

## Cryptococcus

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DOI:

[10.1038/nrmicro.2015.6](https://doi.org/10.1038/nrmicro.2015.6)

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*Document Version*

Peer reviewed version

*Citation for published version (Harvard):*

May, RC, Stone, NRH, Wiesner, DL, Bicanic, T & Nielsen, K 2016, 'Cryptococcus: from environmental saprophyte to global pathogen', *Nature Reviews Microbiology*, vol. 14, no. 2, pp. 106-117.  
<https://doi.org/10.1038/nrmicro.2015.6>

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Checked 13/06/2016

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1 ***Cryptococcus*: from environmental saprophyte to global**  
2 **pathogen**

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14 **Abstract**

15 Cryptococcosis is a globally distributed invasive fungal infection caused by  
16 species within the genus *Cryptococcus* that presents substantial therapeutic  
17 challenges. Although natural human-to-human transmission has never been  
18 observed, recent work has unveiled multiple virulence mechanisms that allow  
19 cryptococci to infect, disseminate within and ultimately kill their human host. In  
20 this Review, we describe these recent discoveries that illustrate the intricacy of  
21 host-pathogen interactions and reveal new details about host immune responses  
22 that either help protect against disease or increase host susceptibility. In  
23 addition, we discuss how this improved understanding of both the host and the  
24 pathogen informs potential new avenues for therapeutic development.

25

26 Cryptococcosis has been recognized since 1894, when the pathologist Otto Busse  
27 and physician Abraham Buschke jointly identified *Cryptococcus* as the cause of a  
28 chronic granuloma of the tibial bone in a 31-year-old woman. However, human  
29 cryptococcosis only became recognized as a major health threat with the onset of  
30 the AIDS pandemic in the 1980s, in which these fungal infections became a  
31 common AIDS-defining illness in patients with catastrophically reduced T-cell  
32 function (**Box 1**). Although cryptococcosis is predominantly a disease of  
33 immunocompromised patients, a recent outbreak of cryptococcosis in otherwise  
34 healthy individuals in North America and Canada (now known as the Pacific  
35 Northwest Outbreak) has focused attention on the capacity of some lineages of  
36 the pathogen to act as primary pathogens (see below).

37 Since its identification, cryptococcosis has been attributed to a single  
38 fungal species, *Cryptococcus neoformans*. However, improved molecular methods  
39 led to a previous variety, *Cryptococcus neoformans* var. *gattii*, being classified as a  
40 novel species, *Cryptococcus gattii*, in 2002<sup>1</sup>. More recently, whole-genome  
41 sequencing-based analyses have highlighted the complex evolutionary history of  
42 this group (**Box 2**) and led to a proposal to further split *C. neoformans* into two  
43 species (*C. neoformans* and *Cryptococcus deneoformans*) and *C. gattii* into a total  
44 of five species (*C. gattii*, *Cryptococcus bacillisporus*, *Cryptococcus deuterogattii*,  
45 *Cryptococcus tetragattii* and *Cryptococcus decagattii*)<sup>2</sup>. However, as detailed  
46 biological comparisons between these five species have not been yet undertaken,

47 we have adopted the simpler distinction into the two species *C. gattii* and *C.*  
48 *neoformans* throughout this article.

49

### 50 ***Cryptococcus* transmission and disease onset**

51 In the environment, cryptococci reside in diverse ecological niches (**Box 3**). Both  
52 *C. neoformans* and *C. gattii* are abundant in decaying material within hollows of  
53 various tree species, although *C. gattii* has been suggested to favour trees with  
54 waxier cuticles (such as *Pseudotsuga menziesii*)<sup>3, 4</sup>. Furthermore, *C. neoformans*  
55 is globally distributed, whereas *C. gattii* has classically been viewed as a tropical  
56 or subtropical fungus. However, increased surveillance has now identified  
57 environmental reservoirs for *C. gattii* in the Northern USA, Canada and Northern  
58 Europe, indicating that this species may also have a wider ecological range than  
59 previously recognized.

60 *C. neoformans* is particularly abundant in avian excreta<sup>4,5</sup> and its  
61 association with feral pigeons could be a major source of infection in densely  
62 populated urban areas. In addition, both *C. neoformans* and *C. gattii* are able to  
63 survive and replicate within free-living amoebae and soil nematodes and it is  
64 possible that these alternative hosts may have an important role in determining  
65 the distribution and virulence of different cryptococcal lineages around the  
66 world (**Box 3**).

67 With the exception of very rare iatrogenic<sup>6</sup> or zoonotic<sup>7</sup> transmission  
68 events, naturally acquired cases of cryptococcosis are believed to start with  
69 inhalation of fungal cells from the environment. Within the lung, *Cryptococcus*  
70 species can cause pneumonia in immunosuppressed patients, but in  
71 immunocompetent hosts the fungal cells are either cleared by the immune  
72 system or establish an asymptomatic latent infection. Upon subsequent  
73 immunosuppression, this latent infection can then disseminate to other tissues,  
74 most notably the central nervous system (CNS). Once established within the CNS,  
75 cryptococcosis causes an overwhelming infection of the meninges and brain  
76 tissue that is frequently accompanied by raised intracranial pressure; without  
77 rapid and effective treatment, CNS infection is invariably fatal. Despite intensive  
78 investigations, it remains unclear whether reactivation and dissemination of  
79 long-term latent pulmonary infection is a more important cause of

80 cryptococcosis in patients than *de novo* acquisition from the environment, but  
81 experiments in animal models indicate that both routes are capable of causing  
82 lethal disease.

83 Exposure to *C. neoformans* is common in humans, as most individuals  
84 produce antibodies against this fungal species by school age<sup>8</sup>. During active  
85 growth, cryptococcal cells are too large to penetrate deep into the human lung  
86 and thus the initial inoculum is believed to comprise either desiccated cells or  
87 spores. The relative contribution of these two cell types to the burden of disease  
88 remains unclear, largely due to technical challenges associated with generating  
89 and purifying spores. However, recent studies have demonstrated that lethal  
90 brain infections can develop from spore inocula, that spores are readily  
91 phagocytosed by host immune cells and, interestingly, that rising humidity  
92 dramatically increases spore viability<sup>9,10,11</sup>. Thus, as with other fungal pathogens  
93 such as *Coccidioides immitis*, environmental conditions may be an important  
94 factor in regulating human cryptococcal exposure.

95

## 96 **Cryptococcal pathogenesis**

97 Traditional virulence factors produced by *Cryptococcus* (such as the capsule and  
98 melanin production) and changes in fungal growth due to the host temperature  
99 (37°C) have been previously reviewed in great detail (see for example references  
100 <sup>12,13</sup>). Therefore, in this section of the Review, we will focus on recently emerging  
101 concepts in cryptococcal pathogenesis.

102

103 ***Fungal morphology.*** Whether derived from spores or yeast cells, upon  
104 inhalation into a mammalian host, all cryptococci transition to or maintain a  
105 yeast form. When grown under laboratory conditions, *Cryptococcus* cells are  
106 round and 5-7 µm in diameter. However, their cell size, structure, and  
107 characteristics can vary dramatically within the host.

108 The best-characterized atypical morphology of *Cryptococcus* cells is the  
109 titan cell<sup>14</sup> (**Figure 1**). Titan cells are greater than 12 µm in diameter (excluding  
110 the capsule), polyploid, have highly cross-linked capsules and a thickened cell  
111 wall<sup>15,16</sup>. Recent studies have shown that titan cells contain elevated levels of  
112 chitin. This polysaccharide is recognized and cleaved by host chitinases, which

113 induces a detrimental adaptive immune response (see below)<sup>17</sup>. Intriguingly, the  
114 polyploidy observed in titan cells enhances genetic adaptation to the stressful  
115 host environment, resulting in increased within-host survival<sup>18</sup>.

