Colworth Medal Lecture

Mechanisms of microbial escape from phagocyte killing

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Abstract

Phagocytosis and phagosome maturation are crucial processes in biology. Phagocytosis and the subsequent digestion of phagocytosed particles occur across a huge diversity of eukaryotes and can be achieved by many different cells within one organism. In parallel, diverse groups of pathogens have evolved mechanisms to avoid killing by phagocytic cells. The present review discusses a key innate immune cell, the macrophage, and highlights the myriad mechanisms microbes have established to escape phagocytic killing.

Introduction

Phagocytosis is arguably one of the most important processes in biology. From single-celled amoebae using phagocytosis for nutrition to phagocytic cells of the vertebrate immune system using phagocytosis to destroy microbes and present antigens, phagocytosis plays an important role in all kingdoms of life. In the present review, we consider the diverse strategies that microbial pathogens use to escape killing by phagocytes. These fall into three broad categories: diversion and modification of the phagosome maturation process, general resistance and persistence within the phagolysosome, and physical escape from either the phagosome or the entire phagocyte.

Phagosome maturation

Phagocytosis is typically defined as the uptake of particles larger than 0.5 μm, which includes most bacteria, fungi, protozoa and other eukaryotic cells. There is, however, a limit to the size of an object that can be phagocytosed [1]. Phagocytosis is initiated via receptor engagement, either via microbial ligands or host ligands deposited on the pathogen surface. PRRs (pattern-recognition receptors) recognize PAMPs (pathogen-associated molecular patterns) on the surface of microbes and these include mannose receptor, Dectin-1, CD14, scavenger receptor A, CD63 and MARCO (macrophage receptor with a collagenous structure). Opsonic receptors recognize host molecules deposited on the surface of microbes and include FcγR variants, FcαR, FcεR, CR1, CR3, CR4 and the integrin $\alpha5\beta1$ [2]. For most microbes, entry into phagocytes probably involves a combination of these receptors recognizing different aspects of the microbial surface. However, the receptor that dominates in this initiation of phagocytosis is likely to play a major role in determining the precise route of phagosome maturation and indeed microbial fate.

Once internalized, the contents of the phagosome must be digested. For antigen-presenting phagocytes such as macrophages and dendritic cells, antigens derived from the degraded microbe are then secondarily presented on the surface of the phagocyte. To achieve this degradation, phagosomes undergo a series of maturation stages, receiving new material from early endosomes, late endosomes and finally lysosomes and also losing molecules no longer required via sorting and recycling endosomes, creating a dynamic phagosome membrane [3]. The molecular detail

Key words: intracellular survival, lysosome, macrophage, pathogen, phagocytosis, phagosome.
Abbreviations used: Arp2/3, actin-related protein 2/3; BCV, *Brucella*-containing vacuole; $C_\beta G$, cyclic β-1,2-glucan; EEA1, early endosome antigen 1; ER, endoplasmic reticulum; GBS, Group B *Streptococcus*; iNOS, inducible nitric oxide synthase; LAMP, lysosome-associated membrane protein; LC3, light chain 3; LLO, listeriolysin O; MNGC, multi-nucleated giant cell; PKC, protein kinase C; PV, parasitophorous vacuole; RNS, reactive nitrogen species; ROS, reactive oxygen species; T3SS, Type III secretion system; TgCDPK, *Toxoplasma gondii* Ca^{2+}-dependent protein kinase; V-ATPase, vacuolar ATPase; WASP, Wiskott–Aldrich syndrome protein; WASH, WASP and SCAR homologue.

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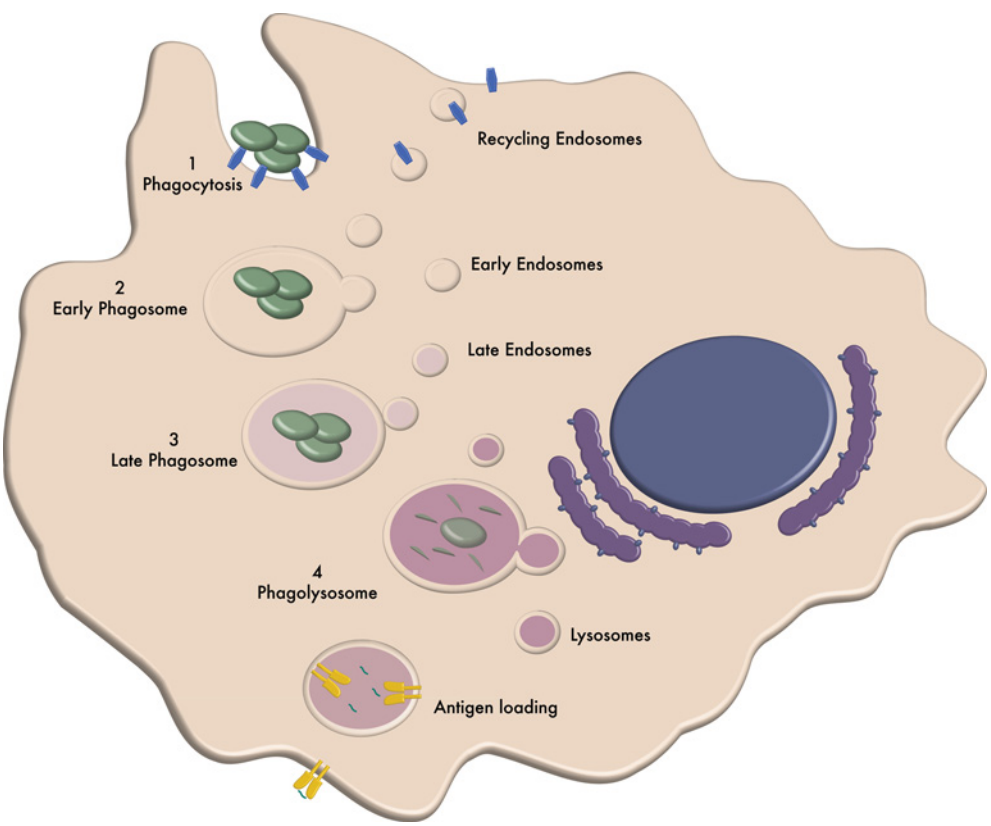
Figure 1 | Phagosome maturation

