

## Review

# The Phagosome: Compartment with a License to Kill

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**Phagosomes are fascinating subcellular structures. After all, there are only a few compartments that are born before our very eyes and whose development we can follow in a light microscope until their contents disintegrate and are completely absorbed. Yet, some phagosomes are taken advantage of by pathogenic microorganisms, which change their fate. Research into phagosome biogenesis has flourished in recent years – the purpose of this review is to give a glimpse of where this research stands, with emphasis on the cell biology of macrophage phagosomes, on new model organisms for the study of phagosome biogenesis and on intracellular pathogens and their interference with normal phagosome function.**

**Key words: endosome, organelle biogenesis, pathogens, phagocytosis, phagolysosome, vacuole**

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## Phagocytosis: An Organelle Is Born!

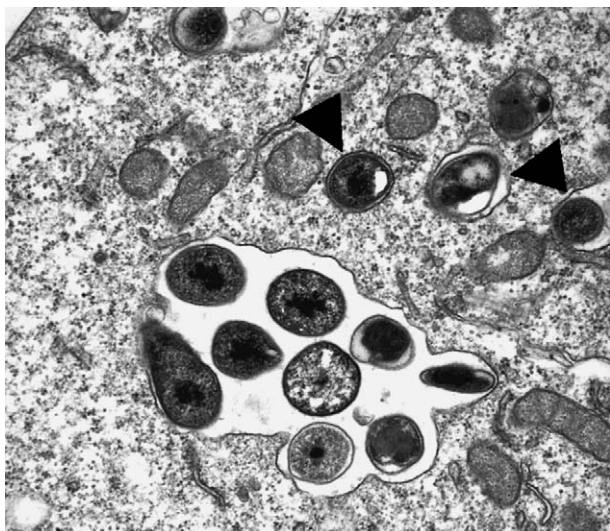
Phagocytosis (Greek, meaning 'cell-eating')<sup>1</sup> describes the ingestion of a particle by a biological cell. Phagocytosis in mammals is a special feature of so-called professional phagocytic cells, i.e. polymorphonuclear leukocytes (also known as neutrophils), dendritic cells and macrophages but is not unique to these cells. When a non-self particle such as a bacterium enters the sterile sections of the body, professional phagocytes are chemotactically attracted, bind the particle, ingest and kill it. In the case of macrophages and dendritic cells, the invader's antigenic molecules are presented to other immune cells. This initiates an adaptive immune response.

<sup>1</sup>The term 'phagocytosis' is used here to describe the process of ingestion *per se*, whereas 'phagosome maturation' refers to the process of intracellular phagosome development after closure of the phagocytic cup. The term 'phagosome' is used for endocytic compartments that contain a non-interfering particle, whereas 'vacuole' describes a compartment containing a particle such as a pathogen that diverts normal phagosome maturation.

A phagosome is formed when the phagocyte wraps a portion of its plasma membrane around the particle, followed by plasma membrane fusion at the tip of the particle and ingestion of the newly produced membrane bag containing the particle (Figure 1). The process of ingestion in most cases follows the 'zipper mode', i.e. particle-ligand macrophage-receptor interactions all around the particle lead to a close apposition of the phagosome membrane (1). Particles ingested through the mannose receptor 'sink' into the phagocytes, and protruding pseudopods are not seen (2). In terms of cell biology, the newly created phagosome is to the cell much like an endocytic compartment with the exception that it contains a particle of usually more than 0.4  $\mu\text{m}$  in diameter. Consequently, viruses of 20–300 nm in diameter are taken up by endocytosis rather than by phagocytosis, and particles exceeding approximately 25  $\mu\text{m}$  in diameter will not be ingested (3). Local particle curvature was recently found to be a defining parameter in the decision of whether phagocytosis would be initiated or not (4).

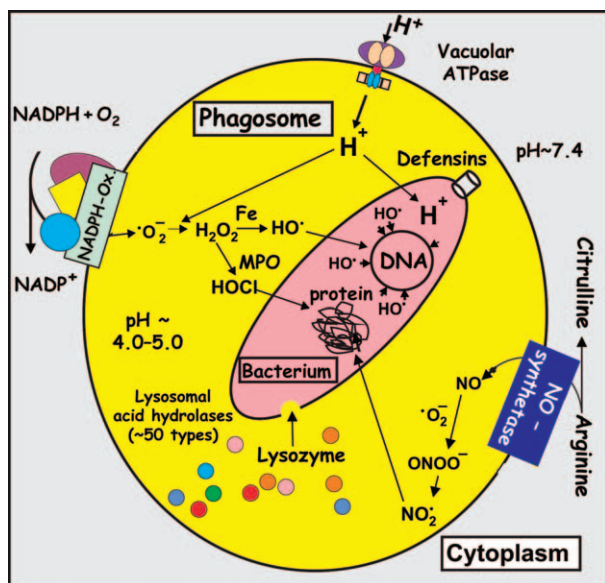
Ligation of suitable phagocyte receptors triggers the flushing of the phagosome contents with antimicrobial compounds: a reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex produces large quantities of superoxide radicals from molecular oxygen, and a nitric oxide synthetase produces NO radicals from arginine. As a central event in phagosome biogenesis, the phagosome lumen is strongly acidified (to a pH  $\sim$ 4.5) by a membrane-embedded proton-pumping adenosine triphosphatase (ATPase) complex, the vacuolar ATPase. In neutrophils, phagosomal myeloperoxidase, in concert with the above enzymes, leads to the formation of hyperchlorous acid and chloramines, hydroxyl radicals and ozone, all of which are strongly biocidal (5). Strong production of superoxides may actually briefly raise the intraphagosomal pH, as they consume protons in their conversion to hydrogen peroxide [(6), for a different view, see Jankowski et al. (7)], and acidification is further influenced by the activity of ion exchangers such as sodium/hydrogen exchanger isoform 1 (NHE-1) and the cystic fibrosis transmembrane conductance regulator  $\text{Cl}^-$  channel (8). As non-oxidative tools, defensin peptides and cationic proteins further support killing of microbes (9).

To what extent these different killing pathways (Figure 2) are used in a given phagosome depends on the port of entry, as superoxide radical production is increased when a particle engages immunoglobulin G receptors (Fc $\gamma$  receptors) compared with engagement of complement or other receptors on the phagocyte surface (10). Finally, dozens of



**Figure 1: Transmission electron micrograph of macrophage phagosomes** Communal vacuoles, containing several bacteria of the species *A. felis*, can be found next to vacuoles containing single bacteria (arrowheads) (courtesy of Bianca Schneider, the author's laboratory, currently at the Max-Planck-Institute for Infection Biology, Berlin).

different types of hydrolases (proteases, DNases, lipases, etc.), which are capable of digesting most biological macromolecules, are released from lysosomes into the phagosome, contributing to the demise and digestion of the



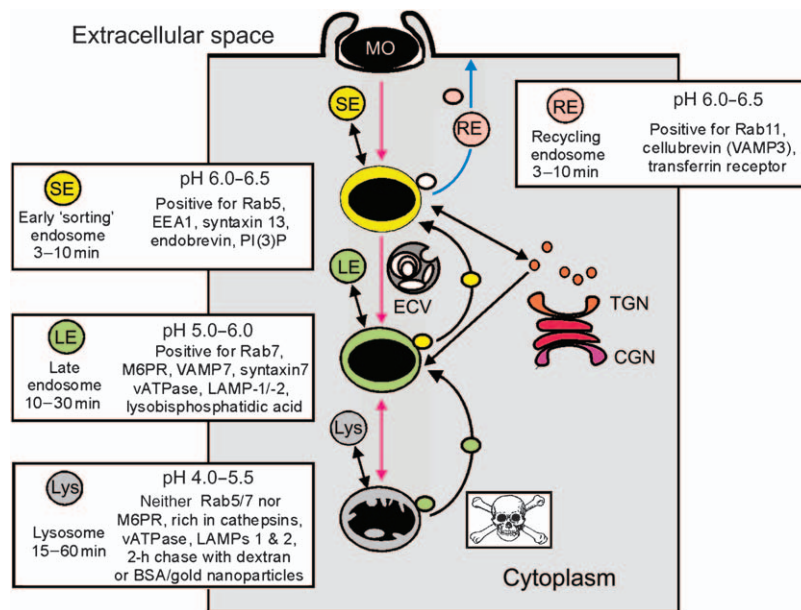
**Figure 2: The killing machinery of professional phagocytes.** The schematic drawing shows a phagolysosome (yellow) in a professional phagocyte (grey). The enclosed micro-organism (pink) is being attacked by various host cell factors. For details, see text. Fe, iron-catalyzed; MPO, myeloperoxidase of neutrophils, NADPH-Ox., NADPH-dependent, superoxide-producing oxidase [Adapted from Haas (2011)].

particle. Resulting monomeric and oligomeric degradation products are likely shuttled through the phagolysosome membrane into the macrophage cytoplasm, although this notion is largely based on parallels in the lysosomal world (11). Even there, the identities of most transporters are not known, while their activities have been demonstrated. The published latex-bead-containing phagosomes (LBP) protein inventory, surprisingly, does not list relevant transporter proteins either (12), indicating that there is a whole area of phagosome biology that has not been touched yet.

