OPINION

Intracellular pathogenic bacteria and fungi — a case of convergent evolution?

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Abstract | The bacterium *Yersinia pestis* and the fungus *Cryptococcus neoformans* are the causative agents of human plague and cryptococcosis, respectively. Both microorganisms are facultatively intracellular pathogens. A comparison of their pathogenic strategies reveals similar tactics for intracellular survival in *Y. pestis* and *C. neoformans* despite their genetic unrelatedness. Both organisms can survive in environments where they are vulnerable to predation by amoeboid protozoal hosts. Here, we propose that the overall similarities in their pathogenic strategies are an example of convergent evolution that has solved the problem of intracellular survival.

Pathogenic microorganisms are often classified as intracellular or extracellular depending on whether their life cycle in the host involves residence or replication inside host cells. Although some extracellular pathogens are found inside cells during their interactions with a host, this categorization has generally proved useful when considering microbial survival strategies in the host. Some obligately intracellular pathogens are completely dependent on host cells for survival and replication; for example, viruses, bacteria, including species of *Ehrlichia*, *Chlamydia* and *Rickettsia*, and eukaryotes, such as *Toxoplasma gondii* and *Plasmodium* spp. Obligately intracellular pathogens often have reduced genomes, as they have lost many of the biosynthetic genes that are needed for a free-living existence. Other microorganisms, such as the prokaryotes *Legionella pneumophila* and *Yersinia pestis* and the eukaryote *Cryptococcus neoformans*, retain the capacity to survive independently of the host, and are referred to as facultatively intracellular pathogens. These organisms tend to have complex life cycles in which their encounter with a vertebrate host is often accidental or only a part of their total interactions with the biota. Interestingly, none of the known human pathogenic fungi is an obligately intracellular pathogen, and many are soil dwellers. It is clear that although no two intracellular microorganisms have identical interactions with their hosts, there are some similar themes in pathogenic strategies. For example, a comparison of the survival strategies of five facultatively intracellular pathogens that can cause lung infections (*Y. pestis*, *C. neoformans*, *Histoplasma capsulatum*, *L. pneumophila* and *Francisella tularensis*) reveals commonalities as well as differences (TABLE 1). The interaction of *L. pneumophila* with amoebae has been extensively studied[1–3]. Of the groups described in TABLE 1, *F. tularensis* is unique, as it replicates within the cytosol of the macrophage after exiting from the phagosome[4–6]. Although *L. pneumophila* is restricted to a vacuole compartment during its intracellular growth phase, the biogenesis of this vacuole is diverted from the normal endocytic pathway until late in the replication cycle[3,7,8]. However, *Y. pestis*, *C. neoformans* and *H. capsulatum* replicate in vacuoles that interact with the endocytic pathway and develop features that are characteristic of mature phagosomes.

In this Opinion article, we compare the intracellular pathogenic strategies of the bacterium *Y. pestis* and the fungus *C. neoformans* to illustrate our central hypothesis: the similarities in intracellular pathogenic strategies reflect the artistry of convergent evolution driven by environmental biotic pressures, such as protozoan predators.

Comparison of *Y. pestis* and *C. neoformans*

Y. pestis and *C. neoformans* are different types of human pathogens (TABLE 2). *Y. pestis*, which is the aetiological agent of plague, is usually transmitted by fleas. Infection through flea bites results in the bubonic form of infection. *Y. pestis* can also be transmitted between human hosts by inhalation of respiratory droplets, which results in pneumonic plague. By contrast, *C. neoformans*, the aetiological agent of cryptococcosis, is not acquired from an infected host, but is acquired from the environment through inhalation of aerosolized spores or desiccated yeast cells that are capable of alveolar deposition. Plague is an ancient disease, whereas cryptococcosis was only described at the end of the nineteenth century. Plague was largely controlled by improvements in hygiene and sanitation, but cryptococcosis has become a major problem in the late twentieth century, primarily in immunocompromised individuals.

