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Antibody action after phagocytosis promotes *Cryptococcus neoformans* and *Cryptococcus gattii* macrophage exocytosis with biofilm-like microcolony formation

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Summary

Antibody-mediated phagocytosis was discovered over a century ago but little is known about antibody effects in phagolysosomes. We explored the consequences of antibody-mediated phagocytosis for two closely related human pathogenic fungal species, *Cryptococcus neoformans* and *Cryptococcus gattii*, of which *C. neoformans* encompasses two varieties: *neoformans* and *grubii.* The interaction between *C. neoformans* varieties *grubii* and *neoformans* and host cells has been extensively studied, but that of *C. gattii* and macrophages remains largely unexplored. Like *C. neoformans*, antibody-mediated phagocytosis of *C. gattii* cells was followed by intracellular replication, host cell cytoplasmic polysaccharide accumulation and phagosomal extrusion. Both *C. gattii* and *C. neoformans* cells exited macrophages in biofilm-like microcolonies where the yeast cells were aggregated in a polysaccharide matrix that contained bound antibody. In contrast, complement-opsonized *C. neoformans* variety *grubii* cells were released from macrophages dispersed as individual cells. Hence, both antibody- and complement-mediated phagocytosis resulted in intracellular replication but the mode of opsonization affected the outcome of exocytosis. The biofilm-like microcolony exit strategy of *C. neoformans* and *C. gattii* following antibody opsonization reduced fungal cell dispersion. This finding suggests that antibody agglutination effects persist in the phagosome to entangle nascent daughter cells and this phenomenon may contribute to antibody-mediated protection.

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Introduction

Cryptococcus neoformans is an encapsulated yeast that is a relatively frequent cause of human disease. *C. neoformans* infection is acquired by inhalation, and host defence against this fungus is critically dependent on innate immune mechanisms including alveolar macrophages (Casadevall and Perfect, 1998). *C. neoformans* has a polysaccharide capsule that interferes with phagocytosis and phagocytic cells are not able to ingest fungal cells without opsonins (Kozel *et al*., 1988; Kozel, 1993). Both antibody to the capsule and complement can be effective opsonins but there are large differences in the efficacy of complement-mediated phagocytosis depending on the strain and the complement source, and antibody-mediated opsonization can promote ingestion through the complement receptor (CR) in the absence of complement components (Netski and Kozel, 2002; Taborda and Casadevall, 2002; Zaragoza *et al*., 2003; Kelly *et al*., 2005). Hence, phagocytosis of *C. neoformans* is thought to be important for host defence but the efficacy of the process is variable and is made complicated by the specific particularities of this encapsulated fungus.

Cryptococcus neoformans strains have historically been grouped into three varieties on the basis of genotypic and phenotypic characteristics, including antigenic differences in their main capsular polysaccharide, glucuronoxylomannan (GXM) (Casadevall and Perfect, 1998). These are var. *neoformans* (serotype D), var. *grubii* (serotype A) and var. *gattii* (serotype B or C) (Casadevall and Perfect, 1998; Taylor *et al*., 2002). However, molecular clock analysis suggests that varieties *gattii* and *neoformans* separated over 30 million years ago (Xu *et al*., 2000) with a phylogenetic distance that may be sufficient to classify them as different species (Ren *et al*., 2005). Hence, for the purposes of this article we assume the existence of two species, *Cryptococcus gattii* and *C. neoformans*, with the latter including two varieties, *neoformans* and *grubii*. Varieties *neoformans* and *grubii* cause meningoencephalitis primarily in immunocompromised individuals, while *C. gattii* does so primarily in immunocompetent individuals (Taylor *et al*., 2002; Narasipura *et al*., 2003). Furthermore, there are differences in

the pathogenesis of cryptococcosis caused by *C. gattii*, which is associated with the formation of pulmonary cryptococcomas (Chen *et al*., 2000) that often require surgical removal (Fisher *et al*., 1993).

Cryptococcus gattii is found mostly in tropical and subtropical habitats where it has been isolated from eucalyptus tree (*Eucalyptus camaldulensis* and *Eucalyptus tereticornis*) (Casadevall and Perfect, 1998; Campbell *et al*., 2005). In contrast, *C. neoformans* varieties *grubii* and *neoformans* can be isolated from all regions of the world and are commonly found in avian excreta (Casadevall and Perfect, 1998; Taylor *et al*., 2002; Raso *et al*., 2004). The overall medical importance of *C. gattii* is increasing given recent outbreaks of *C. gattii*-related disease in humans and animals in Vancouver and Spain suggesting that this organism may be expanding its habitat range, possibly as a result of climatic changes (Baro *et al*., 1998; Hoang *et al*., 2004).

Both *Cryptococcus* varieties *grubii* and *neoformans* are facultative intracellular pathogens that can be extruded from, spread to or lyse macrophages, but relatively little is known about the cellular pathogenic strategy of *C. gattii* (Alvarez and Casadevall, 2006; 2007). Consequently, we sought to characterize the interaction of *C. gattii* with murine macrophages and compare the outcome of phagocytosis to the other cryptococcal varieties. Our results establish that *C. gattii*, like the *C. neoformans* varieties, is a facultative intracellular pathogen with similar pathogenic strategies to the *C. neoformans* varieties, despite their ancient divergence from a common ancestor. Most interestingly, we observed that the degree of cellular dispersal of *C. neoformans* and *C. gattii* following macrophage exocytosis was dependent on the opsonizing agent used. These results illustrate the importance of opsonin type on the outcome of phagocytosis and imply the persistence of antibody-mediated effects during the intracellular life of certain microbes.

Results

Exocytosis and secondary phagocytosis

Prior studies from our group and others have shown that *C. neoformans* exits macrophages after intracellular replication by a process that involves phagosomal extrusion (Ma *et al*., 2006; Alvarez and Casadevall, 2007). Time-lapse imaging revealed that released cells were phagocytosed again but only when opsonic monoclonal antibody (mAb) was present in the media (data not shown) or when yeast cells from a previously lysed macrophage group that contained ingested yeast cells were added to previously uninfected macrophages (Movie S4). To reduce the likelihood of secondary phagocytosis events, all imaging studies described herein were carried out in media without opsonic antibody. This was accomplished by washing the monolayer after the initial phagocytosis to remove free antibody and extracellular yeast cells followed by replacing the media with fresh, antibody-free media.

