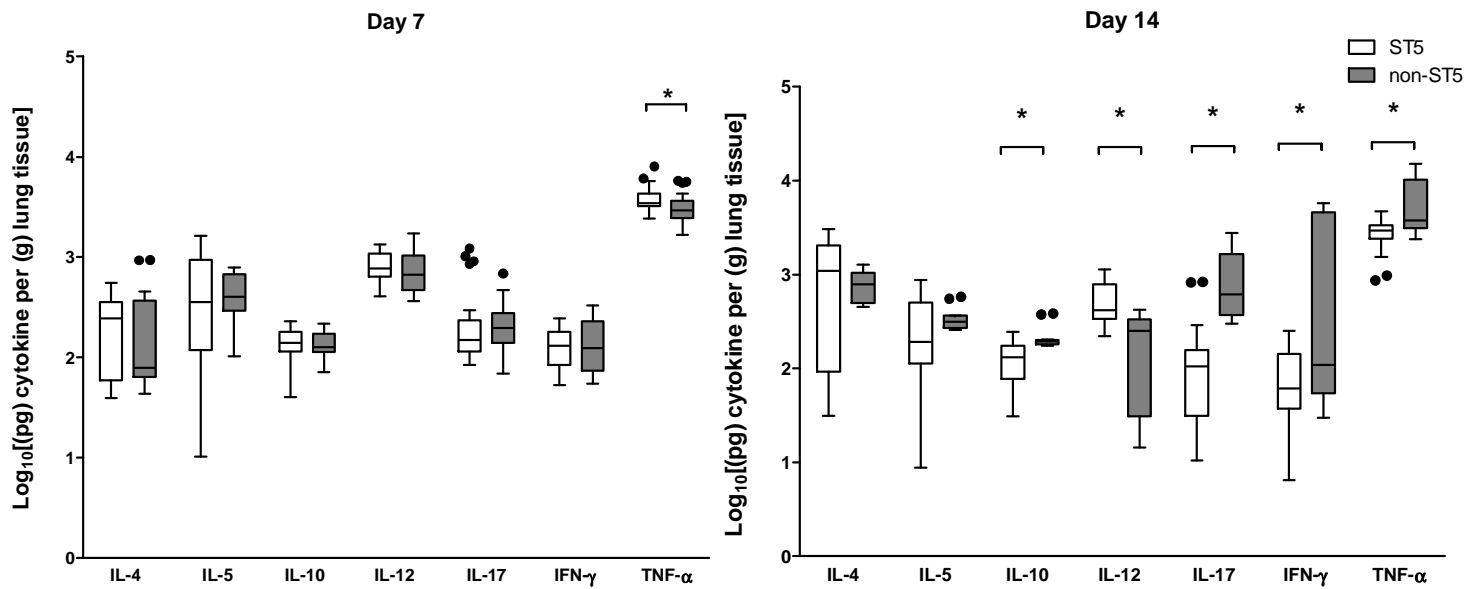
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753 **Figure 3. Fungal burden in mouse lung and brain tissue at days 7, 14 and the point of impending death (mortality experiment) according to**  
 754 **infecting genotype.** *In vivo* virulence (tissue fungal burden and mortality) was assessed in three independent mouse infection experiments. In  
 755 the first two experiments, five A/J mice were infected with each of the eight test *C. neoformans* isolates (five ST5 isolates and 3 non-ST5 isolates,

756 total N=40 mice in each experiment). All mice were sacrificed at day 7 post-infection in the first experiment and at day 14 post-infection in the  
757 second experiment to assess *in vivo* fungal burden in mouse lung and brain. Fungal burden in the lung and brain at either day 7 or day 14 for  
758 each isolate is presented in panel A. In the last experiment, ten A/J mice were infected with each of the 8 test *C. neoformans* isolate (five ST5  
759 isolates and 3 non-ST5 isolates, N=80 mice in total) and monitored daily until the point when 15% of body weight loss, a sign of distress and  
760 impending death, was evident. Fungal burden in lung and brain at point of death was again assessed as before. Here, for each strain, five mice  
761 were randomly selected from the ten test mice for fungal burden investigation. The pooled fungal burden in non-ST5 infections was higher than  
762 in ST5 infections in both lung tissue at all time points, and in brain tissue at the point of sacrifice (Panel B) (For P values see text). Boxplots  
763 (Tukey's method) describe the median and interquartile range, the whiskers demarcate the largest or smallest values that were not outliers  
764 (black dots); outliers are defined as more than 1.5 times the interquartile range from the nearest quartile.



