Antimicrobial mechanisms of phagocytes and bacterial evasion strategies

Ronald S. Flannagan, Gabriela Cosío* and Sergio Grinstein*

Abstract | Professional phagocytes have a vast and sophisticated arsenal of microbicidal features. They are capable of ingesting and destroying invading organisms, and can present microbial antigens on their surface, eliciting acquired immune responses. To survive this hostile response, certain bacterial species have developed evasive strategies that often involve the secretion of effectors to co-opt the cellular machinery of the host. In this Review, we present an overview of the antimicrobial defences of the host cell, with emphasis on macrophages, for which phagocytosis has been studied most extensively. In addition, using *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Legionella pneumophila* and *Coxiella burnetii* as examples, we describe some of the evasive strategies used by bacteria.

Endocytic pathway
The route followed inside the cell by vesicles derived from the plasma membrane by endocytosis, including their membrane-associated cargo and trapped fluid. Vesicles or vacuoles derived from the plasma membrane undergo fusion and fission events that deliver some of their components to lysosomes for degradation, whereas others are recycled.

Program in Cell Biology, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada.
Correspondence to S.G.
e-mail: sga@sickkids.ca
**These authors contributed equally to this work.*
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Professional phagocytes, such as macrophages, neutrophils and dendritic cells, are uniquely qualified to engulf large (≥ 0.5 μm) particles, including microorganisms. The internalization and subsequent destruction of pathogens are key to the innate immune response, and promote antigen presentation and the development of adaptive immunity. After engulfment, the microorganisms are trapped, together with extracellular fluid, in a vacuole, or phagosome, derived from the plasma membrane. Because the nascent phagosomal membrane and its contents are innocuous, they must undergo a drastic conversion to acquire the microbicidal and degradative features associated with innate immunity. This conversion, known as phagosomal maturation, is accomplished through a strictly choreographed sequence of fusion and fission events that involve defined compartments of the endocytic pathway (FIG. 1). Effective phagocytosis therefore requires two components: particle internalization and phagosomal maturation.

Although most bacteria are successfully internalized and eliminated by phagocytes, several pathogens have developed survival strategies that interfere with the internalization and/or maturation processes. Prevention and management of the infections caused by such pathogens would obviously benefit from understanding the manner in which they circumvent and often co-opt the immune response. This, in turn, requires detailed knowledge of the basic mechanisms underlying phagocytosis. To this end, we briefly summarize our current knowledge of phagocytosis and describe salient examples of bacterial species that have evolved distinct strategies to evade killing.

Phagosome formation

The interaction of the microorganism with the phagocyte can be direct, through recognition of pathogen-associated molecules (such as surface carbohydrates, peptidoglycans or lipoproteins) by pattern recognition receptors, or indirect, through mediation by opsonins. Opsonins are host factors, such as immunoglobulin G (IgG), and components of the complement cascade that attach to the pathogen surface, acquiring a conformation that is recognized by phagocytic receptors, such as Fcγ receptors (FcγRs) and complement receptor 3 (CR3)[1]. The signalling that is triggered by the particle varies depending on the nature of the receptors engaged. The pathway elicited by FcγR is best understood. Exposure to multivalent ligands induces clustering of these receptors in the plane of the membrane, initiating phosphorylation of their cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) by Src-family kinases[2]. ITAM phosphorylation recruits and activates the tyrosine kinase SYK, which in turn phosphorylates various substrates[3]. The events that follow SYK activation and culminate in particle engulfment are not as clearly understood. Remodelling of actin is unambiguously required for pseudopod extension and, in the case of FcγR, polymerization is driven by Rac1 and/or Rac2, and cell division control protein 42 (Cdc42)[4].

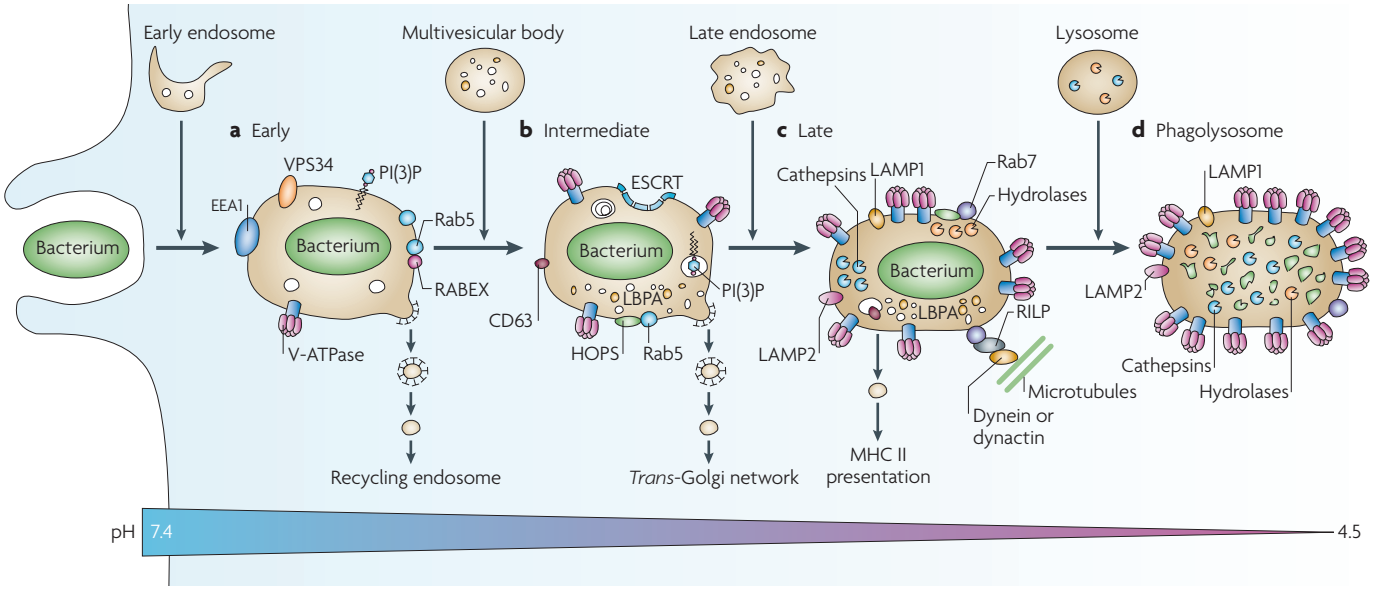

Figure 1 | **Stages of phagosomal maturation.** Shortly after pathogen uptake, the phagosome undergoes a series of transformations that result from its sequential interaction with subcompartments of the endocytic pathway. Different stages of maturation are recognized — early (**a**), intermediate (**b**) and late (**c**) phagosomes — that culminate with the formation of phagolysosomes (**d**). During maturation, the phagosomes acquire various hydrolases and undergo a progressive acidification caused by proton pumping by the V-ATPase. EEA1, early endosome antigen 1; ESCRT, endosomal-sorting complex required for transport; HOPS, homotypic protein sorting; LAMP, lysosomal-associated membrane protein; LBPA, lysobisphosphatidic acid; PI(3)P, phosphatidylinositol-3-phosphate; MHCII, major histocompatibility complex II; RILP, Rab-interacting lysosomal protein.