Y. pestis is a subclone of *Yersinia pseudotuberculosis*, an enteric pathogen that is typically associated with lymphadenitis[9,10]. Although *Y. pestis* has acquired several unique and important virulence factors during its recent evolution from *Y. pseudotuberculosis*[9–11], it is likely that most *Y. pestis* virulence factors were directly inherited from *Y. pseudotuberculosis*. *Y. pestis* was classified as a facultatively intracellular pathogen in the late 1950s (discussed below), but it recently became clear that *Y. pseudotuberculosis* shares the ability of *Y. pestis* to survive and replicate in macrophages[12]. Based on this finding, it was proposed that the ability to replicate in macrophages evolved first in *Y. pseudotuberculosis*, and that this trait was passed on to *Y. pestis*[12]. *Y. pseudotuberculosis* is capable of surviving in water and soil[13,14] and *Y. pestis* can survive in soil[15]. Therefore, both organisms are probably subject to predation by a range of protozoa, including amoebae. It is possible that the ability to survive in phagocytic cells was first selected for in *Y. pseudotuberculosis*,

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Table 1 | **Comparison of intra-macrophage survival strategies of selected respiratory pathogens**

Pathogen	Replicates in macrophage phagosome?	Macrophage phagosome matures?	Macrophage phagosome acidifies?	Major survival mechanisms within macrophages	Escapes macrophage without killing it?	References
Cryptococcus neoformans	Yes	Yes; LAMP1 positive	Yes; only at the early stages	Antioxidants, melanin, capsule, phospholipase and urease	Yes	52,53,79
Histoplasma capsulatum	Yes	Yes	Partially (pH ~6.5); differences have been observed between mouse and human macrophages	CBP and cell wall α-(1,3)-glucan	Unknown	80–82
Yersinia pestis	Yes	Yes; LAMP1 and cathepsin-D positive	Unknown	PhoP-regulated resistance to phagosome stress and Rip proteins in activated macrophages	Unknown	31,51,61
Legionella pneumophila	Yes	Yes; only at the late stages	Yes; only at the late stages	Type IV secretion system directs vacuole biogenesis	No	3,7,8
Francisella tularensis	No (cytosolic replication)	Not applicable	Not applicable	*F. tularensis* pathogenicity island mediates phagosome escape	Unknown	4–6

CBP, calcium-binding protein; LAMP1, lysosome-associated membrane glycoprotein 1.

probably as a strategy to avoid intracellular killing by amoebae, similarly to *C. neoformans* (discussed below). Consistent with this notion, some articles in the Russian literature suggest that both *Y. pseudotuberculosis* and *Y. pestis* can survive in protozoa (reviewed in REF. 16). The idea that *Y. pestis* might survive in the soil of rodent burrows, and that this 'telluric' phase could provide the reservoir necessary for establishment of a pandemic, has recently been reviewed[16].

Early studies by Cavanaugh and Randall[17] and Burrows and Bacon[18] established a model to explain the temperature-regulated transition of *Y. pestis* from an intracellular to an extracellular pathogen during infection. The authors observed that bacteria grown at 28 °C were susceptible to phagocytosis by neutrophils (polymorphonuclear leukocytes; PMNs) and monocytes, and ingested bacteria were killed in PMNs but multiplied in monocytes. Bacteria grown at 37 °C for 3–5 hours became moderately resistant to phagocytosis by PMNs, whereas bacteria grown at 37 °C for more than 9 hours became encapsulated and were maximally resistant to phagocytosis by both PMNs and monocytes. The authors proposed that *Y. pestis*, which is deposited in the dermis during a flea bite, exploits monocytes as a niche in which it can replicate and become protected from the bactericidal action of PMNs. After its release from damaged monocytes, *Y. pestis* had a phagocytosis resistance phenotype, allowing it to counteract phagocytosis and to replicate extracellularly to establish bubonic plague in a susceptible host[17].

Histological studies of tissues infected with *Y. pestis* revealed that the bacterium mainly replicates in an extracellular form, at the late stages of infection (see REF. 19 for a review of older studies and REF. 20 for a more recent example). *Y. pestis* produces a formidable array of virulence factors that promote resistance to phagocytosis and enhance extracellular growth. These factors include antiphagocytic proteins in the form of Yop effectors, adhesins and capsules, all of which are maximally produced at 37 °C[9,10,21–23].

It has been difficult to identify an intracellular phase for *Y. pestis* at the early stages of infection by histological or microscopic examination of infected tissue, probably owing to the small numbers of bacteria present. Despite this challenge, *Y. pestis* has been detected inside alveolar macrophages of animals that were experimentally infected by the pulmonary route[24–26]. In a recent study of pulmonary infection of mice with *Y. pestis*, Lathem *et al.*[20] were unable to visualize the bacteria in lung tissue by microscopy 24 hours post-infection, despite the fact that the lungs contained 10^5–10^6 viable microorganisms. However, 48 hours post-infection, extracellular *Y. pestis* bacteria were clearly visible within the alveoli and small bronchioles[20]. It is becoming clear that in the first 24–36 hours following exposure to *Y. pestis*, there is little inflammation. However, by 48 hours post-infection there is a highly proinflammatory state[20,27]. The possibility that the bacteria are present inside alveolar macrophages in the lungs during the initial low inflammatory state would be consistent with earlier studies[24–26].