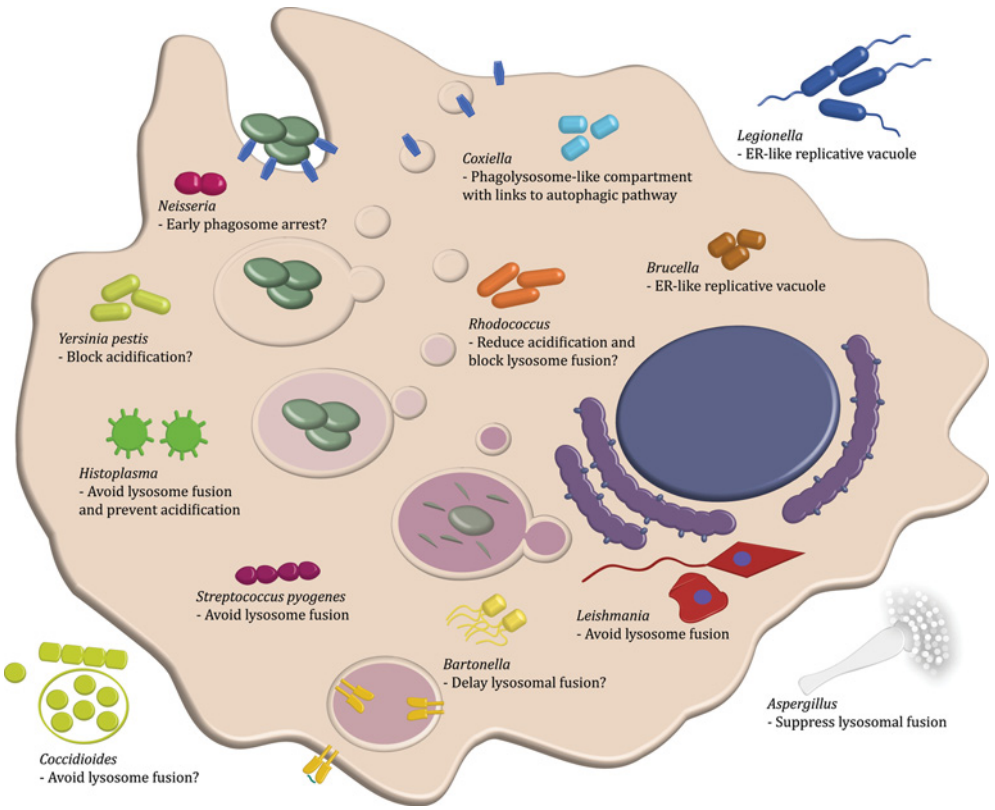
1. Phagocytosis of microbes via phagocytic receptors. Receptor engagement initiates cytoskeletal rearrangements and pseudopod extensions to surround and eventually fuse around the microbe, creating an internal phagosome. 2. The early phagosome rapidly loses plasma membrane associated actin and PtdIns(4,5)P_2. Early phagosomes interact with early endosomes and recycling endosomes. These interactions with early endosomes allow delivery of PtdIns3*P*, syntaxin-13 and the GTPase Rab5. After Rab5 activation (by its unknown guanine-nucleotide-exchange factor) the Rab5 effectors Vps34, Mon1 and Rab7 are recruited. Vps34 recruits proteins with FYVE or PX domains such as sorting nexins, Hrs, p40^{phox} and the early endosome marker EEA1. EEA1 is a bridging molecule that aids vesicle fusions. The Rab5–Mon1–Ccz1 complex is thought to recruit Rab7 and aid activation by competitively displacing the Rab7 GDI (guanine-nucleotide-dissociation inhibitor). 3. The late phagosome becomes more acidic internally (~pH 5.5), sheds some of the early phagosome markers and acquires late markers. Late phagosome markers include Rab7, Mon1, RILP and HOPS (complex of Vps11, 16, 18 and 33) and potentially the Rab7 GEF. RILP (Rab-interacting lysosomal protein) and ORP1L (oxysterol-binding-protein-related protein 1-like) (recruited by Rab7) are dynein adaptors that help to direct late phagosomes towards the microtubule-organizing centre, the collecting place for lysosomes. 4. The phagolysosome is finally formed when late phagosomes fuse with lysosomes that are packed with hydrolases (nucleases, lipases, glycosidases, proteases and cathepsins) and a highly acidic content (~pH 4.5). Lysosomes also deliver membrane proteins LAMP1 and the V-ATPase H^+ ion pump. The phagolysosome is a highly inhospitable intracellular compartment. Eventually phagolysosomes fuse with Golgi vesicles carrying MHCII to allow loading of peptide antigens and subsequent presentation.



involved in progressing from early and late stages through to the mature phagolysosome has been the topic of much research for many years. However, finding suitable control particles to study 'normal' phagosome maturation is challenging. For example, latex beads have been used for this purpose for many reasons, such as the range of particle sizes available and the ability to covalently link molecules to their surface. Indeed, they have been invaluable in probing the early stages of phagosome maturation [4]. However, since these particles are essentially indestructible by phagocytes, they are of limited value in investigating the later phases of phagolysosome development. In particular, it is now clear that phagosomes containing living (or once living) things are processed in a different way to those containing inert particles. For example the tetraspanin CD63 is now used as a marker of mature phagolysosomes (although its exact role is still being determined), yet this marker is not found on phagosomes containing latex beads [5]. To address this, most research efforts now use non-pathogenic microbes alongside latex beads as targets, and this dual approach has led to a comprehensive model for normal phagosome maturation that is summarized in Figure 1.

Figure 2 | Strategies used by different pathogens to modify phagosome maturation

Many pathogens are able to manipulate the phagosome. The pathogens discussed in the main text and a summary of the modification they exert on the phagosome are illustrated.



Despite this highly co-ordinated process that is intended to kill invading microbes, many pathogens have evolved mechanisms to enable their survival within macrophages. Below, we consider the different classes of survival mechanism and highlight some outstanding questions in the field.

Methods of diversion and modification of phagosome maturation: making the environment more comfortable

Many pathogens, whether obligate intracellular pathogens or opportunistic, have evolved mechanisms to pause, arrest or redirect entirely the phagosome maturation pathway (Figure 2). If successful, pathogens are then able to lie dormant within a protected 'Trojan horse', disguised from the immune system. The most widely studied example of such a process occurs in *Mycobacterium tuberculosis*, the causative agent of human tuberculosis. Not only is this organism able to arrest normal phagosome maturation [6], but also other studies suggest that it may be able to escape the phagosome [7,8]. *M. tuberculosis* retains the characteristics of an early phagosome, such as Rab5 [6] and recycling endosome communication [9]; however, acidification is blocked by excluding the V-ATPase (vacuolar ATPase) [10]. *M. tuberculosis* phagosomes also avoid lysosome fusion by disrupting host Ca^{2+} signalling [11]. *Mycobacterium marinum*, a close relative of *M. tuberculosis*, is able to escape the phagosome and use actin-based motility within the cytosol [12]. This huge field of research has recently been comprehensively reviewed [13,14] and will not be considered further in the present article.

Phagosome maturation is also manipulated by the food-borne pathogen *Salmonella enterica*, which arrests phagosome maturation at a late, but not fully mature, stage, creating an SCV (*Salmonella*-containing vacuole) [15–17]. Recent data suggest that this process may be different in different host cell types. For instance, novel *Salmonella* spacious phagosomes have recently been described for B-cell infection [18]. Phagosome escape [19,20] and autophagy [21,22] have also been implicated in *Salmonella* infection. Because the intracellular *Salmonella* field goes beyond the scope of the present review, we will not be covering this pathogen. However, we direct readers to recent in-depth reviews [23,24]. We continue this section exploring some pathogens that are less well known for their ability to alter phagosome maturation.

Neisseria gonorrhoeae

N. gonorrhoeae is a Gram-negative bacterium and the causative agent of gonorrhoea in humans. *N. gonorrhoeae*

is best known for its infection of epithelial cells; however, once gonococci have transversed this cellular layer, they are likely to be engulfed by professional phagocytes such as macrophages.