The development of the phagosome into a final, degradative phagolysosome is a neatly ordered process and parallels endosome maturation (Figure 3). Phagosome biogenesis starts with the formation of a phagocytic cup from specialized plasma membrane domains. Phosphoinositides play a crucial role in this process [reviewed by Yeung et al. (13)]. The newly formed early phagosome develops into a late phagosome after fusion with late endosomes and finally into a phagolysosome by fusion with lysosomes (normally the terminal organelles of the endocytic pathway). Phagosomes containing the same cargo in the same cell type acquire and lose their maturation markers with almost identical kinetics (14), although exceptions to this rule may apply (14,15).

Through the analysis of various established endosome marker molecules, which an endosome picks up and loses on the way to lysosome formation (Figure 3), it has been shown that endosome biogenesis is vectorial in that an early endosome is not able to fuse directly with a lysosome. Rather, early phagosomes fuse mostly 'homotypically' with early endosomes and, with lower frequency, 'heterotypically' with late endosomes (16). Late phagosomes are likely to fuse relatively rapidly with late endosomes and less frequently with lysosomes, which again fuse homotypically. Such vectorial fusion preference also likely occurs with phagosomes; yet, formal evidence has still to be provided. Generally, fusion competence decreases as a phagocytic (or endocytic) compartment matures (16–19). Not completely unexpected, the definition of three endocytic organelles along the degradative endosomal pathway (early or sorting endosome, late endosome and lysosome) considerably oversimplifies the diversity of compartments harbouring various marker molecule combinations (20). Still, this classification is very useful to explain most features seen in the endocytic-phagocytic system and will be applied here for discussion.

Rab proteins (Rab5, 7, 11) are, as with other membranes of the endocytic and exocytic pathways, key constituents or regulators of phagosome fusion machineries and help in the recognition of membranes that should fuse specifically. SNARE proteins (e.g. syntaxins 7, 8 and 13 and VAMPs 3, 7 and 8) as downstream factors then pull the membranes to be fused together to a distance of a few nanometers (21). In addition, phagosome acidification modulates maturation (22), and so do calcium fluxes (23). While the contributions of cytoskeleton elements to phagocytosis, and phagosome



**Figure 3: Schematic simplified view of the endocytic (phagocytic) pathway.** Maturation along the degradative pathway is indicated by red and the recycling pathway by blue arrows. Endosomes mature as they consecutively undergo fusion with membranes of the next stage of maturation, followed by fission of recycling vesicles whose identity is largely unknown (small, coloured vesicles with black arrows in backward direction). In macrophages, same-type endocytic organelles fuse particularly avidly with each other (double-sided black arrows). Traffic between early and late endosomes may be predominately accomplished by a vesicle (202). These are vesicles with multiple internal membranes that contain transmembrane proteins destined for degradation. Micro-organism would not fit into such comparably small vesicles. It is more likely that early phagosomes fuse directly with late endosomes to form late phagosomes. Most of the killing and digestion is accomplished in a late phagosome and in phagolysosomes. Antigen presentation through MHC class II occurs predominately from a late phagosome compartment (not included here). Vesicles with biosynthetic cargo from the TGN can fuse with early and late endosomes. Times indicate the approximate periods of time required for a particle to appear in the respective compartment. For example phagolysosomes can normally be observed starting 15 min after ingestion by macrophages, while most phagosomes have matured into phagolysosomes by 60 min of infection (times can vary between macrophage type and activation status). Other endocytic compartments may exist (20), but this simplified four-compartment view has proven valuable in the discussion of most features seen. CGN, *cis* Golgi network; EEA1, early endosome antigen 1; LE, late endosomes; M6PR, mannose 6-phosphate receptor; MO, micro-organism; MVB, multivesicular body; PI(3)P, phosphatidylinositol 3-phosphate; RE, recycling endosomes; SE, sorting endosomes; TGN, *trans* Golgi network.

maturation in particular, are still poorly defined, recent studies shed some light on the crucial role in phagosome maturation of the polymerization of tubulin (24) and actin (25,26). Furthermore, bidirectional movement of LBPs on microtubules has been directly demonstrated in a cell-free reconstituted system (27). Finally, lipid composition also regulates phagosome maturation (28,29). More than 1000 different lipid species have been identified on LBP from macrophages (J. Brouwers and B. Helms, Utrecht University, and M. Kuehnel and G. Griffiths, European Molecular Biology Laboratory, Heidelberg, unpublished data).

### The Origin of the Phagosome Membrane – What You See Is What You Get

It is generally accepted that, under normal circumstances, most of the nascent phagosome's membrane is derived from the plasma membrane (30,31). Still, several additional membrane sources have been recently proposed to participate in ingestion, particularly when many large particles are

taken up by the same phagocyte. The current prime candidates for additional membrane sources are given below.

#### Sorting endosomes and recycling endosomes

Bajno et al. (32) described the accumulation of VAMP-3-containing vesicles in the vicinity of forming phagosomes, suggesting that recycling endosomes may contribute phagosome membrane. In agreement with such hypothesis, Allen et al. (33) found a particle-specific reduced rate of phagocytosis in VAMP-3 (recycling endosome v-SNARE) knock-out mice, and Niedergang et al. (34) reported focal exocytosis of recycling endosomes at the site of immunoglobulin-mediated particle entry.

#### Late endosomes and lysosomes

Tapper et al. (35) reported calcium-dependent focal exocytosis of macrophage lysosomes in the vicinity of forming phagosomes, possibly indicating the delivery of membrane material to be incorporated in the phagocytic cup. Their findings have been recently extended by the observation that synaptotagmin VII, a protein critical to plasma

membrane resealing after injury, is critical for phagocytosis when large numbers of particles are ingested (36). It does so by helping to mobilize lysosomes to the site of phagocytosis in a  $\text{Ca}^{++}$ -dependent manner. The necessity for late endosomal, VAMP-7-positive, vesicles in immunoglobulin or complement receptor-mediated phagocytosis has also been described (37). A dominant negative form of VAMP-7 or VAMP-7 silencing RNAs inhibited corresponding phagocytosis (37).

### **Endoplasmic reticulum**

The first to propose a role of endoplasmic reticulum (ER) constituents in phagocytosis were Müller-Taubenberger et al. (38). Using a *Dicyostelium* double knock out in the genes for the ER-resident chaperones calnexin and calreticulin, they observed a strongly decreased rate of phagocytosis with an arrest at the stage of cup closure, suggesting a role for these proteins in phagosome biogenesis. Recent proteomics analysis of LBP from *Dicyostelium* (39) confirmed the presence of calreticulin in highly purified LBP fractions.

A contribution of ER-to-macrophage phagocytic cup formation in macrophages was first reported by Gagnon et al. (40) using J774 murine macrophage-like cells. Their evidence was largely based on biochemical data using isolated LBP and *in situ* ER-specific (glucose-6-phosphatase) staining at transmission electron microscopy (EM) level. Their findings were of extraordinary interest to immunologists because the incorporation of ER into a phagosome membrane could help explain the cross-presentation of antigens from a pathogen-containing vacuole on major histocompatibility complex (MHC) class I proteins. All the MHC class I processing and presentation machinery necessary for cross-presentation could exist in an ER-phagosome hybrid organelle, making this process efficient (41–43). Recent studies (44) on dendritic cells further supported an important immunological role of likely phagosome ER components and export of proteins out of the phagosome in immunity. In line with an important role of the ER in phagocytosis, functional studies have implicated ER-SNAREs in phagosome formation (45,46) and suggested (45) that phagocytosis of large (3  $\mu\text{m}$ ) beads requires typical ER-SNAREs, while phagocytosis of smaller (0.8  $\mu\text{m}$ ) beads would be independent of such SNAREs.

