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Microreview

Cryptococcus interactions with macrophages: evasion and manipulation of the phagosome by a fungal pathogen

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Summary

***Cryptococcus* is a potentially fatal fungal pathogen and a leading cause of death in immunocompromised patients. As an opportunistic and facultative intracellular pathogen of humans, *Cryptococcus* exhibits a complex set of interactions with the host immune system in general, and macrophages in particular. *Cryptococcus* is resistant to phagocytosis but is also able to survive and proliferate within the mature phagolysosome. It can cause the lysis of host cells, can be transferred between macrophages or exit non-lytically via vomocytosis. Efficient phagocytosis is reliant on opsonization and *Cryptococcus* has a number of anti-phagocytic strategies including formation of titan cells and a thick polysaccharide capsule. Following uptake, phagosome maturation appears to occur normally, but the internalized pathogen is able to survive and replicate. Here we review the interactions and host manipulation processes that occur within cryptococcal-infected macrophages and highlight areas for future research.**

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Introduction

Cryptococcus species are the causative agents of cryptococcosis. Cases of cryptococcosis are primarily caused by *Cryptococcus neoformans* in immunocompromised individuals, particularly those with AIDS (Park *et al.*, 2009). The second major pathogenic species, *Cryptococcus gattii*, is capable of causing infection in the immunocompetent individuals (Chaturvedi and Chaturvedi, 2011). In cryptococcal infection, one of two things happens: either the host immune response clears the infection, or *Cryptococcus* successfully evades this response and, without treatment, will disseminate with potentially fatal consequences. An additional possible outcome is that cryptococcal infection may be contained but not cleared and becomes latent, demonstrating how this interplay of host and pathogen, which determines the outcome of infection, can be highly protracted (Dromer *et al.*, 2011). Treating these infections is complicated due to patients being diagnosed late in disease (often at the stage of cryptococcal meningoencephalitis) and because of the limitations of current antifungal treatments. The route of infection is through the lungs with desiccated yeast cells or spores being the probable infectious agents (Velagapudi *et al.*, 2009). In the absence of other pathology in the lungs the first cells to encounter cryptococci will be resident macrophages and the outcome of this interaction will likely determine the course of infection.

It is important to note when contemplating the interaction of cryptococci with macrophages in human disease that *Cryptococcus* has not evolved directly to evade or hijack the human immune system (unlike, for example, *Mycobacterium tuberculosis*) and therefore any apparent adaptation to human pathogenesis needs to be considered in the context of the wider ecology of this organism (Steenbergen and Casadevall, 2003). Whatever the evolutionary path of cryptococcocal virulence [and this is a fascinating area (Casadevall, 2012)] it is extremely unlikely that it included selection through a vertebrate host.

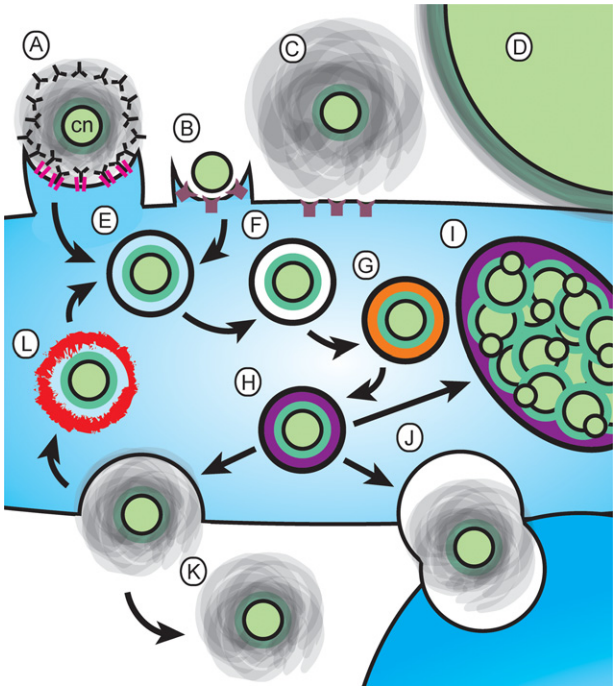

Fig. 1. Interactions of *Cryptococcus* with macrophages.
Cn, *Cryptococcus neoformans* cell.
A. Opsonic phagocytosis. Opsonization of cryptococci is the most efficient route of uptake by macrophages. In this case antibody specific to the cryptococcal capsule activates the Fc receptor and stimulates phagocytosis.
B. Non-opsonic phagocytosis. Pattern recognition receptors (PRRs) on the macrophage directly bind pathogen-associated molecular patterns (PAMPs) on cryptococci. Examples of PRRs that bind cryptococci are the beta-glucan and mannose receptors.
C. The cryptococcal capsule may resist phagocytosis by macrophages by covering PRR binding sites in the cell wall.
D. Titan cryptococcal cells. This cell would be 50 μm in comparison with the smaller cells at 5 μm.
E. Phagocytosis results in a membrane bound vesicle called the phagosomes that matures to contain a highly antimicrobial environment (F–H).
F. The generation of the respiratory burst.
G. Acidification.
H. pH-dependent lysosomal enzymes and antimicrobial peptides.
I–L. Alternative fates of mature phagosome.
I. Intracellular proliferation of cryptococci in the macrophage phagosome.
J. Lateral transfer. Donor cell, top. Acceptor cell, bottom.
K. Vomocytosis.
L. Actin flash.

Phagocytosis of *Cryptococcus* by macrophages

The uptake of particles, including microorganisms, by professional phagocytes is mediated by receptor activation that drives cytoskeletal rearrangements to engulf and internalize the particle. Phagocytic receptors can be divided into opsonic (Fig. 1A) and non-opsonic (Fig. 1B). The opsonic phagocytic receptors are the Fc receptor family, which bind antibody (on macrophages IgG, IgM or IgA), or the complement receptor family. Non-opsonic phagocytic receptors are pattern recognition receptors (PRRs), such as dectin-1 or mannose receptor, which recognize distinct pathogen-associated molecular patterns (PAMPs) on the fungal surface. *In vivo*, it is likely that phagocytosis occurs through multi-hetero-complexes of receptors, producing a complex set of downstream signals.