Although relatively poorly studied, it is clear that *Neisseria* can manipulate phagocytes in several diverse ways. *Neisseria* gonococcal porin is able to reduce ROS (reactive oxygen species) produced by primary human neutrophils and monocytes, thus limiting their antimicrobial activity [25]. The same porin has also been implicated in blocking phagosome maturation [26], since treatment of phagocytes with purified porin (PorB) causes latex bead phagosomes to retain increased amounts of early markers (such as Rab5) and reduced late markers (such as Rab7). *Neisseria* also secretes proteases that digest LAMP (lysosome-associated membrane protein) 1. This process appears important in mediating *Neisseria* survival within epithelial cells and fibroblasts, although it has yet to be investigated in phagocytic cells [27].

Yersinia pestis

Y. pestis is the infamous bubonic-plague-causing Gram-negative bacterium. Upon engulfment, *Y. pestis* up-regulates a putative stress-induced operon. Deletion of the stress-induced operon, or the previously unidentified gene *orfX*, leads to down-regulation of type III secretion, higher intracellular replication and a filamentous morphology of internal bacteria [28]. Transposon mutagenesis has also recently revealed a role for glucose-1-phosphate uridylyltransferase (galU) and the UDP-modifying enzymes WecBC in the intracellular survival of *Y. pestis* [29]. Once intracellular, *Y. pestis*-containing vacuoles seem to fuse with lysosomes [30], acquire cathepsin D and LAMP1 [31], and, in some cases, are positive for the LC3 (light chain 3) autophagosome marker. Despite this, however, the vacuoles maintain a neutral pH [32], suggesting that modification of the *Y. pestis* phagosome is likely to occur, although the precise stages of maturation interrupted are currently unknown.

Legionella pneumophila

L. pneumophila is a Gram-negative facultative intracellular bacterium. Like many of the microbes discussed in the present review, *L. pneumophila* primarily causes respiratory infections and is known to interact with, and replicate within, human alveolar macrophages. Infection with *L. pneumophila* can lead to an acute form of pneumonia known as Legionnaires' disease. *L. pneumophila* is naturally adapted to an intracellular lifestyle as it parasitizes freshwater amoebae, hence outbreaks of legionellosis are often associated with contaminated water supplies [33,34]. Early work by Marcus Horwitz [35] demonstrated that the *Legionella* phagosome is less acidic than other non-pathogen phagosomes and does not fuse with lysosomes. Additionally, within 2 h of uptake by human monocytes, *Legionella* phagosomes associate with mitochondria and the rough ER (endoplasmic reticulum), with the phagosome becoming studded with ribosomes around the membrane [36].

More recent data have shown that the diversion of *Legionella* phagosomes away from the normal route of maturation, preventing them from acquiring LAMP1 or Rab7, occurs rapidly after uptake and requires the *Legionella* product DotA. DotA is an inner membrane protein believed to be required for the formation of a macromolecular complex to direct the phagosome towards a replicative vesicle form. Additionally, the fate of the *Legionella* phagosome seems to be determined within minutes of uptake, as DotA-negative bacteria were seen to already be LAMP1-positive at this premature stage [33]. DotA is part of the Dot/Icm T4SS (Type IV secretion system) transporter [37] and acts to export a series of effector proteins, including RalF. RalF acts as a guanine-nucleotide-exchange factor for the host factor ARF1 (ADP-ribosylation factor 1), which regulates vesicle traffic between the ER and Golgi and is found on *dot/icm* wild-type *Legionella* phagosomes [38].

Once *Legionella* has modified the phagosome appropriately, it then converts into a replicative form. This form is unable to secrete virulence factors and thus the replicative vacuole slowly acquires lysosomal markers and becomes nutrient-depleted. However, the accumulation of ppGpp within this organelle eventually triggers a return to stationary phase and re-expression of virulence factors [39]. After approximately 24 h of replication, cells then burst open, although it is currently unclear whether this is due to the physical limits of phagocyte membranes or apoptosis [33].

Streptococcus pyogenes

S. pyogenes, or Group A *Streptococcus*, is a Gram-positive bacterial pathogen most commonly thought of as an extracellular infectious agent. However, *S. pyogenes* is able to cause serious deep-tissue infections (such as necrotizing fasciitis) in which macrophage reservoirs of persisting bacteria are likely to play an important role in disease progression. A well-known virulence factor of this particular *Streptococcus* is the M1 protein. Recent findings indicate a role for M1 protein in controlling vesicle trafficking and preventing lysosomal fusion of *S. pyogenes* phagosomes, as well as in suppressing the macrophage inflammatory response via NF-κB (nuclear factor κB) signalling [40]. Additionally, the transcription factor Mga has been linked to V-ATPase acidification blocking [41].

Microarray and qRT-PCR (quantitative real-time PCR) studies have identified 145 genes that are significantly altered following uptake. These included the CovS/CovR two-component system that has also been implicated in intracellular survival of the related pathogen *Streptococcus agalactiae* [41,42].

Histoplasma capsulatum

The dimorphic fungus *H. capsulatum* is the causative agent of the life-threatening infection histoplasmosis. During infection, *H. capsulatum* resides within a modified phagosome of macrophages, which are considered to be the primary infected cells within hosts. *Histoplasma* survives within macrophages by modifying the vacuole and keeping it at a pH of 6.5 by

blocking V-ATPase acidification and lysosomal fusion with the phagosome [43]. This incomplete block of acidification is likely to reflect a compromise between enabling fungal utilization of iron from transferrin (at low pH) while still reducing the hydrolytic activity of acid-dependent proteases. Interestingly, *Histoplasma* actively retains a slightly acidic phagosomal pH, even when phagosomal acidification is blocked by the V-ATPase inhibitor bafilomycin [43].

Studies into *Histoplasma* with human and mouse macrophages have highlighted differences between these two cell types, since human macrophages did not need to induce phagosome acidification for effective fungal killing, whereas a low phagosomal pH is critical for the antifungal activity of mouse macrophages [44].

Leishmania spp.

Leishmania are parasitic protozoa that are spread by sandfly vectors in tropical and subtropical regions of the world. *Leishmania* can exist in two life-cycle stages, the promastigote and amastigote, both of which can be phagocytosed by means of receptor-mediated phagocytosis [45]. The receptors used for uptake vary depending on species, stage and serum quality, but generally involve CR2, CR3 or mannose receptor [46,47]. Upon internalization, promastigotes insert LPG (lipophosphoglycan) into the phagosome membrane, which inhibits the depolymerization of F-actin (filamentous actin) and thus blocks lysosomal fusion [47–49]. This delay possibly provides enough time for the parasite to convert into the amastigote form, which is more resistant to hydrolases and thus able to replicate within the phagosome. Persistence and replication in the mature phagolysosome is also aided by cell-surface and secreted glycoconjugates such as GIPLs (glycoinositol phospholipids) and PPG (proteophosphoglycan) and by active inhibition of PKC (protein kinase C), which blocks assembly of the NADPH oxidase, lowering the phagocyte oxidative burst [45,50].