The identity of the guanine nucleotide exchange factors (GEFs) that are responsible for Rac and Cdc42 activation are the subject of debate[5,6]. By contrast, it is generally agreed that downstream effectors, such as Wiskott–Aldrich syndrome protein[7], which in turn interacts with and activates actin-related protein 2/3 (Arp2/3), are actively involved in actin polymerization during FcγR-initiated phagocytosis[8]. In the case of CR3-mediated phagocytosis, the diaphanous-related formin Dia1 (also known as DIAPH1) is thought to initiate actin filament nucleation and elongation[4,9].

Phosphoinositides also provide an important contribution to actin remodelling during phagocytosis. Both phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate accumulate at sites of particle engagement and are instrumental in timing the onset and termination of actin assembly. Whereas phosphatidylinositol-4,5-bisphosphate is essential for the initial polymerization that drives pseudopod formation, its conversion to phosphatidylinositol-3,4,5-trisphosphate seems to be required for pseudopod extension and phagosomal closure[10], at least in part by recruitment of myosin X[11]. The metabolism of other phospholipids by phospholipases A and D is also necessary for successful completion of phagocytosis[12,13], although the precise products and mechanisms involved have not been fully resolved.

During phagocytosis of large or multiple particles, a considerable amount of membrane is internalized, and the cell needs to compensate for the loss of surface area. Paradoxically, capacitance measurements have shown that the plasmalemmal surface in fact increases during phagocytosis[14]. This has been attributed to focal exocytosis of endomembranes at sites of phagocytosis. Recycling[15] and late endosomes[16] are thought to be the main contributors, but even lysosomes have been reported to fuse when the demand for membrane is excessive[17,18]. Rab and ADP-ribosylation factor (Arf) GTPases are thought to be important in directing and tethering the endomembrane organelles to form phagosomes[19,20], whereas SNARE proteins (soluble NSF-attachment protein receptor proteins), including vesicle-associated membrane protein 3 (VAMP3)[15] and VAMP7 (REF. 16) underpin the fusion reaction.

Phosphoinositide
An inositol phospholipid that can be phosphorylated separately or at all possible combinations of the D-3, D-4 and D-5 positions of the inositol ring.

SNARE protein
A member of the soluble *N*-ethylmaleimide sensitive factor attachment protein receptor family that mediates docking and fusion of cellular membranes. Cognate SNARE pairs on the vesicular and target membranes intertwine to form a SNARE pin that brings the membranes into close apposition, driving their fusion.

Phagosome maturation

Maturation starts immediately after, and possibly even before, phagosome sealing. After scission from the surface membrane, the phagosome undergoes sequential fusion with early endosomes, late endosomes and lysosomes[21]. Whether complete fusion of the incoming membranes with the pre-existing vacuole or 'kiss and run' events are involved remains unclear and a combination of both may occur. Regardless, remodelling of the membrane of the phagosome is accompanied by acute changes in the composition of its lumen, which becomes a highly acidic, oxidative and degradative milieu. The steps leading to the formation of the phagolysosome, which is the terminal stage of the maturation sequence, are illustrated in FIG. 1 and discussed in more detail below.

The early phagosome. Newly formed phagosomes rapidly gain many of the properties of early endosomes. They have a propensity to fuse with sorting and recycling endosomes and are refractory to fusion with lysosomes[22,23]. Their lumen is mildly acidic (pH 6.1–6.5) and poor in hydrolytic activity[24].

The small GTPase Rab5A integrates the targeting, tethering and fusion of early endosomes[25], and also seems to be involved in the dynamics of early phagosomes, in which it is activated by the GAPVD1 (GTPase-activating protein and VPS9 domain-containing protein 1) exchange factor after the ingestion of apoptotic cells[26]. Rab5A acts using multiple effectors, including the p150–hVPS34 complex, early endosome antigen 1 (EEA1) and SNARE proteins. The Ser and Thr kinase p150 supports the recruitment of hVPS34, a class III phosphatidylinositol-3-kinase that generates phosphatidylinositol-3-phosphate (PI(3)P) on the early phagosomal membrane[27]. PI(3)P anchors effector proteins, such as EEA1, to the cytosolic face of the phagosome through FYVE and PX domains[28,29]. EEA1, which also interacts directly with Rab5 (REF. 30), is thought to act as a bridge that tethers early endosomes to incoming endocytic vesicles[31], and probably has an equivalent role in phagosomes. Additionally, EEA1 interacts with syntaxin 13 (REF. 32), a SNARE protein required for membrane fusion, and with an *N*-ethylmaleimide-sensitive fusion protein that is essential for the disassembly and reuse of SNARE complexes[33].

Despite repeated rounds of fusion with endomembrane vesicles, the surface area of the phagosomal membrane does not increase perceptibly, and continues to envelop the internalized particle tightly. This probably results from the concomitant occurrence of membrane fission events. Similarly to early endosomes, phagosomes are thought to be able to recycle molecules to the plasma membrane by a process involving coat protein I (COPI), and Arf and Rab GTPases[34]. Rab11A, which was previously known to mediate recycling of endosomes to the plasma membrane, also participates in the retrieval of phagosomal constituents to the plasmalemma[35], a process that is regulated by the Rab-coupling protein[36,37]. In addition, cargo is retrieved to endosomes and the *trans*-Golgi network by a complex of carrier vesicles, tubules and molecular motors[38]. The retromer complex of sorting nexin 1 (SNX1), SNX2, vacuolar protein sorting-associated protein 26A (VPS26A), VPS29 and VPS35, which links cargo selection to tubule generation in endosomes, is likely to play a similar part in phagosomes. SNX4 and EH domain-containing protein 1 (EHD1), two other components that are active in retrieval and tubule stabilization in other systems[39,40], may also contribute to phagosomal maturation.