In vitro, in the absence of opsonin, macrophages are poor at internalizing cryptococci (< 10% of primary human macrophages phagocytose one or more cryptococci within 2 h, even with a multiplicity of infection of 10:1; our own unpublished observations). Unsurprisingly, the most efficient uptake of cryptococci by macrophages is in the presence of specific antibody (Levitz *et al*., 1991; Mukherjee *et al*., 1996; Spira *et al*., 1996; Shapiro *et al*., 2002). Complement is an efficient opsonin in the absence of specific antibody (Davies *et al*., 1982; Levitz and Tabuni, 1991) but there is variation between strains that correlates with capsule size (Zaragoza *et al*., 2003). There is also evidence that the complement phagocytic receptor CR3 may also bind directly to cryptococci (Taborda and Casadevall, 2002). Complement is not present at high levels in the lung but is induced by the presence of cryptococci (Blackstock and Murphy, 1997). In contrast, specific antibody production is slow to develop after initial exposure, but most individuals are seropositive by late childhood (Goldman *et al*., 2001). Uptake via opsonin is therefore likely the route taken *in vivo* but that variation in either strain or antibody production may have consequences for its effectiveness.

At present, little is known about the involvement of non-opsonic receptors in cryptococcosis. Both the mannose and beta-glucan receptors bind cryptococci but their ligands are in the cryptococcal cell wall and are thus are likely to be concealed by capsular polysaccharide (Kozel, 1977). In line with this, dectin-1 (which recognizes beta-glucan) appears to have little role in the macrophage interaction with cryptococci (Nakamura *et al*., 2007). The role of the scavenger receptors in phagocytosis of cryptococci by macrophages has not been studied.

Interestingly, within the lung, surfactant protein D (SP-D) can act as an opsonin of poorly encapsulated cryptococci and this process is actively beneficial to the pathogen, since deletion of SP-D is protective in the mouse (Geunes-Boyer *et al*., 2009; 2012). Opsonization with SP-D shows a small decrease in recruitment of the phagolysosome marker LAMP but a large reduction in the destruction of an acapsular mutant (Geunes-Boyer *et al*., 2012).

Despite the ability of cryptococci to survive within phagocytes, the pathogen has a number of mechanisms that render it inherently resistant to phagocytosis:

i. Polysaccharide capsule (Fig. 1C). The cryptococcal capsule acts by physically and chemically blocking potential sites of macrophage receptor binding (Bose

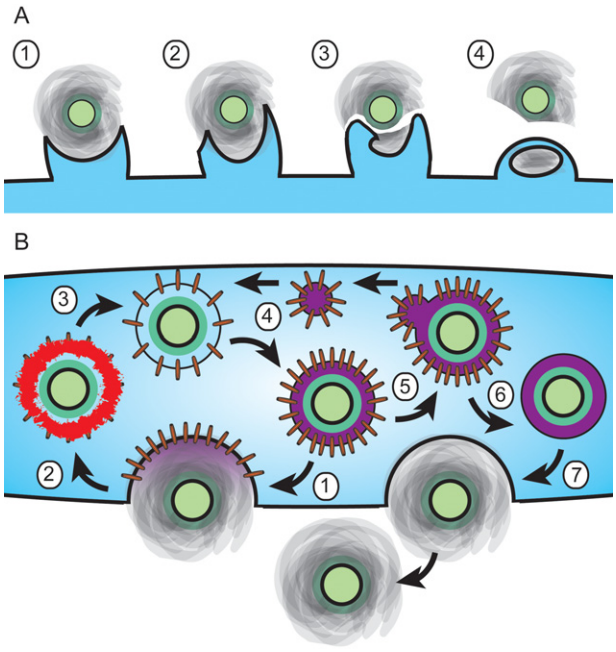

Fig. 2. A. Capsule elasticity may disrupt completion of phagocytosis. 1. Phagocytosis is initiated by receptor activation and the actin cytoskeleton drives the engulfment of the cryptococcal cell. 2. The action of the phagocytic pseudopod deforms the capsule. 3. Cryptococcal capsular polysaccharide that is bound to the phagocytic pseudopod is shed. 4. Cryptococcal cell is released and capsule polysaccharide internalized.
B. Model of actin flash/vATPase regulation of vomocytosis. 1. Phagolysosome fuses with plasma membrane prematurely, releasing soluble contents, in an attempted vomocytosis. 2. Due to presence of vATPase on the phagosome membrane actin flash is induced that prevents the completion of vomocytosis. 3. Phagosome is resealed. 4. Phagosome re-matures including the fusion of vATPase containing vesicles. 5. vATPase is recovered from phagosome by WASH/Arp2/3 complex-dependent budding off of vATPase containing vesicles. 6. Post-maturation phagosome without vATPase. 7. Phagosome fusion with the plasma membrane completes resulting in successful vomocytosis.

et al., 2003). The capsule of *Cryptococcus* is composed of the polysaccharides GXM (glucuronoxylomannan; 90–95%) and GXMGal [glucuronoxylomannogalactan; 5–10% (Doering, 2009)] together with mannoproteins. This capsule is typically 4–6 μm in depth but can be as large as 30 μm or appear non-existent. Acapsular mutants are much more readily phagocytosed than their parental strains (Cross and Bancroft, 1995). One speculative possibility, that to our knowledge has not previously been investigated, is that attempts to phagocytose cryptococci fail because capsular polysaccharide is shed from the fungal cell at the point where the macrophage first attaches to it (Fig. 2A; arising from our unpublished observation that even with antibody there are occurrences of phagocytic engagement and pseudopod formation that are subsequently aborted, in contrast to a failure to engage at all), a hypothesis that is supported by the fact that larger capsules have a reduced Young's modulus (Frases *et al.*, 2009). This may be due to differences in how different phagocytic receptors engage or a consequence of how cryptococci are presented *in vitro* (i.e. in free solution) and would not occur in a limited spatial environment of, for example, aveaoli.

ii. Anti-phagocytic protein 1 (App1). App1 is a secreted factor that blocks complement-mediated, but not Fc-mediated, phagocytosis (Stano *et al.*, 2009). App1 binds to the CD11b component of CR3 and blocks internalization of iC3b coated cryptococci, presumably through steric hindrance (Stano *et al.*, 2009).

iii. Gat201 (Gat204 and Blp1). Analysis of a 1200 gene knockout library identified the transcription factor GAT201 as being required for the anti-phagocytic ability of cryptococci (Liu *et al.*, 2008). The mechanism is currently unknown but two GAT201 regulated genes, Gat204 (also a transcription factor) and Blp1, are required to maintain anti-phagocytic activity (Chun *et al.*, 2011).