In contrast to the above, recent studies by Touret et al. (47) using J774 and RAW264.7 macrophage-like cell lines, primary bone marrow-derived macrophages and dendritic cells did not find evidence for ER involvement in phagosome formation. Their methodology included immunogold EM, *in situ* glucose-6-phosphate staining and EM analysis, analysis of accessibility of extracellular proteins to the ER in a phagocytic cup and testing for accessibility of biosynthetically produced avidin-KDEL to phagocytosed biotinylated latex beads. In line with a critical view of ER contribution, theoretical considerations based on probability calculations suggested that phagosome-ER contribu-

tion to cross-presentation is minimal, if at all existent (48). However, at least some of the contradictions between the studies of Gagnon et al. (40) and Touret et al. (47) may be resolved when assuming that only distinct ER regions contribute to phagosome formation (M. Desjardins, EMBO Conference Series on 'Interface of Cell Biology and Cellular Microbiology', Sant Feliu, Spain 2006; with permission).

In summary, time will tell which type of donor membrane is central to which kind of phagocytosis. It is clear from the above, however, that the source membrane may vary considerably dependent on the phagocytic cell type tested, whether the phagocytes are immortalized or primary cells, and dependent on particle size, chemistry and shape. It could well be the combination of macrophage surface receptors ligated by the particle plus possibly biophysical properties (membrane curvature and changes in local membrane lipid composition and concentration) that trigger the release of certain subcellular 'membrane stores'. ER membranes constitute more than half of the area of intracellular membranes (49) and could be predominately involved when large quantities of particles have to be ingested. That ER membranes can directly contribute to some reactions of phagosome biogenesis is extremely likely, weighing all available evidence.

### **What Is in a Phagosome?**

Phagosomes are not mere membrane bags around a particle meant to entertain the researcher with a number of analysable organelle marker proteins. They are organelles with a mission, and their contents are expected to reflect this and to influence their destiny.

### **Enzymes**

Research into the hydrolase arsenal of lysosomes was very helpful in the analysis of the degradative potential of phagosomes and phagolysosomes. Claus et al. (50) nicely dissected the acquisition of various 'lysosomal' hydrolases by LBP in murine J774 macrophage-like cells. They found the protease cathepsin H predominately in early phagosomes, while cathepsin S was typically present in late phagosomes. Classical 'lysosomal hydrolases' such as  $\beta$ -hexosaminidase,  $\beta$ -glucuronidase or cathepsin B were confirmed to be largely lysosomal in macrophages and to accumulate in phagosomes over a period of several hours (12,50). Yates et al. (22) used a novel assay for the quantification of phagosomal lipase activities based on a fluorogenic triglyceride analogue coupled to silica beads and showed that lipase activity can be detected in LBP as early as 5 min after uptake and increases with a peak activity being reached within 20 min. Similarly, cathepsin L proteolytic activities were quantified by following the dequenching of a particle-coupled, fluorogenic rhodamine-peptide substrate. Kinetics of digestion was the same as for lipase (22).

All these hydrolases are likely transported into phagosomes through vesicles from the *trans* Golgi network even before they can be acquired from lysosomes that contain these hydrolases in high quantities: studies with *Mycobacterium*-containing phagosomes (which are arrested as early endosome-like organelles) clearly demonstrated that cathepsins can enter the phagocytic pathway at an early stage and deliver (immature) hydrolases to phagosomes (51,52). Likely, some proton-pumping ATPase complex is also fed into phagosomes through the secretory pathway (53).

### Small molecules

While the knowledge about which compartmental marker proteins are in or on phagosomes has increased in recent years, studies have now turned to the question as to what small substances are contained in phagosomes? To understand why a given intravacuolar pathogen is going to thrive in this particular environment, it is of outstanding interest to know what this environment is really like. How are these pathogens supplied with nutrients? Are there nutrient carriers in the vacuole membrane? How do relevant ions such as ferric ions reach the bugs and what are their concentrations in a phagosome?

While investigations into this are only starting, the few pieces of information available stem largely from studies on phagosomes containing pathogens (rather than from LBP):

- Grieshaber et al. (54) used ratiometric probes to analyse *Chlamydia trachomatis*-containing vacuoles for the potassium, sodium and calcium content and for vacuolar pH. They concluded that pH was around neutral, and concentrations of the three tested ions in the vacuole were also approximately the same as those in the host (Vero) cell cytoplasm. Together with previous studies showing that substances as small as 500 Da cannot cross the vacuole membrane, this study suggests that passage from the host cell cytoplasm into the vacuole of possibly any molecule smaller than some 500 kDa is possible.
- Wagner et al. (55) performed elemental analyses for chlorine, calcium, potassium, manganese, copper, phosphorus, sulphur and zinc and studied differences in their concentrations at 1 and 24 h of infection and between vacuoles containing different mycobacteria. Beyond producing an inventory of what is in the phagosome, this study provided experimental evidence for the validity of the approach by using a mutant of *Mycobacterium tuberculosis* that cannot produce an extracellular iron-capturing siderophore: iron is a limiting growth factor for many pathogenic bacteria (56). This siderophore mutant failed to accumulate iron in the vacuole over a 24-h period, while iron concentration increased 10-fold in vacuoles with wild-type bacteria.
- Concentrations of ions found in lower concentrations such as manganese can be very relevant for pathogen survival in phagosomes, as one of the best known genetic resistance host factors is natural resistance-

associated macrophage protein 1 (Nramp1), a transporter for divalent metal cations, possibly particularly relevant for iron and manganese transport (57). Accordingly, deprivation of iron and other cations by Nramp1 changes maturation of *Salmonella*-containing phagosomes (58).

- *Toxoplasma gondii*, an intracellular protozoan parasite, may use several strategies in parallel to secure nutrients: this parasite possesses several types of secretory granules named micronemes, rhoptries and dense granules. Some *Toxoplasma* proteins are incorporated into the host cell protein-stripped vacuole membrane and may well serve as transporters for nutrients (59). In essence, the membrane of the parasitophorous membrane has been described as a molecular sieve supplying nutrients while intimate interactions with the ER and mitochondria may secure lipid supply (60). Recently, a surprising model was presented in which *T. gondii*-containing vacuoles possess deep invaginations supported by host microtubules that deliver host endosomes and lysosomes into the parasitophorous vacuole (61). The parasites would then feed on these endocytic organelles, questioning previous conclusions that the *Toxoplasma* vacuole would be completely separated from the endocytic pathway (62).

### Nutrients in activated versus resting macrophages

Investigations into the metabolism of pathogenic microorganisms and their metabolic needs and capabilities *in vivo* have recently been started with interesting results [reviewed by Munoz-Elias and McKinney (63)]. It is probably fair to speculate that 'elementomics' and 'small molecule' research using phagosomes are going to be increasingly important in the future and that further assays will be developed that look inside a phagosome (13,22). This research is also likely to yield further insights into cell-autonomous resistance after immune activation, e.g. Wagner et al. (55) showed in their above study that activation of macrophages with proinflammatory interferon- $\gamma$  (IFN- $\gamma$ ) decreased iron ion concentration in *Mycobacterium*-containing vacuoles at least threefold, while that of copper ions actually increased to fourfold. The concentrations of other ions also varied strongly. Similarly, IFN- $\gamma$  induces expression of indoleamine 2,3-dioxygenase, which transforms L-tryptophan into N-formylkynurenine, thereby depleting tryptophan from infected cells. This conversion results in the production of non-infectious but persistent chlamydia (64). These and further changes in phagosome microenvironments will deserve further analysis in the presence and absence of immune activation.