In addition to budding outwards for the purpose of retrieval, phagosomes divert membrane-associated cargo that is destined for degradation to intraluminal vesicles. Such vesicles are thought to arise from inwards budding and pinching of the limiting membrane of the phagosome, in a manner akin to the generation of multivesicular bodies (MVBs). This initially occurs at a stage we designated as intermediate in FIG. 1, as it possesses features that are not present in early phagosomes, but lacks other features that are typical of late phagosomes (discussed below). As in endosomes, phagosomal membrane proteins destined for degradation are ubiquitinated and associate with the endosomal-sorting complex required for transport (ESCRT)[41]. In MVBs, the final component of the complex, ESCRTIII, forms a lattice that in conjunction with the ATPase VPS4A forces the extrusion of vesicles into the organellar lumen[42,43]. Phosphatidylinositol-(3,5)-bisphosphate synthesized by the FYVE finger-containing phosphoinositide kinase PIP5K3 (PIKfyve kinase) may also be important for vesiculation, as it binds to ESCRTIII[44].

The late phagosome. Once the recycling proteins are removed, the phagosome proceeds to the late stage, which is characterized by a more acidic luminal pH (5.5–6.0) brought about by the acquisition of additional proton-pumping V-ATPases[21]. The late phagosome is also enriched in proteases and lysosomal-associated membrane proteins (LAMPs), which are either imported from the Golgi complex or acquired by fusion with late endosomes. Little is known about late phagosome dynamics[21]. The small GTPase Rab7A is a characteristic marker of this organelle, and is known to mediate the traffic between phagosomes and late endosomes or lysosomes[45,46]. The VpsC–homotypic protein sorting (HOPS) complex, which mediates the transition from Rab5A- to Rab7A-positive endosomes[47], probably serves a similar function in phagosome maturation. However, whereas VpsC–HOPS does regulate vesicular traffic and fusion during lysosome biogenesis, it is not needed for Rab7A recruitment[48,49]. Regardless of how it is acquired, Rab7A recruits several effectors to the vacuolar membrane. One such effector, Rab-interacting lysosomal protein (RILP), promotes the centripetal movement of late phagosomes and lysosomes by bridging the membrane to the dynein–dynactin motor complex process[46,50]. Fusion of endosomes and lysosomes is facilitated by bringing the organelles in close apposition so that SNAREs such as VAMP7 and VAMP8 can complete membrane coalescence[51,52], and physical proximity is equally likely to favour fusion of phagosomes. Although necessary, Rab7A and RILP are not the only mediators of late phagosome maturation. Phosphatidylinositol-3-kinase antagonists block phagosome maturation despite the acquisition of Rab7A and RILP[53], implying that a separate, inositide-dependent event is also essential.

Retrieval and disposal of membrane components also occur at this stage. Similarly to late endosomes, late phagosomes contain lysobisphosphatidic acid (LBPA), a unique lipid found in luminal vesicles of MVBs. Programmed cell death 6-interacting protein (PDCD6IP; also known as ALIX), which binds LBPA and can link ESCRTI and ESCRTIII[54] in endosomes, is speculated to participate in the inward budding process.

Phagolysosome. The maturation process culminates with the formation of the phagolysosome, the ultimate microbicidal organelle. Phagolysosomes are endowed with a complete, sophisticated armamentarium to eliminate and

Multivesicular body
(MVB). A defined stage in the transit between early endosomes and late endosomes or lysosomes. MVBs are characterized by a limiting membrane that encloses internal vesicles rich in lysobisphosphatidic acid, CD63 and phosphatidylinositol-3-phosphate. Proteins destined for degradation are sorted to internal vesicles of MVBs.

degrade microorganisms (discussed below). They are generated by fusion with lysosomes through a Rab7A-dependent process and are highly acidic (luminal pH values as low as 4.5 have been reported). Insertion of additional V-ATPases and tightening of the H^+ 'leak' account for the accentuated acidification. Phagolysosomes can be differentiated from late phagosomes by their paucity of LBPA or PI(3)P-enriched internal membranes[55,56], by their elevated mature cathepsin content and by their lack of mannose-6-phosphate receptors[57].

Microbicidal activity of the phagosome

During the course of maturation, phagosomes acquire a full arsenal of antimicrobial features (FIG. 2), which are described individually below.

Acidification of the phagosome. The V-ATPases that acidify the phagosomal lumen consist of a cytoplasmic V_1 complex that hydrolyses ATP and transfers the energy to a membrane-embedded V_0 complex that translocates H^+ across the bilayer[58]. Phagosomal acidification creates a hostile environment that impedes microbial growth[59]: not only does it directly impair the metabolism of some bacteria, but it also favours the activity of many hydrolytic enzymes of the phagocyte that have acidic pH optima. In addition, the transmembrane H^+ gradient generated by the V-ATPase is used to extrude essential microbial nutrients from the phagosomal lumen. The V-ATPase also facilitates the generation of superoxide (O_2^-) by transporting H^+ in an unaccompanied (and therefore electrogenic) manner, thereby counteracting the negative charges translocated by the NADPH oxidase. The products of the oxidase can subsequently combine with H^+ in the lumen of the phagosome, generating more-complex reactive oxygen species (ROS) (discussed below).

Phagosomal acidification is not only a consequence of phagolysosome formation, but seems to be an integral element of the maturation process, as it directly controls membrane traffic[60,61]. Dissipation of the pH gradient across the phagosomal membrane by addition of weak bases or by interference with V-ATPase activity arrests maturation, preventing the formation of phagolysosomes. Evidence derived from the endocytic pathway suggests that acidification is required for the assembly of COPI complexes[62] and for the recruitment of ARF6 and cytohesin 2 (also known as ARNO) (REF. 63).