Coxiella burnetii

The Q fever agent, *C. burnetii*, replicates within a modified phagosome in many host cell types. It is an intracellular bacterial pathogen able to create a large phagolysosome-like compartment. The mature *Coxiella* phagosome looks like a large phagolysosome, but is actually a modified compartment [51], which recruits the autophagosome marker LC3. If autophagy, or the host signalling factors cAMP and protein kinase A, are pharmacologically inhibited, *Coxiella* replication is reduced [52,53]. This modified phagosome recruits the small GTPases Rab5 and Rab7 and inhibits lysosomal fusion (as measured by the presence of cathepsin D) [54], although, at present, the link between autophagy and phagosomal modification remains unclear.

Bartonella henselae

B. henselae is a facultative intracellular pathogen causing 'cat-scratch' disease and vasculoproliferative disorders in humans. The pathogen interacts with macrophages and endothelial cells and survives within them [55], although little is known about the intracellular niche it occupies. At 2 h after phagocytosis, the *Bartonella* phagosome remains negative for endocytic or lysosomal markers and is not acidic, suggesting that the bacterium is able to radically alter phagosomal maturation. However, by 24 h, normal maturation and bacterial killing has occurred, suggesting that *B. henselae* slows, rather than blocks, phagosome maturation [56].

Rhodococcus

Rhodococcus equi causes severe pneumonia in horses and tuberculosis-like symptoms in AIDS patients. Following uptake, this Gram-positive facultative intracellular pathogen transiently acquires maturation markers such as PtdIns3*P*, EEA1 (early endosome antigen 1) and Rab5, but not cathepsin D or V-ATPase [57,58]. This non-acidic phagosome can be maintained for 48 h in a manner that is dependent on the presence of the bacterial VAP virulence plasmid and KasA, which is essential for long-chain mycolic acid synthesis [59].

Coccidioides spp.

Coccidioides immitis and *Coccidioides posadasii* are dimorphic fungi found in regions of southern U.S.A. and pockets of South America where Valley fever (coccidioidomycosis) is endemic. Coccidioidomycosis mostly remains asymptomatic, but can cause serious complications and disseminated disease (such as meningitis) in immunodysfunctional individuals [60].

Coccidioides can be found in the soil in a mycelial form that produces infectious arthoconidia. Arthoconidia transform into large spherules (containing 100–300 endospores) rapidly after inhalation by humans. Both arthoconidia and endospores can be phagocytosed upon inhalation, although phagocyte activity against them appears to be fungistatic rather than fungicidal. Even in immunocompetent individuals, neutrophils will kill only up to 30 % of invading endospores. Spherules, on the other hand, are too large to be phagocytosed (having a 60–100 μm diameter) so no defence can be utilized until the endospores are released. Following uptake, endospores appear to inhibit lysosome fusion [61] and eventually lyse the host cell as the endospores continue to develop into new spherules while within the phagosome [60]. Interestingly, if macrophages are primed by immune T-cells before infection, these cells then become capable of effectively killing *Coccidioides* [62,63].

Brucella spp.

The Gram-negative facultative intracellular pathogens of the genus *Brucella* cause the zoonotic bacterial disease brucellosis, mostly seen in developing countries where it has a high prevalence. There are many pathogenic species of *Brucella*, although *B. melitensis* is the most common cause of human infection. Infection can cause abortion, infertility and septicaemia in animals and fever or debilitation in humans.

B. melitensis can invade and replicate within phagocytic and non-phagocytic cells of humans. This ability is crucial to disease progression. Some 90 % of *Brucella* are ingested and

killed by macrophages, but the small proportion left alive is sufficient to replicate with macrophages, allowing persistence within the host niche [64,65]. The last 10 years has seen an explosion of *Brucella* research that has greatly advanced our understanding of this host–pathogen relationship. The O-chain of the non-classical *Brucella* LPS (lipopolysaccharide) appears to be necessary for *Brucella* to enter cells via lipid rafts and prevent lysosome fusion [66]. In the mouse macrophage cell line J774, clinical strains of *B. melitensis* do manipulate vesicle trafficking, but this does not involve the autophagy pathway as seen in epithelial cell models [67–69]. This manipulation creates a modified phagosome known as a BCV (*Brucella*-containing vacuole) that eventually fuses with the ER. The sequence of events leading to becoming an ER-like vacuole are complex and key issues (such as whether lysosomal fusion occurs) are unresolved [65,70]. Intracellular survival of *Brucella* requires a number of virulence factors such as the T3SS (Type III secretion system) VirG and cell envelope components such as OPG (osmoregulated periplasmic glucan) and C_{β}Gs (cyclic β-1,2-glucans) [71]. Interestingly, other C_{β}Gs are used by legume endosymbionts to enable intracellular survival [72]. It has now been shown that *Brucella* C_{β}G affects BCV maturation (including blocking lysosome fusion in epithelial cells) by modulating lipid rafts in pathogen (and possibly host) membranes [64,71].

Aspergillus spp.

Several species of fungus within the genus *Aspergillus* are capable of causing serious human disease. The most common by far is *Aspergillus fumigatus*, a species that is crucial for the decomposition of organic matter in the environment, but which is also an opportunistic pathogen causing several different human infections. Infections typically start following inhalation of asexual conidia into the lungs. If not successfully cleared, this initial infection can progress into invasive aspergillosis, the most severe and life-threatening form of aspergillosis, often associated with high mortality in immunocompromised individuals [73].

Following inhalation, most *A. fumigatus* conidia are efficiently phagocytosed and killed by alveolar macrophages. However, recent data have shown that a minority of conidia are capable of suppressing fusion between phagosomes and lysosomes, allowing the fungus to germinate and ultimately lyse the macrophage [74]. The underlying mechanism of this process is currently unknown, although the species variant DHM (dihydroxynaphthalene)-melanin coating on *A. fumigatus* and *A. flavus* conidia appears to be important for diverting maturation away from lysosome fusion [75]. Moreover, the hydrophobin RodA appears to suppress the immune response to inhaled conidia, including macrophage maturation. Hydrophobin molecules are believed to mask the conidia of many inhaled environmental fungi [76].

The related species *Aspergillus terreus* is a less common cause of IBPA (invasive bronchopulmonary aspergillosis). However, once established, *A. terreus* infection is often fatal. Exposure to alveolar macrophages results in rapid phagocytosis, possibly due to the high surface exposure of β-1,3-glucan and galactomannan. Despite rapid uptake, however, conidia show long-term persistence in macrophages even in immunocompetent hosts. Unlike *A. fumigatus*, the phagosome became acidified both in cell culture and in wild-type mice, indicating that *A. terreus* probably persists despite the harsh phagolysosome conditions, although seems to be able to germinate only if the phagosomal pH rises [77].