### Kiss and Run – Not Just a Question of Morality

While 'complete' fusions, i.e. complete mixing of the fusion partners' membranes and their luminal contents was

assumed to be the general mode of fusion, it has become clear that 'incomplete' or partial fusions can also occur. During kiss and run, the two lipid bilayers intermingle transiently, likely when the SNARE fusion machinery (21) is still in place. Contents can now be exchanged, while there is likely little exchange of membrane constituents. After a short mixing of contents ('kiss'), the fusion vesicle retracts ('run') to possibly re-fuse with the target membrane. This process, termed 'kiss and run' (65), has also been described for fusion between phagosomes and endocytic organelles:

Originally, Wang and Goren (66) used macrophages whose lysosomes had been simultaneously loaded with the two differently sized fluorescent marker molecules sulforhodamine (molecular weight 607, red fluorescent) and fluoresceinated dextran (molecular weight 40000, green fluorescent). The macrophages were fed yeast particles, which were delivered to phagolysosomes. Surprisingly, these phagolysosomes did not turn simultaneously red and green by receipt of lysosome material. Rather, they first turned red, then green. Wang and Goren interpreted their observations as a transient fusion during which the two partner membranes formed only a very small transient pore, which preferentially permitted the passage of small over large molecules. These very suggestive experiments have later been extended, with respect to endosomes and synaptic vesicles (67–70) and with respect to phagosomes (65,71).

Possibly, kiss-and-run fusion of lysosomes has a simple biological reason: most of the digestion in a macrophage seems to occur in late endosomes rather than in lysosomes (72,73), which would make the lysosome a gland for digestive juices rather than a stomach (late endosome). Kiss-and-run fusion between lysosomes and late phagosomes could lead to parsimonious injection of digestive hydrolases while it removes the necessity to recycle the complete fused-in lysosome membrane from a lysosome–phagosome hybrid (74). Likely, full and partial fusion events exist in parallel, at least when it comes to endosome maturation in fibroblasts (75).

The existence of a kiss-and-run mode of fusion is today largely unchallenged (70,76), while the question remains how much this fusion mode contributes to each type of fusion (e.g. endosome–phagosome, *cis* Golgi vesicles with medial Golgi membranes). From what is known so far, the endocytic and phagocytic pathways are particularly skilled professionals when it comes to kissing and running, and even there, complete fusion events have been observed directly (77).

## Entering Latex Infections

Desjardins and colleagues optimized LBP purification from macrophages (17,18) based on a protocol used to purify *Acanthamoeba* phagosomes (78). This procedure uses the low buoyancy of polystyrene (latex) in sucrose gradients to enrich LBP in a single ultracentrifugation step. Using such preparations, Desjardins et al. (17,18) applied methods of

proteomics to analyse the composition of LBP and identified more than 140 proteins, many of which had previously been found on phagosomes using other phagocytic probes and detection methods (12). The list of identified proteins hence included the usual suspects, lysosomal cathepsin proteases, trafficking-regulating Rab proteins and lysosome-associated membrane proteins. But additionally, proteins involved in apoptosis regulation and a collection of ER proteins were identified. This study was an important starting point for a lot of research into phagosome function. One of the particularly remarkable results of this investigation was that a follow-up study suggested that portions of the macrophage ER contribute directly through fusion with the plasma membrane to the formation of the phagocytic cup and to antigen cross-presentation through MHC class I (see above). In the years since the publication of the LBP inventory (12), Desjardins' group has identified a total of 800 proteins on the LBP in J774 murine macrophages (M. Desjardins, University of Montréal, Montréal, Canada, personal communication). Further analysis is required to dissect the sequential appearance and disappearance of these phagosome constituents during maturation.

Although LBP can be very easily handled, latex beads are, however, non-degradable, the nature of the 'latex receptors' is ill-defined, and naturally occurring latex bead infections are unheard of. Some studies suggest, however, that the fate of a phagosome and the particle contained therein can be regulated by the degradability of the particle (79–83) and its hydrophilicity (83,84). A 'state-of-digestion-sensing system' is also suspected to exist in *Acanthamoeba* (85) and *Dictyostelium* (86). If the chemical nature of phagosome cargo is relevant to phagosome development, then it is not surprising that, in some systems, the maturation kinetics of LBP seems slower than that of phagosomes containing degradable contents [e.g. the kinetics of Rab5 acquisition and loss in J774 cells; Jahraus et al. (19) versus Fernandez-Mora et al. (87); reviewed by Griffiths (72)].

The maturation pathway of an LBP also likely depends on the biological surface molecules that it recruits before it is ingested. Heterogeneity of the phagosome population can be somewhat circumvented by offering the phagocyte covalently surface-bound purified and defined ligands, such as immunoglobulins or glycosylated serum albumin (84). Similarly, chemically activated beads can be excellent carriers for purified bacterial factors to explore their contribution to phagosome biogenesis, as exemplified by studies with *Listeria monocytogenes* surface internalins (88). An interesting, more recent alternative to LBP is paramagnetic bead-containing phagosomes, which can be isolated from homogenates using magnets. These beads, too, are amenable to chemical surface modification (52,89).

While purification of phagosomes containing inert beads seems so easy, phagosomes containing microorganisms

are much harder to purify (90–94). Hence, not a single proteome of a phagosome containing a pathogen has so far been published, although protocols to isolate them to various degrees of purity have been published. However, a partial proteome of a phagosome from a pathogen has been reported, again, containing latex beads: Okada et al. (95,96) identified 159 different proteins on LBP from *Entamoeba histolytica*, largely proteins that are known to regulate vesicular trafficking, hydrolases and actin cytoskeleton components. A number of proteins have been identified on LBP from *Dictyostelium* (39,97) and from *Tetrahymena* (98) as well.

In summary, while we can learn much from the synthetic bead–phagosome systems, conclusions must be drawn cautiously when it comes to pathobiological relevance of the observed features. The field requires the detailed molecular analysis of micro-organism (pathogen)-containing phagosomes as well, if at all possible purified in parallel to phagosomes containing avirulent mutants. This will make comparisons between various phagosome compositions much easier, as the specific protocols for LBP versus micro-organism-containing phagosome differ strongly.

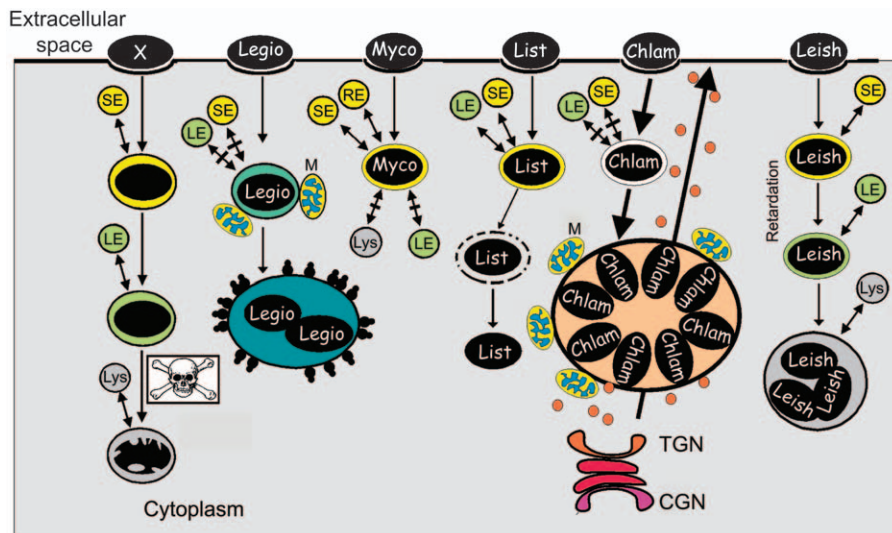
### Leaving the Beaten Path Is a Common Strategy

As described above, a phagosome is not just a transport membrane bag with a passive passenger but rather a compartment whose exact composition and even destiny can critically depend on what it contains. While a regular, harmless particle encounters the degradative pathway to the lysosome, some ‘intracellular pathogens’ have evolved structures and mechanisms, which allow them to leave the usual pathway, thus avoiding an encounter with much or all of the macrophage killing and degradation machinery,

including the exposition to reactive oxygen metabolites and acidification [also reviewed by Knodler et al. (99), Rosenberger and Finlay (100) and Scott et al. (101)].

When it comes to the classification of the different types of intracellular pathogens and their vacuoles, a number of arguments can be brought forward for the following four categories (Figure 4; Table 1).