iv. Titan cells (Fig. 1D). Titan cryptococcal cells are 50–100 μm in diameter (with a capsule of varying thickness but typically 50% of the total diameter; the normal cell diameter is 3–10 μm) and make up around 20% of the population of cryptococci on exposure to the host lung (Okagaki *et al.*, 2010; Zaragoza *et al.*, 2010). Titan cell morphology is dependent on the protein kinase A signalling pathway as is capsule regulation (Okagaki *et al.*, 2011). Inhibition of titan cell formation results in a reduction of virulence and dissemination in the mouse model (Crabtree *et al.*, 2012). Titan cells are resistant to phagocytosis but, interestingly, are able to confer this resistance to neighbouring normal sized cryptococci (Okagaki and Nielsen, 2012). The addition of large inert particles does not reproduce the effect and the mechanism is currently unknown (Okagaki and Nielsen, 2012).

Maturation of phagosomes containing cryptococci

After phagocytosis a single membraned vesicle that contains the pathogen is formed (Fig. 1E). This phagosome undergoes a maturation process through the fusion of different classes of endocytic vesicles to produce an increasingly antimicrobial environment. At a basic level the stages of maturation can be considered to be: Production of a respiratory burst, acidification of the phagosome and addition of a complex cocktail of antimicrobial peptides, proteases and other degradative enzymes (Fig. 1F–H respectively; Fairn and Grinstein, 2012). The direct respiratory burst in the macrophage phagosome is weak when compared with neutrophils and may have little role in microbe killing directly but is instead both an intra- and inter-cellular signalling process (Nordenfelt and

Tapper, 2011), although it should be noted that there maybe secondary reactive oxygen production that is directly antimicrobial (West *et al*., 2011). Likewise, although phagosome acidification has a profound effect on the viability of many microbes, it also acts as a critical step to allow subsequent fusion of antimicrobial lysosomes, and to allow effective function of their acid-dependent enzymes. Notably, the occurrence and timing of phagosome maturation events likely varies, depending on the route of uptake and activation state of the macrophage (Huynh *et al*., 2007).

Precise mapping of the passage of cryptococci through the phagosomal pathway has not yet been undertaken, but by examining known markers the major steps of maturation have been observed. The early endosome marker EEA1 is recruited within 10 min after phagocytosis and is still present after 1 h (Wozniak and Levitz, 2008; Qin *et al*., 2011). Both MHC II and the tetraspanin CD63 are recruited early in phagosome development (Artavanis-Tsakonas *et al*., 2006), with CD63 recruitment being coincident with (and depending upon) phagosome acidification (Artavanis-Tsakonas *et al*., 2006). The lysosomal associated membrane protein LAMP1 is recruited between 1 and 3 h after uptake (Levitz *et al*., 1999; Wozniak and Levitz, 2008; Johnston and May, 2010; Qin *et al*., 2011). The presence of phagolysomal markers does not, however, prove that there is antimicrobial activity associated with this stage of phagosome maturation (e.g. degradative enzymes) within the phagosome. Further work is needed to show that phagosomes containing cryptococci exhibiting these markers are functionally antimicrobial.

Autophagy is not strictly part of the phagosomal pathway but has been increasingly implicated in its function, particularly in reference to intracellular pathogens (Deretic, 2012). Autophagy is the cellular process by which cells breakdown and recycle their components within characteristic double membraned lysosomes. A number of autophagy components (*Atg2a*, *Atg5*, *Atg9a*, *Atg12* and *LC3*) were identified recently as playing a role in phagocyte/*Cryptococcus* interactions, via an siRNA screen in *Drosophila* S2 cells (Qin *et al*., 2011). Similarly, cryptococcal infection of murine macrophages activates autophagy and LC3-positive vesicles appear in close proximity to phagosomes containing cryptococci, although the molecular relationship between these two pathways is currently unknown (Qin *et al*., 2011).

Intriguingly, blocking autophagy by reducing levels of ATG5 recues the ability of RAW or J774 macrophages to restrict cryptococcal growth (Qin *et al*., 2011; Nicola *et al*., 2012). In contrast, bone marrow-derived macrophages from Atg5–/– mice only show improved control of cryptococci when they are unactivated; in the presence of interferon gamma and lipopolysaccharide there were no differences between knockout and control cells (Nicola *et al*., 2012). A possible explanation for this is the varying 'basal activation' states of the different cell types.

Intracellular replication of *Cryptococcus* in the phagosome

The growth of cryptococci within macrophages is one of the earliest characteristics reported for the cryptococcal host–pathogen interaction (Diamond and Bennett, 1973; Fig. 1I). The ability of cryptococci to grow within the phagosome relies on being able to both survive antimicrobial attack and scavenge sufficient nutrients to proliferate.

Many of the resistance factors studied in *Cryptococcus* are concerned with preventing damage by reactive oxygen species (ROS). The capsule appears protective against ROS (and perhaps other antimicrobials) and protection is proportional to the size of the capsule (Zaragoza *et al*., 2008). A number of additional cryptococcal genes have a described role in protecting against reactive oxygen and nitrogen species (Sod1, Aox1, Fhb1, Tsa1 and Ure1; Brown *et al*., 2007), while cryptococci are also capable of producing melanin that can potentially neutralize cationic antimicrobial peptides through its negative charge (Doering *et al*., 1999). Melanin is generated by the enzyme laccase (Eisenman *et al*., 2007), which is regulated by PKC through diacylglycerol and the phytoceramide pathway in *Cryptococcus* (Heung *et al*., 2004; Shea *et al*., 2006). Mutants in regulators of this pathway have very interesting mouse infection phenotypes [ΔIsc1: failure to disseminate (Shea *et al*., 2006); ΔGcs1: contained in granulomas (McQuiston *et al*., 2010)] that suggest a more complex immune modulatory action, perhaps through cryptococcal enzymes and lipid messengers acting on host pathways.