116 In addition to the large titan cells, unusually small cryptococcal cells have  
117 also been observed<sup>19,20</sup> (**Figure 1**). These so-called “drop” or “micro” cells are  
118 only 2-4 μm in size, despite having a thickened cell wall, and appear adapted for  
119 growth within macrophages. At present, little is known about this cell type,  
120 although they appear to be relatively metabolically inactive and therefore may  
121 have an important role during the latent stage of disease.

122 In the environment or under laboratory conditions, cryptococci can also  
123 grow as hyphae (during sexual reproduction) or pseudohyphae, but (unlike  
124 other pathogenic fungi) these morphologies are not seen in human infections<sup>21</sup>.  
125 Recent studies overexpressing the transcription factor Znf2, a “master regulator”  
126 that triggers the transition from yeast to hyphal growth, showed that the hyphal  
127 form elicits a robust protective immune response and is readily cleared by the  
128 host<sup>22,23</sup>, perhaps explaining why filamentous morphologies are not seen in  
129 mammalian infections. Interestingly, however, hyphal cryptococci are protected  
130 from predation by free-living amoebae<sup>24</sup> and thus mammalian and amoebal  
131 hosts presumably exert opposing selective pressures on this aspect of  
132 cryptococcal morphology (with mammalian hosts favouring the existence of the  
133 yeast forms and amoebae favouring hyphal forms).

134

135 ***Fungal ageing.*** Even within a clonal infection, not all cryptococcal cells are  
136 equal. For example, the age of individual cryptococcal cells has emerged as a  
137 factor that impacts survival in the host and subsequent pathogenesis<sup>25</sup>. Older  
138 cells present in the initial infection, referred to as founder cells, are better able to  
139 resist phagocytosis and killing by phagocytes and are resistant to antifungal  
140 drugs. This increased resistance to phagocyte killing and antifungals is  
141 potentially due to changes in cell wall structure<sup>26</sup>, and results in the  
142 accumulation of founder cells in the brain at a higher frequency than young  
143 cells<sup>27</sup>.

144

145 **Population-wide signals.** In bacterial infections, quorum sensing is a well-  
146 known mechanism that regulates virulence according to population density.  
147 Interestingly, emerging data suggest that quorum sensing may also have an  
148 important role during cryptococcal pathogenesis. For example, a quorum sensing  
149 effect, mediated by an oligopeptide with 11 amino acids, was identified using  
150 mutations in the global repressor TUP1. Notably, although TUP1 is present in  
151 several species, the quorum sensing effect mediated by this oligopeptide appears  
152 only to occur in *C. neoformans*<sup>28</sup>. However, more recently a different signaling  
153 molecule, pantothenic acid, has been demonstrated to mediate quorum sensing  
154 both between different cryptococcal strains and between cryptococci and other,  
155 relatively distantly related, fungal species<sup>29</sup>. The adhesin Cfl1 has also been  
156 shown to modulate colony morphology in a paracrine manner<sup>30</sup>. Activation of the  
157 hyphal regulator Znf2 (discussed above) induces expression of this adhesin,  
158 some of which is shed into the environment and triggers neighboring cells to  
159 activate Znf2, leading to a positive feedback loop. Thus cryptococci may  
160 communicate locally using a range of chemical messengers<sup>31</sup>.

161 Perhaps most unique is the observation that light-sensing pathways may  
162 also be important for virulence in *Cryptococcus* since deletion of either *Bwc1* or  
163 *Bwc2*, which encode two transcription factors that control fungal responses to  
164 light, reduces virulence in a murine model of infection<sup>32</sup>. In the dark, BWC1 and  
165 BWC2 bind to DNA and repress genes involved in filamentation. However, upon  
166 light activation, they release this inhibition leading to filamentation and  
167 upregulation of UV-resistance pathways. Thus, it is possible that an additional  
168 function of these two proteins is to detect darkness and prevent inappropriate  
169 filamentation within the host, which would induce a potent immune response  
170 and pathogen clearance.

171

## 172 **Host immunity and pathogen subversion**

173 One of the most remarkable discoveries of recent years has been the extent to  
174 which cryptococci are able to manipulate the host immune response to dampen  
175 inflammation, avoid killing by phagocytic cells and ultimately disseminate into  
176 the CNS.

177

178 **Inflammatory perturbation.** In general, environmental fungi trigger a potent  
179 inflammatory response upon entry into the human host. By contrast, cryptococci  
180 appear to be immunologically inert, driving much lower levels of inflammatory  
181 cytokine release *in vitro* than other human fungal pathogens such as *C. albicans*<sup>33</sup>.  
182 This immunological masking relies on a variety of pathogen traits (**Figure 1**).

183 Firstly, the complex carbohydrates glucuronoxylomannan (GXM) and  
184 galactoxylomannan (GalXM), which make up most of the cryptococcal capsule,  
185 are extensively shed during infection and directly dampen inflammation by  
186 suppressing the pro-inflammatory NF- $\kappa$ B pathway and driving down levels of  
187 pro-inflammatory cytokines such as TNF<sup>34</sup>. In addition, emerging data indicate  
188 that cryptococcal chitin, and derivatives thereof, can also act to alter host  
189 inflammatory responses during infection<sup>17</sup>. Secondly, *Cryptococcus* blocks  
190 dendritic cell maturation by reducing both MHC class II-dependent antigen  
191 presentation and inhibiting the production of the pro-inflammatory cytokines  
192 interleukin (IL)-12 and IL-23<sup>35</sup>. Lastly, via a series of as-yet poorly characterized  
193 steps, cryptococci are able to partially “repolarize” the immune response, at least  
194 in mice, from a strong Th1 response towards a weaker Th1 or often a Th2  
195 response that is less effective at fungal clearance<sup>17,36-38</sup>.

196 Collectively, these mechanisms generate an environment that is  
197 dominated by anti-inflammatory markers such as IL-4 and IL-33<sup>39,40,41</sup> which, as  
198 a consequence, reduce cryptococcal killing by the immune system<sup>38,42</sup>. Therefore,  
199 modulating natural immune responses to cryptococcal infection towards a more  
200 pro-inflammatory profile offers one potential avenue for treatment. However,  
201 such approaches need to be carefully managed in order to avoid the potentially  
202 fatal “immune overreactions” that can accompany overt inflammation, which can  
203 be just as life-threatening as the original infection (**Box 4**).

204

205 **Avoidance and escape from phagocytes.** Following entry into the lung, the first  
206 immune cell typically encountered by cryptococci is a phagocyte such as an  
207 alveolar macrophage or dendritic cell. However, cryptococci are predisposed to  
208 avoid killing by these cells, due to their long evolutionary history of exposure to  
209 environmental amoebae (**Box 3**). Several cryptococcal virulence factors such as  
210 capsule synthesis, melanization and urease secretion combine to protect the

211 fungus from the harsh environment within phagocytic cells by neutralizing  
212 reactive oxygen species and pH, allowing it to survive and proliferate within such  
213 cells (**Figure 2**)<sup>43</sup>.

214 More recently, it has also become clear that cryptococci exhibit a  
215 remarkable strategy to escape from within phagocytes. This process, which has  
216 been labeled vomocytosis or extrusion, involves inducing the fusion of the  
217 phagosomal membrane with the plasma membrane, which results in the  
218 expulsion of the fungi from the phagocyte<sup>44-48</sup>. In addition, either this process, or  
219 a closely related one, can drive the direct “lateral transfer” of cryptococci  
220 between host cells <sup>44,45</sup>. However, the underlying mechanisms of both of these  
221 remarkable processes remain unknown.