Evidence supporting a role for intra-macrophage survival of *Y. pestis* in plague comes from studies of strains carrying mutations in the *phoP* gene. PhoP is a transcription factor that is known to play an important part in intra-macrophage survival of *Salmonella* species[28,29]. *Y. pestis phoP* mutants are defective for survival in macrophages[30,31] (discussed in more detail below), and are attenuated in bubonic[30] and pneumonic (J.B.B., unpublished observations) mouse infection models, which suggests that transient residence of the bacteria in phagocytic cells has an important role in plague pathogenesis.

Detecting intracellular *Y. pestis* in tissues at the early stages of experimental bubonic plague infections will prove challenging owing to the incredibly low infectious dose of this pathogen. Following intradermal infection of rats, Sebbane *et al.*[32] were unable to detect intracellular forms of the bacterium in either lymph vessels that drained the inoculation site or primary lymph nodes. It could be argued that a logical time to look for intracellular *Y. pestis* would be immediately following natural inoculation by a flea bite. However, several factors make this a complicated undertaking. First, technical hurdles must be overcome to capture the infected tissue at the appropriate time point. Second, fleas can occasionally canulate blood vessels in the dermis, allowing *Y. pestis* to initiate septicaemic plague[33]. *Y. pestis* internalized by blood monocytes could be transported away from the initial site of infection, making detection difficult. Finally, it has been proposed that during a blood meal, infected fleas deposit fragments of *Y. pestis* biofilms into the dermis[34,35]. Because bacteria embedded in a biofilm are more resistant to uptake or killing by phagocytes, this process could limit the uptake of *Y. pestis* by macrophages in the early stages of bubonic plague[35].

Large clumps of *Y. pestis* in biofilm fragments are potentially resistant to

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Table 2 | **Comparison of *Yersinia pestis* and *Cryptococcus neoformans***

	Y. pestis	*C. neoformans*
Kingdom	Bacteria	Fungi
Size	Gram-negative rod of 1 μm	Yeast cell of 4–100 μm
Genome	4.6 Mb	20 Mb
Ecological niche	Soil of rodent burrows?	Bird excreta and trees
Infection	Flea bite and inhalation	Inhalation
Environment-associated hosts	Enzootic and epizootic in burrowing rodents and their fleas	None known; possibly amoebae
Potential hosts	Protista, insects (fleas), rodents and other mammals	Protista, slime mould, insects, birds, amphibians, reptiles and mammals
Major virulence factors	• Type III secretion system • Ybt siderophore system • Pla protease • Psa pili • Transcriptional regulators	• Polysaccharide capsule • Melanin synthesis • Phospholipase • Urease • Mannitol synthesis

phagocytosis, similarly to *C. neoformans*, which uses its large size and extensive capsule to reduce ingestion by phagocytosis. Although the rat bubonic plague model used by Sebbane *et al.*[32] did not yield evidence for intracellular residence of *Y. pestis* at early stages of infection, another group[36] reported extensive intracellular residence of *Y. pestis* in the spleen in a mouse bubonic plague infection model. These investigators reported that after *Y. pestis* disseminated to the spleen following subcutaneous inoculation, the bacteria were largely present in spleen macrophages 3 days post-infection, whereas 5 days post-infection significantly higher numbers of extracellular bacteria were detected[36]. In summary, an intracellular phase for *Y. pestis* is likely to be of greatest importance during the early stages of infection. In addition, the extent to which *Y. pestis* survives within macrophages *in vivo* might also depend on the tissue site as well as the specific host.

C. neoformans has the capacity to replicate in macrophages[37] and histological sections often depict yeast forms inside macrophages in granulomatous inflammation[38], but the crucial role of intracellular residence in pathogenesis was not recognized until the late 1990s[39]. *C. neoformans* is a free-living, soil-dwelling organism that can survive in extracellular and intracellular spaces during mammalian infection. The relative contributions of intracellular and extracellular replication to the overall virulence phenotype are unknown, but there is evidence that both are important. Extracellular growth in the meninges and ventricular spaces, combined with the release of capsular polysaccharide into tissues, is associated with brain oedema. By contrast, intracellular growth and survival might be required for extrapulmonary dissemination, crossing of the blood–brain barrier and latency[40]. Furthermore, intracellular residency could provide the fungus with a refuge against antifungal drugs and immune clearance mechanisms.