Appropriate immune balance is critical for eradicating cryptococcal infection. Cryptococcal meningoencephalitis is commonly associated with the loss of the Th1 response (Siddiqui *et al*., 2005). Immune balance modifies intracellular proliferation *in vitro*, with individual Th2 cytokines resulting in increased intracellular proliferation of cryptococci in comparison with those treated with Th1 or Th17 cytokines (Voelz *et al*., 2009). *In vivo* models recapitulate this difference, since intracellular cryptococci are abundant in WT (C57/BL6) or IFNγ–/– mice but very rare in IL-4–/– mice (Arora *et al*., 2011). Interestingly, administration of TGF-β shows an order of magnitude difference in overall fungal burden when administered in the acute or chronic phases of infection with an increase with treatment in the acute phase and a reduction with treatment in the chronic phase in comparison with control (Shao *et al*., 2005). A more detailed study of the role of macrophages *in vivo* in these phenomena, especially in the naturally resistant rat host, would be highly desirable.

There have now been a number of studies that have directly measured the inter-species and inter-strain variation in intracellular proliferation of cryptococci (Ma *et al*., 2009; Voelz *et al*., 2009; 2010; Byrnes *et al*., 2010; 2011; Alanio *et al*., 2011). There is significant inter-strain variation, with proliferation rates varying by almost a log between the fastest and slowest proliferating strains (Ma *et al*., 2009). The reason for this variation remains unknown, although rapid intracellular growth in an *in vitro* cell culture model is a defining feature of a hypervirulent outbreak of *C. gattii* disease in the Pacific North-west and strongly correlates with virulence in the mouse model (Ma *et al*., 2009; Byrnes *et al*., 2010; 2011).

To date the relationship between *in vitro* culture and *in vivo* studies in the mouse of intracellular proliferation and human disease progression has not yet been demonstrated. A first step in determining this relationship would be to relate observations with human isolates to experimental data. A recent approach using flow cytometry took clinical isolates and analysed their interaction with macrophages through a number of *in vitro* assays. Comparison of data from these assays to the clinical parameters available for the respective patients suggested two correlations when compared with the reference strain (H99). Isolates showing poor uptake by macrophages AND a low intracellular burden after 2 h were associated with a poor response to antifungal therapy (non-sterilization of CSF after 2 weeks) (Alanio *et al*., 2011). In contrast, isolates showing high uptake and a high intracellular burden after 2 h were associated with increased mortality at 3 months. Both associations were lost when intracellular burden at 24 h was used.

Lysis of macrophages by *Cryptococcus*

The lysis of host cells by pathogens is a common cause of pathology and an important route of escape from the intracellular environment of host cells for many organisms. *Cryptococcus* does not possess recognized pore-forming proteins (PFPs) that are a common method for lysing host cells. It is tempting to suggest that the cryptococcal phospholipase Plb1, a known virulence factor, is a candidate for breaking down host membranes, but its activity is very unlikely to cause discernable physical damage to the phagosome or plasma membrane bilayer. Similarly, it is possible that cryptococci mechanically disrupt host cells through proliferation within the phagosome and perhaps via production of large amounts of polysaccharide capsule, although neither possibility has yet been experimentally tested. It is also known that macrophages can undergo apoptosis in response to intracellular cryptococcal signalling via the alternative NF-κB pathway (Ben-Abdallah *et al*., 2012). However, macrophage lysis in response to intracellular cryptococci is morphologically distinct from apoptosis, suggesting that other host lysis pathways exist.

Transfer of cryptococci between macrophages

The lateral transfer of cryptococci between macrophages is an extremely rare event that allows pathogens to move directly between host cells (Fig. 1J) (Alvarez and Casadevall, 2007; Ma *et al*., 2007). The mechanism of transfer is unknown, although it is actin-dependent and does not occur with latex beads or killed cryptococci, but the most likely models are transfer along a transient membrane tunnel (Onfelt *et al*., 2004) or co-ordinated, synchronous, exocytosis and endocytosis.

Vomocytosis (non-lytic expulsion)

The vomocytosis of cryptococci is a unique process [although an apparently similar process has been recently described for *Candida albicans* (Bain *et al*., 2012)] by which the fungal cell is expelled from the macrophage without lysis (Fig. 1K) (Alvarez and Casadevall, 2006; Ma *et al*., 2006). Morphologically distinct, but possibly related processes have been described for *Chlamydia* spp. (Hybiske and Stephens, 2007) and *Orientia tsutsugamushi* (Schaechter *et al*., 1957). Vomocytosis occurs through an exocytic mechanism whereby the phagosome fuses with the plasma membrane releasing the cryptococcal cell (Johnston and May, 2010), although the molecular basis for the specific exocytosis of phagosomes containing cryptococci is not known. Exocytosis is a highly regulated process that requires a large number of effector and signalling proteins. It is highly unlikely that *Cryptococcus* provides the machinery that drives exocytosis. Phagosome exocytosis has been long assumed to occur (although has not been explicitly described) to expel harmless undigested material once the phagosome has done its work and it is this pathway that cryptococci may be using (Carnell *et al*., 2011). Therefore, a possible explanation, which is yet to be formally tested, is that cryptococci directly or indirectly modulate macrophage signalling to ectopically induce exocytosis of the phagosome.

The non-lytic escape of a pathogen from immune cells has a number of important implications for the progression of disease. For *Cryptococcus* the two most significant are the crossing of tissue barriers in the host (macrophages acting as an infectious Trojan horse, e.g. from the lungs into the bloodstream and across the blood brain barrier) and in maintaining a latent disease that can subsequently be reactivated under immunocompromised conditions. There are a number of proposed mechanisms for *Cryptococcus* crossing of tissue barriers (Dromer and Levitz, 2011). Support for a model whereby macrophages are responsible for the transfer of cryptococci across the

blood brain barrier comes from the injection of infected mouse monocytes into a second acceptor mouse increases the number of cryptococci within the brain (Charlier *et al*., 2009). However, this has yet to be directly observed *in vivo*. A pulmonary infection model in rat provides laboratory evidence for a persistent asymptomatic infection that may represent latency but the mechanistic role of macrophages (although cryptococci are present intracellularly in macrophages) and specifically vomocytosis in the maintenance of latency (by moving into and out of host cells without triggering a larger immune response) has not been explained (Goldman *et al*., 2000).