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767 **Figure 4. Genotype-specific cytokine concentrations from lung homogenate of A/J mice at 7 and 14 days post infection with  $5 \times 10^4$  *C.***

768 ***neoformans* cells/mouse.** Five mice were infected with each strain from each genotype at each time points. ST5 strains induced significantly

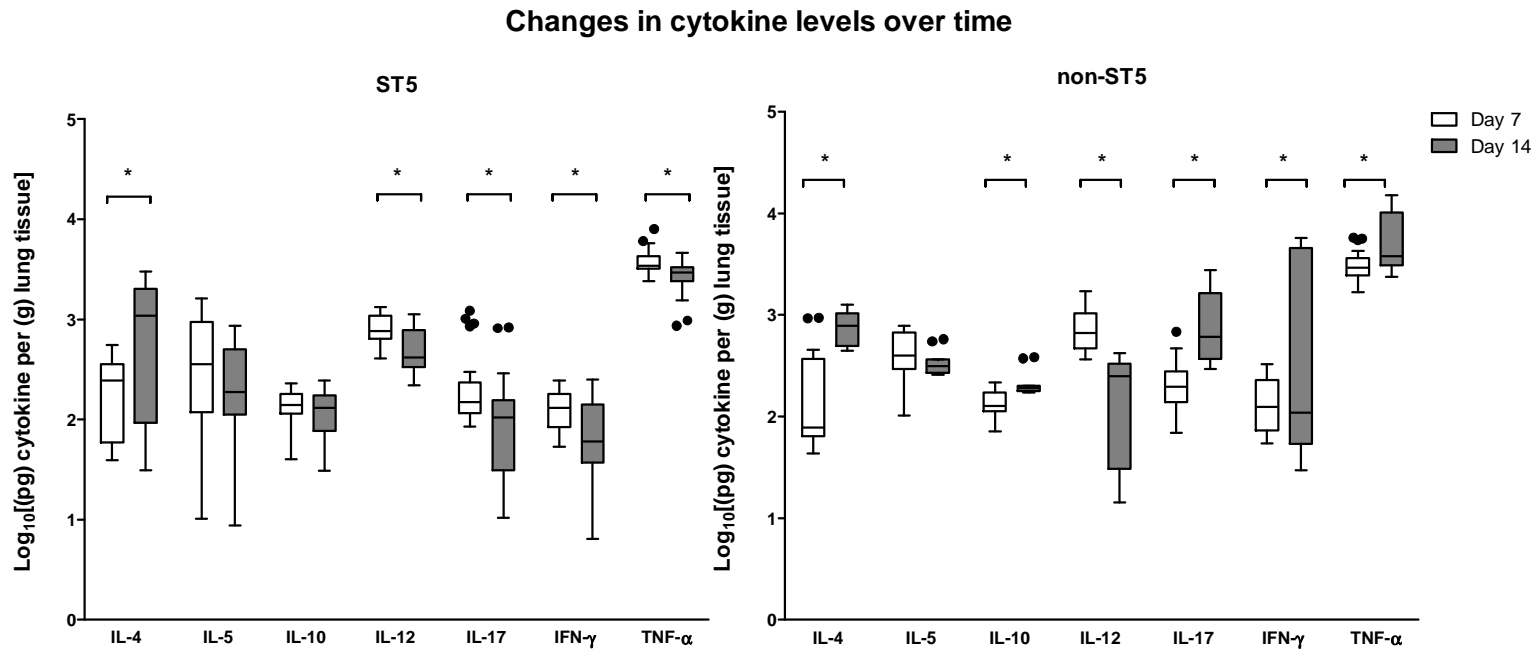
769 higher levels of TNF- $\alpha$  at day 7, suggesting an earlier and more profound initial inflammatory response in infected mice. By day 14 mice infected

770 with non-ST5 strains have higher levels proinflammatory cytokines, probably a result of ST5 yeasts being cleared more rapidly from infected

771 mice. The horizontal line within the box indicates the median; boundaries of the box indicate the 25<sup>th</sup> and 75<sup>th</sup> percentile and the whiskers

772 indicate the highest and lowest values of the results; outliers are denoted as black dots (Tukey's method). Data are standardized as picograms of  
773 cytokine per gram lung tissue. Asterisks indicate statistically significant differences (Mann-Whitney test).