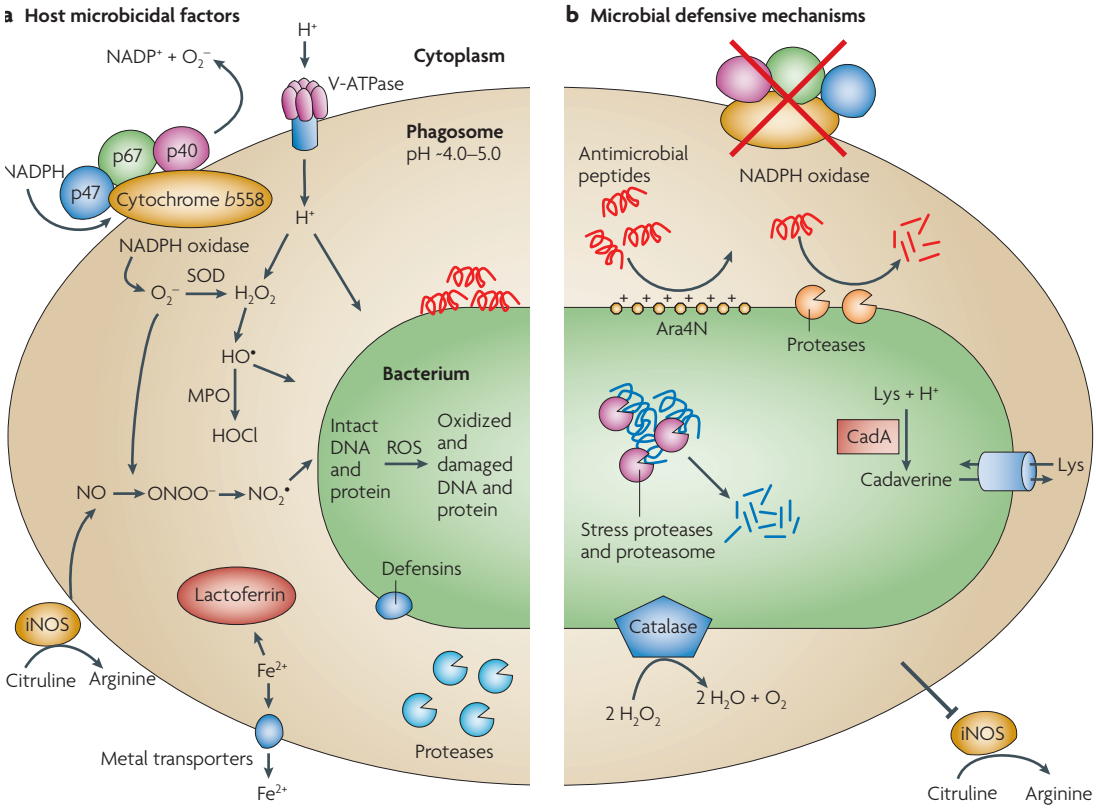

Figure 2 | **The microbicidal arsenal of phagocytes versus the defensive mechanisms of the microorganism.** The host microbicidal mechanisms (**a**) include the NOX2 (also known as CYBB) NADPH oxidase, the inducible NO synthase (iNOS), iron scavengers and exporters, such as lactoferrin and natural resistance-associated macrophage protein 1 (NRAMP1; also known as SLC11A1), plus antimicrobial peptides and proteins that permeabilize and degrade the bacteria. Bacterial defensive mechanisms (**b**) include modification of their surface to resist or break down antimicrobial peptides, and expression of enzymes, such as catalase, that convert reactive species to less harmful compounds or prevent recruitment of the protein complexes that synthesize reactive nitrogen species (RNS) or reactive oxygen species (ROS) (see the main text for details). SOD, superoxide dismutase.

Azurophil or primary granule
A specialized neutrophil granule, also called a peroxidase-positive granule, that resembles lysosomes, in that it contains degradative enzymes, such as β-glucuronidase, cathepsins, elastase, lysozyme and myeloperoxidase, as well as antimicrobial peptides, such as defensins.

Specific or secondary granule
A specialized neutrophil granule, also called a peroxidase-negative granule, that exists as a heterogeneous continuum of granules with varying amounts of lactoferrin, collagenase, heparanase, lysozyme and antimicrobial cathelicidins.

Reactive oxygen and nitrogen species. Professional phagocytes destroy pathogens in part through ROS generated directly or indirectly by the NOX2 (also known as CYBB or gp91phox) NADPH oxidase. Because ROS production is most prominent in neutrophils, most of our knowledge of NOX2 biology is derived from this cell type. The importance of ROS in pathogen elimination is highlighted by individuals with mutations that cause partial or total inactivation of the oxidase[64]. These patients suffer from chronic granulomatous disease, which is characterized by severe recurrent infections that can result in death[65]. NOX2 is a multisubunit complex, consisting of a transmembrane heterodimer (CYBB and CYBA) that after activation assembles with three cytosolic subunits (neutrophil cytosol factor 4 (NCF4), NCF1 and NCF2)[64]. Rac1 and Rac2 are also required for activation of the enzyme[66,67]. The active oxidase transfers electrons from cytosolic NADPH to molecular oxygen, releasing O_2^- into the phagosomal lumen[68]. Within the phagosome, O_2^- can dismutate to H_2O_2, which can in turn react with O_2^- to generate hydroxyl radicals and singlet oxygen[69]. H_2O_2 can also be converted by myeloperoxidase into hypochlorous acid and chloramines[70]. Collectively, these highly reactive, toxic ROS effectively kill intraphagosomal microorganisms. The NOX2 NADPH oxidase has also been invoked in the activation of proteolytic enzymes by indirectly altering the ionic composition of phagosomes[71,72], but this role remains controversial[73].

Similarly to ROS, nitric oxide ($NO^{\bullet}$) and the reactive nitrogen species (RNS) derived from it are important antimicrobial effectors. RNS are prominent in macrophages, in which they have been studied in greatest detail. The activity of the inducible nitric oxide synthase, or NOS2, the isoform most relevant to phagocytes[74], is regulated at the transcriptional level; RNS production requires *de novo* synthesis of the protein in response to proinflammatory agonists[74]. The synthase functions as a dimer: one subunit transfers electrons from NADPH to FAD, then to FMN (flavin mononucleotide) and to the haem iron of the adjacent subunit, to produce $NO^{\bullet}$ and citrulline from L-arginine and oxygen[75]. Unlike superoxide, $NO^{\bullet}$ is synthesized on the cytoplasmic side of phagosomes, but has the ability to diffuse across membranes to reach intraphagosomal targets[76]. In the luminal environment, where it encounters ROS, $NO^{\bullet}$ can undergo either spontaneous or catalytic conversion to a range of RNS, including nitrogen dioxide ($NO_2^{\bullet}$), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), dinitrosyl iron complexes, nitrosothiols and nitroxyl (HNO)[74]. ROS and RNS synergize to exert highly toxic effects on intraphagosomal microorganisms. They interact with numerous microbial targets, such as thiols, metal centres, protein tyrosine residues, nucleic acids and lipids[77]. As a result, proteins are inactivated and lipids are converted by oxidative damage. In addition, microbial DNA can undergo irreparable damage. Together, these reactions can impair bacterial metabolism and ultimately inhibit replication.

Antimicrobial proteins and peptides. A set of proteins that antagonize bacterial growth complement the phagosomal inventory of antimicrobial tools (TABLE 1). They can be grossly subdivided into those that prevent growth and those that compromise the integrity of the microorganism. Growth prevention can be accomplished by limiting the availability of essential nutrients inside the phagosome. To this end, phagocytes secrete scavengers into the lumen or insert transporters into the phagosomal membrane. This has been investigated in more detail in neutrophils, which are equipped with specialized granules (azurophil or primary granules, specific or secondary granules and gelatinase granules) and secretory vesicles that when stimulated release their contents extracellularly and/or into the phagosome[78]. One such scavenger is lactoferrin, a glycoprotein contained in neutrophil granules that is released into the phagosome lumen, where it sequesters iron that is required by some bacteria[79]. The other strategy is illustrated by natural resistance-associated macrophage protein 1 (NRAMP1; also known as SLC11A1), an integral membrane protein expressed in late endosomes and lysosomes that is recruited to the phagosomal membrane soon after pathogen uptake. NRAMP1 exerts a bacteriostatic effect by extruding divalent cations, such as Fe^{2+}, Zn^{2+} and Mn^{2+} from the phagosomal lumen[80]. Fe^{2+} and Zn^{2+} are cofactors of microbial housekeeping enzymes, and Mn^{2+} is required by superoxide dismutase, a key protective enzyme expressed by certain pathogens.