Using a flow cytometry analysis an attempt has been made to examine vomocytosis *in vivo* (Nicola *et al*., 2011). Primary mouse macrophages were infected with cryptococci *in vitro* and then introduced into mouse lungs for 24 h, before being re-isolated to allow an estimation of vomocytosis to be made. Based on this method, the authors suggest that vomocytosis rates may be higher *in vivo* than *in vitro*, although this may be influenced by confounding factors such as assortment of cryptococci following host cell division, or transfer of cryptococci between cells. Thus, recapitulating these findings using direct observation *in vivo* is required to confirm these data.

Vomocytosis occurs with both *C. neoformans* and *C. gattii*, with a large variation in incidence between strains, and it is absent or very rare with killed cryptococci (Alvarez and Casadevall, 2006; Ma *et al*., 2006; Voelz *et al*., 2009; 2010; Johnston and May, 2010). Acapsular mutants are deficient in vomocytosis, presumably because their survival in macrophages is poor (although this has not been formally tested). Interestingly, cryptococci that lack the virulence factor phospholipase B1 (or a protein required for its secretion, Sec14) show reduced rates of vomocytosis (Chayakulkeeree *et al*., 2011). Plb1 is a secreted phospholipase that is required for normal eicosanoid production in *Cryptococcus* (Noverr *et al*., 2003). Eicosanoids act as immune modulatory compounds in animals and the cryptococcal eicosanoids and eicosanoid pathway enzymes may thus directly regulate host immune signalling. In support of this, it is known that treating macrophages with Th1 or Th2 cytokines alters the rate of vomocytosis (Voelz *et al*., 2009).

Vomocytosis is dependent on an undisrupted microtubule cytoskeleton but it is not known if microtubules directly provide the driving force for expulsion (Alvarez and Casadevall, 2006; Ma *et al*., 2006). Interestingly, the host actin cytoskeleton is not required for vomocytosis (Alvarez and Casadevall, 2006; Ma *et al*., 2006; Johnston and May, 2010) but prolonged observation via timelapse microscopy revealed repeated cycles of polymerized actin ('actin flashes') on cryptococcal phagosomes many hours after the completion of phagocytosis (Figs 1L and 2B). These flashes are generated through the canonical WASP-Arp2/3 pathway, last from seconds to hours and, when observed in three-dimensions, appear to be dynamic cages around the phagosome (Johnston and May, 2010).

Actin flashes appear to be triggered by phagosome permeabilization and act to prevent successful vomocytosis, allowing the phagosome to reseal. Permeabilization of cryptococcal phagosomes has been previously reported (Tucker and Casadevall, 2002) and has been suggested to be responsible for the presence of cryptococcal capsule throughout the macrophage (although this may also be due to the budding off of vesicles from the phagosome or via macropinocytosis of capsular material shed into the extracellular media). Analysis of phagosome integrity across a population of macrophages indicates that cryptococcal phagosomes lose integrity soon after phagocytosis (Johnston and May, 2010), suggesting that macrophages can successfully block vomocytosis much of the time but that attempted vomocytosis still results in loss of the soluble contents of the phagosome (Fig. 2B). This is likely to be of great advantage to the pathogen, since it provides a mechanism to remove the antimicrobial contents of the phagosome without losing its intracellular niche. In addition, it probably provides a valuable entry route for cytosolic nutrients that are otherwise inaccessible.

Several sets of data indicate that phagosomal pH may be a vital and complex regulatory signal after phagosome maturation and before vomocytosis. If the pH within the phagosome is artificially raised, by the use of weak bases such as chloroquine and ammonium chloride, vomocytosis rates are increased (Ma *et al*., 2006; Nicola *et al*., 2011). In contrast, preventing acidification in the first place by using inhibitors of the vATPase (the H^+ pump responsible for acidifying the phagosome), such as concanamycin A, triggers a *reduction* in the rate of expulsion (Ma *et al*., 2006).

The regulation of the phagosome post maturation is not well understood in mammalian macrophages but recent data from the free-living amoeba *Dictyostelium discoideum* provides a possible explanation. Exocytosis of indigestible material in *Dictyostelium* [a process strikingly similar to vomocytosis (Clarke *et al*., 2002)] requires the removal of the vATPases from the phagosome prior to exocytosis (Carnell *et al*., 2011). Its removal occurs through budding of the vATPase on small vesicles, a process regulated by the Arp2/3 complex activator WASH. By blocking WASH activity, removal of the vATPase and consequently the exocytosis of indigestible material in *Dictyostelium* is reduced. Remarkably, performing the same experiment in macrophages results in a 50% reduction in cryptococcal vomocytosis (Fig. 2B; Carnell *et al*., 2011), suggesting that this process most likely 'hijacks' normal signalling events that control exocytosis of phagosomal remnants.

Future research

The study of the molecular and cellular biology of the interaction of *Cryptococcus* with macrophages has shown this to be a highly complex pathogen with a number of seemingly unique features. There is still much to be learnt not only about *Cryptococcus* but also about the biology and functionality of macrophages in their handling of pathogens. Our understanding of the mechanistic detail of the interaction of cryptococci with the immune system is still to be elucidated and there may well be significant features of the interaction to be identified. Furthermore, there is added complexity in that the majority of studies do not take into account variations introduced by the stage of disease, the presence of other immune components or the site of the interaction within the body. Vomocytosis is a prime example: Is the escape of the intracellular niche beneficial or harmful to *Cryptococcus* and does this depend upon site and stage of disease? Therefore, a key priority for the near future is identifying new approaches and models to validate these *in vitro* findings *in vivo* and in the context of clinical cryptococcosis – a significant hurdle, but one that must be overcome in order to generate real improvement in the prevention and treatment of cryptococcal infections.

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References

Alanio, A., Desnos-Ollivier, M., and Dromer, F. (2011) Dynamics of *Cryptococcus neoformans*-macrophage interactions reveal that fungal background influences outcome during cryptococcal meningoencephalitis in humans. *MBio* **2:** 4.

Alvarez, M., and Casadevall, A. (2006) Phagosome extrusion and host-cell survival after *Cryptococcus neoformans* phagocytosis by macrophages. *Curr Biol* **16:** 2161–2165.

Alvarez, M., and Casadevall, A. (2007) Cell-to-cell spread and massive vacuole formation after *Cryptococcus neoformans* infection of murine macrophages. *BMC Immunol* **8:** 16.

Arora, S., Olszewski, M.A., Tsang, T.M., McDonald, R.A., Toews, G.B., and Huffnagle, G.B. (2011) Effect of cytokine interplay on macrophage polarization during chronic pulmonary infection with *Cryptococcus neoformans*. *Infect Immun* **79:** 1915–1926.