222 Although cryptococci employ several mechanisms to resist phagocytosis  
223 (such as through production of titan cells<sup>15,49</sup> and the assembly of a thick  
224 polysaccharide capsule), fungal uptake by phagocytes can still occur. However, if  
225 uptake does occur, cryptococci perturb both phagosome maturation<sup>50</sup> and  
226 modify the phagosome membrane in order to allow nutrient exchange and  
227 ultimately escape from within the host cell<sup>51,52</sup>. Notably, these effects are  
228 dependent on fungal virulence factors such as laccase and phospholipase B1.  
229 These enzymes have been classically thought of as having direct structural roles  
230 in melanin synthesis and membrane lipid modification, respectively, but the  
231 observation that they also mediate escape from phagocytosis suggests that  
232 laccase and phospholipase B1 may also have more subtle roles in modifying host  
233 signaling events<sup>36,53,54</sup>.

234

235 ***Dissemination and entry into the CNS.*** A key feature of cryptococcal  
236 pathogenesis involves the exit of *Cryptococcus* from the lungs into peripheral  
237 blood circulation and entry into the CNS compartment. The CNS is both an  
238 immune privileged site and a highly sterile environment and thus *Cryptococcus*  
239 must have evolved potent methods to traverse the blood-brain barrier (BBB)  
240 and subsist in the CNS.

241 There are three proposed mechanisms that *Cryptococcus* could utilize to  
242 penetrate this impervious barrier. First, the yeasts could force their way  
243 between the tight junctions of the endothelial cells in a process known as

244 paracytosis, by using proteases such as Mpr1 to promote transmigration<sup>55</sup>  
245 (**Figure 2**). Impressively, when the *MPR1* gene was introduced into  
246 *Saccharomyces cerevisiae*, a fungus not normally able to penetrate the BBB, *S.*  
247 *cerevisiae* gained the ability to cross endothelial cells in an *in vitro* transwell  
248 assay, although the target of Mpr1 remains unknown. Additional studies utilizing  
249 powerful intravital imaging techniques demonstrated that cryptococci cross the  
250 BBB by inducing an embolic event in the microvasculature that lines the brain<sup>56</sup>.  
251 In essence, the initial “capture” of yeast within the brain is therefore passive,  
252 with the relatively large yeast cells becoming trapped at points where the blood  
253 vessel narrows. However, following the initial passive arrest, cryptococcal  
254 migration into the brain tissue is an active process, since it occurs only with live  
255 fungal cells and is dependent on the secretion of the cryptococcal enzyme  
256 urease<sup>57</sup>. To date, the part played by urease in this process remains enigmatic,  
257 although since urease produces ammonia, which is toxic towards mammalian  
258 cells, it is possible that urease acts to locally weaken the endothelial vessel wall,  
259 facilitating fungal entry.

260 The second mechanism of BBB penetration is transcytosis<sup>58</sup> (**Figure 2**).  
261 Hyaluronic acid situated on the surface of the cryptococcal cell binds to CD44 on  
262 the luminal endothelium, attaching the fungus to the host cell<sup>59</sup>. This binding  
263 then induces protein kinase C-dependent actin remodeling in the host cell,  
264 leading it to engulf the attached *Cryptococcus*<sup>60</sup>. Interestingly, recent work has  
265 revealed that the high levels of inositol present in the brain act as a trigger for  
266 this process, increasing hyaluronic acid expression in the fungus<sup>61</sup>.

267 Finally, *Cryptococcus* is postulated to cross the BBB by a third method  
268 involving “hitchhiking” within host phagocytes, in a process termed the “Trojan  
269 Horse” hypothesis (**Figure 2**). This hypothesis is supported by the observation  
270 that depletion of alveolar macrophages in mice significantly reduces  
271 cryptococcal dissemination to the CNS<sup>62</sup>, while infecting monocytes *in vitro* and  
272 transferring the cells into naïve hosts substantially increases cryptococcal  
273 accumulation in the brain compared to transferring *Cryptococcus* directly; both  
274 studies support the notion that phagocytes act as fungal carriers that breach the  
275 BBB<sup>63</sup>. Although paracytosis, transcytosis, and Trojan Horse models are all  
276 fundamentally different, it is reasonable to conclude that elements of each of

277 these models are readily observed and likely occur in concert during natural  
278 infection.

279 Not much is known about the physiology of *Cryptococcus* after it has  
280 traversed the BBB. However, a recent study of the transcriptome of cryptococcal  
281 yeasts isolated from cerebrospinal fluid (CSF) samples of patients offers some  
282 clues<sup>64</sup>. Most notably, *Cryptococcus* is remarkably metabolically active in the CSF  
283 *in vivo*, showing strong up-regulation of stress response genes and genes  
284 encoding enzymes that are involved in core metabolic processes; this is  
285 somewhat surprising, given that the CSF is a relatively nutrient depleted  
286 medium. By contrast, *Cryptococcus* growing in *ex vivo* CSF does not seem to be  
287 metabolically active, suggesting that the permanent cycling of CSF *in vivo* leads to  
288 a significantly higher nutrient content in the CSF than suggested by the analysis  
289 of *ex vivo* samples.

290 The modified fungal metabolism observed within the CSF is likely to have  
291 significant implications for pathogenesis. For instance, capsule synthesis is  
292 energetically highly demanding and there is a positive correlation between  
293 capsular size and severity of clinical disease<sup>65</sup>. Therefore, these data suggest that  
294 yeasts in a more active metabolic state may drive more aggressive CNS  
295 infections. Furthermore, fungal cells in different metabolic states are likely to  
296 give rise to different immune responses, which may also impact disease severity.  
297 In agreement with this possibility, the presence of a CSF inflammatory response  
298 consisting of an interplay of robust Th1 (IFN- $\gamma$  and IL-6), Th2 (IL-4 and IL-10)  
299 and Th17 (IL-17) cytokines has recently been shown to be highly predictive of  
300 more rapid clearance of infection and consequently improved survival in  
301 patients with HIV-associated cryptococcal meningitis<sup>66</sup> (**Box 4**).

302

303 **Division of labour.** The extent to which cryptococci can exploit phagocytic cells  
304 as a host has been strongly highlighted by the unusual cluster of cryptococcal  
305 disease now known as the Pacific Northwest Outbreak<sup>67</sup>. Although  
306 cryptococcosis is typically a disease of immunocompromised hosts, almost all of  
307 the human and animal cases within the Pacific Northwest Outbreak were  
308 immunocompetent hosts who became infected with near clonal strains of *C.*  
309 *gattii* from the VGII lineage. Both the epidemiology and etiology of these

310 infections differ from “classical” cryptococcosis (typically caused by *C.*  
311 *neoformans* in HIV-positive individuals)<sup>33,68</sup>, which has led to vigorous efforts in  
312 order to establish the underlying mechanism driving virulence in the *C. gattii*  
313 VGII lineage.