More-direct mechanisms deployed by phagosomes to disrupt the integrity of pathogens involve the defensins, cathelicidins, lysozymes, and assorted lipases and proteases (TABLE 1). The defensins, which are subdivided into α and β subgroups, are small, disulphide-bridged polypeptides of ≈10 kDa that in neutrophils are stored within azurophil or primary granules[81]. Defensins bind to negatively charged molecules on the microbial surface. They subsequently induce membrane permeabilization of Gram-positive and Gram-negative bacteria by forming multimeric ion-permeable channels[81]. Cathelicidins are also small proteins of ≈10 kDa that neutrophils store as proforms in secondary granules[82]. The precursors are converted to active species by elastase, a primary granule protein they probably encounter in the phagosomal lumen. Cathelicidins act by permeabilizing the cell wall and inner membrane of Gram-positive bacteria and the outer and inner membranes of Gram-negative bacteria[82].

Phagosomes are also equipped with an assortment of endopeptidases, exopeptidases and hydrolases that degrade various microbial components. The endopeptidases are made up of cysteine and aspartate proteases, whereas the exopeptidase pool consists of cysteine and serine proteases[83]. Endopeptidases, particularly the C1 family of cysteine proteases, are especially important, because they efficiently generate substrates for the exopeptidases[83]. Not all the proteases are acquired simultaneously by the maturing phagosome, implying that they are delivered by distinct organelles. Cathepsin H is predominant in early phagosomes, whereas cathepsin S is typically present in late phagosomes[84].

Table 1 | **Proteins and peptides with antimicrobial activity**

Antimicrobial activity	Protein or peptide	Refs
Bacteriostatic		
Nutrient deprivation	Lactoferrin	71
	NRAMP1 (also known as SLC11A1)	72
Bactericidal		
Membrane permeabilization	Defensins	73
	Cathelicidins	74
Hydrolysis		
Carbohydrates	Lysozyme	158
	β-hexosaminidase	159
	β-glucuronidase	159
Lipids	Phospholipase A2	160
Proteins	Cysteine proteases*: cathepsins B, C, H, K, L, O, S and W	159,161
	Aspartate proteases*: cathepsins D and E	159,161
	Serine proteases*: cathepsin G	159,161
	Carboxypeptidases‡: lysosomal carboxypeptidase (cathepsin A), cathepsin B (dipeptidase), cathepsin X, lysosomal carboxypeptidase B, prolylcarboxypeptidase and peptidyl dipeptidase B	159,161
	Aminopeptidases‡: cathepsin H, dipeptidyl peptidase I (cathepsin C), dipeptidyl peptidase II and tripeptidyl peptidase	159,161

*Endopeptidases. ‡Exopeptidases. NRAMP1, natural resistance-associated macrophage protein 1.

Hydrolases that target carbohydrates (for example, α-hexosaminidase, β-glucuronidase and lysozyme) and lipids (for example, phospholipase A2) are also delivered to the phagosomes.

Bacterial resistance to phagocyte killing

Despite the presence of these antimicrobial host factors, many pathogens can survive inside the host cell. Such pathogens, which include bacteria, fungi and viruses, have evolved a multitude of strategies to counteract host defences. For simplicity, we confine the remainder of this Review to bacterial pathogens. Some bacterial species interfere with the ability of phagocytes to engulf them[85,86], either by scavenging, inhibiting or even degrading opsonic antibodies or complement[87–89], or by directly impairing the phagocytic machinery of macrophages and neutrophils[85,86,90]. Other bacteria have become resistant to one or more of the antimicrobial factors of phagocytes (FIG. 2). Some species have developed metabolic pathways to counteract acid accumulation inside phagosomes or have acquired uniquely resistant proteins to withstand the low pH[91,92]. Yet other bacteria protect themselves by actively degrading[93] or shielding themselves[94,95] from the antimicrobial peptides and proteins produced by phagocytes, or by expressing detoxifying enzymes, such as catalase, that neutralize ROS and/or RNS[96,97]. Alternatively, some bacterial species prevent RNS and ROS formation by impairing recruitment of the proteins that mediate their synthesis[98,99]. Other species have devised means of overcoming the scarcity of iron by secreting specialized iron-scavenging molecules called siderophores, which sequester and target the cation for bacterial use[100], or by expressing iron storage[101] or transport proteins[102]. Lastly, many bacteria improve their intraphagosomal survival by mounting a vigorous stress response to dispose of and replace damaged proteins[103].

Although most bacteria use one or more of these resistance mechanisms, only a select group of bacteria are 'professional' intracellular pathogens. These species survive and replicate inside phagocytes, effectively avoiding attack by their antimicrobial factors. To accomplish this feat, such pathogens have evolved multiple strategies towards one common goal: to perturb phagosomal maturation. These different strategies are exemplified by the mechanisms used by *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Legionella pneumophila* and *Coxiella burnetii*. These bacteria parasitize host cells by arresting or reprogramming phagosomal maturation, by escaping maturing phagosomes or by withstanding the microbicidal properties of the phagolysosome.

M. tuberculosis*: inhibition of phagosomal maturation.* The pathogenicity of *M. tuberculosis* is largely attributed to its ability to survive within macrophages by arresting phagosomal maturation[104]. This bacterium is exquisitely adapted to life within macrophages and not only arrests phagolysosome formation but can also escape the phagosome[105] and modulate other macrophage defences to promote its survival[106,107]. Phagosomal escape, a previously unappreciated facet of intracellular *M. tuberculosis*, requires the expression of a novel bacterial secretion system, ESX[105], which is lacking in avirulent mycobacteria (reviewed in REF. 108). Phagocytosis of *M. tuberculosis* by macrophages occurs through the engagement of various receptors, including CR3 (REF. 109). However, unlike other particles that are engulfed by the same receptors, the *Mycobacterium*-containing phagosome fails to progress and become a phagolysosome and is instead arrested at an early stage[110] (FIG. 3a). Arrested *M. tuberculosis*-containing phagosomes are characterized by the presence of Rab5A, but the recruitment of Rab5A effectors, such as EEA1 and hVPS34, is impaired[110,111], and as a result, PI(3)P does not accumulate. *M. tuberculosis* uses a range of protein and lipid effectors to alter PI(3)P signalling[112,113] (TABLE 2). The mycobacterial phosphoinositide lipoarabinomannan[112], a component of the cell wall that is shed from live bacteria and becomes distributed throughout the endocytic network[114], prevents the increase in cytosolic [Ca^{2+}] that normally accompanies phagocytosis and that is thought to be required to activate hVPS34 through calmodulin[112]. *M. tuberculosis* further impairs cytosolic Ca^{2+} flux by inhibiting sphingosine kinase, which converts sphingosine to sphingosine-1-phosphate, which in turn promotes Ca^{2+} efflux from the endoplasmic reticulum (ER)[115,116]. *M. tuberculosis* also produces the phosphatase SapM, which specifically hydrolyses PI(3)P[113]. This combined strategy effectively depletes PI(3)P from early phagosomes and prevents the transition to the late and phagolysosomal stages.