Opsonization of C. neoformans *and* C. gattii

The opsonic requirements for *C. neoformans* have been extensively studied (Kozel, 1993; Kelly *et al*., 2005; Macura *et al*., 2007). In general, antibody to GXM is opsoninc for all strains but there is great variation in the efficiency of complement-mediated phagocytosis with mouse or guinea pig sera being opsonic for strain H99 but not as efficiently for 24067 (Zaragoza *et al*., 2003). Prior studies had shown that IgM could promote complement-independent phagocytosis of *C. grubii* and *C. neoformans* via the CR3 and CR4 receptor by a mechanism that presumably involved a change in capsule structure that facilitates direct interactions of the capsular polysaccharide with both receptors (Taborda and Casadevall, 2002). This phenomenon also occurred with IgG and $F(ab)_2$ fragments, and consequently IgG phagocytosis occurred through both Fc and CR receptors even in the absence of complement (Netski and Kozel, 2002). As the capsular polysaccharide of *C. gattii* is more highly substituted than that of the other varieties (Cherniak *et al*., 1995) we sought to establish whether antibody-mediated complement-independent phagocytosis through CR also occurred with this variety, especially when considering comparative studies of macrophage fungal cell interactions involving the different cryptococcal varieties and species. A dose-dependent relationship was observed with IgM mAb 12A1 in phagocytosis assays, whereby increasing concentrations of IgM promoted an increase in phagocytosis by J774.16 cells in the absence of complement (Fig. 1), as noted previously to occur with serotypes A and D (Taborda and Casadevall, 2002). For comparison, strain 24067 was used as a positive control and phagocytosis indices were similar for both the *C. gattii* I23 and *C. neoformans* 24067 strains (data not shown). To confirm that ingestion occurred via the CRs, phagocytosis was performed in the presence of antibodies to CR3 and CR4, and resulted in significantly reduced phagocytic indices (Fig. 1). Hence, the ingestion mechanism for *C. neoformans* and *C. gattii* cells opsonized with antibody was similar.

C. gattii *replicates in macrophage-like cells*

Cryptococcus gattii replicated in J774.16 cells at similar rates to *C. neoformans* and *grubii*, that is, every 2–3 h on average (data not shown). Macrophages infected with *C. neoformans* and *grubii* are known to contain vesicles

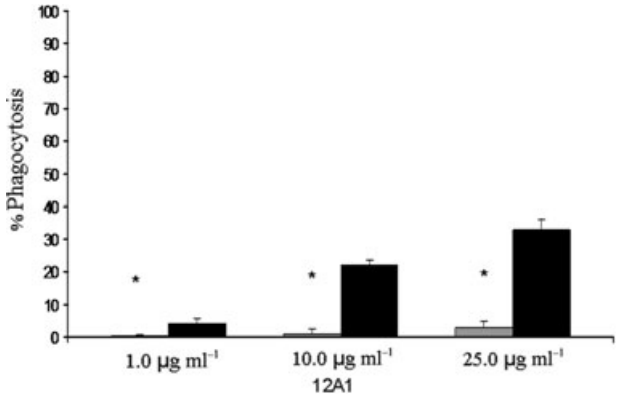

Fig. 1. IgM-mediated complement-independent phagocytosis of *C. gattii* via CR3 and CR4. Phagocytosis assays with various concentration of IgM (12A1) were performed and a dose-dependent opsonization of *C. gattii* and subsequent phagocytosis was observed (black bars). Blockage of CR with anti-CR ablates phagocytosis of *C. gattii* (grey bars with asterisk on top). Line bars represent data carried out in triplicates, ± SD.

with capsular polysaccharides (Tucker and Casadevall, 2002). To investigate whether the same phenomenon followed macrophage infection with *C. gattii*, J774.16 cells infected with *C. gattii* were permeabilized and incubated with conjugated Alexa 546-18B7, which binds GXM. The cells were then examined in a fluorescence microscope for the presence of cytoplasmic vesicles containing polysaccharide. As in previous studies, vesicles positive for polysaccharide were identified starting at 18 h post infection (Fig. 2A). A group of control-uninfected cells gave no positive signal even when overexposed (Fig. 2B). In addition to replication and polysaccharide shedding, macrophages containing all *C. gattii* strains were observed to extrude the *C. gattii* cells.

Interaction with primary macrophages

To ascertain whether the results obtained with macrophage-like J774.16 cells also occurred with primary macrophages we compared the outcome of infection of alveolar and peritoneal macrophages from BALB/c mice.

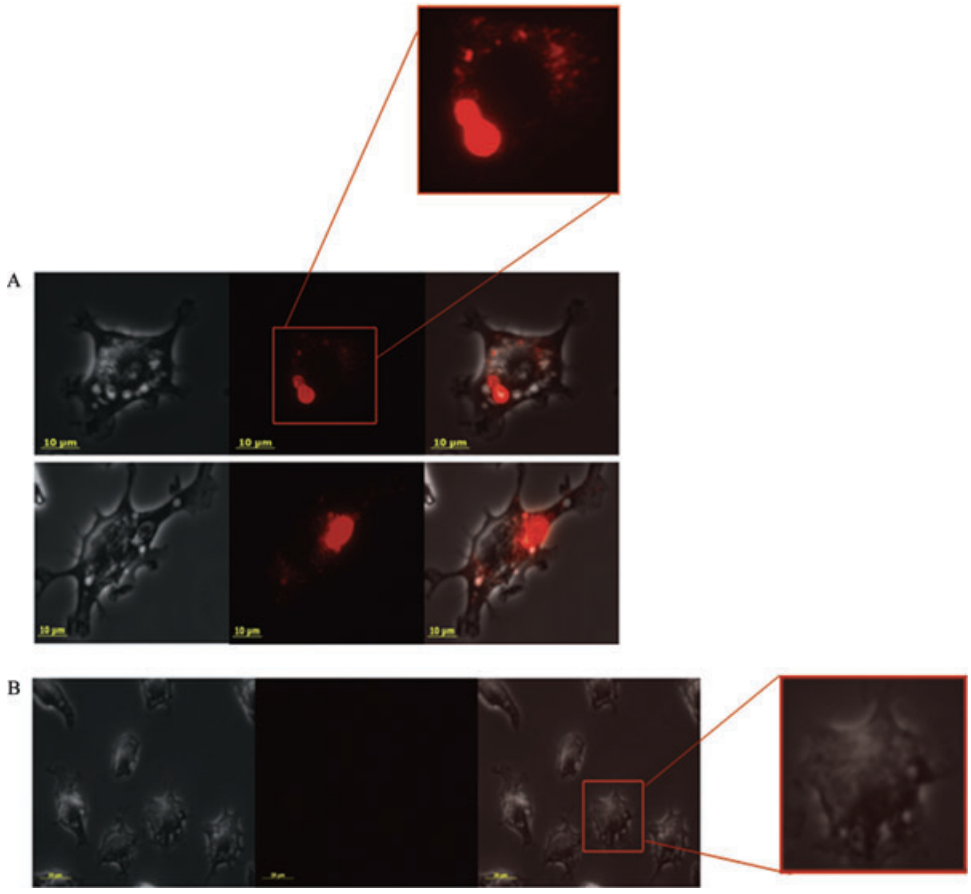

Fig. 2. Intracellular polysaccharide shedding by *C. gattii* cells. Organized left to right are phase, fluorescence and merged phase and fluorescence images taken at 40×.
A. Intracellular shedding of cryptococcal polysaccharide from *C. gattii* cells into J774.16 cells. Top: 5.0 s exposure, bottom: 4.0 s exposure.
B. J774.16 cells lacking intracellular cryptococcal cells showed no fluorescence, even when overexposed (20.0 s).