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776 **Figure 5. Genotype-specific changes in cytokine concentrations from lung homogenate of A/J mice infected with *C. neoformans* between day**  
777 **7 and day 14 post-infection.** Box and whisker plots (Tukey's method) compare levels of each cytokine between day 7 and day 14 for each  
778 genotype. Data are standardized as picograms of cytokine per gram lung tissue. Asterisks indicate statistically significant differences (Mann-  
779 Whitney test).

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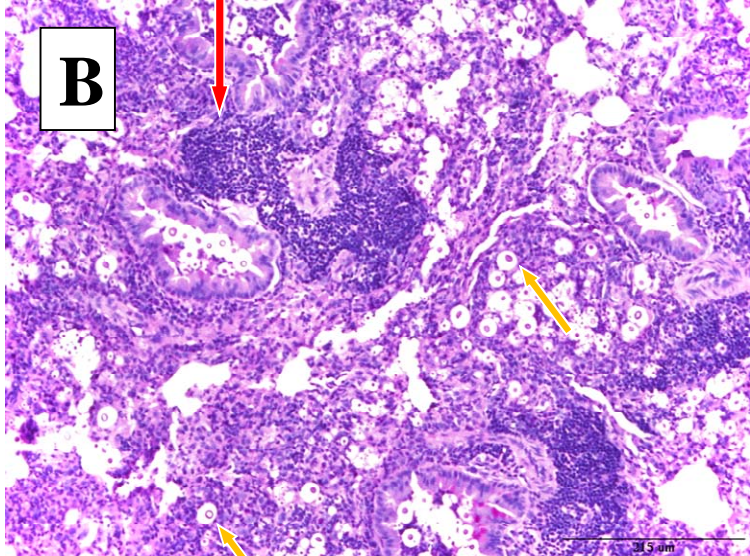
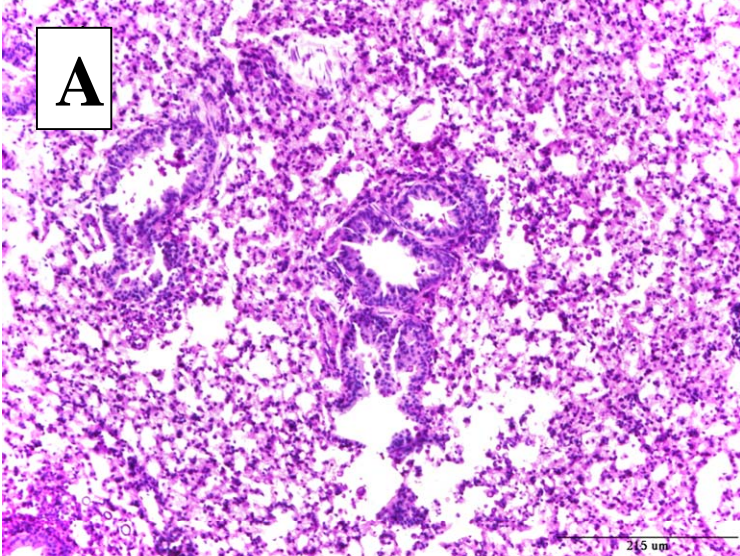
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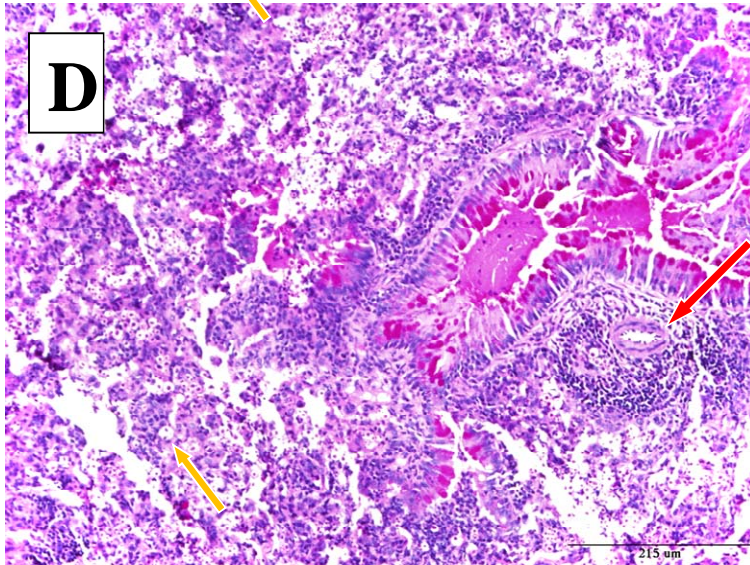
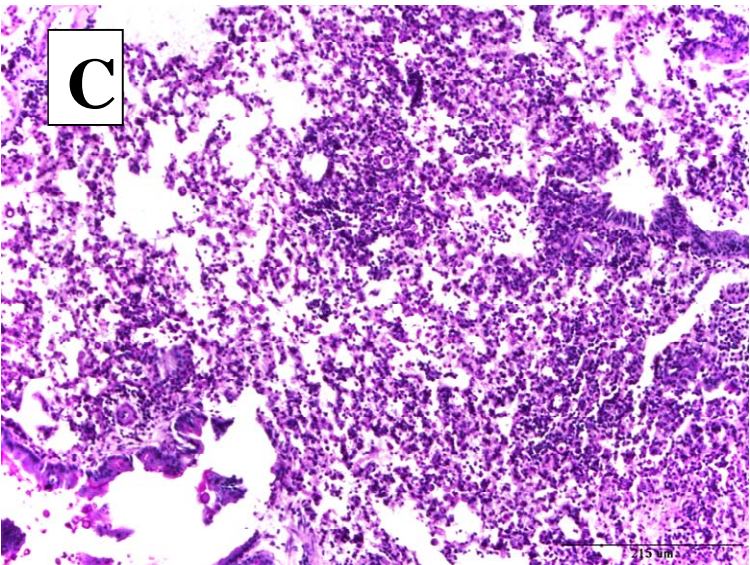
**Day 7**