- (i) Pathogens in this group (*L. monocytogenes*, *Shigella flexneri*, *Francisella tularensis* and *Rickettsia* spp.) disrupt the membrane of the maturing phagosome to escape into the cytoplasm. This protects these pathogens from encounter with lysosome agents and allows them to metabolize cytoplasmic nutrients (102,103).
- (ii) These pathogens arrest phagosome maturation at a prephagolysosome stage and therefore avoid encounter with lysosomal contents. These microbes may still be in touch with an intact upstream endocytic machinery and are likely to use this route to secure nutrient supply. Examples for this strategy are pathogenic mycobacteria [foremost, *M. tuberculosis*, (104)], which arrests maturation at an early endocytic stage, and *Rhodococcus equi* (87), which arrests phagosome maturation during transition from an early to a late endocytic stage.
- (iii) These pathogens redirect phagosome maturation during or soon after uptake and are contained in a vacuole without endocytic character. Often, these vacuoles intersect with the secretory pathway (105). Examples are given below (see also Table 1):
  - *Legionella pneumophila*, whose vacuole intercepts secretory vesicles from ER exit sites and develops into a compartment that shares many features with the rough ER, including the possession of ribosomes (106). Early, but not late, in infection, these phagosomes are closely associated with mitochondria (107).



**Figure 4: How different pathogenic bacteria escape killing in macrophages.** For details, see text. Fusion competence is indicated by double-sided arrows and inhibition of fusion by crossed double-sided arrows. Interactions with early sorting endosomes (SE), recycling endosomes (RE), late endosomes (LE), and lysosomes (Lys) are depicted. CGN, cis Golgi network; Chlam, *Chlamydia*; Legio, *Legionella*; Leish, *Leishmania*; List, *Listeria*; M, mitochondrium; Myco, *Mycobacterium*; TGN, trans Golgi network; X, harmless bacterium.

**Table 1:** Intracellular pathogens and their vacuoles<sup>a</sup>

Micro-organism	Clinical manifestations	Vacuole compartmentation	Representative trafficking reference
<b>Phagosomes with features of the endocytic pathway</b>			
<i>Anaplasma phagocytophilum</i> <sup>b</sup> <i>Coxiella burnetii</i> <sup>b</sup>	Human granulocytic ehrlichiosis Human Q fever	Not classically endocytic Acidic, LAMP1-positive vacuole; spacious through homotypic vacuole fusion	Lin and Rikihisa (203) Howe et al. (204)
<i>Ehrlichia chaffensis</i> <sup>b</sup>	Illness in humans, reaching from asymptomatic infection to severe morbidity and death in some cases	Corresponds to an early endocytic compartment	Lin and Rikihisa (203)
<i>Ehrlichia risticii</i> <sup>b</sup>	Equine diarrhoeal disease (Potomac horse fever), mortality 30%	Corresponds to an early endocytic compartment	Wells and Rikihisa (205)
<i>Histoplasma capsulatum</i> <sup>c</sup>	Broad spectrum of disease activities; progressive disseminated infections particularly in immunocompromised patients	No phagolysosome formation, pH ~6.5, likely arrested endocytic organelle with connection to upstream compartments	Strasser et al. (206)
<i>L. donovani</i> <sup>b</sup>	Kala azar (severe liver and spleen swelling)	Retarded phagosome maturation, eventually a late endocytic organelle	Desjardins and Descoteaux (71)
<i>Mycobacterium avium</i> complex (MAC) <sup>c</sup>	Bird infection but can cause human tuberculosis in immunocompromised patients	Hybrid of sorting and recycling endosome compartment	Schaible and Kaufmann (64)
<i>M. bovis</i> (BCG) <sup>c</sup>	Bovine and human tuberculosis; the experimentally frequently used strain 'BCG' is an attenuated strain of <i>M. bovis</i> (risk level 1)	Hybrid of sorting and recycling endosome compartment	Gutierrez et al. (198)
<i>Mycobacterium marinum</i> <sup>c</sup>	Cutaneous 'swimming-pool granulomas' in humans, fish pathogen	Hybrid of sorting and recycling endosome compartment	Barker et al. (207)
<i>M. leprae</i> <sup>b</sup>	Tuberculoid and lepromatous leprosy	Not known in detail; likely as with <i>M. avium</i> or <i>M. tuberculosis</i>	Sibley et al. (144)
<i>Mycobacterium</i> <i>pseudotuberculosis</i> <sup>c</sup>	Paratuberculosis, possibly intestinal Crohn's disease	Non-lysosomal endocytic compartment with some late endocytic characteristics	Kuehnel et al. (208)
<i>M. tuberculosis</i> <sup>c</sup>	Human tuberculosis, most common cause of death seen with any infectious disease. Chronic, disseminated disease	Hybrid of sorting and recycling endosome compartment	Vergne et al. (123)
<i>Nocardia asteroides</i> <sup>c</sup>	Nocardiosis; human acute or chronic suppurative infections. Spreads to nervous system and skin	Non-lysosomal compartment, not acidic	Black et al. (209)
<i>R. equi</i> <sup>c</sup>	Subacute or chronic abscessating bronchopneumonia in foals or human immunocompromised patients	Endocytic compartment arrested between early and late endosomes, not acidic	Fernandez-Mora et al. (87)
<i>Salmonella enterica</i> serovar Typhimurium <sup>c</sup>	Salmonellosis, severe enteric fever	Late endocytic compartment that does not fuse with lysosomes, acidic	Holden (210)
<b>Phagosomes with features of the exocytic pathway</b>			
<i>B. abortus</i> <sup>c</sup>	Brucellosis in swine; chronic infection with recurrent bacteraemia; also severe infections in humans	Some characteristics of ER, while typically endocytic to start with. Later in infection likely involvement of autophagic elements	Celli et al. (113)
<i>Chlamydia pneumoniae</i> <sup>b</sup>	Possibly causing arteriosclerosis	Intersects with sphingomyelin transport vesicles from TGN, barely contact with endocytic system	Wolf and Hackstadt (108)
<i>Chlamydia trachomatis</i> <sup>b</sup>	Most commonly transmitted sexual disease agent in the USA	Intersects with sphingomyelin transport vesicles from TGN, barely contact with endocytic system	Hackstadt et al. (211)

Table 1: Continued

Micro-organism	Clinical manifestations	Vacuole compartmentation	Representative trafficking reference
<i>L. pneumophila</i> <sup>c</sup>	Legionaire's disease, Pontiac fever	ER-like compartment with possibly autophagic components. May become late endocytic later in development. Early association with mitochondria	Roy and Tilney (212)
<b>Phagosomes that have unknown or mixed localization</b>			
<i>A. felis</i> <sup>c</sup>	Some cases of cat-scratch disease, lymphadenopathy	Non-canonically endocytic, undefined compartment formed after lipid-raft-dependent macropinocytosis	Schneider et al. (111)
<i>B. henselae</i> <sup>c</sup>	Most cases of cat-scratch disease, bacillary angiomatosis	Endothelial vacuoles are non-endocytic, macrophage phagosomes are late endocytic after longer times of infection	Kyme et al. (159)
<i>T. gondii</i> <sup>b</sup>	Toxoplasmosis	Nascent vacuole membranes are 'stripped' of most host proteins during entry; pathogen incorporates its own proteins into largely protein-depleted membrane; association with host mitochondria	Charron and Sibley (213)

ER, endoplasmic reticulum; TGN, *trans* Golgi network.

<sup>a</sup>Pathogens that exit their vacuoles to multiply in the host cytoplasm have not been considered.

<sup>b</sup>Obligate intracellular micro-organism.

<sup>c</sup>Facultative intracellular micro-organism.