314 The ability of the *C. gattii* VGII lineage to establish disease in individuals  
315 with a fully functional immune system seems to stem from a capacity to replicate  
316 extremely rapidly within host phagocytes (**Figure 2**), presumably overwhelming  
317 the host before adaptive immunity can be triggered<sup>69</sup>. Recent data has revealed  
318 that this rapid proliferation is, in turn, driven by a remarkable “division of  
319 labour” mechanism. In response to reactive oxygen species generated by the  
320 phagocyte, intracellular cryptococcal cells adopt different fates; some  
321 cryptococcal cells cease growth and acquire an unusual morphology  
322 characterized by extensive tubularisation of their mitochondria, whereas  
323 neighboring cells do not undergo this morphological transition. Notably, via a  
324 mechanism that remains unclear, the cells that undergo the morphological  
325 switch then protect neighboring cryptococci from the antimicrobial activity of  
326 the host phagocyte, enabling these cells to replicate rapidly, maximizing the  
327 proliferative capacity of the population as a whole<sup>70</sup>. These data highlight the  
328 *Cryptococcus*—phagocyte interaction as a key aspect of infection that may offer  
329 powerful opportunities for therapeutic intervention in both *C. neoformans* and *C.*  
330 *gattii* infections.

331

### 332 **Anti-cryptococcal therapeutics**

333 Despite its global distribution, treatment of cryptococcosis remains a major  
334 challenge, relying on a limited arsenal of decades-old therapeutic agents.  
335 Furthermore, therapeutic outcomes are generally poor and even with  
336 amphotericin-based therapy (to target *Cryptococcus*) and widespread access to  
337 anti-retroviral therapy (to target HIV, since most patients are  
338 immunocompromised HIV-positive patients), acute (3-month) mortality  
339 following cryptococcal meningoencephalitis remains 35-40%, both in resource-  
340 rich and resource-poor settings<sup>71,72</sup>.

341

342 **Currently used drugs.** Only three classes of antifungal agents are currently used  
343 to treat cryptococcosis: polyenes (amphotericin B), azoles (fluconazole) and the  
344 pyrimidine analogue flucytosine (5FC) (**Figure 3**).

345 The cornerstone of treatment of cryptococcal meningoencephalitis is  
346 amphotericin B deoxycholate (AmBd), developed in the 1950s, which exerts its  
347 fungicidal effect both by binding to ergosterol in the cryptococcal cell wall  
348 (generating pores in the cell membrane) and by inducing cell death via oxidative  
349 damage<sup>73-75</sup>. AmBd is sometimes combined with 5-FC. The mechanism of action  
350 of 5-FC is deamination by the fungal enzyme cytosine deaminase into 5-  
351 fluorouracil (5-FU), which then acts via two pathways: 5-FU can be converted by  
352 cellular pyrimidine processing enzymes into 5-fluorodeoxyuridine  
353 monophosphate, which inhibits thymidylate synthetase and blocks DNA  
354 synthesis; or 5-FU can be converted into 5-fluorouridine triphosphate, which is  
355 incorporated into RNA, thereby disrupting protein synthesis and leading to  
356 growth arrest. AmBd and 5-FC act synergistically to produce the fastest rates of  
357 fungal clearance from CSF<sup>76</sup> and combination therapy results in a significant  
358 improvement in 10-week survival compared to treatment with AmBd alone<sup>77</sup>.  
359 This combination remains the recommended 'gold standard' induction treatment  
360 in international treatment guidelines<sup>78</sup> but presents significant challenges in  
361 resource poor settings, since AmBd must be administered intravenously and has  
362 notable toxicities. In addition, neither AmBd nor 5-FC are widely available in  
363 countries where cryptococcosis is most prevalent<sup>79</sup>.

364 To circumvent the problems associated with AmBd and 5-FC combination  
365 therapies, the combination of fluconazole with 5-FC (which can both be  
366 administered orally) and shorter (1-week) AmBd-based induction treatment is  
367 being compared to the standard 2-week induction regimens in a multi-site phase  
368 III African trial<sup>80</sup>. Fluconazole is being tested because it has good oral  
369 bioavailability and excellent CSF penetration; these properties also make it  
370 recommendable for maintenance therapy after initial treatment. Fluconazole  
371 inhibits the fungal cytochrome P450 enzyme, 14 $\alpha$ -demethylase, which is  
372 required for conversion of lanosterol to ergosterol, an essential component of  
373 the fungal cell membrane. However, fluconazole is as a fungistatic (rather than

374 fungicidal) making it is less effective at pathogen clearance and not  
375 recommended for initial therapy.

376

377 **Drug resistance.** Resistance to antimicrobials is a growing issue in infectious  
378 disease and cryptococcosis is no exception. While environmental resistance is  
379 rare, acquired resistance has been observed with all three classes of antifungals  
380 in use against *Cryptococcus* species.

381 Polyene resistance is uncommon but has been reported in *C. neoformans*,  
382 with mutations in sterol synthesis and therefore alteration of the target site noted  
383 in isolates with extensive exposure to AmB<sup>81</sup>. For 5-FC, single mutations at  
384 varying points along the 5-FU intracellular pathways lead to *in vitro* and clinical  
385 resistance. Therefore, monotherapy with 5-FC is not appropriate due to rapid  
386 selection of resistant *Cryptococcus* leading to treatment failure; the drug is thus  
387 always combined with either AmB or fluconazole. Fluconazole, like 5-FC, is  
388 fungistatic, making it liable to evolution of secondary resistance during  
389 prolonged treatment<sup>82</sup>. A key mechanism of resistance against fluconazole is the  
390 selection of intrinsically resistant cryptococcal sub-populations<sup>83</sup> that carry  
391 specific chromosomal disomies<sup>84</sup> and thus overexpress the *ERG11* gene (which  
392 encodes the fluconazole target enzyme lanosterol-14 $\alpha$ -demethylase<sup>85</sup>) or have  
393 enhanced drug efflux by the ATP Binding Cassette (ABC) transporter-encoding  
394 gene *C. neoformans* AntiFungal Resistance 1 (*CnAFR1*)<sup>86</sup>.

395

396 **New drugs.** Given the ongoing high global incidence and mortality from  
397 cryptococcal meningoencephalitis, the dearth of drugs, together with toxicity and  
398 the potential for development of resistance, there is an urgent need for new  
399 drugs. Recent activity in this area has begun to highlight potential routes either  
400 for the discovery of novel antifungals or for the repurposing of existing  
401 molecules showing anti-cryptococcal activity (**Figure 3**).

402 An ideal antifungal drug should be fungal-specific, to avoid host cell  
403 toxicity; this is challenging, given that fungal cellular processes are more closely  
404 related to mammals than those that are targeted by common antimicrobials,  
405 such as the ones used to target bacterial pathogens. Furthermore, an ideal  
406 antifungal drug should target either a virulence factor or a fungal component

407 essential for fungal viability. Such a drug should be fungicidal when used alone  
408 or when combined with the widely available fluconazole, should have good oral  
409 bioavailability (allowing it to be readily administered even in resource-poor  
410 settings) and be able to enter cryptococcal niches within the host (such as  
411 phagocytes and the CNS).

412 One obvious target of such a drug is the cryptococcal cell wall.  
413 Unfortunately, the latest class of antifungals active against the cell wall, the  $\beta$ -  
414 1,3-D-glucan synthase inhibitors (echinocandins), have no significant anti-  
415 cryptococcal activity. However, synthesis of another cell wall component,  
416 glycosylphosphatidylinositol (GPI)-anchored mannoproteins, is inhibited by the  
417 orally-active experimental molecule E1210, which has *in vitro* activity against  
418 *Cryptococcus* and other medically-relevant fungi (such as *Candida* and  
419 *Scedosporium* species) and is currently in pre-clinical development<sup>87</sup>.

420 Further along the development pipeline is VT-1129, an orally-available  
421 ergosterol synthesis inhibitor which shows good CNS penetration and is  
422 fungicidal in murine models of *Cryptococcus* infection. VT-1129 blocks the  
423 activity of CYP51, an essential enzyme in the pathway to produce ergosterol, and  
424 is currently entering human clinical trials <sup>88</sup>. Also in Phase I trials is the  
425 arlyamidine T-2307, which targets the fungal mitochondrial membrane<sup>89</sup>. T-  
426 2307 is a fungicidal injectable compound that shows comparable efficacy to AmB  
427 in murine models of infection.