Methods of escaping phagosomes and phagocytes: knowing when to bail out

Cryptococcus neoformans

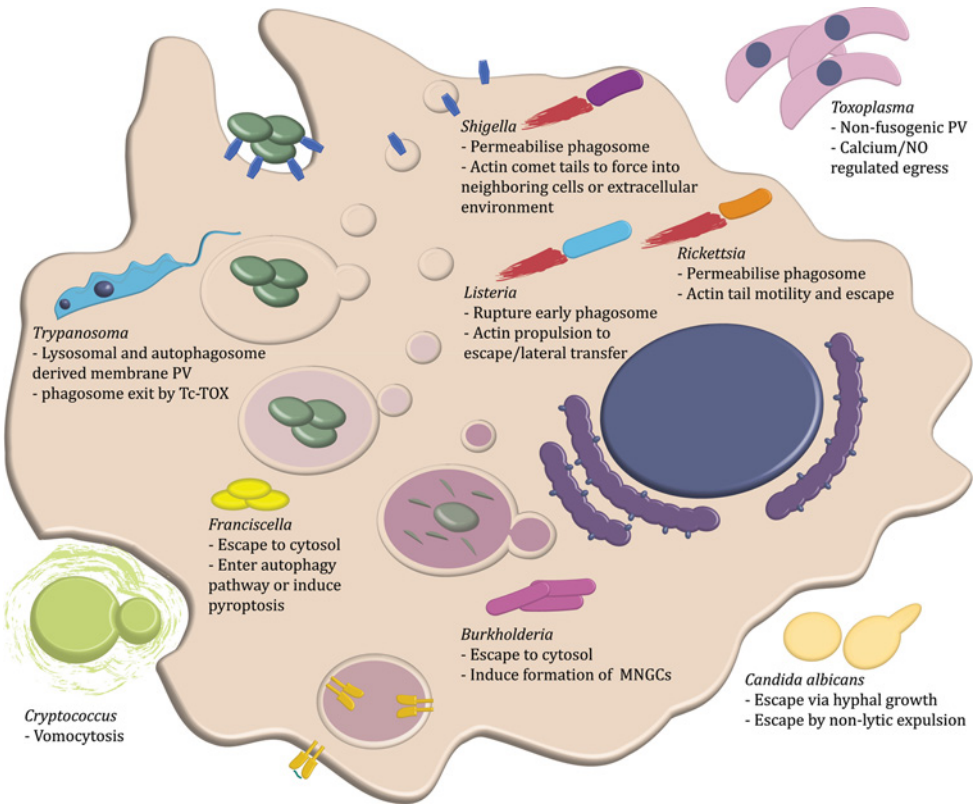
C. neoformans is a lethal fungal pathogen of immunocompromised individuals infecting approximately one million people worldwide per year, with an overall mortality rate of 60 % [78]. Found primarily in soil, some trees and pigeon excreta, *C. neoformans* initially infects the human lung before disseminating to and infiltrating the host central nervous system, leading to cryptococcal meningoencephalitis [79]. During cryptococcosis, macrophages are vital in the innate immune response. However, cryptococci are well adapted to parasitize these phagocytic effector cells [80]. It is well known that cryptococci are able to replicate within the macrophage phagosome and escape in a non-lytic manner (vomocytosis) [81,82]. The molecular detail that regulates and allows this replication and escape is yet to be defined.

Intracellular replication of cryptococci in macrophages was first observed in 1973 [83]. Early hypotheses to explain this suggested that the cryptococcal phagosome was diverted from becoming a phagolysosome [84], a model that has not yet been extensively tested. A detailed examination of the cryptococcal phagosome over the duration of infection has still not been completed, although some markers have been investigated. Cryptococcal phagosomes acquire EEA1 within 10 min of uptake by dendritic cells or macrophages [85,86] and subsequently acquire LAMP1, MHCII and the tetraspanin CD63 (often used as a mature phagosome marker) [85–88]. CD63 recruitment requires acidification of the phagosome [5] which is also, surprisingly, a prerequisite for intracellular survival of cryptococci [85,88]. The presence of some late phagosome markers has led to the hypothesis that cryptococci persist within a normal phagolysosome, rather than modifying maturation processes. However, the presence of membrane markers is not in itself evidence for normal phagosomal contents and further work is required in this area.

C. neoformans is well equipped to avoid phagocytosis and to persist within a phagolysosome. The thick polysaccharide capsule [89], App1 (antiphagocytic protein 1) [90], the transcription factor Gat201 [91,92] and the formation of enormous titan cells [93,94] have all been implicated in phagocytosis prevention. Once internalized, cryptococci are able to utilize many virulence factors for persistence and replication within phagocytes. ROS and RNS (reactive nitrogen species)

Figure 3 | Strategies used by pathogens to escape the phagosome and phagocyte

There are a surprising number of pathogens that are able to escape either the phagosome or the phagocytic cell entirely. Summarized are the mechanisms of escape used by the pathogens discussed in the main text.



are absorbed or destroyed by the capsule [95] as well as protective factors such as Sod1 (superoxide dismutase 1), AOX1 (aldehyde oxidase 1), Fhb1 (flavohaemoglobin 1), Tsa1 (thiol-specific antioxidant 1) and Ure1 (urease 1) [96], whereas the pigment melanin has been linked to antimicrobial peptide neutralization [97]. Interestingly, it is likely that *Cryptococcus* acquired many of these adaptive virulence traits to combat digestion by amoeboid predators in their natural environment [98], in an analogous way to *Legionella pneumophila*.

Vomocytosis is the unique non-lytic escape mechanism of cryptococci from host cells [81,82] (Figure 3). Only recently has a similar process been described for *Candida albicans* [99] and *Candida kruzei* [100]. An escape mechanism that leaves both the host cell and the pathogen alive has major implications for infection progression and is likely to be important for tissue dissemination and possibly also as a mechanism for re-activation after latency. Vomocytosis has been confirmed *in vivo* within mice and possibly occurs more frequently *in vivo* than *in vitro* [101]. The current model for vomocytosis implies that cryptococci escape via exocytic fusion of the phagosome with the plasma membrane, thus releasing the fungus [87]. Moreover, vomocytosis requires microtubule activity, but not actin polymerization. However, actin and the WASP (Wiskott–Aldrich syndrome protein)–Arp2/3 (actin-related protein 2/3) nucleating complex are involved in prevention of vomocytosis by the formation of dynamic actin cages, or 'flashes', around the fungal phagosome. Although strains with high rates of vomocytosis induce more actin flashes, vomocytosis still occurs, suggesting that actin flashes may actually be a reaction invoked by vomocytosis attempts that will hold off expulsion for a short period, but will eventually fail to contain this pathogen [87].

Cryptococcal phagosomes are also seen to permeabilize rapidly after phagocytosis. This permeabilization seems to be necessary and possibly even triggers actin flashes [87,102]. The secreted phospholipase B_1 is essential for vomocytosis [103]. The exact role of this phospholipase is still under investigation, but it is tempting to predict a role in permeabilization of the cryptococcal phagosome. The reason for permeabilizing the phagosome is currently unknown; however, it is likely to aid neutralization of the phagosome, thus inactivating antimicrobial proteases and allowing nutrients from the host cell to enter. One possible role for actin flashes is thus in resealing the phagosome after pathogen-induced permeabilization.

The pH of the phagosome has a major impact on intracellular parasitism by cryptococci. Chloroquine or ammonium chloride treatment to increase the phagosomal

pH increases the rate of vomocytosis, but if acidification is blocked from occurring in the first instance (by using V-ATPase inhibitors), vomocytosis is suppressed [81,101]. Thus it seems that *Cryptococcus* either requires acidification to initiate vomocytosis (which seems to be true for replication) or vomocytosis only occurs from phagosomes that have been appropriately matured.