- *Chlamydia* spp., an obligate intracellular bacterium, remains in a vacuole for its entire developmental cycle. This vacuole intersects with the trafficking route of sphingomyelin vesicles between the Golgi and the plasma membrane and collects sphingolipids (108) and cholesterol (109). Vacuoles containing *Chlamydia psittaci* can be associated with mitochondria, possibly for the acquisition of lipids by the bacteria (110).
  - *Afipia felis* that enters macrophages by lipid-raft-mediated macropinocytosis (111) into a compartment of unknown composition: neither endocytic nor Golgi- or ER-typical marker proteins were identified on *Afipia*-containing vacuoles early in infection (112).
  - *Brucella abortus* whose vacuoles briefly interact with endocytic organelles but that do not fuse with late endocytic and lysosomal organelles and develop into an ER-like compartment (113).
- (iv) The pathogens in this group force the maturation machinery to slow down but will not permanently inhibit phagolysosome formation. Pathogen growth may actually become dependent upon eventual phagolysosome formation. Examples are the eukaryotic pathogen *Leishmania mexicana* (114) and the obligate intracellular bacterium *Coxiella burnetii* (115).

Avoidance of phagolysosome formation by a pathogen might not necessarily be the major cause for survival and multiplication but, in some cases, could be an epiphenomenon. It is challenging to find a suitable experimental setup to test whether delivery to a phagolysosome would in itself be deleterious to a given pathogen. However, one case is

known where experimental acidification of a pathologically neutral pH phagosome did not lead to a significant increase in bacterial killing (116).

At first sight, the escape from phagosomes into the macrophage cytoplasm might seem the best strategy to promote pathogen survival and possibly multiplication by protecting the microorganisms from noxious phagolysosomal acid and hydrolases. In contrast, it seems that macrophages possess a number of defence factors against cytoplasmic microorganisms, such as restriction of appropriate nutrients and the expression of antibacterial peptides in the macrophage cytoplasm (117). Hence, this strategy requires some genetic and physiological preadaptations and intracellular bacteria, which normally are not released into the cytoplasm, like *Salmonella*, do also not multiply when microinjected into macrophage cytoplasm (102).

## How Do They Go Astray?

As diverse as the compartment characteristics of the various intracellular pathogens are, so also are the ways in which the pathogens establish them. Three types of mechanisms work in most cases with every bug adding some of its own flavour.

### **Mechanism 1: bacterial protein injection machines and their effector proteins**

Not so long ago, the first bacteria-to-host protein secretion machinery was uncovered. More recently, genome

sequencing projects have identified additional similar secretion devices. Briefly, in addition to conventional protein secretion pathways [type I and II (118)], bacteria can use pilus-like (type III) or conjugation apparatus-like structures (type IV) to transfer specific proteins from their cytoplasmic space into the host cell cytoplasm (119). Inactivating mutations in genes corresponding to type III secretion system components usually lead to either attenuation of virulence in these pathogenic bacteria or complete loss of virulence. Prominent pathogens of humans, animals or plants possessing type III secretion systems are *Salmonella* spp., *Shigella* spp., *Pseudomonas aeruginosa*, *Erwinia* spp. and *Xanthomonas* spp. The structurally similar type IV system is produced by *L. pneumophila*, *B. abortus*, *Bartonella henselae*, *Agrobacterium tumefaciens*, *Helicobacter pylori* and others. This review does not intend to give an in-depth introduction into the various host cell manipulation machines, but there have recently been excellent reviews covering this topic (119,120). To illustrate the general principles, I mention only a few well-characterized examples of secreted bacterial effector proteins: protein tyrosine phosphatases from *Yersinia* and *Salmonella*, ubiquitin-like cysteine proteases from *Vibrio* and *Yersinia*, a Cdc42-activating protein from *Shigella*, a guanosine nucleotide-exchange factor for small guanosine triphosphatases (GTPases) from *Salmonella* and *Pseudomonas*, a *Legionella* guanosine nucleotide-exchange factor for ADP ribosylation factor and a host cell dephosphorylating enzyme from *Helicobacter* (119,120).

The particular mechanistic elegance of these bacterial effectors is that many of them reprogramme host cell signalling processes either by directly possessing a key regulatory activity that changes host physiology or by modulating such host cell key activities. This is particularly intriguing as the bacteria themselves do not possess such regulators, i.e. evolutionary pressure likely lead to the establishment of 'stolen' DNA from their hosts in the pathogen genomes (121). In other instances, pathogens evolved 'eukaryotic' regulators independently of the host, and these proteins share no obvious amino acid sequence homology with the mammalian counterparts. *Salmonella* produces such proteins that functionally, but not structurally, mimic ras-like host GTPases (122).

#### **Mechanism II: secretion of diffusible substances**

Gram-positive bacteria do not have type III or type IV secretion systems. Perhaps the best investigated system for interference with phagosome maturation in this group is that of pathogenic ('slow-growing') mycobacteria whose phagosomes share characteristics of recycling endosomes (Rab11/TfR positive) and of sorting endosomes (Rab5 positive) (104,123). The established vacuole still communicates with the host cell surface through the early endocytic system (124). Recent research has highlighted the role of glycolipids such as lipoarabinomannans (LAMs) and cord factor (trehalose dimycolate) in interference with phagosome maturation (123,125,126) in addition to a role

of proteins (127,128). Several hypotheses as to how glycolipids may change phagosome maturation have been proposed. (i) both LAM and cord factor contain very long (~C<sub>50</sub>) hydrophobic mycolic acid (alkyl) chains, which could intercalate into the phagosome membrane and change the membrane's biophysical properties in a way that trafficking is disturbed (125). Alternatively, these glycolipids could (ii) inhibit the recruitment of factors needed for the progression of phagosome maturation [such as early endosome antigen 1 (126)]. (iii) When *Mycobacterium* is taken up through its surface glycolipid LAM, which is an excellent ligand for the macrophage mannose receptors, this partially determines avoidance of phagolysosome formation and induces anti-inflammatory signalling (129).

Another organism that uses amphiphilic, carbohydrate-containing compounds to interfere with the phagosome maturation machinery is the eukaryotic pathogen *Leishmania donovani*. Strains that produce lipophosphoglycan (LPG) on their surface block phagosome–endosome and endosome–endosome fusion and hence retard phagosome maturation, while isogenic non-LPG producers do not (71). Attaching isolated LPG to the mutant surface restored the maturation block. LPG consists of a lipid covalently attached to a very long carbohydrate moiety. It has been proposed that the lipid moiety is the major active ingredient by inserting into membranes and interfering with membrane properties; yet the carbohydrate portion is also necessary for activity and possibly leads to disturbance of 'lipid raft' structures in host phagosomes and, hence, may disrupt signalling events (130).

Virulent *Streptococcus pyogenes* have been described to possess a virulence factor transmission system that is positioned somewhere between a type III secretion system and transfer of substances by mere diffusion: streptolysin O, a toxin secreted by these bacteria, can form large holes and arc-like structures in cholesterol-containing membranes. This toxin is instrumental in the transfer of bacterial oxidized nicotinamide adenine dinucleotide (NAD<sup>+</sup>)-glycohydrolase into the mammalian cytoplasm [in this case, transfer of epithelial cells (131)]. There, NAD<sup>+</sup>-glycohydrolase influences signalling pathways to support extracellular persistence of *Streptococcus* (132).

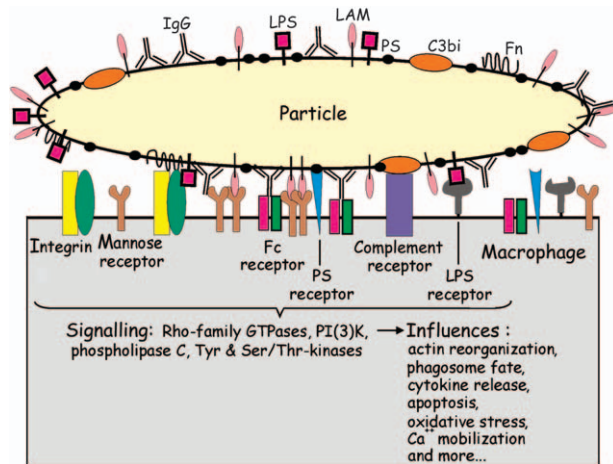
#### **Mechanism III: use of hospitable receptors**

Many investigations into the unusual biogenesis of pathogen-containing vacuoles have studied how a single relevant receptor influences phagosome fate (129,133,134,135). Such receptors can either directly recognize microbial components (the mannose receptor ligates target mannose or fucose residues) or host compounds that have been deposited on the microbe to label the particle as foreign ('opsonized' particles). Opsonizing agents can be complement factors, specific antibodies or, in a wider interpretation, any host protein that sticks to a micro-organism [e.g. the secreted *Drosophila* macroglobulin complement-related (Mcr) protein (136)]. Ligation of phagocytic receptors leads

to reorganization of the actin cytoskeleton beneath the particle attachment site hand-in-hand with activation of Rho-type GTPases and continual ligation of phagocytic receptors around the particle (137).