428 Given the lack of market forces driving pharmaceutical development for a  
429 neglected disease such as cryptococcal meningoencephalitis, an alternative,  
430 cheaper and more expedient strategy in drug development is the repurposing of  
431 drugs not originally developed for antifungal use. Recently developed high-  
432 throughput screening techniques have advanced the repurposing effort. One  
433 such powerful tool is chemical-genetic profiling, whereby large collections of  
434 cryptococcal knockout mutants, for which the function of a particular pathway is  
435 compromised, are screened against a library of small molecules<sup>90</sup>, and the  
436 growth behavior of the screened strain (i.e. increased or decreased  
437 susceptibility) is then recorded. This technique was recently performed with  
438 1448 knockout mutants of *C. neoformans* and demonstrated distinct differences  
439 in drug susceptibility between this species and the model organism

440 *Saccharomyces cerevisiae* which, until now, has been the standard choice for such  
441 screens<sup>90</sup>. As proof of principle, this approach has identified a number of  
442 molecules that synergize strongly with fluconazole to inhibit ergosterol  
443 synthesis in *C. neoformans* and which are now being further investigated for  
444 potential clinical applicability. Moreover, this method has the additional  
445 advantage of providing information on the mechanism of action of lead  
446 compounds and can therefore identify both potential new drugs and potential  
447 new drug targets.

448 A more classical approach is to screen for compounds that trigger fungal  
449 lysis (detected by the release of adenylate kinase, a cytosolic enzyme, into the  
450 medium) or alter ATP content<sup>91</sup> (a particularly effective approach for identifying  
451 compounds that are antifungal under starvation conditions). This strategy has  
452 identified a collection of off-patent drugs<sup>92</sup> with anti-cryptococcal activity that  
453 are additive or synergistic with fluconazole. These include drugs as diverse as  
454 amiodarone (a cardiac anti-arrhythmic drug), phenothiazines (widely used  
455 antipsychotics) and tamoxifen (an estrogen antagonist used in the treatment of  
456 breast cancer). Illustrating the utility of these approaches, tamoxifen in  
457 combination with fluconazole, decreased the *C. neoformans* burden in the brain  
458 by  $\sim 1 \log_{10}$  CFU per gram of brain tissue, in a mouse model of infection<sup>93</sup>. Finally,  
459 another candidate that has emerge from repurposing screens is the anti-  
460 depressant sertraline (also known as Zoloft®), a drug that is fungicidal, has high  
461 CNS penetration, and appears to target fungal protein synthesis through an  
462 unknown mechanism<sup>94</sup>. Sertraline is currently being evaluated in combination  
463 with AmBd and fluconazole in a phase II/III clinical trial<sup>95</sup>.

464

## 465 **Outlook**

466 The last five years have seen a remarkable revolution in our understanding of  
467 cryptococcosis. A deeper understanding of the natural ecology and an  
468 appreciation of the genetic and phenotypic diversity of this group of pathogens is  
469 transforming our understanding of cryptococcal pathogenesis. Meanwhile, huge  
470 progress has been made in understanding the host immune response to infection  
471 and how this process is hijacked by cryptococci to drive latency, dissemination  
472 and proliferation. However, despite these advances, cryptococcosis remains a

473 major worldwide killer, causing hundreds of thousands of deaths per year and  
474 the anti-cryptococcal drug arsenal remains limited. To address this, there is  
475 renewed focus on translational research to discover and develop new  
476 therapeutic agents and to evaluate new therapeutic strategies in a clinical setting.  
477 Whilst progress is being made in this respect, more is urgently required, and  
478 advances in understanding of the pathogenesis of *Cryptococcus spp* offer new  
479 opportunities for developing therapeutics beyond the traditional approaches of  
480 killing the fungal cell or preventing its replication. In particular, the rapidly  
481 expanding understanding of the *Cryptococcus*-host interface opens up new  
482 avenues for potential therapy development; for instance, in modifying host  
483 inflammatory responses, augmenting phagocytic clearance of the fungus,  
484 disrupting population signaling or preventing migration to the CNS. Together,  
485 such approaches offer the hope of significantly reducing the huge global burden  
486 of infection and making fatal cryptococcosis a disease of the past.

487

#### 488 **Acknowledgements**

489 The authors gratefully acknowledge the help of Shichina Kannambath in  
490 preparing Figure 3 and apologize to those colleagues in the field whose work  
491 could not be included in this review due to space constraints. RCM is supported  
492 by funding from the European Research Council, Medical Research Council,  
493 Lister Institute and Royal Society. DLW received support from NIH T32 training  
494 grant AI007313, a University of Minnesota Doctoral Dissertation Fellowship, and  
495 a Dennis W. Watson Fellowship. KN is supported by funding from the National  
496 Institutes of Health. TB is supported by funding from the Wellcome Trust and the  
497 Medical research Council (UK). NS is supported by a Wellcome Trust Strategic  
498 Award in Medical Mycology and Fungal Immunology to the University of  
499 Aberdeen.

500

501 **Box 1. Clinical cryptococcosis.**

502 **Epidemiology.** Since cryptococci are capable of extended latency within host  
503 cells<sup>43</sup> and most humans encounter the organism in early childhood<sup>8</sup>, it has been  
504 assumed that most clinical cases represent reactivation of a longstanding,  
505 asymptomatic infection (triggered, for instance, by falling CD4<sup>+</sup> T-cell counts in  
506 HIV-infected individuals). The proportion of clinical disease representing  
507 reactivated latent disease versus primary infection is unknown in HIV-positive  
508 individuals, but a study in patients with cryptococcosis following solid-organ  
509 transplantation found only 52% of infections to be due to reactivation<sup>96</sup>,  
510 suggesting that the classical view of cryptococcosis as a reactivating infection  
511 may not be accurate.

512 Emerging data are also highlighting the heterogeneity of cryptococcal  
513 disease worldwide, as illustrated by the prevalence of serum cryptococcal  
514 antigen (CrAg) in HIV-positive cohorts in different countries (see the figure,  
515 which displays the highest recorded prevalence per country). In addition, it is  
516 now clear that there is also considerable global heterogeneity in the fungal  
517 population structure. For example, *C. neoformans* var *grubii* (serotype A) is the  
518 predominant global cause of HIV-associated cryptococcal meningoencephalitis,  
519 but in China this organism frequently infects apparently immunocompetent  
520 hosts<sup>97</sup>. Similarly, particular lineages of *C. neoformans* vary both in virulence<sup>98-100</sup>  
521 and in their ability to infect immunocompromised or immunocompetent  
522 individuals<sup>101</sup>. In the near future, intensive whole genome sequencing efforts for  
523 both cryptococcal isolates and affected patients offers the possibility of being  
524 able to explain the relative contribution of host and pathogen genotypes  
525 underlying these global patterns of disease.