Artavanis-Tsakonas, K., Love, J.C., Ploegh, H.L., and Vyas, J.M. (2006) Recruitment of CD63 to *Cryptococcus neoformans* phagosomes requires acidification. *Proc Natl Acad Sci USA* **103:** 15945–15950.

Bain, J.M., Lewis, L.E., Okai, B., Quinn, J., Gow, N.A., and Erwig, L.P. (2012) Non-lytic expulsion/exocytosis of *Candida albicans* from macrophages. *Fungal Genet Biol* **49:** 677–678.

Ben-Abdallah, M., Sturny-Leclere, A., Ave, P., Louise, A., Moyrand, F., Weih, F., *et al*. (2012) Fungal-induced cell cycle impairment, chromosome instability and apoptosis via differential activation of NF-kappaB. *PLoS Pathog* **8:** e1002555.

Blackstock, R., and Murphy, J.W. (1997) Secretion of the C3 component of complement by peritoneal cells cultured with encapsulated *Cryptococcus neoformans*. *Infect Immun* **65:** 4114–4121.

Bose, I., Reese, A.J., Ory, J.J., Janbon, G., and Doering, T.L. (2003) A yeast under cover: the capsule of *Cryptococcus neoformans*. *Eukaryot Cell* **2:** 655–663.

Brown, S.M., Campbell, L.T., and Lodge, J.K. (2007) *Cryptococcus neoformans*, a fungus under stress. *Curr Opin Microbiol* **10:** 320–325.

Byrnes, E.J., 3rd., Li, W., Lewit, Y., Ma, H., Voelz, K., Ren, P., *et al*. (2010) Emergence and pathogenicity of highly virulent *Cryptococcus gattii* genotypes in the northwest United States. *PLoS Pathog* **6:** e1000850.

Byrnes, E.J., Li, W., Ren, P., Lewit, Y., Voelz, K., Fraser, J.A., *et al*. (2011) A diverse population of *Cryptococcus gattii* molecular type VGIII in southern Californian HIV/AIDS patients. *PLoS Pathog* **7:** e1002205.

Carnell, M., Zech, T., Calaminus, S.D., Ura, S., Hagedorn, M., Johnston, S.A., *et al*. (2011) Actin polymerization driven by WASH causes V-ATPase retrieval and vesicle neutralization before exocytosis. *J Cell Biol* **193:** 831–839.

Casadevall, A. (2012) Amoeba provide insight into the origin of virulence in pathogenic fungi. *Adv Exp Med Biol* **710:** 1–10.

Charlier, C., Nielsen, K., Daou, S., Brigitte, M., Chretien, F., and Dromer, F. (2009) Evidence of a role for monocytes in dissemination and brain invasion by *Cryptococcus neoformans*. *Infect Immun* **77:** 120–127.

Chaturvedi, V., and Chaturvedi, S. (2011) *Cryptococcus gattii*: a resurgent fungal pathogen. *Trends Microbiol* **19:** 564–571.

Chayakulkeeree, M., Johnston, S.A., Oei, J.B., Lev, S., Williamson, P.R., Wilson, C.F., *et al*. (2011) SEC14 is a specific requirement for secretion of phospholipase B1 and pathogenicity of *Cryptococcus neoformans*. *Mol Microbiol* **80:** 1088–1101.

Chun, C.D., Brown, J.C., and Madhani, H.D. (2011) A major role for capsule-independent phagocytosis-inhibitory mechanisms in mammalian infection by *Cryptococcus neoformans*. *Cell Host Microbe* **9:** 243–251.

Clarke, M., Kohler, J., Arana, Q., Liu, T., Heuser, J., and Gerisch, G. (2002) Dynamics of the vacuolar H(+)-ATPase in the contractile vacuole complex and the endosomal pathway of Dictyostelium cells. *J Cell Sci* **115** (Part 14): 2893–2905.

Crabtree, J.N., Okagaki, L.H., Wiesner, D.L., Strain, A.K., Nielsen, J.N., and Nielsen, K. (2012) Titan cell production enhances the virulence of *Cryptococcus neoformans*. *Infect Immun* **80:** 3776–3785.

Cross, C.E., and Bancroft, G.J. (1995) Ingestion of acapsular

Cryptococcus neoformans occurs via mannose and beta-glucan receptors, resulting in cytokine production and increased phagocytosis of the encapsulated form. *Infect Immun* **63:** 2604–2611.

Davies, S.F., Clifford, D.P., Hoidal, J.R., and Repine, J.E. (1982) Opsonic requirements for the uptake of *Cryptococcus neoformans* by human polymorphonuclear leukocytes and monocytes. *J Infect Dis* **145:** 870–874.

Deretic, V. (2012) Autophagy as an innate immunity paradigm: expanding the scope and repertoire of pattern recognition receptors. *Curr Opin Immunol* **24:** 21–31.

Diamond, R.D., and Bennett, J.E. (1973) Growth of *Cryptococcus neoformans* within human macrophages *in vitro*. *Infect Immun* **7:** 231–236.

Doering, T.L. (2009) How sweet it is! Cell wall biogenesis and polysaccharide capsule formation in *Cryptococcus neoformans*. *Annu Rev Microbiol* **63:** 223–247.

Doering, T.L., Nosanchuk, J.D., Roberts, W.K., and Casadevall, A. (1999) Melanin as a potential cryptococcal defence against microbicidal proteins. *Med Mycol* **37:** 175–181.

Dromer, F., and Levitz, S.M. (2011) Invasion of *Cryptococcus* into the central nervous system. In *Cryptococcus: From Human Pathogen to Model Yeast*. Heitman, J., Kozel, T.R., Kwon-Chung, K.J., Perfect, J.R., and Casadevall, A. (eds). Washington, DC: ASM Press, pp. 465–471.

Dromer, F., Casadevall, A., Perfect, J.R., and Sorrel, T. (2011) *Cryptococcus neoformans:* latency and disease. In *Cryptococcus: From Human Pathogen to Model Yeast*. Heitman, J., Kozel, T.R., Kwon-Chung, K.J., Perfect, J.R., and Casadevall, A. (eds). Washington, DC: ASM Press, pp. 431–439.