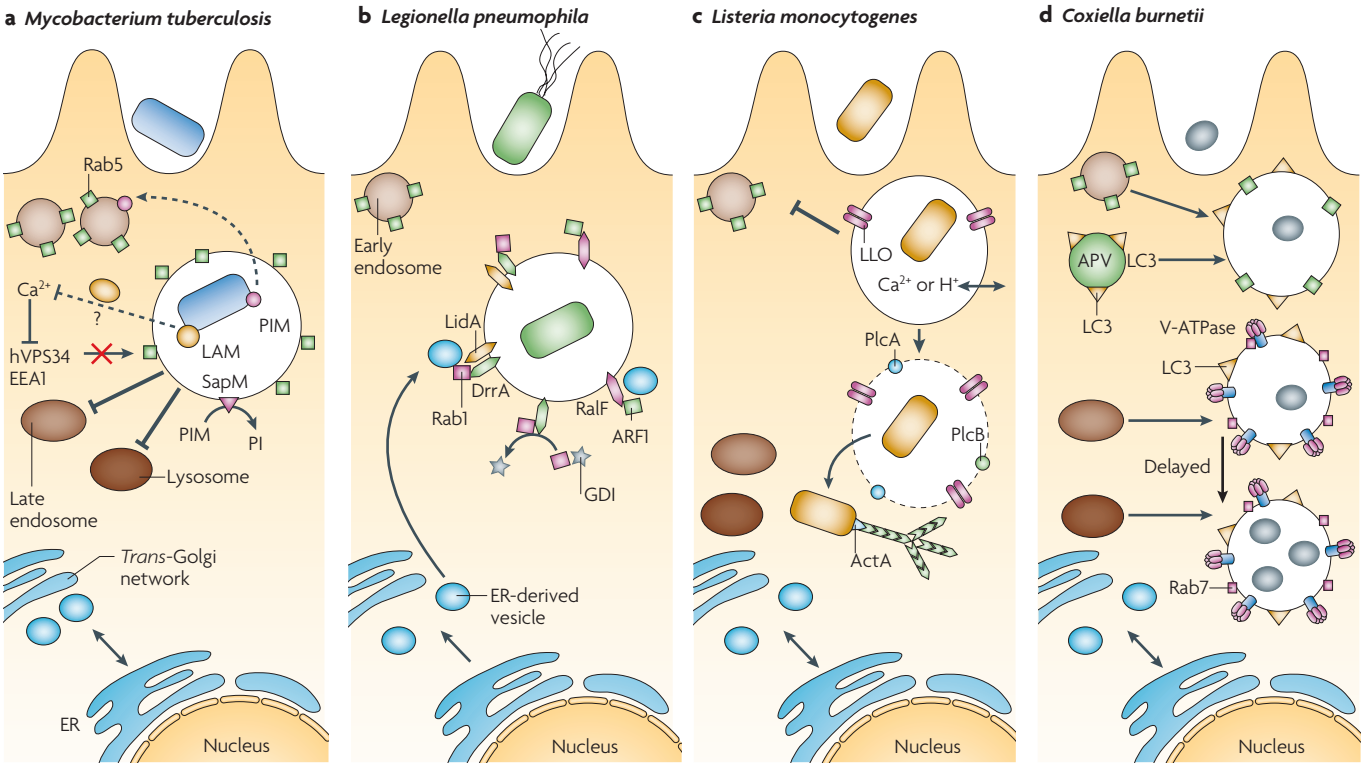

Figure 3 | **Strategies used by professional intracellular bacterial pathogens to modulate phagosome maturation.** **a** | *M. tuberculosis*. After internalization, the bacterium uses an array of effector molecules, including the lipids phosphatidylinositol mannoside (PIM) and lipoarabinomannan (LAM), and the phosphatidylinositol-3-phosphate (PI(3)P) phosphatase SapM to arrest phagosome maturation at an early stage. **b** | *L. pneumophila*. This bacterium impairs fusion of the *Legionella*-containing vacuole with endolysosomal compartments, and instead promotes fusion with endoplasmic reticulum (ER)-derived membranes. **c** | *L. monocytogenes*. This pathogen evades phagolysosomal fusion after internalization by escaping the phagosome through secretion of listeriolysin O (LLO) and two phospholipases, PlcA and PlcB. Once in the cytoplasm, *L. monocytogenes* replicates and becomes motile by using actin 'comet tails' generated by the effector ActA. **d** | *C. burnetii*. Phagosomes containing this bacterium undergo delayed maturation as they fuse with autophagocytic vesicles (APVs) bearing LC3. The delay enables *C. burnetii* to acquire features that allow it to replicate in a membrane-bound compartment that resembles phagolysosomes. EEA1, early endosome antigen 1; GDI, guanine nucleotide dissociation inhibitor; LAM, lipoarabinomannan.

Activation of macrophages increases their ability to eradicate intracellular *M. tuberculosis* and other organisms[117,118]. This is highlighted by the observation that interferon-γ (IFNγ)-stimulated macrophages demonstrate enhanced bacterial clearance; in stimulated cells, *M. tuberculosis*-containing phagosomes are sequestered by autophagic compartments that ultimately fuse with lysosomes[119]. This autophagic response can be enhanced by Toll-like receptor ligands[120] and the activation of immunity-related p47 guanosine triphosphatase protein[121]. Immunity to pathogens such as mycobacteria is in part attributable to the activation of the inflammasome, a multiprotein complex that facilitates the killing of intracellular bacteria and is required for interleukin-1β (IL-1β) processing. IL-1β enables macrophages to overcome the arrested maturation of the *M. tuberculosis*-containing phagosome[122,123] through an unknown mechanism that may involve restored PI(3)P production and subsequent maturation of the phagosome. Interestingly, the bacteria have also evolved a way to counteract the inflammatory response: *M. tuberculosis* secretes ZmpA, a predicted zinc metalloprotease that inhibits IL-1β processing by the host cells[123]. Intracellular survival of the bacteria therefore depends on an ongoing, multilevel tug of war between the pathogen and host macrophage.