526 **Susceptibility.** In contrast to other systemic fungal infections (such as  
527 candidiasis), relatively little is known about genetic risk factors for  
528 cryptococcosis. However, recent allelic association studies have shown that  
529 apparently immunocompetent individuals with cryptococcosis are significantly  
530 more likely to have defects in mannose-binding lectin<sup>102</sup> or be homozygous for  
531 the “232I” allele of the Fcγ receptor 2B (FcγR2B)<sup>103</sup>, although these  
532 polymorphisms are relatively common and thus, on their own, are clearly not  
533 sufficient to render an individual fully susceptible to cryptococcosis. Therefore,

534 subtle defects in the innate immune response to fungi may underlie at least some  
535 cases of *C. neoformans* infection in otherwise healthy individuals. Similarly, in  
536 HIV-positive patients, allelic variation in a different FcγR, FcγR3A, also correlates  
537 with susceptibility<sup>104</sup>. In this case, individuals with a higher affinity receptor  
538 variant are at greater risk of infection, perhaps indicating that efficient uptake of  
539 the pathogen may actually aid dissemination and drive more severe disease. This  
540 is particularly striking since the same is true from the pathogen perspective;  
541 cryptococcal strains that are more avidly phagocytosed drive more aggressive  
542 disease and carry a higher risk of death in patients<sup>105</sup>. Thus, excessive  
543 phagocytosis as a result of either host or pathogen variation appears to drive  
544 cryptococcal dissemination, strongly supporting the “Trojan Horse” model of  
545 pathogen spread (see the main text).

546 **Diagnosis.** Diagnosis of cryptococcosis relies on detection either of the organism  
547 itself or its shed capsular GXM polysaccharide in serum or CSF. This has been  
548 hugely facilitated by the introduction of the point-of-care lateral flow  
549 cryptococcal antigen assay, which is cheaper and more sensitive than earlier  
550 serological tests<sup>106</sup>. This test can detect very early dissemination and has  
551 facilitated cohort studies across the world, revealing a prevalence of  
552 cryptococcal antigens in HIV-infected patients ranging between 2 and 21%. As an  
553 increasing proportion of cases of cryptococcal meningoencephalitis are now  
554 presenting as “unmasking” of latent infection following therapy (i.e. the  
555 appearance of clinical symptoms following immune reconstitution by  
556 antiretroviral treatment), wider implementation of a ‘screen-and-treat’ approach  
557 is cost effective as a public health intervention and has been demonstrated to  
558 reduce mortality in African HIV cohorts in the first year on ART<sup>107</sup>.

559

## 560 **Box 2: The evolutionary history of cryptococci**

561 The two *Cryptococcus* species, *C. gattii* and *C. neoformans*, probably diverged  
562 from a common environmental saprophyte ancestor around 30-40 million years  
563 ago<sup>108,109</sup> (see the figure). For *C. neoformans*, extensive genetic data now  
564 indicates a common origin in sub-Saharan Africa<sup>5,110,111</sup>. The observation that  
565 most non-African *C. neoformans* populations are near-clonal supports a model in  
566 which recombining African populations of cryptococci occasionally dispersed to

567 other parts of the globe. Coalescence analyses indicate that almost all of these  
568 events have occurred within the last 5000 years, suggesting the potential  
569 involvement of human or avian migrations in this process<sup>5</sup>.

570 Probing the origin and diversity of *C. gattii* has proven more challenging.  
571 There is a growing consensus that the evolutionary origins of this species lie  
572 within Australia and South America, since most dispersed lineages of *C. gattii* are  
573 near clonal (such as the lineage responsible for the Pacific Northwest Outbreak)  
574 but always cluster with Australian and South American isolates during  
575 phylogenetic analyses, with an estimated origin within the last 50 thousand  
576 years <sup>112-114</sup>. A recurrent theme therefore appears to be that local populations of  
577 *C. gattii* in endemic areas (such as Brazil) undergo continual recombination,  
578 which occasionally results in a novel recombinant lineage that disperses and  
579 expands rapidly by means of clonal growth (either asexual cell division or same-  
580 sex mating) <sup>112,113,115</sup>.

581 Both species of *Cryptococcus* have a bipolar mating system in which cells  
582 are either mating type a (MATa) or mating type alpha (MAT $\alpha$ ) (reviewed in <sup>116</sup>).  
583 Classical mating involves genetic exchange between a MATa and MAT $\alpha$  strain,  
584 followed by normal Mendelian segregation of alleles. However, both species of  
585 cryptococci are also capable of same-sex mating in which two strains of the same  
586 mating type are able to exchange genetic material <sup>115,117</sup>. In addition, diploid and  
587 aneuploid strains are not uncommon<sup>118,119</sup>, and inter- and intra-species hybrids  
588 can be found both in the environment and in patients<sup>120,121</sup>. Thus the global  
589 population structure of these pathogens reflects a complex mix of “diversity  
590 generating” recombination and aneuploidy, coupled with highly clonal  
591 amplification steps during dispersion events.

592

### 593 **Box 3: The evolution of virulence in cryptococci**

594 Opportunistic pathogens represent an evolutionary enigma: why has natural  
595 selection driven the acquisition of often highly specific virulence factors when  
596 the majority of the population remain as exclusively environmental organisms  
597 for their entire existence? This conundrum is particularly pertinent for  
598 cryptococci, which are abundant in the environment and yet are remarkably well  
599 suited to survive in a human host.

600           A compelling hypothesis to resolve this conundrum is that of “accidental  
601 pathogenesis”<sup>122</sup>. This hypothesis proposes that cryptococcal pathogenesis does  
602 not result from direct selection for virulence within a mammalian host, but  
603 rather by the evolution of traits (which happen to be advantageous in mammals)  
604 in response to other selective pressures in both environmental and animal  
605 niches. So, for instance, the complex polysaccharide capsule, laccase activity and  
606 ability to synthesize melanin, which are all *Cryptococcus* virulence factors, are  
607 likely to offer protection against environmental pressures such as desiccation or  
608 exposure to ultraviolet light <sup>123</sup>, or aid in the colonization of plant hosts <sup>124</sup>.  
609 Similarly, cryptococci can replicate not only within vertebrate phagocytes, but  
610 also within free-living phagocytic amoebae<sup>125</sup> (see the figure). Despite the  
611 enormous evolutionary distance between vertebrates and amoebae, many of the  
612 mechanisms used by phagocytic white blood cells to kill pathogens (e.g. the  
613 generation of reactive oxygen species or secretion of antimicrobial peptides) are  
614 identical to those used by amoebae to digest ingested prey. Thus, over millions of  
615 years, cryptococci have been selected to evolve strategies that facilitate fungal  
616 growth and persistence within amoebae that coincidentally also enable their  
617 survival within phagocytes. Such strategies include not only stress-tolerance  
618 approaches, such as resistance to reactive oxygen species<sup>126</sup>, but also elaborate  
619 mechanisms to regulate expulsion from host cells<sup>46,47</sup>.

620           In addition, *Cryptococcus* has a remarkable ability to perturb adaptive  
621 immunity, preventing complete fungal clearance and resulting in latent  
622 infections.<sup>19,127</sup>. Perhaps the ability to remain latent without perturbing its host  
623 is the strongest evidence for host adaptation by *Cryptococcus*. Since only higher  
624 vertebrates have adaptive immune systems, *Cryptococcus* species probably  
625 evolved these properties under the selective pressures of reptilian, avian or  
626 mammalian hosts within the environment, which also explains the diverse range  
627 of animals that can succumb to cryptococcosis.

628           Taken together, these observations suggest that interactions with both  
629 soil microorganisms (such as amoebae and nematodes) and vertebrates likely  
630 have a critical role in the virulence potential of *Cryptococcus* (reviewed in <sup>128</sup>).  
631 Intriguingly, laboratory studies have shown that selection pressure by amoebae  
632 can rapidly select for resistant, pseudohyphal forms of cryptococci<sup>23</sup>. These

633 forms are attenuated in mammalian hosts and consequently frequently revert to  
634 yeast upon entry into a vertebrate host. Thus, rapid microevolutionary events  
635 may have an important role in driving cryptococcal pathogenesis in different  
636 hosts.