In summary, there is evidence that both *Y. pestis* and *C. neoformans* are facultatively intracellular pathogens and that the intracellular phase of infection could make a substantial contribution to mammalian pathogenesis.

Entry into cells

Y. pestis is taken up efficiently by macrophages when the bacteria are cultured at ambient temperatures (25–28 °C). Uptake can occur in the absence of complement or antibody opsonization, which is in stark contrast to *C. neoformans*, for which little or no ingestion occurs in the absence of opsonins. It is likely that a specific surface structure mediates this efficient uptake process, but such a factor remains unidentified. Uptake of non-opsonized bacteria is reduced when the bacteria are cultured at 37 °C, owing to the production of antiphagocytic Yop proteins, pili or capsule. Opsonization with antibody that recognizes the bacterial surface increases uptake of *Y. pestis* grown at 37 °C[41,42]. It is unknown if the mechanism of uptake affects intracellular survival or replication of *Y. pestis* in macrophages. Unlike the cells of *Y. pestis*, *C. neoformans* cells are surrounded by an antiphagocytic polysaccharide capsule, and cell phagocytosis is further inhibited by the ability of cryptococcal cells to rapidly increase the size of the capsule *in vivo*.

Both *Y. pestis* and *C. neoformans* can invade non-phagocytic cells, such as epithelial and endothelial cells[43–45]. It has been proposed that in *Y. pestis* this process allows the bacteria to invade an epithelial layer that overlies lymphoid tissue[44]. It is unknown if replication of the bacterium occurs following the internalization of *Y. pestis* into non-phagocytic cells, and the contribution of non-professional phagocytes to plague pathogenesis remains unclear. By contrast, the importance of the *C. neoformans* interaction with non-phagocytic cells for pathogenesis is well established. *C. neoformans* binds to, and induces its uptake by, brain endothelial cells to begin a process that leads to transcellular crossing of the blood–brain barrier[46]. Although the mechanism by which *C. neoformans* induces its own uptake by endothelial cells is unknown, contact with fungal cells induces actin and cytoskeletal rearrangement in the host cell[47].

Ingestion of *C. neoformans* by mammalian phagocytic cells can have a variable outcome depending on the species used. In mouse and human macrophages, *C. neoformans* replicates *in vitro*, whereas rat macrophages can inhibit intracellular replication[37,48,49]. Mouse macrophages differ in their ability to inhibit *C. neoformans* intracellular replication and, at least for Balb/c and CBA/J mice, there is a correlation between murine susceptibility to pulmonary infection and the permissiveness of alveolar macrophages to intracellular replication[50]. The factors responsible for these differences are not understood, but, at least in a comparison between rat and mouse macrophages, there was an association between greater phagocytic burst and lysozyme production with control of intracellular replication[48]. It therefore seems that mammalian macrophages can inhibit *C. neoformans* if they produce sufficient amounts of antimicrobial compounds.

Phagosome biology

Following internalization into macrophages, both *Y. pestis* and *C. neoformans* reside in partially or fully mature phagosomes[31,51–53]. The *Y. pestis*-containing phagosome undergoes fusion with late endosomes or lysosomes for at least the first 8 hours post-infection, as shown by the presence of lysosome-associated membrane glycoprotein 1 (LAMP1) or cathepsin D in these compartments[31]. The phagosome initially forms tightly around the bacterium, but at around 8 hours post-infection begins to enlarge, and the formation of spacious phagosomes is associated with the start of bacterial replication[31]. An expansive phagosome that contains *C. neoformans* is also observed owing to the presence of the capsule, which might reflect a common mechanism of