In addition to these truly ingestion-promoting receptors, there are 13 different TOLL-like receptors [e.g. TLR4 ligating bacterial lipopolysaccharide, TLR9 ligating bacterial CpG, or TLR5 ligating bacterial flagellin (138)], which signal into the macrophage, some from the macrophage surface, some from inside the phagosome. Their ligation may also influence phagosome maturation kinetics, although this is still a matter of debate (138,139).

Given the complex surface of both pathogen and host cells, it is obvious that many different types of receptors and ligands should be engaged simultaneously (Figure 5). In consequence, the nascent phagosome contains a plethora of macrophage receptors (140) at variable proportions that may influence phagosome fate to variable degrees. Some ligand–receptor interactions, such as immunoglobulin–Fc $\gamma$  receptor complexes, may signal ‘dominantly’ over others. Ligating Fc receptors can, e.g. turn *Toxoplasma* from a pathogen that enters mammalian cells actively and creates its own unusual compartment into a particle that is ‘passively’ ingested and ends up in a phagolysosome where the pathogen is killed (134). Similar data were



**Figure 5: Schematic drawing of receptors on a macrophage surface meeting a collection of ligands on the particle side.**

Only a few receptor–ligand pairs relevant to phagocytosis are shown, and receptors that cannot trigger uptake of a particle by themselves (e.g. TOLL-like receptors) have been excluded. Ligation and, in particular, clustering of receptors leads to signalling events, release of Ca<sup>++</sup> from intracellular stores and actin polymerization underneath the particle. Note that not all indicated signalling events are implicated in each phagocytic event. C3bi, complement protein fragment C3bi; Fn, fibronectin; IgG, immunoglobulin G; LAM, mycobacterial lipoarabinomannan; LPS, lipopolysaccharide; PS, phosphatidylserine; Ser/Thr, serine/threonine; Tyr, threonine.

reported for *Afipia* (112), *Chlamydia* (141), *Escherichia* (142), *Salmonella* (143) and *Toxoplasma* (135). Other phagosome systems do not seem to be particularly sensitive to previous opsonization of the phagocytic probe, e.g. phagosomes containing *Mycobacterium leprae* (144) or *Mycobacterium avium* (145).

How does one explain these differences in the susceptibility of different pathogen phagosomes to opsonization before uptake? One way is the different ways in which these microorganisms manipulate their host cells. For example, the unusual fate of *E. coli*-containing phagosomes seems to be determined at a very early step of their biogenesis when the bacteria ligate plasma membrane CD48. Determination may here be a more passive event from a bacterial point of view. In this case, phagolysosome formation can be strongly enhanced by forcing the microbes to use an immunoglobulin-mediated port of entry, which feeds into the degradative system (142). Conversely, the fate of a *Legionella* vacuole is determined through the bacterial type IV secretion system used to microinject effector proteins. This system is much less sensitive to opsonization, possibly because the secreted bacterial effector proteins quickly divert the maturation pathway toward an ER-like compartment, independent from the ‘correct’ phagocyte receptor composition.

While most studies on phagocytic uptake naturally focused on professional phagocytic cells, Joiner et al. (134) and later Downey et al. (146) used an interesting model system, Chinese hamster ovary (CHO) cells, transformed with the gene encoding Fc $\gamma$ RII receptor. The resulting transgenes avidly devoured yeast cell wall particles (zymosan), sheep red blood cells (146) or *Legionella* (147) but only when the particles were coated with immunoglobulin G. In other words, all the additional genetic information needed to turn CHO epithelial cells into professional phagocytes was a suitable surface receptor gene. Such a system is most useful, as macrophages are notoriously poorly transfectable, whereas CHO cells are not, so that this model allows for much easier host cell genetics.

Not all microorganisms are taken up by receptor-mediated phagocytosis. Sometimes, in professional as well as in non-professional phagocytic cells, microorganisms use macropinocytosis as hitchhikers, usually after initiating macropinocytotic activity (‘trigger-mode’). Macropinocytosis normally is a mechanism to sample large quantities of the immediate environment of the cell, leading to large (0.2–5.0  $\mu$ m) vesicles and aiding antigen presentation by macrophages and dendritic cells. Some microbial pathogens (*Afipia*, *Brucella*, *Francisella*, *Legionella*, *Salmonella* and *Shigella*) and viruses (adenovirus and HIV) can use macropinosome can be a crucial determinant for whether a bug enters a degradative compartment or not.

## Genetic Approaches to Phagosomology

### Pathogen genes

When an intracellular pathogen enters a eukaryotic cell, the change in environment is dramatic and the pathogen has to adapt to the new environment quickly. Sensing the changed environments and signal transduction is often mediated by bacterial regulatory two-component systems (153), leading to substantially altered transcription of genes related to intracellular survival (154,155). Numerous studies have applied pathogen gene cloning and mutation to the analysis of infected host cells and to phagosome trafficking. One such study led to the identification of *B. abortus* transposon mutants whose phagosome maturation had been normalized (156), demonstrating a tight connection between avoidance of phagolysosome formation and bacterial multiplication.

Two particularly interesting recent studies have developed novel, large-scale, unbiased methods for direct selection of mutants with trafficking defects from a pool of mutant bacteria. The first of these techniques was designed to select for (*M. tuberculosis*) mutants that cannot prevent phagolysosome formation. In this protocol (157), macrophage lysosomes are preloaded with iron dextran, followed by macrophage infection with a mutant pool. Lysates are run over a paramagnetic column, and magnetic organelles (i.e. lysosomes, late endosomes and phagolysosomes) are pulled out followed by plating of the retained material onto nutrient agar. Over several rounds of selection, mutants with more normalized phagosome maturation should be enriched. In the case of *M. tuberculosis*, the affected genes included putative transporter and lipid synthesis genes (158). The corresponding mutants could not prevent phagosome acidification and were attenuated for multiplication in macrophages.

The second study used selection of bacterial [*Mycobacterium bovis* bacille Calmette–Guérin (BCG)] mutants that failed to inhibit phagosome acidification (158) rather than phagolysosome formation. In this protocol, infected macrophages were stained with LysoTracker, a lysosomotropic fluorescent dye, which labels acidic compartments. Macrophages were lysed, and the lysate was sorted in a fluorescence-activated cell sorter (FACS) to obtain a subfraction of acidic phagosomes, i.e. phagosomes that are positive for LysoTracker and, hence, contain mutants that cannot (fully) inhibit phagosome acidification. Remarkably, none of the mutants was identified in both, the magnetic and the FACS-based screens was identical, likely because of the very complex phenotypes, limited sample size and possibly only partial overlap between the mutants that cannot block phagosome acidification and those that do not block phagolysosome formation (a later step in maturation).

Unfortunately, biosafety considerations restrict the applicability of a FACS-based approach. In the study by Stewart

et al. (158), the BCG vaccination strain of *M. bovis* was used, which is of the lowest genetic engineering risk level. However, both methods should be applicable to almost all other phagosome-modifying pathogens, and the Pethe et al. strategy (157) has already been used to identify *B. henselae* (159) and *R. equi* (T. Sydor and A.H. unpublished data) phagolysosomal mutants.

### Mouse genes

Host genetic factors involved in host–pathogen interactions (and hence, phagosome-related questions) are usually analysed using mice with gene deletions ('knock outs'). Examples for host genes that can be decisive for pathogen and phagosome fate studied with such approach are following:

- The Nramp (Bcl<sup>f</sup>, Ity<sup>f</sup> and Lsh<sup>f</sup>) locus that mediates mouse resistance to some pathogens such as *Mycobacterium*, *Salmonella* or *Leishmania* (160). The Nramp protein is likely a manganese transporter system in the phagosome/endosome membrane (161).
- The family of IFN- $\gamma$  induced p47 GTPases (162): such GTPases contribute to the killing of intravacuolar pathogens such as *M. tuberculosis* [e.g. LRG-47 by promoting phagolysosome formation (163)] or *T. gondii* [e.g. Interferon-inducible GTPase I by promoting phagosome membrane disruption (164)].
- The nitric oxide synthase (NOS2) that mediates killing of intracellular pathogens (163) mediated by nitric oxide compound (in particular, peroxynitrite).
- The multisubunit NADPH oxidase complex that produces superoxide radicals upon suitable phagocytic stimulus. Knock-out mutants in the p47<sup>phox</sup> subunit are more sensitive to staphylococcal infection (165).