637 The paradigm of ‘accidental pathogenesis’ extends beyond cryptococci to  
638 other fungal<sup>129</sup> and even bacterial pathogens<sup>130</sup>, such as *Aspergillus*, *Blastomyces*  
639 and *Legionella* species, and highlights two important issues. Firstly, as pathogens  
640 adapt to changing environments due to global warming, we may see additional  
641 instances of “accidental pathogenesis” through the selection of new traits that  
642 promote both environmental survival and pathogenesis in humans. Secondly, we  
643 should be alert to the fact that changes in human behavior and habitat use (e.g.  
644 increased tourist access to remote rainforest or desert areas) may expose us to  
645 novel potential pathogens that have been predisposed to infection via selection  
646 through environmental predators.

647

#### 648 **Box 4. Host immunity: too little or too much?**

649 Poor inflammatory responses to cryptococci, such as those in patients with  
650 advanced HIV infection, lead to life-threatening meningoencephalitis.  
651 Consequently, immune profiling of patient peripheral T-cell responses and CSF  
652 cytokines has shown that those mounting a pro-inflammatory immune response  
653 are more likely to clear the pathogen and survive infection<sup>66</sup>. Moreover,  
654 augmenting pro-inflammatory immune responses using adjunctive IFN- $\gamma$   
655 improves fungal clearance<sup>131</sup>. Conversely, individuals producing anti-cytokine  
656 antibodies that interfere with appropriate inflammatory responses are known to  
657 be at enhanced risk of infection<sup>132</sup>.

658 Although a potent immune response to *Cryptococcus* is clearly essential  
659 for fungal clearance, too strong a response can also be harmful. For instance, a  
660 low level of anti-inflammatory activity driven by both Th2 and regulatory T  
661 cells<sup>133,134</sup> prevents complete immune paralysis. This is also the case for the  
662 classical antifungal cytokine IL-17, which is essential for resistance to  
663 cryptococcosis<sup>135</sup> but whose effects must also be regulated by IL-23 in order to  
664 prevent damage to the host due to excessive inflammation<sup>136</sup>. Thus a “successful”  
665 immune response to cryptococcal infection appears to be a complex blend of

666 Th1, Th2 and Th17 responses, which must be counter-regulated to prevent  
667 either runaway fungal growth or damaging levels of inflammation.

668 This critical role for “restraining” inflammatory signaling is particularly  
669 highlighted by the problem of immune reconstitution inflammatory syndrome  
670 (IRIS). This life-threatening inflammatory reaction occurs in some HIV-infected  
671 patients during antiretroviral therapy (ART) and is attributable to the newly  
672 reconstituted immune system “overreacting” to residual pathogen antigen.  
673 Consequently, the timing of clinical intervention is critical; early introduction of  
674 ART is important to restore cell-mediated immunity, but if introduced too early  
675 (during the initial 2 weeks following induction of antifungal treatment) at a time  
676 of high fungal load, the risk of death is increased<sup>137</sup>. Development of IRIS is  
677 particularly likely in patients whose initial pro-inflammatory response to  
678 cryptococcal infection is poor, resulting in high residual antigen burden<sup>138</sup>.  
679 Coupled with an exaggerated baseline CNS chemokine response, this results in  
680 aberrant CNS immune responses following ART initiation, resulting in IRIS.

681 Excessive inflammation can also occur following withdrawal of immune  
682 suppression in solid organ transplant recipients, as well as in apparently  
683 immunocompetent patients. In such situations, steroids are often administered  
684 alongside antifungals. It remains unclear, however, whether steroids are  
685 beneficial in other contexts: a multi-centre clinical trial to address this issue  
686 (investigating the effect of adjunctive dexamethasone in patients with HIV-  
687 associated cryptococcal meningoencephalitis) has been terminated early and  
688 results are awaited<sup>139</sup>.

689

690

691

692 **Figure 1. Inflammatory signaling in response to cryptococcal infection.**

693 Cryptococci inevitably shed microbial molecules that contain pathogen  
694 associated molecular patterns (PAMPS). Such fungal molecules are typically cell  
695 wall or capsular components such as chitin,  $\beta$ -glucan or glucuronoxylomannan  
696 (GXM), which are detected by immune sentinel cells, most notably dendritic cells  
697 (DCs). DC activation then summons T-cell help, inducing CD4<sup>+</sup> T-cells to secrete  
698 cytokines that induce a T helper cell 1 (Th1) response (such as interleukin (IL)-  
699 12 and IL-23). Th1 cells produce pro-inflammatory cytokines (such as IFN- $\gamma$ )  
700 that ultimately control fungal infection. However, some fungal PAMPs can  
701 influence DC activation, including modulating the levels of MHC-II or NF- $\kappa$ B  
702 signaling. This leads to the generation of a Th2 response (mediated by the  
703 production of cytokines such as IL-4 and IL-13); this anti-inflammatory  
704 environment impacts the ability of macrophages to mediate fungal clearance.

705

706 **Figure 2. Infection establishment and dissemination within the human**

707 **host.** Cryptococcal cells typically enter the human host through the lung. Here  
708 they are recognized by patrolling phagocytes but can avoid uptake either by  
709 growing into very large “Titan” cells, or by relying on the antiphagocytic  
710 properties of the fungal capsule. If uptake occurs, however, cryptococci are able  
711 to survive and persist within phagocytes. For most strains, a failure in host  
712 immune function is then required to allow intracellular proliferation. However,  
713 the unusual Pacific Northwest Outbreak (PNO) strains of *C. gattii* can proliferate  
714 within immunocompetent host cells by exploiting a poorly-characterized  
715 “Division of Labour” mechanism: in response to reactive oxygen species  
716 generated by the phagocyte, some cryptococcal cells acquire an unusual  
717 morphology characterized by extensive tubularisation of their mitochondria,  
718 which increases survival of neighboring cells (via a mechanism that remains  
719 unclear). *Cryptococcus* proliferation within phagocytes ultimately leads either to  
720 host cell lysis or to a novel non-lytic escape mechanism termed vomocytosis.  
721 Upon replication in the lung, cryptococci are able to disseminate to other tissues,  
722 including the central nervous system (CNS). Entry into the CNS can occur in  
723 three ways: by squeezing between host endothelial cells (paracytosis), which

724 involves the fungal protease Mpr1; by moving directly through endothelial cells  
725 (transcytosis), in a process that is mediated by hyaluronic acid in the fungal  
726 capsule and the host receptor CD44; or by “hitching a ride” within migrating  
727 phagocytes, in a process termed the “Trojan horse” hypothesis.

728

729 **Figure 3: Current and future therapies for cryptococcosis.** Schematic  
730 representation of a cryptococcal cell, showing key current and potential  
731 therapeutic targets and examples of antifungal drugs acting at each site. Drugs in  
732 current clinical use are shown in red, novel drugs are shown in blue and  
733 repurposed drugs are shown in green. The three classes of antifungal agents  
734 currently used to treat cryptococcosis are polyenes (amphotericin B), azoles  
735 (fluconazole) and the pyrimidine analogue flucytosine (5-FC) Amphotericin B  
736 deoxycholate (AmBd) acts by binding to ergosterol in the cryptococcal cell wall,  
737 generating pores in the cell membrane, and by inducing cell death via oxidative  
738 damage. 5-FC is deaminated by the fungal enzyme cytosine deaminase into 5-  
739 fluorouracil (5-FU), which then inhibits thymidylate synthetase and blocks DNA  
740 synthesis or is converted into 5-fluorouridine triphosphate, which is  
741 incorporated into RNA and disrupts protein synthesis. Fluconazole inhibits the  
742 fungal cytochrome P450 enzyme, 14 $\alpha$ -demethylase, which is required for  
743 conversion of lanosterol to ergosterol, an essential component of the fungal cell  
744 membrane. E1210 inhibits the synthesis of the cell wall component  
745 glycosylphosphatidylinositol (GPI)-anchored mannoproteins. VT-1129 blocks  
746 the activity of CYP51, an essential enzyme in the pathway to produce ergosterol.  
747 The arlyamidine T-2307 targets the fungal mitochondrial membrane. Tamoxifen  
748 (an estrogen antagonist used in the treatment of breast cancer) targets  
749 calmodulin and the anti-depressant sertraline appears to target fungal protein  
750 synthesis through an unknown mechanism.