Eisenman, H.C., Mues, M., Weber, S.E., Frases, S., Chaskes, S., Gerfen, G., and Casadevall, A. (2007) *Cryptococcus neoformans* laccase catalyses melanin synthesis from both D- and L-DOPA. *Microbiology* **153** (Part 12)**:** 3954–3962.

Fairn, G.D., and Grinstein, S. (2012) How nascent phagosomes mature to become phagolysosomes. *Trends Immunol* **33:** 397–405.

Frases, S., Pontes, B., Nimrichter, L., Rodrigues, M.L., Viana, N.B., and Casadevall, A. (2009) The elastic properties of the *Cryptococcus neoformans* capsule. *Biophys J* **97:** 937–945.

Geunes-Boyer, S., Oliver, T.N., Janbon, G., Lodge, J.K., Heitman, J., Perfect, J.R., and Wright, J.R. (2009) Surfactant protein D increases phagocytosis of hypocapsular *Cryptococcus neoformans* by murine macrophages and enhances fungal survival. *Infect Immun* **77:** 2783–2794.

Geunes-Boyer, S., Beers, M.F., Perfect, J.R., Heitman, J., and Wright, J.R. (2012) Surfactant protein D facilitates *Cryptococcus neoformans* infection. *Infect Immun* **80:** 2444–2453.

Goldman, D.L., Lee, S.C., Mednick, A.J., Montella, L., and Casadevall, A. (2000) Persistent *Cryptococcus neoformans* pulmonary infection in the rat is associated with intracellular parasitism, decreased inducible nitric oxide synthase expression, and altered antibody responsiveness to cryptococcal polysaccharide. *Infect Immun* **68:** 832–838.

Goldman, D.L., Khine, H., Abadi, J., Lindenberg, D.J., Pirofski, L., Niang, R., and Casadevall, A. (2001) Serologic evidence for *Cryptococcus neoformans* infection in early childhood. *Pediatrics* **107:** E66.

Heung, L.J., Luberto, C., Plowden, A., Hannun, Y.A., and Del Poeta, M. (2004) The sphingolipid pathway regulates Pkc1 through the formation of diacylglycerol in *Cryptococcus neoformans*. *J Biol Chem* **279:** 21144–21153.

Huynh, K.K., Kay, J.G., Stow, J.L., and Grinstein, S. (2007) Fusion, fission, and secretion during phagocytosis. *Physiology (Bethesda)* **22:** 366–372.

Hybiske, K., and Stephens, R.S. (2007) Mechanisms of host cell exit by the intracellular bacterium Chlamydia. *Proc Natl Acad Sci USA* **104:** 11430–11435.

Johnston, S.A., and May, R.C. (2010) The human fungal pathogen *Cryptococcus neoformans* escapes macrophages by a phagosome emptying mechanism that is inhibited by Arp2/3 complex-mediated actin polymerisation. *PLoS Pathog* **6:** e1001041.

Kozel, T.R. (1977) Non-encapsulated variant of *Cryptococcus neoformans*. II. Surface receptors for cryptococcal polysaccharide and their role in inhibition of phagocytosis by polysaccharide. *Infect Immun* **16:** 99–106.

Levitz, S.M., and Tabuni, A. (1991) Binding of *Cryptococcus neoformans* by human cultured macrophages. Requirements for multiple complement receptors and actin. *J Clin Invest* **87:** 528–535.

Levitz, S.M., Farrell, T.P., and Maziarz, R.T. (1991) Killing of *Cryptococcus neoformans* by human peripheral blood mononuclear cells stimulated in culture. *J Infect Dis* **163:** 1108–1113.

Levitz, S.M., Nong, S.H., Seetoo, K.F., Harrison, T.S., Speizer, R.A., and Simons, E.R. (1999) *Cryptococcus neoformans* resides in an acidic phagolysosome of human macrophages. *Infect Immun* **67:** 885–890.

Liu, O.W., Chun, C.D., Chow, E.D., Chen, C., Madhani, H.D., and Noble, S.M. (2008) Systematic genetic analysis of virulence in the human fungal pathogen *Cryptococcus neoformans*. *Cell* **135:** 174–188.

Ma, H., Croudace, J.E., Lammas, D.A., and May, R.C. (2006) Expulsion of live pathogenic yeast by macrophages. *Curr Biol* **16:** 2156–2160.

Ma, H., Croudace, J.E., Lammas, D.A., and May, R.C. (2007) Direct cell-to-cell spread of a pathogenic yeast. *BMC Immunol* **8:** 15.

Ma, H., Hagen, F., Stekel, D.J., Johnston, S.A., Sionov, E., Falk, R., *et al*. (2009) The fatal fungal outbreak on Vancouver Island is characterized by enhanced intracellular parasitism driven by mitochondrial regulation. *Proc Natl Acad Sci USA* **106:** 12980–12985.

McQuiston, T., Luberto, C., and Del Poeta, M. (2010) Role of host sphingosine kinase 1 in the lung response against Cryptococcosis. *Infect Immun* **78:** 2342–2352.

Mukherjee, S., Feldmesser, M., and Casadevall, A. (1996) J774 murine macrophage-like cell interactions with *Cryptococcus neoformans* in the presence and absence of opsonins. *J Infect Dis* **173:** 1222–1231.

Nakamura, K., Kinjo, T., Saijo, S., Miyazato, A., Adachi, Y., Ohno, N., *et al*. (2007) Dectin-1 is not required for the host defense to *Cryptococcus neoformans*. *Microbiol Immunol* **51:** 1115–1119.

Nicola, A.M., Robertson, E.J., Albuquerque, P., Derengowski Lda, S., and Casadevall, A. (2011) Nonlytic exocytosis of *Cryptococcus neoformans* from macrophages occurs *in vivo* and is influenced by phagosomal pH. *MBio* **2:** 4.

Nicola, A.M., Albuquerque, P., Martinez, L.R., Dal-Rosso, R.A., Saylor, C., De Jesus, M., *et al*. (2012) Macrophage autophagy in immunity to *Cryptococcus neoformans* and *Candida albicans*. *Infect Immun* **80:** 3065–3076.

Nordenfelt, P., and Tapper, H. (2011) Phagosome dynamics during phagocytosis by neutrophils. *J Leukoc Biol* **90:** 271–284.