The molecular details that precede vomocytosis are still under investigation. The most likely explanation is 'hijacking' of the exocytic pathway normally used to expel indigestible material from host cells. In the social amoeba *Dictyostelium discoideum*, exocytosis of digested material requires that the phagosome be devoid of V-ATPase, which is achieved by vesicle budding from the mature phagosome. This budding is controlled by the cytoskeletal protein WASH (WASP and SCAR homologue). Blocking WASH activity inhibits this exocytosis from amoebae and, intriguingly, also reduces cryptococcal vomocytosis rates from macrophages [104].

Cryptococcus is also able to move from one cell to another via the process lateral transfer [105], a rare event that has not been extensively investigated. Transfer requires live cryptococci and may reflect either a vomocytosis event followed by rapid phagocytosis or another mechanism requiring a transient membrane tunnel between the two host cells in question [106].

Listeria monocytogenes

L. monocytogenes is a Gram-positive bacterium capable of replication within many types of host cell [107], by escaping from the phagosome into the cytoplasm. Phagosome escape requires multiple steps, proceeding from phagosome permeabilization to phagosome rupture. LLO (listeriolysin O) is a pore-forming toxin that permeabilizes the phagocytic vacuole and is essential for phagosome escape [108]. Cross-talk between the host cell and *Listeria* is required to complete phagosome rupture. For instance, the host GILT (γ-inducible lysosomal thiol reductase) is required for activation of the pathogen's LLO [109]. Host CFTR (cystic fibrosis transmembrane conductance regulator) is used to escape into the cytosol by increasing Ca^{2+} levels, likely to aid LLO pore formation and possibly inducing Ca^{2+}-dependent activation of host calpains via LLO [107,110]. LLO must be tightly regulated and its expression restricted to the intraphagosomal phase as overexpression can lead to host cell lysis and exposure to the immune system. As such, LLO is induced by the low pH and high Ca^{2+} conditions in the *Listeria*-modified phagosome [107]. It is by a combination of host (activated by bacterial factors) and bacterial phospholipases that the phagosome membrane is completely degraded and ruptures to allow *Listeria* escape into the cytosol [111,112]. But the activity *Listeria* is probably best known for is its ability to transfer between cells once in the cytosol via characteristic 'actin rocketing', initiated by the *Listeria* surface protein ActA [113–115], a phenomenon that has provided remarkable insights into host actin dynamics [114,116–118].

Shigella flexneri

The Gram-negative bacterium *S. flexneri* is able to invade many host cell types by use of a T3SS [119,120]. Once internalized, *Shigella* disrupt the phagosome membrane via the combined efforts of the bacterial effectors IscB [121], IpaB and IpaC [122]. Once in the cytoplasm, *Shigella* are also able to multiply and induce actin 'comet tails' in an analogous way to *Listeria*, by using the bacterial protein IcsA to initiate actin polymerization and propel themselves around the cytosol and eventually into a neighbouring cell. IscA mimics the Cdc42-dependent activation of N-WASP (neuronal WASP) to initiate actin polymerization via Arp2/3 [123]. Lateral transfer in this way results in a double-membraned vacuole surrounding the *Shigella* once in the next cell. This double membrane is also ruptured, by IscB, and the cycle begins again [121]. If all else fails, *Shigella* are also able to cause MxiI-induced pyroptosis of macrophages (a form of necrosis) triggering inflammation and enabling invasion of more epithelial cells [124].

Rickettsia

The genus *Rickettsia* was first recognized after the type species *Rickettsia ricketsii* was identified as the cause of Rocky Mountain spotted fever [125]. All *Rickettsia* species are obligate intracellular pathogens that primarily inhabit endothelial cells. Once internalized, they rapidly escape the phagosome by using the secreted phospholipase A_2 to destabilize the phagosome membrane [126]. Once in the cytoplasm, *Rickettsia* are able to replicate and produce actin tails via the activity of the bacterial protein RickA, which has domains homologous with the WASP family proteins, enabling Arp2/3 complex activation [127]. As with other pathogens, this actin-based motility allows direct cell–cell transfer of *Rickettsia* without exposure to the host's immune system. For a review on SFG (spotted fever group) and TG (typhus group) *Rickettsia*, see [128].

Burkholderia pseudomallei

B. pseudomallei, is a Gram-negative bacterial pathogen causing melloidosis in southeast Asia and northern Australia. The bacteria are able to induce their own uptake by phagocytic and non-phagocytic cells by manipulation of the host cytoskeleton [129]. Once internal, *Burkholderia* replicates in the cytosol of cells and can also induce cell–cell fusion to create MNGCs (multi-nucleated giant cells). The molecular detail of *Burkholderia* intracellular survival is still poorly understood. To enable invasion and exit from the phagosome, *Burkholderia* utilizes an Inv/Mxi-Spa-like T3SS apparatus [129], but otherwise does relatively little to perturb normal phagosome maturation, instead residing within a mature phagosome [130]. To survive in the harsh phagosome environment, the σ factor RpoS is used to initiate MNGCs and inhibit iNOS (inducible nitric oxide synthase) to enhance intracellular replication [131]. After escape into the cytosol, *Burkholderia* uses RpoS and its T3SS to induce actin-associated membrane protrusions. The superoxide dismutase

SodC is required for intracellular survival and virulence by offering protection from ROS produced by macrophages and neutrophils. However, there is a difference in susceptibility of the SodC mutant to extracellular ROS and ROS produced within cells [132].

Francisella tularensis

The causative agent of tularaemia, *F. tularensis* enters macrophages via pathogen-induced CR3-dependent asymmetric spacious pseudopod loops ('looping phagocytosis') [133]. *Francisella* can survive and replicate within many different cell types and host species. Entry via looping phagocytosis subverts the oxidative burst, although the phagosome still acquires EEA1, LAMP1, LAMP2 and Rab7 very rapidly (within the first 30 min of uptake) [134,135]. V-ATPase acidification also occurs quickly and is essential for phagosomal escape [136]. Cathepsin D is not present within *Francisella*-containing phagosomes and so it is presumed that lysosomal fusion is minimal. Escape into the cytosol occurs rapidly (30–60 min), although how rapid depends on the receptor employed for uptake [137,138]. Once in the host cytosol, *Francisella* re-enters the endosomal autophagic pathway (24–48 h) [138]. The relevance of this link to autophagy is still undetermined as *Francisella* are known to repress several autophagy-related proteins [139]. Interestingly, the route of initial uptake has a significant bearing on *Francisella*, with complement-dependent uptake leading to slower escape from the phagosome [136]. By manipulation of the macrophage inflammasome, *Francisella* are able to eventually induce pyroptosis and thus be released from the host cell [140]. For an in-depth review of *F. tularensis* infection, see [141].