As observed with J774.16 cells, intracellular replication in alveolar BALB/c macrophages infected with *C. neoformans* varieties *neoformans* and *grubii* and *C. gattii* led to host cell lysis, or phagosomal extrusion as previously described (Alvarez and Casadevall, 2006; Ma *et al*., 2006). For *C. neoformans* we observed that the yeast cell intracellular replication in alveolar macrophages required at least 4 h after ingestion by primary cells whereas in peritoneal macrophages or J774.16 cells fungal buds were noted to emerge within 1–2 h after ingestion. Regardless of whether there was a time lag or not, intracellular replication led to lysis of the host macrophage or to extrusion of the cryptococcal-containing phagosome. Hence, the interactions between *C. neoformans* var. *neoformans* and *grubii*, and *C. gattii* with primary macrophages and J774 were similar, although alveolar macrophages were transiently less permissive for intracellular replication.

C. neoformans *and* C. gattii *extrusion results in microcolony formation*

Extrusion of mAb (IgG)-opsonized *C. neoformans* and *C. gattii* resulted in the release of a clump of yeast cells that remained attached to one another and at times to the macrophage cell from which it was extruded. These cells then continued to replicate extracellulary resulting in the growth of the clump (Fig. 3A). Like *C. gattii* cells, *C. neoformans* cells were able to clump but to a minor degree as compared with *C. gattii*, where clumping was more prominent (Movies S1 and S2). Additionally, the yeast cells appeared encased within a polysaccharide matrix as indicated by the strong fluorescence when stained with a capsule-binding mAb followed by fluorescein- or rhodamine-conjugated secondary antibody (Fig. 3B–E). In contrast, complement-opsonized *C. neoformans* var. *grubii* cells (strain H99) disseminated freely following extrusion and the individual free floating cells continued to divide in the extracellular milieu as single cells (Fig. 4; see Movie S3). Unfortunately, it was not possible to examine the outcome of complement-mediated phagocytosis for *C. neoformans* strain 24067 or the *C. gattii* strain NIH 198 because mouse, guinea pig and pooled human sera each were not opsonic for these strains. Further, macrophages were not observed to have ingested these strains when examined using live time-lapse microscopy for up to 24 h (data not shown).

Clumps of microcolonies from extracellular media were collected and compared with *C. gattii* cells grown in feeding and SAB broth. Interestingly, when *C. gattii* cells passaged though macrophages were collected and transferred to fungal media the clumps maintained their integrity (Fig. 5A), whereas overnight *C. gattii* cultures grown in feeding media and sabouraud dextrose broth did not have microcolony formations (Fig. 5B and C). Additionally, when *C. gattii* were grown in the presence of mAb 18B7 clumping occurred (Fig. 5D) as opposed to when yeast cells were grown in the presence of 20% guinea pig or pooled human sera (Fig. 5E). The same observations were made using strain *C. neoformans* strain H99. As the experimental design involved the removal of the initial supernatant containing antibody opsonin followed by washing of the monolayer and replacement with fresh media without antibody, it is probable that the formation of microcolonies following intracellular passage is a result of antibody-mediated agglutination resulting from residual capsule bound antibody. To test this we stained extruded cells with goat anti-mouse IgG_1 to ascertain whether the opsonic antibody persisted in the capsule of extruded yeasts. The results showed the presence of mAb in cryptococcal capsules post extrusion (Fig. 6A and B). As macrophages were not permeabilized and the media contained no antibody, only extruded extracellular cryptococcal cells that retained bound mAb from the initial opsonizing conditions were stained by the secondary antibody. Extruded yeast cells that had undergone budding in the extracellular milieu were not stained at the site of the new bud suggesting that the staining was specific for those yeast cells that were initially coated with mAb (data not shown). Control experiments with yeast cells that were incubated with the secondary antibody alone did not stain (data not shown). We noted that some macrophage-like cells that were incubated with mAb for 1 h, permeabilized and then incubated with a secondary antibody were stained suggesting that the cells were also endocytosing the mAb (Fig. 6C). Control cells that were not incubated mAb 18B7 did not stain at all (data not shown). Hence, it appears that some cells take up mAb and that it is then found in round, vesicle-like structures in the cytoplasm.

To determine if the antibodies that were still bound to the capsule after phagosomal residence maintained their capability of promoting phagocytosis, macrophages that had been incubated with antibody-opsonized yeast cells for approximately 10 h were lysed and the collected yeast cells were added to a fresh monolayer of macrophage-like cells without any other opsonin present. Some yeast cells originating from the lysed macrophages were ingested implying that the bound antibody retained opsonic activity after prolonged phagosomal residence (Movie S4).

Discussion

Given that cells of *C. neoformans* varieties *neoformans* and *grubii* are capable of replicating within macrophages (Tucker and Casadevall, 2002) we considered whether *C. gattii* was also an intracellular pathogen. However, before carrying comparative intracellular replication