L. monocytogenes*: a phagosomal escape artist.* Listeriosis, a potentially fatal disease caused by the Gram-positive bacterium *L. monocytogenes*[124], is frequently contracted through the consumption of contaminated foods. *L. monocytogenes* is internalized by both non-phagocytic cells and professional phagocytes[124], which is crucial for bacterial propagation and dissemination. Uptake by epithelial cells is mediated by the surface proteins internalin A and internalin B (InlA[125] and InlB[126]), which function as ligands for the adhesion molecule E cadherin[127], the hepatocyte growth factor receptor Met[128] and the complement receptor, C1qR[129]. However, phagocytosis of *L. monocytogenes* by macrophages is mediated by scavenger receptors that recognize lipoteichoic acid, a component of the Gram-positive bacterial cell wall[130]. In addition, the surface of *L. monocytogenes* can become decorated with the complement components C1q[131] and

Table 2 | **Effector molecules that contribute to the survival of professional intracellular bacterial pathogens**

Pathogen	Effector	Cellular target	Effector function	Refs
Mycobacterium tuberculosis	SapM	PI(3)P	Hydrolyses PI(3)P to PI, thereby inhibiting phagosomal maturation	105
	ZmpA	Inflammasome	Blocks processing of IL-1β	110
	Phosphatidylinositol mannoside	Endosomal pathway	Mechanism unknown, but promotes fusion of endosomes with *M. tuberculosis*-containing phagosomes	162
	Lipoarabinomannan	Unknown	Blocks cytosolic Ca^{2+} fluxes, impairing hVPS34 activation	104
Legionella pneumophila	Drra (also known as SidM)	Rab1 GDI and Rab1	Displaces Rab1 from host GDI and functions as Rab1 GEF	121,122
	LidA	Rab1	Binds Rab1 to enhance recruitment of activated Rab1 to LCV	119
	RalF	ARF1	ARF1-specific GEF that activates ARF1	123
	LepB	Rab1	Rab1 GAP that promotes GTP hydrolysis	121
Listeria monocytogenes	Listeriolysin O	Phagosomal membrane	Generates pores to perturb ion gradients and facilitate escape	138,141
	Phospholipase (PlcA)	Phagosomal membrane	Phosphoinositol-specific phospholipase that facilitates phagosome escape	142
	Phospholipase (PlcB)	Phagosomal membrane	Broad range phospholipase that facilitates escape	142
Coxiella burnetii	Largely unknown; AnkF was recently identified as a type IV secretion system protein	Unknown	Type IV secretion system-secreted effector with ankyrin repeat motifs	124

GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GDI, guanine nucleotide dissociation inhibitor; IL, interleukin; LCV, *Legionella*-containing vacuole; PI(3)P, phosphatidylinositol-3-phosphate.

C3 (REF. 132), which are ligands for macrophage complement receptors. Lastly, InlB also functions as a ligand for the C1q receptor[129].

L. monocytogenes, a facultative intracellular pathogen, survives intracellularly by modifying and subsequently escaping from phagosomes (FIG. 3c). To this end, the bacteria use a sophisticated combination of effectors. The cholesterol-dependent cytolysin listeriolysin O (LLO) creates pores in the phagosomal membrane as early as 5 minutes after infection[133]. The effect of LLO is restricted to the phagosome, as it needs to be activated by acidification and/or by the host enzyme GILT (IFNγ-inducible lysosomal thiol reductase) that is found inside the phagosome[134]. Secretion of LLO inhibits the maturation of phagosomes[135] owing to a loss of luminal H^+ and Ca^{2+}, which are thought to be required for fusion with endosomes and/or lysosomes[136]. *Listeria* also expresses two membrane-active phospholipase C enzymes, phosphoinositol-specific phospholipase C (PI-PLC; encoded by *plcA*) and broad-range phospholipase C (PC-PLC; encoded by *plcB*). Together with LLO, PI-PLC and PC-PLC cause the breakdown of the membrane of the *L. monocytogenes*-containing phagosome and thereby enable the bacteria to escape and take up residence in the cytosol[136,137], where bacterial replication occurs[138].

Cytosolic *L. monocytogenes* replicates efficiently, and has a generation time of ~30 minutes, owing to the expression of genes that enable nutrients to be used directly from the host cell[139]. In the cytosol, *L. monocytogenes* becomes motile by usurping the host's actin cytoskeletal machinery. The bacterial surface protein ActA induces the assembly of spectacular actin 'comet tails' by recruiting host cell Arp2/3 complexes, G actin and vasodilator-stimulated phosphoprotein (VASP) family members (reviewed in REF. 140). Although this motility is not required for phagosomal escape, ActA contributes substantially to *L. monocytogenes* dissemination during infection[141].

Although *L. monocytogenes* was previously thought to reside primarily in the cytosol, under some circumstances, it replicates in macrophages, inside large, LAMP1-positive vacuoles called spacious *Listeria*-containing phagosomes (SLAPs)[142]. The formation of SLAPs is strictly dependent on low levels of LLO production and the recruitment of the autophagy protein LC3 to the phagosome[142]. SLAP formation in macrophages allows *L. monocytogenes* to replicate slowly (generation time >8 hours) without destroying the infected cell[142]. This newly discovered facet of the *L. monocytogenes* life cycle could contribute to the development of chronic *L. monocytogenes* infections.

L. pneumophila*: reprogramming the phagosomal maturation pathway.* *L. pneumophila* is a Gram-negative bacterium that is found ubiquitously in aquatic environments, growing in biofilms or within freshwater protozoa[143]. In humans, it can survive and replicate within professional phagocytes[144] by redirecting the maturation of phagosomes to create a unique intracellular niche suited for bacterial replication (FIG. 3b).

After inhalation of *L. pneumophila*, the major outer membrane protein on the surface of the bacteria effectively fixes complement[145], thereby promoting phagocytosis by macrophages through complement receptors[146] and leading to the formation of *Legionella*-containing vacuoles (LCVs). Internalized *L. pneumophila* rapidly modulates the maturation of the LCV, avoiding interaction with the default endolysosomal pathway[147,148]. Shortly afterwards,

Autophagy
A complex cellular process by which intracellular components, including entire organelles, are sequestered in double-membrane vesicles or vacuoles called autophagosomes that eventually fuse with lysosomes, bringing about the degradation of their contents.