Toxoplasma gondii

T. gondii is a globally distributed intracellular parasite that causes typically mild infections, but can lead to significant pathology *in utero* or in immunocompromised individuals [142]. *Toxoplasma* resides within a PV (parasitophorous vacuole) made by invaginating the host cell membrane as it actively invades many different types of host cell (thus keeping plasma membrane characteristics) and resists phagosome–lysosome fusion [3,45]. *Toxoplasma* is able to replicate within this non-acidic vacuole, unless it has been previously opsonized by antibody, leading to normal maturation, fusion and killing. This is a prime example of phagocytic entry route having huge implications on the intracellular fate of pathogens [143]. The non-fusogenic PV also associates with host cell mitochondria and ER [144] Intracellular replication can generate between 32 and 128 parasites in one cell, at which point *T. gondii* exits cells either by orchestrated egress or simple mechanical lysis of host cells. Which of the two exit routes dominates under natural conditions is still under debate [142]. Since *T. gondii* exit from macrophages seems to be related to preceding active invasion, it too is affected by Ca^{2+} and appears to be morphologically similar to invasion when microscopically recorded [145]. It has also been demonstrated that by inducing the activity of *T. gondii* NTP hydrolase, host cell ATP is depleted which then acts as a trigger for parasite exit. More recently, TgCDPK (*T. gondii* Ca^{2+}-dependent protein kinase) 1 has been identified as a key regulator of invasion, replication and egress. TgCDPK1 regulates the secretion of specialized organelles of the parasite, the micronemes, in response to increased intracellular Ca^{2+} levels. Micronemes store many virulence factors, including the perforin-like protein TgPLP1 that facilitates permeabilization of the PV [146,147]. TgCDPK3 also plays a role in microneme secretion, permeabilization of the PV and initiation of gliding motility, but is able to sense when the parasite is intracellular from the higher K^+ ion concentration [148]. Gliding motility via the unique actomyosin motor (glideosome) aids invasion, egress and travel through tissue. In the last 12 months, a flurry of papers on Ca^{2+}-dependent [148,149] and NO (nitric oxide)-induced [150] *T. gondii* egress have been published. New methods for separation of intracellular parasites and parasites that have egressed from cells [151] are likely to open new doors for the genetic exploration of other *T. gondii* factors responsible for egress from host cells.

Trypanosoma cruzi

The flagellated protozoan pathogen *T. cruzi* causes American trypanosomiasis or Chagas' disease. This protozoan has many morphological forms in its life cycle and can penetrate any nucleated cell type, although the host receptor is still unknown. Even though many can be killed in the human host, a small population will persist and stay with the host for their lifetime [152]. This persistent infection seems reliant on *T. cruzi*'s ability to replicate within cells and avoid immune discovery. The mechanisms of entry and phagosome manipulation are unusual. Whereas *T. cruzi* can be phagocytosed by macrophages, it can also induce uptake from non-phagocytic cells. Remarkably, *T. cruzi* enters cells in an actin-independent manner, involving the recruitment of lysosomes to the plasma membrane entry point [153]. Entry seems to involve *de novo* microtubule polymerization (possibly explaining lysosome recruitment) and LC3-decorated autophagosome membranes may contribute to the PV [154,155]. Lysosome recruitment could be a result of *T. cruzi* hijacking the plasma membrane lesion repair mechanism. Lysosome exocytosis releases acid sphingomyelinase, an enzyme whose function is to repair plasma membrane lesions and which has been found to be required for *T. cruzi* entry [45,152].

Once within a PV, *T. cruzi* rapidly escapes the PV by using Tc-TOX, a pore-forming molecule that is active at low pH. Tc-TOX activity is mediated by a *trans*-sialidase present on the trypomastigote form of *T. cruzi* [156,157].

Candida spp.

Several species within the ascomycete fungal genus *Candida* are able to cause invasive infections of humans. The most common, *C. albicans*, can be engulfed by host phagocytes but rapidly undergoes filamentation, growth and eventually lysis of the host cell. During intracellular growth, *Candida albicans*

is able to inhibit ROS generation by macrophages, although the mechanism is unknown [158]. Mature *C. albicans* phagosomes show only minimal acquisition of late-stage markers such as lysobisphosphatidic acid and V-ATPase. In addition, *C. albicans* appears to induce the removal, by recycling vesicles, of LAMP1 and cathepsin D from the phagosome [159]. *C. albicans* also expresses catalase, which plays an important role in virulence and hydrogen peroxide resistance [160], although it is notable that *C. albicans* still requires some ROS to act as a signal to initiate arginine biosynthesis and hyphal growth escape [161–163]. *C. albicans* has very recently been discovered to be capable of non-lytic escape, in a similar way to *Cryptococcus*, although how this expulsion is achieved is unknown [99].

In contrast with its dimorphic relative, *Candida glabrata* (the second most common cause of candidiasis) grows as a yeast, but remains able to replicate within macrophages. As an emerging opportunistic infection of humans, little is known about its pathogenicity. While internal, *C. glabrata* is able to suppress apoptosis of macrophages, thus protecting itself from immune detection. The *C. glabrata* phagosome recruits LAMP1 and low levels of EEA1, but not Rab5 or cathepsin, and the contents are only weakly acidic [164].

Methods to persist within phagosomes: making the best of a bad situation

Streptococcus agalactiae

S. agalactiae, also referred to as GBS (Group B *Streptococcus*), is the leading cause of pneumonia, sepsis and meningitis in neonatal humans [165]. This Gram-positive opportunistic pathogen is a commensal of the human gastrointestinal tract and is thought to asymptomatically colonize the genitourinary tract of approximately one-third of the female adult population [166,167]. Of the newborns delivered to colonized females, up to 70 % will subsequently become colonized, with approximately 1 % of infected newborns developing a serious illness [168,169]. Many questions remain about the combination of virulence factors and individual variables of the immune system that must combine to result in disease. An increasing occurrence of GBS infections in immunocompromised adults, the elderly and diabetic adults has been witnessed in recent years, possibly demonstrating that similar neonatal deficiencies of the immune system are replicated under these circumstances [170]. There are many complex deficiencies of the neonatal immune system that render newborns, especially pre-term infants, vulnerable to infection.

The phagocytic cells of the human immune system provide a crucial clearance mechanism for invasive GBS. Opsonophagocytosis is the most efficient mechanism used by these cells to kill GBS, since non-opsonized bacteria are poorly killed following uptake [165]. As such, GBS have a myriad of factors to mimic host molecules and interrupt the complement cascade [171]. It has long been documented that GBS are able to survive within macrophages for a prolonged period of time [165]. This ability is conserved across a collection of clinical isolates [42], despite the fact that GBS appear to reside within a mature LAMP1-positive phagosome [172]. Interestingly, phagosomal acidification seems to be necessary for the survival of internalized GBS, probably by acting as a stress signal to induce survival genes, since there is a marked fall in the survival of GBS when macrophages are treated with concanamycin A (an inhibitor of V-ATPase acidification). The two-component system CovS/R is required for this acid-sensing and intracellular survival of GBS [42].

GBS have many virulence factors that can attribute to their ability to persist intracellularly (Figure 4). The GBS cofactor superoxide dismutase (SodA) is known to provide a defence mechanism against the oxidative burst of the phagosome. By conversion of singlet oxygen radicals into oxygen and hydrogen peroxide, SodA allows for the complete metabolism of these molecules by peroxidases [173]. The reduced oxidative burst of neonatal phagocytes compared with adult cells gives further reason for the expected prolonged intracellular survival of GBS in newborn macrophages [166,170]. An important GBS shield against hydrogen peroxide and oxygen radicals of oxidative burst killing is the orange carotenoid pigment. This pigment is unique to haemolytic streptococci and neutralizes toxic molecules via free radical scavenging. GBS also produces a large amount of the oxygen metabolite scavenger glutathione [166,171]. Despite this ability to survive intracellularly, replication within the phagosome has not been documented for GBS, suggesting that GBS are able to persist rather than multiply within phagocytic cells.