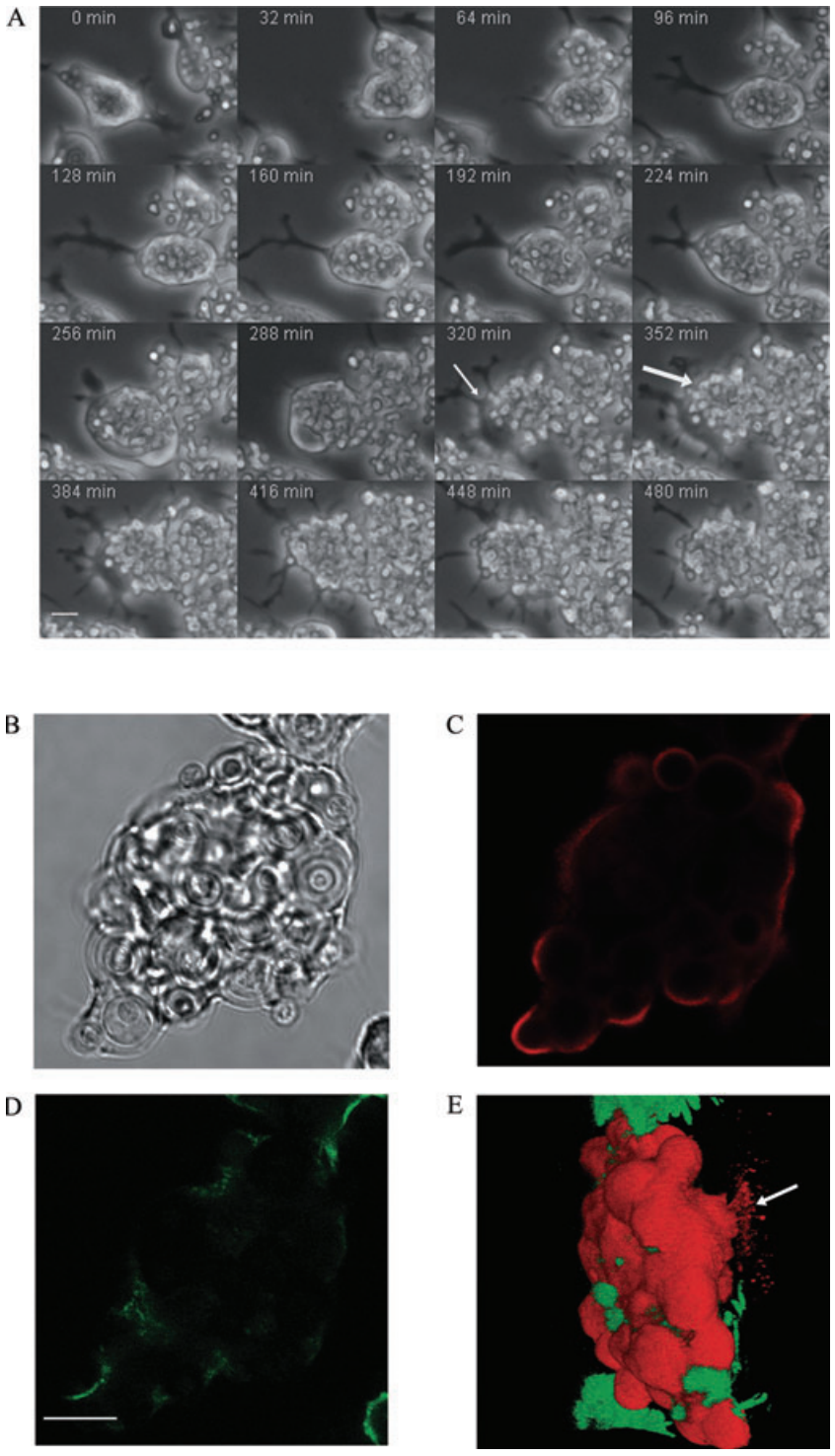

Fig. 3. *C. gattii* forms microcolony following macrophage exit. Following extrusion or lysis of macrophages, *C. gattii* exits in microcolony form as shown in (A) where the thick arrow points to the forming microcolony while the thin one indicates the macrophage that extruded. The yeast cells appear to be closely apposed to one another in an apparent polysaccharide matrix, as shown in a 3D composite (E) of combined Z-steps from (C) and (D) to recognize the polysaccharide capsules. In (E) the arrow points to polysaccharide residues and indicates the bottom end of the sample where the coverslip is located. The yeast cells continue to reproduce in such form, adding to the diameter of the microcolony. (B) Differential interference contrast (DIC) image. (A) was captured at 10× while (B–E) were captured at 63× with oil.

studies between the cryptococcal species we needed to ascertain whether the mode of ingestion of antibody-opsonized *C. gattii* cells was similar to the other varieties. Prior studies had shown that both IgM- and IgG-opsonized cells of varieties *neoformans* and *grubii* could be ingested through the CR by a complement-independent mechanism (Taborda and Casadevall, 2002). *C. gattii* opsonized with IgM was also ingested through CR. For these experiments IgM was used to avoid phagocytosis through Fc-gamma. Hence, the outcome of phagocytosis of *C. gattii* was comparable to that of the *C. neoformans* var. *grubii* and *neoformans*

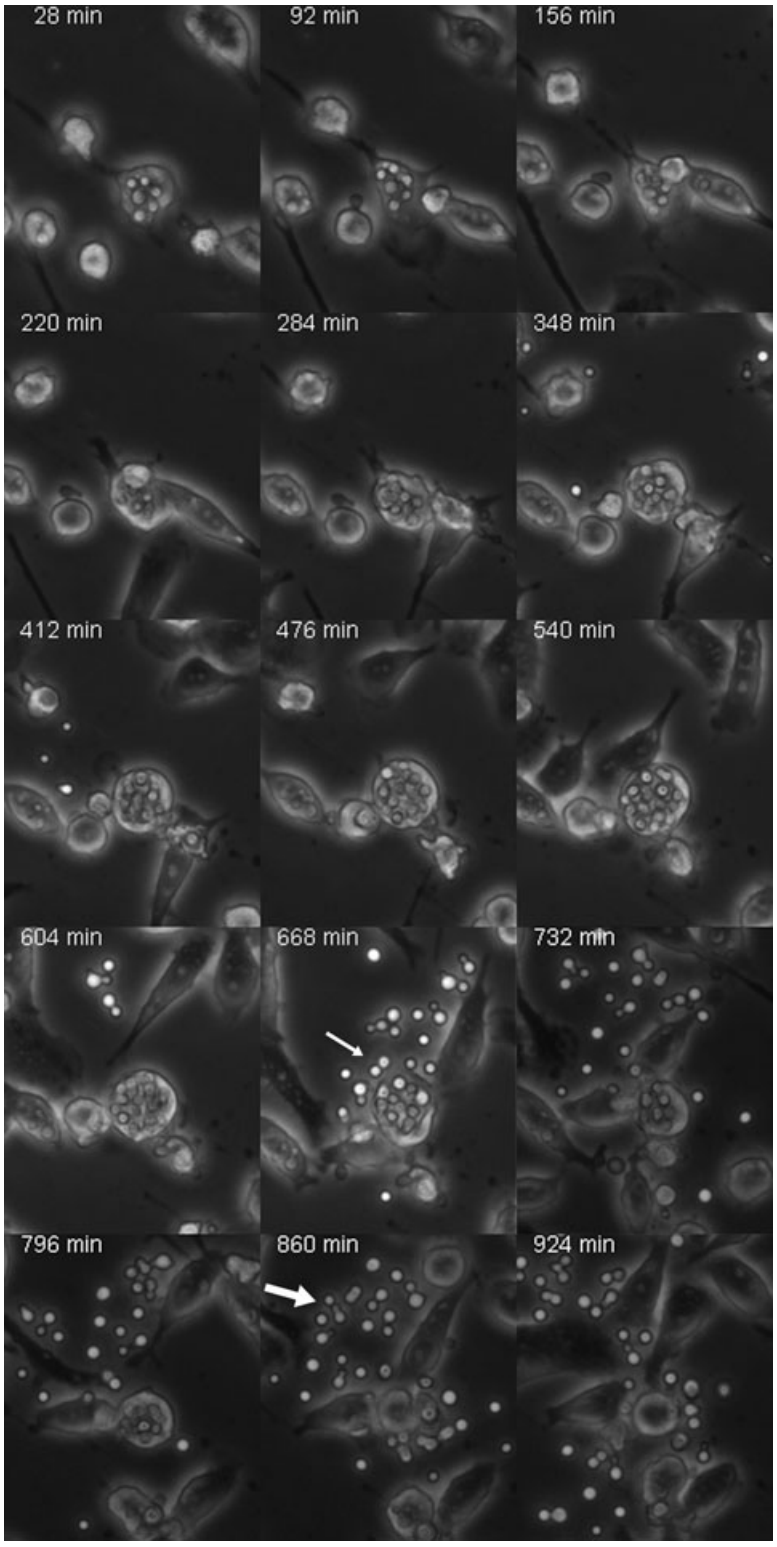

Fig. 4. *C. neoformans* gets extruded as dispersed cells following complement opsonized phagocytosis. *C. neoformans* strain H99 was opsonized with complement, incubated for 4 h with macrophage-like cells and taken for time-lapse imaging as indicated in *Experimental procedures*. Following phagocytosis and intracellular residence the yeast cells were extruded as dispersed cells out to the extracellular media as the white arrows point to. Images were collected at 10×.