751

752

753 **GLOSSARY**

754 *Pacific Northwest Outbreak* – an unusual cluster of cryptococcal disease in  
755 otherwise healthy (rather than immunocompromised) individuals. First  
756 identified on Vancouver Island, British Columbia, in 1999 (and hence originally  
757 called the Vancouver Island Outbreak), both the causative organism and cases of  
758 human and animal disease have now expanded into mainland Canada and the  
759 northwestern USA, prompting a renaming of the outbreak.

760  
761 *Iatrogenic* – caused by medical treatment. For instance, infections due to  
762 contaminated surgical instruments.

763  
764 *Zoonotic* – a disease transmitted from non-human animals to people

765  
766 *Diploid* – having two homologous sets of chromosomes, one from each parent

767  
768 *Aneuploid* – having an ‘unbalanced’ set of chromosomes; for instance, having only  
769 a single copy of one chromosome in an otherwise diploid genome.

770  
771 *Polyloid* – having multiple (more than two) sets of homologous chromosomes

772  
773 *Founder* – the initial (small) group of individuals that seeds a new population.  
774 For instance, the inoculum that starts an infection, or the first individuals to  
775 arrive on a new island habitat.

776  
777 *Quorum sensing* – the regulation of gene expression or behavior in response to  
778 changes in the local population size.

779  
780 *Paracrine* – a signal that acts close to where it is produced; for instance, on  
781 neighbouring cells.

782  
783 *Filamentation* – the growth of an organism by elongation without division.

784  
785 *MHC Class II* – molecules that are expressed on the surface of professional  
786 antigen presenting cells (such as macrophages and dendritic cells) and present  
787 extracellular antigens to the immune system to coordinate an immune response

788  
789 *Th1/Th2 response* – A broad characterization of the differentiation of CD4<sup>+</sup>  
790 helper T cells (Th). Th1 responses are generally provoked by intracellular  
791 pathogens and Th2 responses are typically involved in the elimination of  
792 parasitic worms, harmful allergic responses, and dampening of Th1-mediated  
793 inflammation. In the context of cryptococcal infection, Th1 responses are widely  
794 thought to be protective and Th2 responses are detrimental.

795  
796 *Melanization* – the production of the dark, insoluble pigment melanin, which  
797 provides protection from high energy radiation and reactive oxygen molecules.

798  
799 *Blood-brain barrier* – a specialized endothelial barrier that prevents the entry of  
800 cells or large molecules into the central nervous system

801

802 *Paracytosis* – transitioning between tissues by moving between, rather than  
803 through, adjacent cells.  
804  
805 *Transcytosis* - transitioning between tissues by moving directly through cells,  
806 rather than between adjacent cells.  
807  
808 *Hyaluronic acid* – an abundant, high molecular weight polysaccharide that forms  
809 part of the extracellular matrix, particularly in neural tissue.  
810  
811 *Cerebrospinal fluid (CSF)* – a clear fluid produced in the brain which bathes the  
812 central nervous tissue and is slowly turned over.  
813  
814 *Fungistatic* – an antimicrobial agent that prevents fungal growth, but does not  
815 kill the organism  
816  
817 *Fungicidal* – an antimicrobial agent that kills fungi, rather than simply preventing  
818 growth  
819  
820 *Coalescence analysis* – an evolutionary analysis method in which genetic drift is  
821 “played backwards” in order to calculate common ancestry of individuals within  
822 a population and thereby estimate lineage branch points within an evolutionary  
823 phylogenetic tree.  
824  
825 *Bipolar mating* – a system to control sexual reproduction that relies on a single  
826 genetic locus at which individual organisms can carry one of two alleles,  
827 effectively generating a species with two sexes.  
828  
829 *Regulatory T-cell* – a type of T-cell that functions to regulate the immune system,  
830 typically by suppressing the function of proinflammatory effector T-cells.

831  
832

833 **Online only**

834

835 **Author bios**

836 Kirsten Nielsen received her PhD from North Carolina State University and then  
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842

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858

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861 and cryptococcosis. Initially, with Tom Harrison, she conducted clinical trials in  
862 treatment of HIV-associated cryptococcal meningitis in Southern Africa. More  
863 recently her group have been exploring the relationship between pathogen  
864 phenotype and genotype with clinical presentation and outcome of human

865 cryptococcosis, host genetic susceptibility to cryptococcal infection and the  
866 evolution and mechanisms of fluconazole resistance in *Cryptococcus* sp.

867

868 Neil Stone is a Specialist Registrar physician in Infectious Diseases and  
869 Microbiology and is a Wellcome Trust Clinical Research Fellow at the Institute of  
870 Infection and Immunity at St. George's, University of London, with an interest in  
871 neglected infections in the developing world and cryptococcal meningitis in  
872 particular. He is currently undertaking a clinical PhD under the supervision of  
873 Dr Tihana Bicanic and Professor William Hope, investigating the evolution of  
874 fluconazole resistance in a cohort of patients with cryptococcal meningitis in  
875 Tanzania, East Africa.

876

### 877 **Online Summary**

878 • Cryptococcosis is a widespread opportunistic fungal infection of humans  
879 and other animals.

880 • *Cryptococcus* species that infect humans likely evolved as “accidental  
881 pathogens” in response to environmental selective pressure.

882 • Recent genomic analyses have highlighted the evolutionary history of  
883 *Cryptococcus* species and narrowed down the geographical origin of an unusual,  
884 hypervirulent outbreak.

885 • Despite being accidental pathogens, cryptococci display a remarkable  
886 ability to manipulate the human immune response in order to facilitate disease  
887 establishment and spread.

888 • Detailed *in vivo* and *in vitro* characterization of *Cryptococcus* species has  
889 started to elucidate the details of multiple mechanisms of pathogenesis that  
890 likely have important roles in disease severity. These include changes in fungal  
891 morphology, interactions with host phagocytes and mechanisms that allow  
892 *Cryptococcus* to disseminate from the lung to the CNS.

893 • Renewed efforts to develop improved therapeutic approaches have  
894 highlighted potential new drugs and potential new uses for old drugs in the fight  
895 against cryptococcal disease.

896

897 **ToC blurb**

898 Recent studies have elucidated multiple virulence mechanisms used by  
899 *Cryptococcus* to infect, disseminate within and ultimately kill their human host.  
900 In this Review, May *et al.* describe these recent developments in understanding  
901 host-fungal interactions, discuss how they affect disease severity and debate  
902 current and future therapeutic interventions against cryptococcosis.

903

#### 904 **Subject categories**

905 Biological sciences / Microbiology / Fungi / Fungal pathogenesis

906 [URI /631/326/193/2542]

907 Biological sciences / Microbiology / Fungi / Fungal immune evasion

908 [URI /631/326/193/2545]

909 Biological sciences / Microbiology / Fungi / Fungal host response

910 [URI /631/326/193/2544]

911 Biological sciences / Microbiology / Antimicrobials / Antifungal agents

912 [URI /631/326/22/1292]

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