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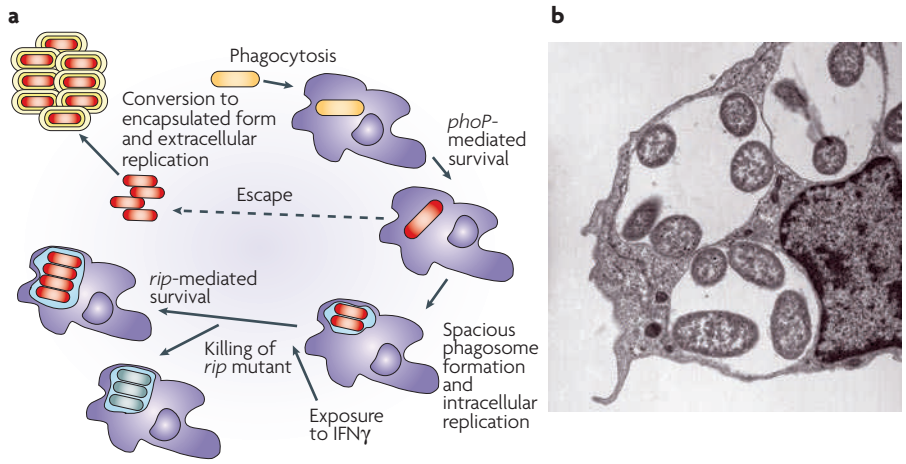

Figure 1 | **Model for the interaction of *Yersinia pestis* with macrophages. a** | A schematic of the interaction of *Y. pestis* with macrophages. *Y. pestis* grown at ambient temperature (28 °C) is shown in yellow. *Y. pestis* is phagocytosed by a macrophage and uses products of *phoP*-regulated genes to survive in a phagosome. The bacterium converts to a phagocytosis-resistant form (red) in the phagosome, and subsequently escapes the macrophage by an unknown mechanism (dashed arrow) or replicates within a spacious phagosome (light blue). Exposure of the macrophage to interferon-γ (IFNγ) results in activation. *Y. pestis* uses products of the *rip* operon to continue to replicate in the activated macrophage until its escape. Alternatively, *rip* mutant bacteria are killed (green). *Y. pestis* that escapes macrophages becomes encapsulated (yellow outer layers) and replicates to form aggregates of extracellular bacteria. **b** | A thin-section electron-microscopy image of *Y. pestis* in spacious phagosomes of a primary murine macrophage (magnification of x10,000).

intracellular survival. The mechanism of spacious-phagosome formation by *Y. pestis* has not been identified, but unidirectional fusion of endocytic compartments with the phagosome could occur. It is unknown to what extent the *Y. pestis* phagosome acidifies. A study that examined the acidification of macrophage phagosomes that contained *Y. pseudotuberculosis* indicated that the bacterium actively prevented acidification of the vacuole below pH 6 (REF. 54). If the same is true for *Y. pestis*, it could explain how the bacterium can survive in a vacuole that is undergoing fusion with late endosomes or lysosomes, as acidification of phagosomes is required to activate the full bactericidal activity of the compartment[55].

The cryptococcal phagosome in human monocytes acidifies to a pH of 5.1 (REF. 53). The fact that no difference in pH was measured between phagosomes that contained live or dead fungi led to the conclusion that, unlike *H. capsulatum*, *C. neoformans* does not actively regulate phagosomal pH[53]. However, subsequent studies revealed that after several hours the pH inside the cryptococcal phagosome increased. This was attributed to a loss of phagosomal membrane integrity, as the phagosome became leaky to large molecules, such as dextran[52]. Although the mechanism for this phagosome compromise is not well understood, there is indirect evidence that fungal phospholipases could be involved in this process, as phospholipase-deficient mutants have a transient intracellular growth defect[56]. Phagosomal acidification to approximately pH 5 does not necessarily create a non-permissive environment for *C. neoformans*. In fact, *C. neoformans*, like many fungi, is far more tolerant of acidic than basic conditions and, in common with the intracellular bacterial pathogen *Salmonella enterica* subsp. *enterica* serovar Typhimurium (S. Typhimurium), survives partial phagosomal acidification. Consistent with this observation, diprotic, weak bases that increase phagosomal pH are fungicidal for *C. neoformans* in macrophages by mechanisms that are dependent on pH[57,58]. Evidence for maturation of the *C. neoformans* phagosome through fusion with late endosomal components comes from the observation that phagosomes are decorated with LAMP1, a glycoprotein that is considered to be a marker of phagosome–lysosome fusion[53].