strains, possibly occurring through the same mechanism of capsule structure rearrangement and exposure of CD18 binding sites, thus allowing direct interactions with CR (Taborda and Casadevall, 2002). Additionally, the finding that *C. gattii* released polysaccharide intracellularly like the other cryptococcal varieties suggests a similar mechanism of intracellular pathogenesis as described before (Feldmesser *et al.*, 2000; 2001; Tucker

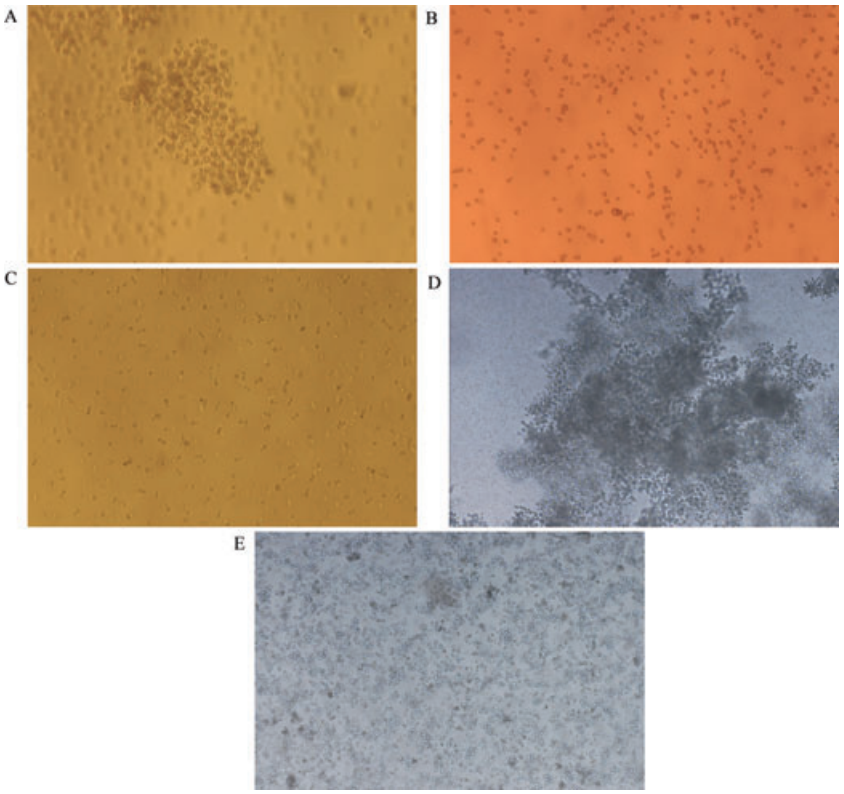

Fig. 5. *C. gattii* remains in a microcolony following phagosome extrusion. Following 20 h incubation of *C. gattii* with macrophage-like cells, careful collection of the extracellular media demonstrates clumps of *C. gattii* microcolonies can be found (A). Overnight incubation of *C. gattii* in feeding media indicates that no microcolonies form (B). Overnight incubation of *C. gattii* in sabouraud dextrose broth indicates that no microcolonies form. Overnight incubation of *C. gattii* with mAb 18B7 (100 μg ml^{-1}) shows clumping formation (D), whereas overnight incubation with 20% pooled human sera does not. Image in (A) was collected at 48× and images in (B–E) were collected at 15×. Difference in media colour is not relevant to results and is due to a difference in lighting.

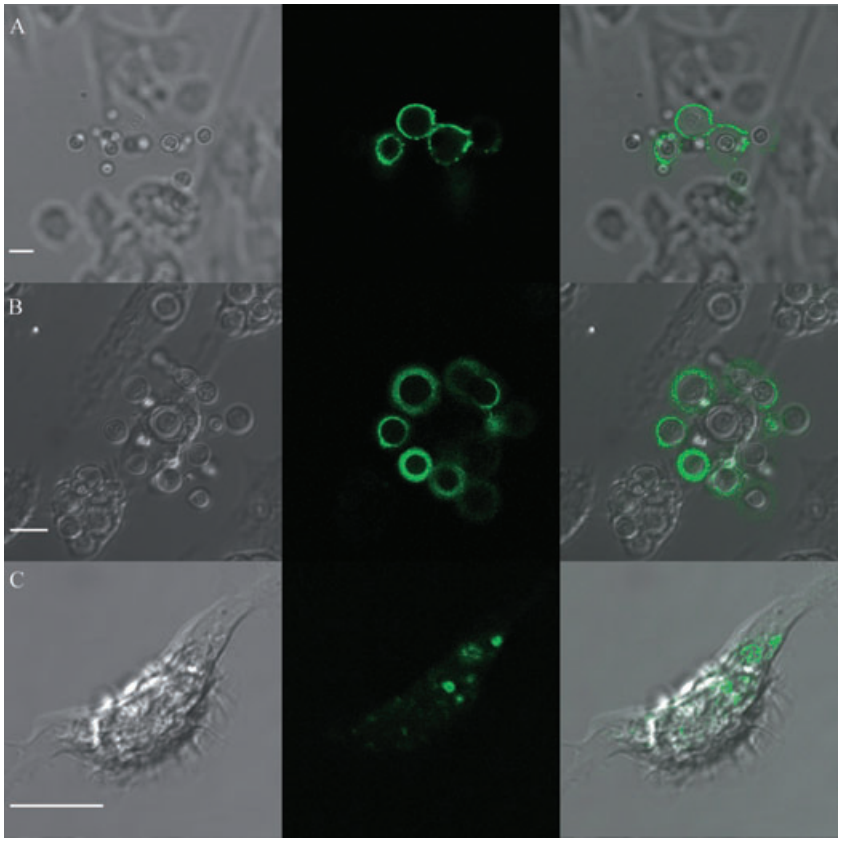

Fig. 6. Monoclonal antibody (mAb) 18B7 remains attached to yeast cells post extrusion. Yeast cells were opsonized with mAb 18B7 and phagocytosis was carried out as indicated in the experimental procedures section. Extruded *C. gattii* strain NIH 198 remained stained with fluorescein-labelled goat anti-mouse IgG while new buds after extrusion appeared not stained (A). Note that in these images the macrophages are not lysed and hence the intracellular yeast does not stain with the secondary antibody. Likewise, *C. neoformans* strain H99 was remained opsonized by mAb post extrusion (B). Macrophage-like cells that were incubated with and endocytosed mAb demonstrated the presence of the endocytosed mAb after 1 h incubation (C). Controls in which no primary mAb was added demonstrated no staining (data not shown). The panels are composed of DIC (left), FITC (middle) and overlaid images (right). Confocal images were collected at 63× with oil.

and Casadevall, 2002). Using live microscopy phase imaging we observed that all *C. gattii* strains I23, NIH 198 and NIH 444 replicated within J774.16 cells at approximately the same rate as serotypes A and D. Intracellular replication usually led to extrusion of the cryptococcal phagosome or lysis of the host macrophage. Hence the outcome of *C. gattii* ingestion by J774.16 cells is comparable to that described for var. *neoformans* and var. *grubii* in prior studies (Feldmesser *et al*., 2001; Taborda and Casadevall, 2002; Tucker and Casadevall, 2002; Alvarez and Casadevall, 2006), and confirmed here again.