SarI–COPII-coated secretory vesicles derived from the ER fuse with the LCV in a process that in part requires the host cell GTPase Rab1, which, together with another host GTPase, ARF1, regulates vesicular transport between the ER and the Golgi complex. The extent and timing of Rab1 recruitment to the LCV are tightly regulated through effector proteins delivered by the Dot–Icm type IV secretion system (T4SS)[149]. The T4SS effector DrrA[150] (also known as SidM[151]) binds in a phosphatidylinositol-4-phosphate-dependent manner to the LCV[152], and displaces the guanine nucleotide dissociation inhibitor that stabilizes the GDP-bound form of Rab1 (REFS 153,154). DrrA also facilitates nucleotide exchange[150,151], generating the active, GTP-bound form of Rab1, which is required for vesicular fusion. A second T4SS effector, LidA, functions synergistically with DrrA to enhance Rab1 recruitment to the LCV[151]. Subsequently LepB, another T4SS effector, deactivates Rab1 by promoting GTP hydrolysis[153].

L. pneumophila also manipulates the activity of ARF1 through the T4SS effector RalF, which operates as an ARF1-specific GEF[155]. Together, DrrA, LidA and RalF recruit active Rab1 and ARF1 to the LCV, thereby promoting and regulating the fusion of ER-derived vesicles to the LCV[150,153,155]. As the ER-derived vesicles interact with the LCV, *L. pneumophila* simultaneously disrupts the normal microtubule-dependent organellar transport of the host cell through the secretion of additional virulence factors, such as AnkX[156]. It is noteworthy that mutants lacking DrrA or RalF can survive in macrophages, implying that multiple, redundant mechanisms can lead to formation of the LCV. *L. pneumophila* therefore possesses a vast, incompletely characterized arsenal of effectors that perturb many aspects of vesicular transport in host cells.

Ultimately, *L. pneumophila* replicates intracellularly within a large, acidic vacuole with some of the properties of lysosomes[157,158]. Although there is disagreement as to whether the acidification is required for efficient replication[158,159], it is clear that fusion with the ER is crucial to allow the bacteria sufficient time to develop resistance to the vacuolar environment. Interaction of ER-derived membranes with the LCV has been associated with host cell autophagy[160], and it has been suggested that *L. pneumophila* can delay autophagolysosome formation, allowing enhanced survival[161].

The effectors discussed above are only a fraction of those required for successful culmination of the bacterial replication process. More than 80 different T4SS effectors have been implicated[162], in addition to several other proteins that are secreted by the bacteria through Icm–Dot-independent mechanisms[163]. Several of these effectors possess motifs that are commonly identified in eukaryotic proteins, suggesting that *L. pneumophila* has the potential to manipulate additional host cell processes[156,163].

C. burnetii*: weathering the storm.* *C. burnetii*, the causative agent of Q fever, is a highly infectious, Gram-negative, obligate intracellular pathogen[164]. It has a biphasic developmental cycle that consists of an infectious small-cell variant (phase 1 *Coxiella*) and a large-cell variant (phase 2 *Coxiella*) that replicates intracellularly[164]. In contrast to the other intracellular bacterial pathogens described above, *C. burnetii* resides in an acidified lysosome-like compartment, in which it replicates in the presence of several antimicrobial factors[165] (FIG. 3d).

Phagocytosis of phase I *Coxiella* occurs after engagement by the bacterium of the leukocyte response integrin (αVβ3)[166], which activates a cell signalling cascade that ultimately induces localized actin polymerization[167], propelling internalization and formation of the *Coxiella* phagosome. By contrast, internalization of avirulent phase II *Coxiella*, through engagement of αVβ3, CR3 or through hydrophobic binding mediated by the bacterial lipopolysaccharide[164], does not elicit the same signalling cascade[168].

After sealing, the *Coxiella* phagosome interacts with the default endocytic pathway[165]. Simultaneously, *C. burnetii* begins to alter the maturation programme, thereby conferring properties of autophagosomes to the vacuole[169,170]. Specifically, the autophagic protein LC3 is recruited to the *Coxiella* phagosome, delaying its fusion with lysosomes[171] and giving the bacteria time to initiate the transition to the replication-competent, large-cell variant[170].

Within 48 hours of infection, *C. burnetii* resides in a large spacious compartment that contains several lysosomal proteins, including the V-ATPase[165]. This compartment, termed the replicative *Coxiella* vacuole, is acidic (pH ≈4.8)[172], which is a requirement for *C. burnetii* replication[165], even though acidification is normally thought to be an important bacteriostatic or bactericidal component of the phagolysosome. Physiological studies of *C. burnetii* reveal that it behaves similarly to an acidophile, as it requires a low pH for certain metabolic activities[173]. Within the replicative vacuole, the bacteria also encounter other antimicrobial agents. Yet *C. burnetii* seems to be well adapted to this biological niche, and probably uses an assortment of virulence factors to nullify the antimicrobial effects. Most of these factors remain to be identified, but one known example is the induction of the SOS DNA repair system that protects the pathogen from chromosomal damage owing to ROS exposure[174]. Similarly to *L. pneumophila*, *C. burnetii* also encodes a functional T4SS. Furthermore, the recent identification of several candidate effectors with ankyrin-repeat homology domains[156] will stimulate an even greater interest in *C. burnetii* pathogenesis.

Concluding remarks

The confluence of microbiology and cell biology was made possible to a large extent by the development of biochemical and imaging techniques that improved the sensitivity and spatio-temporal resolution of events that take place during the infectious sequence. Elucidation of the full genome sequences of a number of pathogens, together with the increased sensitivity and accuracy of proteomic analyses and the implementation of powerful intravital imaging will further accelerate the pace of knowledge acquisition in the new discipline of cellular microbiology. As a consequence, more fascinating insights into the interaction between phagocytes and pathogens are certain to emerge.

SarI–COPII-coated secretory vesicle
A vesicle derived from the endoplasmic reticulum (ER) through coating with the coatomer protein complex II (COPII) protein complex, a process initiated at specialized ER exit sites by the GTPase SarI.

Type IV secretion system
A macromolecular apparatus used by bacteria to secrete effector molecules. This secretion system is ancestrally related to bacterial DNA conjugation systems, and is often expressed by pathogenic bacteria, which contributes to their virulence.

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DATABASES

Entrez Genome Project: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj
Coxiella burnetii | *Legionella pneumophila* | *Listeria monocytogenes* | *Mycobacterium tuberculosis*

UniProtKB: http://www.uniprot.org
ActA | ARF6 | ARNO | Cdc42 | CYBA | DIAPH1 | DrrA | EEA1 | EHD1 | GAPVD1 | IFNγ | IL-1β | InlA | InlB | LAMP1 | LepB | LidA | NCF1 | NCF2 | NCF4 | NOS2 | NOX2 | NRAMP1 | PIP5K3 | Rac1 | Rac2 | Rab5A | Rab7A | Rab11A | RalF | RILP | SNX1 | SNX2 | SNX4 | SYK | VAMP3 | VAMP7 | VAMP8 | VPS4A | VPS26A | VPS29 | VPS35

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