Noverr, M.C., Cox, G.M., Perfect, J.R., and Huffnagle, G.B. (2003) Role of PLB1 in pulmonary inflammation and cryptococcal eicosanoid production. *Infect Immun* **71:** 1538–1547.

Okagaki, L.H., and Nielsen, K. (2012) Titan cells confer protection from phagocytosis in *Cryptococcus neoformans* infections. *Eukaryot Cell* **11:** 820–826.

Okagaki, L.H., Strain, A.K., Nielsen, J.N., Charlier, C., Baltes, N.J., Chretien, F., *et al*. (2010) Cryptococcal cell morphology affects host cell interactions and pathogenicity. *PLoS Pathog* **6:** e1000953.

Okagaki, L.H., Wang, Y., Ballou, E.R., O'Meara, T.R., Bahn, Y.S., Alspaugh, J.A., *et al*. (2011) Cryptococcal titan cell formation is regulated by G-protein signaling in response to multiple stimuli. *Eukaryot Cell* **10:** 1306–1316.

Onfelt, B., Nedvetzki, S., Yanagi, K., and Davis, D.M. (2004) Cutting edge: membrane nanotubes connect immune cells. *J Immunol* **173:** 1511–1513.

Park, B.J., Wannemuehler, K.A., Marston, B.J., Govender, N., Pappas, P.G., and Chiller, T.M. (2009) Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS* **23:** 525–530.

Qin, Q.M., Luo, J., Lin, X., Pei, J., Li, L., Ficht, T.A., and de Figueiredo, P. (2011) Functional analysis of host factors that mediate the intracellular lifestyle of *Cryptococcus neoformans*. *PLoS Pathog* **7:** e1002078.

Schaechter, M., Bozeman, F.M., and Smadel, J.E. (1957) Study on the growth of Rickettsiae. II. Morphologic observations of living Rickettsiae in tissue culture cells. *Virology* **3:** 160–172.

Shao, X., Rivera, J., Niang, R., Casadevall, A., and Goldman, D.L. (2005) A dual role for TGF-beta1 in the control and persistence of fungal pneumonia. *J Immunol* **175:** 6757–6763.

Shapiro, S., Beenhouwer, D.O., Feldmesser, M., Taborda, C., Carroll, M.C., Casadevall, A., and Scharff, M.D. (2002) Immunoglobulin G monoclonal antibodies to *Cryptococcus neoformans* protect mice deficient in complement component C3. *Infect Immun* **70:** 2598–2604.

Shea, J.M., Kechichian, T.B., Luberto, C., and Del Poeta, M. (2006) The cryptococcal enzyme inositol phosphosphingolipid-phospholipase C confers resistance to the antifungal effects of macrophages and promotes fungal dissemination to the central nervous system. *Infect Immun* **74:** 5977–5988.

Siddiqui, A.A., Brouwer, A.E., Wuthiekanun, V., Jaffar, S., Shattock, R., Irving, D., *et al*. (2005) IFN-gamma at the site of infection determines rate of clearance of infection in cryptococcal meningitis. *J Immunol* **174:** 1746–1750.

Spira, G., Paizi, M., Mazar, S., Nussbaum, G., Mukherjee, S., and Casadevall, A. (1996) Generation of biologically active anti-*Cryptococcus neoformans* IgG, IgE and IgA isotype switch variant antibodies by acridine orange mutagenesis. *Clin Exp Immunol* **105:** 436–442.

Stano, P., Williams, V., Villani, M., Cymbalyuk, E.S., Qureshi, A., Huang, Y., *et al*. (2009) App1: an antiphagocytic protein that binds to complement receptors 3 and 2. *J Immunol* **182:** 84–91.

Steenbergen, J.N., and Casadevall, A. (2003) The origin and maintenance of virulence for the human pathogenic fungus *Cryptococcus neoformans*. *Microbes Infect* **5:** 667–675.

Taborda, C.P., and Casadevall, A. (2002) CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are involved in complement-independent antibody-mediated phagocytosis of *Cryptococcus neoformans*. *Immunity* **16:** 791–802.

Tucker, S.C., and Casadevall, A. (2002) Replication of *Cryptococcus neoformans* in macrophages is accompanied by phagosomal permeabilization and accumulation of vesicles containing polysaccharide in the cytoplasm. *Proc Natl Acad Sci USA* **99:** 3165–3170.

Velagapudi, R., Hsueh, Y.P., Geunes-Boyer, S., Wright, J.R., and Heitman, J. (2009) Spores as infectious propagules of *Cryptococcus neoformans*. *Infect Immun* **77:** 4345–4355.

Voelz, K., Lammas, D.A., and May, R.C. (2009) Cytokine signaling regulates the outcome of intracellular macrophage parasitism by *Cryptococcus neoformans*. *Infect Immun* **77:** 3450–3457.

Voelz, K., Johnston, S.A., Rutherford, J.C., and May, R.C. (2010) Automated analysis of cryptococcal macrophage parasitism using GFP-tagged cryptococci. *PLoS ONE* **5:** e15968.

West, A.P., Brodsky, I.E., Rahner, C., Woo, D.K., Erdjument-Bromage, H., Tempst, P., *et al*. (2011) TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. *Nature* **472:** 476–480.

Wozniak, K.L., and Levitz, S.M. (2008) *Cryptococcus neoformans* enters the endolysosomal pathway of dendritic cells and is killed by lysosomal components. *Infect Immun* **76:** 4764–4771.

Zaragoza, O., Taborda, C.P., and Casadevall, A. (2003) The efficacy of complement-mediated phagocytosis of *Cryptococcus neoformans* is dependent on the location of C3 in the polysaccharide capsule and involves both direct and indirect C3-mediated interactions. *Eur J Immunol* **33:** 1957–1967.

Zaragoza, O., Chrisman, C.J., Castelli, M.V., Frases, S., Cuenca-Estrella, M., Rodriguez-Tudela, J.L., and Casadevall, A. (2008) Capsule enlargement in *Cryptococcus neoformans* confers resistance to oxidative stress suggesting a mechanism for intracellular survival. *Cell Microbiol* **10:** 2043–2057.

Zaragoza, O., Garcia-Rodas, R., Nosanchuk, J.D., Cuenca-Estrella, M., Rodriguez-Tudela, J.L., and Casadevall, A. (2010) Fungal cell gigantism during mammalian infection. *PLoS Pathog* **6:** e1000945.