When *C. neoformans* strain H99 was opsonized with complement (guinea pig or pooled human sera), phagosomal extrusion of *C. neoformans* led to rapid dispersal of the yeast cells as they floated away from the host macrophage as single cells. In contrast, phagosomal extrusion of mAb-opsonized yeast cells of both *C. neoformans* and *C. gattii* led to clumping of the extruded yeast cells in the form of a microcolony where dozens of yeast cells were enmeshed in a polysaccharide matrix. This microcolony at times remained attached to the cell and had a biofilm-like appearance. This clumping could result from one or two mechanisms: residual presence of mAb in the phagosome and/or fungal cell intracellular gene-expression changes that alter cell surface characteristics promoting cellular adhesion (Fan *et al*., 2005). Evidence for the latter explanation comes from the observation that *C. neoformans* cells can manifest flocculance during certain growth conditions (Li *et al*., 2006). In our experimental conditions there was no antibody present in the macrophage media during imaging, and this eliminates the explanation that clumping reflects agglutination from the presence of antibody in the extracellular media. However, the finding that yeast cells extruded from macrophages contained capsule-bound mAb from the initial opsonization protocol suggests that the mAb can remain bound during phagolysosomal formation and maturation, and could promote intracellular aggregation. The finding that some cells take up mAb from solution raises the additional possibility that intracellular antibody deposits may find their way to the cryptococcal phagolysosome where they could potentially mediate cellular aggregation. Nevertheless, it is possible that the phenomenon of microcolony formation following antibody-mediated phagocytosis reflects both the presence of residual antibody that promotes agglutination and gene-expression changes in the phagosomal compartment. The finding that yeast cells originating from lysed macrophages can be phagocytosed by previously uninfected macrophages suggests that antibody remains bound to the yeast cell following phagocytosis and extrusion and remains functional, in that it can still mediate phagocytosis. It is not surprising that the phagocytic efficiency is much lower the second time around given that there will be an antibody dilution effect as a result of ongoing cryptococcal budding. Further, it has been experimentally observed and previously documented that during budding the nascent yeast cells will not acquire the antibody originally present on the mother cell (Pierini and Doering, 2001). Thus, as there is no antibody in the medium, some of the residual antibody will be lost to the solution by the law of mass action. The notion that antibody survives in the harsh environment of the phagosome was surprising and we hypothesize either that these molecules have been selected for that purpose or that lysosomal contents are too diluted in the voluminous cryptococcal phagosome to destroy immunoglobulins.

The difference in exocytosis for mAb- and complement-opsonized yeast cells is striking given that until extrusion the only apparent difference between antibody- and complement-mediated phagocytosis was that antibody was a more effective opsonin. The finding of free cellular dispersal following extrusion after complement-mediated phagocytosis is in sharp contrast to the reduced dispersal observed following antibody-mediated phagocytosis. This observation raises the possibility that reduced dispersion following extrusion is a mechanism of antibody-mediated protection *in vivo*.

As all of the *C. gattii* strains appeared to have similar post-extrusion clumping, we continued our studies with strain NIH 198. Both *C. neoformans* and *C. gattii* were reported to differ in biofilm formation *in vitro*, with no biofilms observed for *C. gattii* strains in polystyrene surfaces, an observation that was attributed to the inability of *C. gattii* polysaccharide to attach to polystyrene. The extrusion strategy of *C. gattii* from macrophages had characteristics of biofilm formation including the formation of microcolonies that were encased in a polysaccharide matrix that at times remained attached to the host cell. Consequently, we conclude that *C. gattii*, like *C. neoformans*, can form biofilms in conditions that include intracellular residence in mammalian phagocytic cells. Hence, the two species of *C. neoformans* are both ingested by, replicate in and are extruded from murine macrophages in a similar fashion. The finding that *C. neoformans* and *C. gattii* have such similar pathogenic strategies for macrophages of an animal that did not exist at the time of their divergence over 30 million years ago is remarkable. Conservation of pathogenic strategy among these cryptococcal species is in contradistinction to other intracellular pathogens such as *Mycobacterium tuberculosis* that are believed to have emerged as a human pathogen relatively recently. The similarity in the intracellular strategy of *C. gattii* and *C. neoformans* despite their distant divergence is consistent with and supportive of the view that this pathogenic mechanism may have arisen as a result of selection by amoeboid predators in soils (Steenbergen *et al*., 2001).

Cryptococcal disease caused by *C. gattii* is often associated with pulmonary inflammatory masses that require

surgical resection. In this regard, the finding that *C. gattii* yeast cells remain attached after macrophage exit may also occur *in vivo* and this event could reduce local dissemination and promote granulomatous inflammatory responses.

Both alveolar and peritoneal macrophages were permissive to *C. neoformans* intracellular replication but these cells differed in that cryptococcal budding commenced much later in the alveolar macrophages. These findings parallel results with other microbes which have shown that alveolar macrophages are more effective antimicrobial cells than peritoneal macrophages against *Mycobacterium intracellulare* (Gangadharam and Pratt, 1983) and *Blastomyces dermatitides* (Sugar *et al*., 1986). Alveolar and peritoneal macrophages are known to exhibit a variety of differences which might account for this difference in relative permissiveness to *C. neoformans* intracellular replication. In this regard, alveolar macrophages from BALB/c mice have been reported to produce more nitric oxide (NO) than peritoneal macrophages (Kohut *et al*., 2004). As NO is known to inhibit *C. neoformans* replication this difference may account for the delay in fungal replication.

In summary, our results demonstrate that *C. gattii* is a facultative intracellular pathogen for macrophages *in vitro* and like *C. neoformans* has similar post-phagocytic interactions with macrophages. The finding that the exit of mAb-opsonized *C. neoformans* and *C. gattii* cells is associated with the formation of biofilm-like microcolonies implies that the action of antibody persists after phagocytosis, with the ability to remain opsonic for yeast cells and to mediate their re-ingestion by macrophages. This in turn extends the range of antibody-mediated actions for *C. neoformans* and *C. gattii* to the phagolysosomal compartment and the post-extrusion state where antibody may play a more complex role in pathogenesis than originally thought.

Experimental procedures

Yeast strains and culture conditions

Cryptococcus gattii (Serotype B) strains I23, NIH 198 and NIH 444 were obtained from Uma Banerjee (Calcutta, India), Thomas Mitchell (Durham, NC, USA) and American Type Culture Collection (ATCC, Rockville, MD, USA) respectively. *C. neoformans* var. *neoformans* strain 24067 was obtained from the ATCC. *C. neoformans* var. *grubii* strain H99 was obtained from John Perfect (Durham, NC, USA). All strains were cultured in Sabouraud (SAB) dextrose broth (Difco) at 30°C with agitation (150–180 r.p.m.) and collected and washed three times in PBS prior to use on all experiments.