Avoidance of intracellular killing

Both *Y. pestis* and *C. neoformans* survive in phagosomes by interfering with phagosomal microbicidal mechanisms. Both microorganisms overcome phagosomal oxidative and non-oxidative killing and iron deprivation, but achieve this outcome through different mechanisms. The identification of PhoP-regulated genes in *Y. pestis* that are required for survival in macrophage phagosomes showed that the bacterium adapts to the phagosomal environment by increasing its resistance to antimicrobial peptides and by overcoming the stress of magnesium limitation[31]. The ability to obtain iron from host sources is crucial for virulence in many pathogens. In *Y. pestis*, the *ybt* genes encode a siderophore system that is essential for virulence during the extracellular growth phase of the bacterium[59]. Two additional transport systems, the Yfe and Feo systems, seem to function as redundant ferrous iron transporters that are capable of iron acquisition during intracellular growth of *Y. pestis* in macrophages[60].

Macrophages activated with interferon-γ (IFNγ) show dramatically increased bactericidal activity owing to the production of increased reactive oxygen and nitrogen species. Bacterial pathogens might also have to adjust their metabolic activities to survive in activated macrophages. Once *Y. pestis* has established a productive infection of murine macrophages, it can continue to survive and replicate even after the infected macrophages are activated with IFNγ[61]. The chromosomally encoded *rip* operon is required for survival and replication of *Y. pestis* in activated macrophages[61]. The three protein products of the *rip* operon (RipC, RipB and RipA) are predicted to encode metabolic enzymes, and it has been proposed that these enzymes allow *Y. pestis* to adjust its metabolism in response to macrophage activation[61]. Homologues of the Rip proteins are found in other facultatively intracellular bacteria, including *S.* Typhimurium, *Salmonella enterica* subsp. *enterica* serovar Enteritidis and *Mycobacterium tuberculosis*[61]. The structure of the RipC homologue in *M. tuberculosis* (CitE) has been solved[62], which revealed that CitE might function as a novel citrate lyase that produces acetyl coenzyme A during fatty-acid biosynthesis[62].

C. neoformans avoids intracellular killing through several mechanisms that work in concert. Two obvious differences between the *C. neoformans* phagosome and those of other intracellular pathogens, such as bacteria and protozoa, are that fungal cells are larger and the volume of the phagosome rises rapidly after phagocytosis by capsular enlargement. A larger phagosome volume inevitably leads to dilution of microbicidal lysosomal products, which could reduce their activity. The *C. neoformans* fungal cell wall contains the enzyme laccase, which catalyses the formation of melanin polymer. Melanized cells are protected against a range of insults, including oxygen- and nitrogen-derived oxidants and microbicidal peptides. However, laccase itself

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seems to provide considerable protection in the phagosome by promoting the oxidation of phagosomal iron to Fe(III), with a consequent reduction in microbicidal oxidant formation.

C. neoformans is thought to have a remarkably robust antioxidant system that can protect it against many stresses, including attack by phagocytic cells[63]. For example, it has four catalase genes, which, even when deleted, do not confer increased susceptibility to oxidants *in vitro*, attesting to the existence of other parallel antioxidant mechanisms[64]. By contrast, two enzymes that confer resistance to nitrogen-related oxidants, flavohaemoglobin denitrosylase and *S*-nitrosoglutathione (GSNO) reductase, are important for virulence and intracellular survival[65]. Another important system for the resistance of oxidative stress comprises the thioredoxin proteins, which seem to be important for virulence and survival in macrophages[66]. There are two genes for superoxide dismutase, both of which are important for virulence and resistance to phagocytic cells[67–69]. Yet another non-enzymatic antioxidant system is the production of mannitol, which can protect cryptococcal cells from neutrophil oxidative killing by scavenging for oxygen-related oxidants[70]. In addition, sphingolipid synthesis, and an enzyme that is involved in the metabolism of fungal inositol sphingolipids, confers resistance against macrophage antimicrobial mechanisms and promotes intracellular survival[71].

A unique aspect of the *C. neoformans* intracellular pathogenic strategy is the release of large numbers of polysaccharide-filled vesicles into the cytoplasm of infected macrophages[49,52]. These vesicles seem to bud from the phagosomal membrane, and are not found in cells that have ingested acapsular cryptococcal strains. The mechanism of vesicle formation and the fate of cytoplasmic vesicles are unknown. However, they accumulate in such large numbers that they may commandeer large amounts of cellular membrane material, and this could compromise host cell survival. *C. neoformans* has recently been shown to export its polysaccharide to the cellular exterior in vesicles[72]. Consequently, it is conceivable that the cytoplasmic vesicles that accumulate in infected cells are related to, or form from, these fungal vesicles. Proteomic analysis has shown that these export vesicles also harbour multiple virulence-associated proteins, which, combined with their polysaccharide and phospholipid contents, suggests that they can function as virulence-factor delivery bags[73]. Whether the polysaccharide-containing vesicles are released from infected macrophages to affect neighbouring cells is unknown. Similarly, in bacteria, mycobacterial lipids are released from infected macrophages[74].