Blastomyces dermatitidis

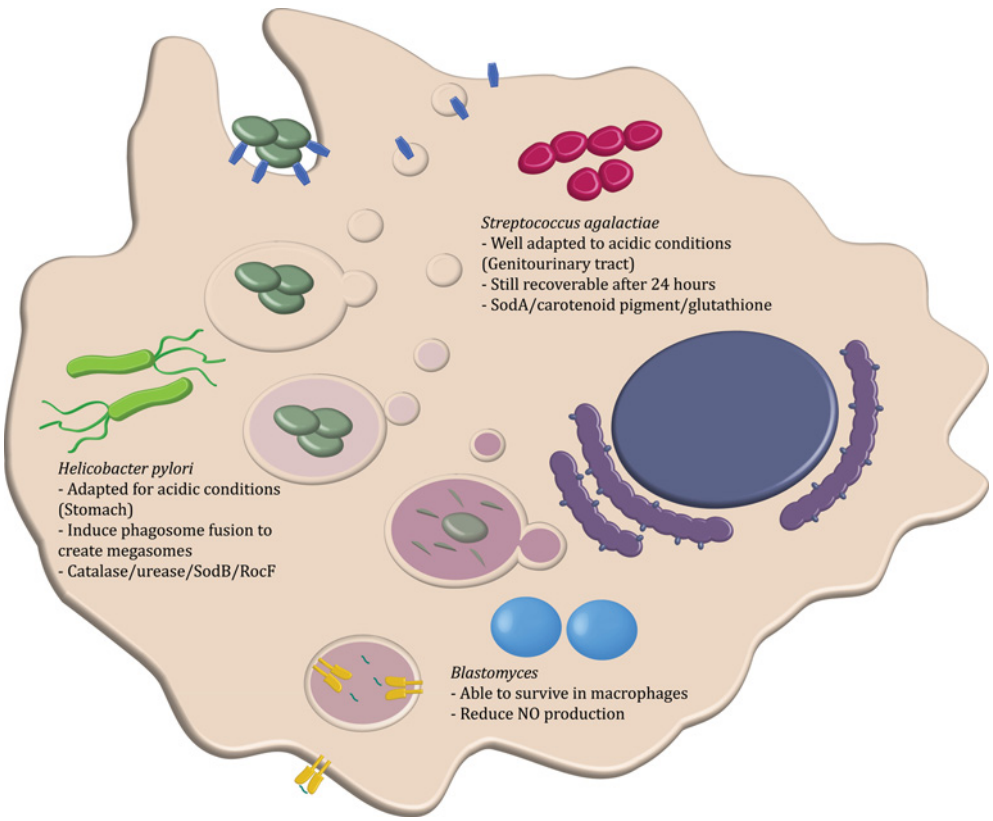
The dimorphic fungus *B. dermatitidis* is found in soil in the Mississippi and Ohio regions of North America [174]. The disease it causes, blastomycosis, affects mostly the lung and skin and occurs following inhalation of spores (conidia) which germinate to form budding yeast within the body. *B. dermatitidis* is very capable of avoiding killing by alveolar macrophages [175]. The antifungal role of NO remains elusive, but other fungal work (*Coccidioides*, *Cryptococcus* and *Histoplasma*) has demonstrated that iNOS promotes fungal clearing by macrophages. *Blastomyces* is able to reduce NO in media and reduce the amount of NO released by macrophages. However, this is likely to occur via a novel mechanism of inhibiting the iNOS enzyme rather than neutralization or sequestering the substrate arginine [176], although the mechanistic details are still to be worked out.

Helicobacter pylori

H. pylori is a microaerophilic spiral-shaped motile bacterium infamous for causing gastric and duodenal ulcers. *Helicobacter* is able to persist in the gastric mucosa and avoid killing by resident phagocytic cells. If engulfed by patrolling macrophages, *Helicobacter* is able to persist in macrophages

Figure 4 | **Pathogens that persist within the phagosome**

Pathogens that are able to persist within the phagosome are presented. What is known about the mechanisms of this persistence is summarized next to the illustration of each pathogen.



and form 'megasomes', by homotypic fusion of multiple *H. pylori*-containing phagosomes [177].

The tools *Helicobacter* has to enable persistence within the phagosome are numerous. Many are directed towards defence against the oxidative burst of macrophages. *H. pylori* urease is required for megasome formation, ROS defence and acid neutralization. Urease deletion can also alter EEA1 acquisition in J774 macrophages [178]. Another important molecule produced by *Helicobacter* is catalase that has been found to be partly responsible for the intracellular longevity seen in macrophages. Catalase (KatA) and superoxide dismutase (SodB) work together to convert superoxide into water and oxygen via hydrogen peroxide [179,180]. Protection from RNS is achieved in several ways. The arginase RocF converts the host iNOS substrate arginine into urea, which can then be converted into ammonium by urease, thus depleting arginine required for NO production [179,181]. Neutrophil-activating protein (NapA) and alkyl hydroperoxide reductase (AphC) are also used in RNS defence [182,183].

H. pylori is also able to delay phagocytosis by activating alternative PKCζ, possibly to allow for up-regulation of virulence factors required for phagosome survival and reducing respiratory burst activation by PKCα [184].

When *Helicobacter*-containing phagosomes were compared with those of *Escherichia coli*, Rab7 and EEA1 were retained on phagosomes, and CD63, LAMP1 and LAMP2 were acquired normally. CagA (cytotoxin-associated gene A)-negative *H. pylori* seem to acquire more EEA1 (relative to Rab5). There also seem to be strain differences in timing of Rab7 acquisition and megasome formation. It is proposed that the persistence of EEA1 and Rab7 could be due to an altered ability to remove them from the phagosome surface via vesicle budding [185]. Earlier studies hinted at a diversion from phagosomal fusion with lysosomes, but, as detailed above, the lysosomal markers do seem to be present; however, this LAMP acquisition is apparently not always seen [178,186]. Past recordings of low lysotracker co-localization could be due to the many factors that *Helicobacter* produces to neutralize its immediate environment. Maturation is disrupted, but slightly, and the relevance of these observations to pathogen survival is unclear.

Conclusions and perspectives

In the present review, we have discussed some of the mechanisms used by microbes to evade phagocyte killing. The clearance of pathogens by phagocytosis and subsequent

digestion is the primary role of macrophages. Despite this, many pathogens have evolved ways to arrest, divert or escape the phagosome. The list of microbes that are not completely eradicated by macrophages is constantly growing. Advances in live-cell imaging, fluorescence microscopy and molecular probes have all aided research of these host–pathogen interactions. The need to carefully consider the phagosome chemistry when choosing molecular probes and fluorescent dyes is becoming more apparent [187]. The phagosome maturation field is a vibrant and growing one, unlikely to slow any time soon.

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