Murine macrophages and phagocytosis assay

Macrophage-like murine J774.16 cells, originally derived from a reticulum sarcoma (Ralph *et al*., 1975), were used for all experiments, except for those specifically performed with primary cells. These macrophage-like cells were grown at 37°C with 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Gibco), 10% NCTC-109 medium (Gibco), 10% heat-inactivated (56°C for 30 min) fetal calf serum (Gemini Bio-products, Woodland, CA, USA), and 1% non-essential amino acids (Mediatech Cellgro, Washington, DC, USA). For *in vitro* phagocytosis and subsequent microscopic imaging macrophages were stimulated with murine IFN-γ (50 μ ml^{-1}) (Roche Molecular Biochemicals) and 0.3 μg ml^{-1} lipopolysaccharide (LPS) (Sigma) and 5×10^4 J774.16 cells were plated overnight in 96-well tissue culture plates and on poly lysine coverslip-bottom MatTek plates (Ashland, MA, USA) respectively. For the pre- and post-phagocytosis confocal imaging studies, 5×10^5 macrophage-like cells were plated in 35 mm tissue culture plates. Cells were then incubated overnight at the same growth conditions, 37°C with 10% CO_2 to allow attachment to the plates. The following day, *C. gattii* was added in a 1:1 ratio (for IgM opsonic efficacy studies) and a 10:1 ratio (for time-lapse and confocal imaging studies with all strains used herein), in the presence of IFN-γ and LPS at the concentrations stated above, for approximately 1 h (2 h for IgM opsonic efficacy studies). Extracellular yeast cells were then washed off and plates were taken for fixing (see IgM opsonic studies below), staining (see immunofluorescence below), time-lapse imaging (see time-lapse imaging below) or incubated for up to 20 h for further confocal studies (see confocal imaging below).

For alveolar macrophage isolation, female BALB/c mice were sacrificed by asphyxiation with CO_2, their tracheas were cannulated with angiocaths (20 GA 1.16 IN) (Becton Dickinson, Sandy, UT, USA), and the lungs were lavaged 10 times with sterile HBSS containing 1 mM EGTA (Sigma, St Louis, MO, USA) without phenol red (Life Technologies, Grand Island, NY, USA). Lavage fluids were pooled, and cells were collected by centrifugation. Red blood cells were lysed by incubating the cellular suspension in 0.17 M NH_4Cl at room temperature for 5 min. The cells were then collected by centrifugation at 1200 r.p.m., washed with HBSS and re-suspended in Dulbecco's minimal essential medium (Life Technologies) with 10% heat-inactivated fetal calf serum (Bioproducts for Science, Indianapolis, IN, USA). Cells were then placed in MatTek plates at a density of 1×10^5 per well in 0.1 ml of Dulbecco's minimal essential medium-10% heat-inactivated fetal calf serum and allowed to attach at 37°C and 10% CO_2 for 1 h. For peritoneal cell collection the same protocol was followed with the exception that the cells were collected by lavaging the peritoneal cavity. As there was no bleeding in this procedure, no erythrocyte lysis step was required and the cells were counted, plated in MatTek plates in the same media mentioned above, and allowed to adhere for the same time period prior to infection with yeast cells (as carried out above) and subsequent time-lapse microscopic imaging.

Antibodies

Monoclonal antibodies 18B7 (IgG1), 12A1 (IgM) and 3E5 (IgG1) have been described previously and bind to the cryptococcal major capsular polysaccharide GXM (Casadevall *et al*., 1992; Mukherjee *et al*., 1993; 1995a; Yuan *et al*., 1995). 18B7 facilitates the phagocytosis of *C. neoformans* by J774.16 cells via Fc receptor and CR3 and CR4 receptors (Mukherjee *et al*., 1995b; Taborda and Casadevall, 2002). Monoclonal antibodies 18B7

and 3E5 were purified by protein G affinity chromatography (Pierce, Rockford, IL, USA).

Opsonic efficiency of IgM

To test the opsonic efficacy of IgM for *C. gattii*, phagocytosis assays were performed as described previously (Taborda and Casadevall, 2002). *C. gattii* cells were added in a 1:1 ratio, along with various concentrations of 12A1 (1.0, 10.0 and 25.0 μg ml^{-1}), 0.3 μg ml^{-1} LPS and 50 units ml^{-1} murine IFN-γ. The macrophage-like cells were then incubated for 2 h, fixed with MeOH, stained with Giemsa and the phagocytosis index was determined for each well. Blockage of CR phagocytosis was carried out as described (Taborda and Casadevall, 2002) by adding 10 μg ml^{-1} (0.5 μg well^{-1}) antibody to CD18, CD11b and CD11c (Pharmingen, San Diego, CA, USA) for 1 h at 4°C, prior to infection with *C. gattii*.

Immunofluorescence of internalized C. gattii *cells*

Immunofluorescence of internalized *C. gattii* was carried out as previously described, but with minor modifications (Tucker and Casadevall, 2002). Briefly, macrophages (J774.16 cells) were infected with *C. gattii* strains NIH 198 or I23 and phagocytosis was carried out in MatTek plates for fixed time periods (2, 4, 6, 8 and 18 h) prior to washing with PBS and staining for immunofluorescence. Macrophages were fixed with 3.7% formaldehyde in 1× fixation buffer pH 7.2 (5 mM KCl/137 mM NaCl/4 mM $NaHCO_3$/0.4 mM KH_2PO_4/1.1 mM Na_2HPO_4/2 mM $MgCl_2$/5 mM Pipes, pH 7.2/2 mM EGTA/5.5 mM glucose) for 5 min at 37°C. Macrophages were then permeabilized using 0.5% Triton X-100 for 20 min at 25°C. To reduce background autofluorescence the monolayers were incubated with 0.1 M glycine for 10 min, followed by three washes with blocking solution (1% BSA/PBS). Conjugation of Alexa 488 dye to mAb 18B7 was performed according to the manufacturer's instructions (Molecular Probes). Alexa 488 mAb 18B7 was added at a concentration of 39.9 nM and the reaction was incubated overnight at 4°C. The macrophage monolayers were then washed three times with blocking solution and immersed in mounting medium (50% glycerol and 50 mM n-propyl gallate in PBS) to reduce quenching of fluorescence. Samples were visualized using an Axiovert 200 M inverted microscope, and photographed with an AxiocamMR camera controlled by the Axio Vision 4.4 software (Carl Zeiss MicroImaging, NY, USA). Images were processed in Axio Vision 4.4 and Adobe Photoshop CS2 for Windows (San Jose, CA, USA).

Confocal imaging

Phagocytosis was carried out as indicated above and after 20 h, macrophages and *C. gattii* were fixed with 4% paraformaldehyde for 10 min followed by a 5 min permeabilization with 1% Triton X-100. Labelling of actin from macrophages was achieved by using Phalloidin-Alexa 488 (Molecular Probes), and was followed by labelling of *C. gattii*'s capsular polysaccharide with 18B7 that was conjugated to Alexa 546, according to the manufacturer's instructions (Molecular Probes).