**Day 14**

**BMD1338**  
**VNI- $\gamma$ /ST5**



**BMD1415**  
**VNI- $\delta$ /ST4**



783 **Figure 6. Periodic Acid Schiff (PAS) staining of pulmonary tissue from mice infected with BMD1338 and BMD1415 on days 7 and 14.** The two  
784 strains represent ST5 and non-ST5, respectively. A/J mice were inoculated intranasally with  $5 \times 10^4$  yeast cells. Lung specimens were harvested at  
785 days 7 and 14 for histopathological examination. Photomicrographs were obtained at 200X magnification; the scale bar represents 215  $\mu\text{m}$ ). (A-  
786 B): lung sections from mice infected with BMD1338 (VNI- $\gamma$ /ST5) at day 7 and day 14, respectively. (C-D): lung sections from mice infected with  
787 BMD1415 (VNI- $\delta$ /ST4) at day 7 and day 14, respectively. Perivascular infiltration (red arrows) and necrosis are more marked by day 14 for both  
788 strains. Encapsulated yeasts (yellow arrows), notable for the larger cell size and capsule thickness of BMD 1338 compared with BMD1415.

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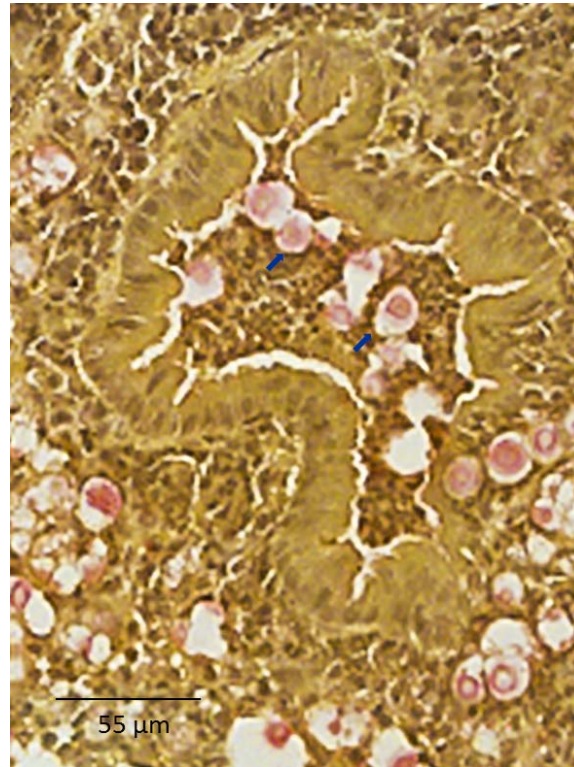
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795 **Figure 7. Mucicarmine staining of capsular material in paraffin-embedded mice pulmonary tissue.**

796 Uninflated lung specimens were harvested from mice as described in the methods. Mucicarmine staining was performed to visualize the  
797 cryptococcal capsule. Photomicrographs were captured at 400X magnification with scale bar indicating 55μm. Capsular polysaccharide is stained  
798 pink (indicated by blue arrows), demonstrating diffuse localization consistent with extensive capsule production by yeasts in the alveolar space.

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