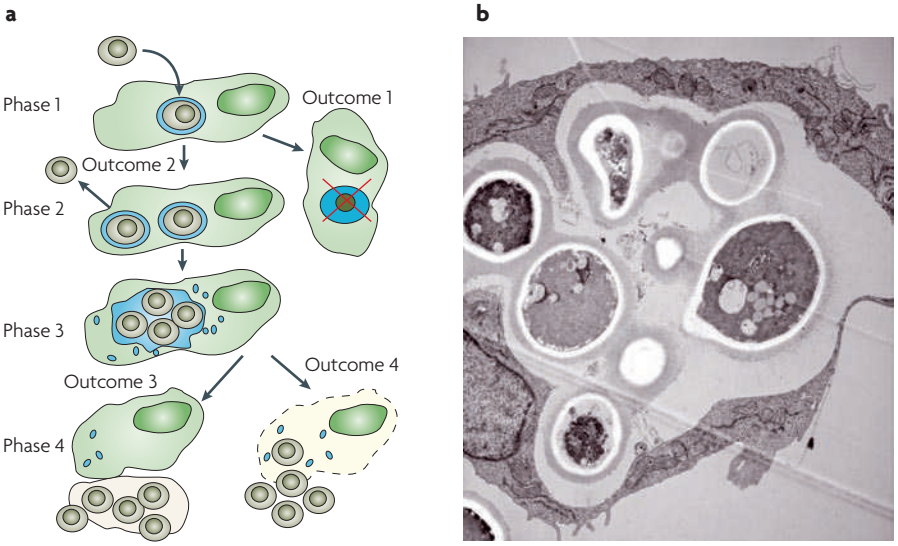

Figure 2 | **Model for the interaction of *Cryptococcus neoformans* with macrophages.** **a** | A schematic of the interaction of *C. neoformans* with macrophages. The process is arbitrarily shown as five phases with four possible outcomes. In phase 1, *C. neoformans* is opsonized with specific antibody or complement and then ingested. After ingestion, macrophages can kill or inhibit the replication of *C. neoformans* (outcome 1). In phase 2, *C. neoformans* replicates inside macrophages and occasionally escapes to the extracellular space (outcome 2). In phase 3, a giant phagosome forms from intracellular replication or homotypic phagosomal fusion. In phase 4, two possible outcomes result in the exit of *C. neoformans* cells to the extracellular space through phagosome extrusion or host cell lysis. **b** | An electron micrograph of a macrophage with numerous yeast cells in a giant phagosome (magnification of x5,000).

Escape from phagocytic cells

Cavanaugh and Randall proposed that the death of macrophages that harbour phagocytosed *Y. pestis* provided an escape mechanism for the bacteria[17]. Although plausible, there is no other evidence for a mechanism by which *Y. pestis* exits macrophages. By contrast, *C. neoformans* ingestion by macrophages has at least three outcomes: death of the fungus, to produce fungal progeny, which are released by host cell lysis, and fungal exit from the infected cell through single-cell exocytosis or phagosomal extrusion. Unchecked replication can lead to catastrophic lysis of the macrophage and the release of viable fungal cells. However, perhaps the most remarkable outcome is the exit of *C. neoformans* cells from the infected macrophage, either singly or in a massive burst following the extrusion of a phagosome that contains numerous yeast forms. *C. neoformans* exocytosis seems to be associated with homotypic phagosome fusion and actin depolymerization. *C. neoformans* also has the capacity to spread from one host cell to another without exiting to the extracellular space[75,76]. Whether this phenomenon is a form of phagosomal extrusion into a neighbouring cell or a specific fungal strategy to infect neighbouring cells in metazoan organisms is unknown.