To test if mAb 18B7 remained attached to *C. neoformans* and *C. gattii* capsules post extrusion we carried out phagocytosis as indicated above and washed the extracellular yeast cells off after 1 h incubation. The plates were supplemented with 1 ml of feeding media and the macrophages were incubated with the intracellular yeasts for 10–15 h to optimize the frequency of extrusion events. The medium was then carefully removed from the plates and 4% paraformaldehyde was immediately added for 10–15 min in order to fix the macrophages and yeast cells. The cells were carefully washed twice with PBS followed by incubation with goat anti-mouse IgG1 conjugated to FITC at 1 μg ml^{-1} to determine if mAb 18B7 remained attached to the yeast cells post extrusion. Cultured (pre-infection) yeast cells were also incubated with the same concentration of the goat anti-mouse IgG1 conjugated to FITC as a negative control. To determine if mAb was endocytosed by macrophages from solution, mAb 18B7 (50 g ml^{-1}) was incubated with macrophage-like cells for 1 h followed by fixation with 4% paraformaldehyde (10 min), permeabilization with 1% Triton X-100 (5 min) and staining with goat anti-mouse IgG1 conjugated to FITC (5 μg ml^{-1}), with PBS washes in between each step. As a control, goat anti-mouse IgG1 conjugated to FITC was added to cells that had not been incubated with mAb 18B7.

All samples were washed with PBS prior to suspending in mounting medium (50% glycerol and 50 mM n-propyl gallate in PBS) and visualization using a Leica AOBS laser scanning confocal microscope. Regular and Z-series images were collected using a 63×/1.4 Oil objective. Figures were generated using Adobe Photoshop CS2.

Time-lapse imaging

For live cell imaging, phagocytosis assays were performed as described (Tucker and Casadevall, 2002). Briefly, 5×10^4 macrophages or J774.16 cells were plated on polylysine-coated coverslip-bottom MatTek plates and allowed to adhere overnight. The medium was then removed and replaced with fresh media containing *C. gattii* cells, strain NIH 198 (yeast to macrophage ratio of 10:1) or *C. neoformans* (for both macrophage-like and primary macrophage studies using a *C. neoformans* to macrophage ratio of 1:1), along with mAb against the cryptococcal capsule (18B7 or 3E5, 50–100 μg ml^{-1}) or 20% guinea pig or pooled human serum (to opsonize H99). In most experiments macrophages were activated with 0.3 μg ml^{-1} LPS (Sigma) and 50 units ml^{-1} murine IFN-γ. Macrophages and yeast cells were then incubated together for 60 min to allow for completion of phagocytosis, washed twice with fresh media, replenished with 2 ml of feeding media and imaged under the microscope.

Microscopic studies were carried out by taking time-lapse movies at 10× (40× primary macrophage studies) using the Axiovert 200 M inverted microscope described above. This microscope was housed in a Plexiglas box and the temperature was stabilized at 37°C with a forced air heater system. The plate lid was kept in place to prevent evaporation, and 5% CO_2 was delivered to a chamber locally at the culture dish. Images were taken every 4 min and were then compiled into movies to carry out imaging analysis.

To test if mAb was still functional in promoting phagocytosis following the yeast escape out to the extracellular milieu, macrophages were incubated with *C. neoformans* H99 as indicated above, but in 35 mm tissue culture plates. After incubating *C. neoformans* with macrophages for 1 h, the extracellular yeast cells were washed off and the remaining macrophages and ingested yeast cells were supplemented with feeding media

(1 ml). This was followed by incubation for 10 h at 37°C 10% CO_2. Extracellular yeasts were then washed off with feeding media and cells were lysed for a maximum of 1 h (to ensure optimal lysis, as predetermined by live microscopic monitoring of cells undergoing lysis) in autoclaved dH_2O (1 ml). This was followed by the collection of the dH_2O supernatant which comprises of yeast cells that were residing within macrophages. The supernatant was then spun down at the same speed used to collect the yeast cells (3500 r.p.m. for 5 min) which were then added to a fresh (non-infected) macrophage monolayer in the absence of antibody opsonins to test the phagocytic capacity of the J774.16 cells for the fungal cells recovered from lysed macrophages. This was followed by time-lapse imaging to look for and record ingested yeast cells.

Imaging of microcolonies

Cryptococcus gattii (NIH 198) and *C. neoformans* (H99) were incubated with macrophages as indicated above, for 20 h. This was followed by careful collection of the extracellular medium which was then placed on a coverslip-bottom MatTek dish and observed under an inverted Zeiss axiovert light microscope. As controls, overnight cultures of the aforementioned strains grown in feeding media and Sabouraud dextrose broth were also placed in MatTek dishes to be observed under the microscope. Further, overnight incubation of the aforementioned strains in feeding media containing antibody solution (100 $\mu g\ ml^{-1}$) or 20% serum (guinea pig or pooled human sera) were used as controls. Images were collected using a Nikon D70 coupled via an adaptor to the inverted microscope. Images were collected at 48× (32× from objective + 16× from 1.5 magnifying factor from Nikon D70 CCD chip) for samples collected post infection and at 15× (10× from objective and 5× from Nikon D70) for samples from overnight cultures.

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Supplementary material

The following supplementary material is available for this article online:

Movie S1. Antibody-opsonized *C. gattii* extrusion is followed by microcolony formation. Following phagocytosis, antibody-opsonized *C. gattii* strain NIH 198 replicated inside and was extruded by macrophage-like J774.16 cells. Extrusion was followed by the formation of clumped microcolonies that continued to grow for the duration of the experiment. Images were collected at 10× and seven frames per second.

Movie S2. Antibody-opsonized *C. neoformans* extrusion is followed by microcolony formation. Following phagocytosis, antibody-opsonized *C. neoformans* strain H99 replicated inside and was extruded by macrophage-like J774.16 cells. Extrusion was followed by the formation of clumped microcolonies that continued to grow for the duration of the experiment. Images were collected at 10× and seven frames per second.

Movie S3. Complement-opsonized *C. neoformans* extrusion is followed by yeast dispersal. Following phagocytosis, complement-opsonized *C. neoformans* strain H99 replicated inside and was extruded by macrophage-like J774.16 cells. Extrusion was followed by free yeast dispersal into the extracellular medium. Images were collected at 10× and seven frames per second.

Movie S4. Phagocytosis of yeast cells from lysed macrophages. Following phagocytosis of *C. neoformans* and incubation of macrophages and *C. neoformans* (strains H99), macrophages with ingested antibody (IgG)-opsonized yeasts were osmotically lysed and the yeasts were collected and added to a fresh monolayer of macrophage-like cells. As evidenced in this movie, ingestion took place, suggesting that antibody remained functional in mediating phagocytosis. Arrow at the end of movie points to internalized yeast cell which is in contrast to extracellular yeasts on the bottom of movie. Images were collected at 40× and compiled at seven frames per second.

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