The case for convergent evolution

Convergent evolution is a process by which genetically distant organisms manifest similar traits in adaptation to similar environments or selection pressures. An example of convergent evolution is the anatomical adaptations to the problem of flight by birds, bats and insects. *Y. pestis* and *C. neoformans* are thought to have diverged 1.5–2.0 billion years ago, when eukaryotes emerged. Because animals do not appear in the evolutionary record until approximately 400–500 million years ago, any similarities in intracellular pathogenic strategy must have arisen long after the divergence of prokaryotes and eukaryotes. Mammalian phagocytic cells, such as macrophages, have a formidable array of antimicrobial properties, and can be envisaged as a specific ecological niche in an animal host.

By comparing the interaction of *Y. pestis* and *C. neoformans* with host cells, it is evident that both microorganisms have found

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Box 1 | **Important questions to address in the future**

- Does *Yersinia pestis* inhibit the acidification of its phagosome?
- How does *Y. pestis* escape from macrophages?
- Does *Y. pestis* replicate in human macrophages?
- If *Y. pestis* can survive in amoebae, is the mechanism the same as that used for survival in macrophages?
- How are the polysaccharide-containing phagosomes in *Cryptococcus neoformans*-infected macrophages formed?
- What mechanism is responsible for the leakiness of the *C. neoformans* phagosome?
- How does *C. neoformans* exit macrophages without lysing the host cell?

similar overall solutions to the problem of intracellular survival, although their molecular and cellular mechanisms differ. Both *Y. pestis* and *C. neoformans* survive and replicate in a phagosome that undergoes fusion with late endosomes and or lysosomes to form spacious phagosomes (FIGS 1,2). Although the mechanism of spacious-phagosome formation might differ for each microorganism, the outcome is similar in that lysosomal contents would be diluted in a manner that could interfere with phagosomal microbicidal activity. Furthermore, surface structures play important parts in the resistance of these pathogens to the harsh conditions of their phagosomes. In *C. neoformans*, the polysaccharide capsule is essential for survival in macrophages. In *Y. pestis*, resistance to antimicrobial peptides is achieved by modification of its surface lipopolysaccharide, an essential process for intracellular growth. Additional studies on *Y. pestis* are needed to extend this comparison to properties of phagosome acidification and mechanisms of macrophage escape (TABLE 1).

Given that the divergence of prokaryotes and eukaryotes is ancient and predated the emergence of animals, it is reasonable to posit that selection forces other than mammalian pathogenicity are responsible for the ability of these phylogenetically unrelated microorganisms to survive in phagocytic cells. When considering possible selection forces that might have acted on these microorganisms, it should be noted that protista are also an ancient lineage and that both bacteria and fungi are likely to have interacted with protozoa long before the emergence of animals.

It is now established that several human pathogenic microorganisms that are facultatively intracellular are resistant to killing by amoebae, including *C. neoformans*, *H. capsulatum*, *L. pneumophila* and *F. tularensis*[77,78]. Given that amoebae are ubiquitous in the environment, it is likely that these protozoa have a role in the evolution of traits that allow for survival in macrophages. It is conceivable that *Y. pseudotuberculosis* also acquired the ability to survive in phagocytes as a strategy to avoid predation by soil or fresh-water amoebae, and passed this ability on to *Y. pestis*. This hypothesis is considered provocative and worthy of future study (BOX 1). In conclusion, we propose that *Y. pestis* and *C. neoformans* acquired their intracellular survival strategies by a process of convergent evolution, possibly in response to interactions with other hosts in the environment, such as amoebae. It is worth noting that some similarities between specialized animal cells, such as macrophages and amoebae, might also reflect convergent evolutionary solutions to the problem of controlling intracellular microorganisms.

Microbial invasion of a host cell poses a problem of survival for both the host and microorganism. Consequently, similarities in the intracellular pathogenic lifestyle of phylogenetically divergent microorganisms could represent the twin convergence of host and microorganism as they each seek to survive the encounter and gain the upper hand. We recognize that similarities between very different microorganisms, such as *Y. pestis* and *C. neoformans*, can appear to be superficial and simplistic. However, given that most, if not all, intracellular microorganisms have different and unique pathogenic strategies, identifying evolutionary patterns requires us to take a step back and look at the process from some distance. In doing so, we hope to see patterns such as those that led Alfred Wegener to propose continental drift in 1912 from the similarities in the coastal outlines. Although acceptance of continental drift had to wait until the theory of plate tectonics was outlined in the 1960s, the crucial hint came from a superficial inspection of coastal outlines. We therefore hope that this comparison of a bacterial and a fungal pathogen provides a useful example of how to approach the bewildering complexity of intracellular pathogenic strategies.

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DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
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FURTHER INFORMATION

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