The ingestion of a pathogen by a macrophage leads to comprehensive signalling, not only during uptake (Figure 5) but also following particle uptake and out of the phagosome. This signalling can change the transcriptional profile of the infected cell considerably and can be monitored using oligonucleotide microarrays. This has been demonstrated for *M. tuberculosis* (Mtb) infecting either resident macrophages or macrophage preactivated with IFN- $\gamma$ . While infection with Mtb reduced the transcription of 288 genes, Mtb plus IFN- $\gamma$  induced the transcription of 925 genes and IFN- $\gamma$  alone of 615 genes (166). The transcription of even more genes was repressed, and there was some congruence in the gene sets between the different samples. A particularly interesting gene was NOS2, which was induced 28-fold by IFN- $\gamma$  alone but more than 400-fold when IFN- $\gamma$  was coupled to an Mtb infection. LRG-47, a p47 GTPase (see above), was induced fourfold under all above conditions. Studies like this will help to develop regulatory maps of macrophage responses to intravacuolar pathogens at different immune states and, hence, will hint to new genes whose contribution to infection and phagosome biogenesis will have to be analysed.

A very important recent technical advance was made with the advent of the silencing RNA (RNAi). Using RNAi, host cells can be genetically manipulated much more reliably than was previously possible using antisense RNA. Unfortunately, this experimental approach has not yet been used frequently with professional phagocytic cells because of the poor transfectability of macrophages and the danger of their immune activation by transfection with recombinant DNA purified from bacteria (167). Examples for studies that overcame these technical difficulties were on the role of coronin-1 (168) and of the SNARE VAMP-7 (37) in the phagocytosis through immunoglobulin receptors.

While mice provide the best qualifications as models for many infectious diseases, they are not suitable for unbiased screening of thousands of host mutants in genes that are relevant to phagosome biogenesis. Two such models, *Drosophila* and *Dictyostelium*, previously used as developmental models, filled the gap to serve as genetically manageable infection models.

### ***Drosophila* genes**

So-called Schneider (S2) cells from *Drosophila* are macrophage-like 'plasmacyte' cells whose genes can be silenced relatively easily using double-stranded RNA (dsRNAs) (169). These cells can be used as models to study pathogens, e.g. *Candida*, *Listeria* and *Mycobacterium*:

- In a recent high-throughput study, 21 300 dsRNAs were used to individually screen genes possibly relevant in infection with *Mycobacterium fortuitum*. This approach leads to the identification of *Peste*, a CD36 family member, as critical for the phagocytosis process (170):
- More than 7000 different short interfering RNA (siRNA) types were used to screen for *Drosophila* genes involved in phagocytosis of the pathogenic yeast *Candida albicans* by S2 cells. One hundred and eighty-four candidate genes were identified, some of which were required for phagocytosis of this particular yeast but not for the bacteria *E. coli* or *Staphylococcus aureus* or for latex particles (135). This finding suggests that, as in man, *Drosophila* has different pathogen pattern recognition factors. An example for a *Candida* phagocytosis-specific factor was the product of the gene *Mcr*, which is secreted by S2 cells and binds to only certain particles to support their uptake.
- siRNAs, representing approximately half the *Drosophila* genome, were selected based on their homology with genes from humans and/or *Caenorhabditis elegans* (171). Using these, the authors identified 116 genes whose knock down lead to one of the following phenotypes: defect in uptake of pathogenic *Listeria*, increased sensitivity to phagosome disruption by *Listeria*, decreased bacterial intracellular multiplication or even increased bacterial multiplication or phagosome escape. Although bacterial expression of the phagosome-membrane-

disrupting protein Listeriolysin O requires 37°C in broth culture (172), the bacteria unexpectedly escaped from *Drosophila* phagosomes at 25°C, induced actin filament production and even multiplied in S2 cells (173).

- The LBP proteome of *Drosophila* S2 cells has been analysed, and a total of some 800 proteins have been identified. In addition, 617 resident proteins of early LBP in S2 have been identified (M. Desjardins, University of Montréal, personal communication). This is a first step to an ordered and complete road map of phagosome maturation, if only for a certain type of phagosome (LBP). Such inventory, together with the power of siRNA analysis in *Drosophila*, will allow to analyse the roles of many proteins in phagocytosis and phagosome maturation.

### ***Dictyostelium* genes**

A well-investigated non-mammalian professional phagocyte is the slime mold *Dictyostelium*, which feeds on environmental bacteria (174). Gotthardt et al. (97) dissected the maturation of LBP in *Dictyostelium* and defined three steps of phagosome maturation: first, coronin and a lysosomal glycoprotein, LmpB, appear on the phagosome, second, lysosomal enzymes are transferred in at least two bursts of delivery accompanied by recycling of plasma membrane proteins, and third, digestion followed by quantitative retrieval of hydrolases from the phagolysosomes and preparation for exocytosis [unlike macrophages, *Dictyostelium* quickly exocytoses indigestible material (86)]. In a follow-up proteomics study, Gotthardt et al. (39) identified almost 200 phagosome proteins. They have ordered the proteins by their temporal appearance and have identified five clusters and 24 functional groups of proteins, which they have assigned to distinct stages of the maturation pathway. As a further result, the authors have identified heterotrimeric G proteins as key players in the phagocytic uptake (39).

*Dictyostelium* was used to genetically identify phagocytic receptors (175), proteins involved in progress of phagocytosis (176) and host resistance genes (177) and to analyse phagosomes containing pathogenic *Legionella* (178–180) or *Mycobacterium* (180). Targeted host cell mutations were used to identify factors involved in phagosome maturation (181), and transcriptome analysis of *Dictyostelium* cells infected with *Legionella* yielded 131 differentially expressed genes (182). The advanced genetics in *Dictyostelium* can now be used to specifically look at the role of further defined genes in phagosome biogenesis (183). Unfortunately, as is with S2 cells, *Dictyostelium* does not multiply at temperatures above 27°C, making the study of pathogens difficult whose virulence regulation demands 37°C.

Two other developmental biology models, zebrafish and the nematode worm *C. elegans*, are now also used as models for infection biology (184,185), and it should be only a question of time until they will be harnessed to understand phagosome biogenesis in these organisms.

## The Phagosome Melting Pot: Phagosomes in a Test Tube

Oates and Touster (186, 187) isolated phagosomes from the amoeba *Acanthamoeba* containing either latex beads or yeast cell wall zymosan or red blood cells and fused them with *Acanthamoeba* lysosomes *in vitro*. They were the first to describe cell-free fusion of endocytic and phagocytic organelles, one of the first membrane fusion events reconstituted biochemically. Several cell-free fusion assays to study phagosome–endosome fusion in mammalian cells have later been published, one based on antigen–antibody binding (90), an assay based on biotin–streptavidin affinity (19) and a fluorescence energy transfer-based reaction (188). The laboratory of the author has recently established a microscopic assay based on fluorescence label colocalization (U. Becken and A. Haas, Cell Biology Institute, University of Bonn, Germany). In spite of the potential of such assays, very few experiments have been published that investigate the fusion between isolated pathogen-containing phagosomes and endocytic organelles (94, 188–190). So, the question of ‘why do *in vitro* fusion assays?’ is justified. Three major advantages are given below.

### ***In vitro* fusion advantage I: ‘mix and match’**

Peyron et al. (188) showed that isolated *Mycobacterium smegmatis*-containing phagosomes from neutrophils showed little fusion with lysosomes *in vitro*, as suggested from infection experiments with intact cells. A cytosol preparation from neutrophils, which had previously been activated by phorbol myristate acetate and a calcium ionophore for 10 min, caused considerable increased fusion efficiency. This is an excellent example where a cell-free fusion reaction opens the door for a biochemical fractionation assay to find host factors that promote phagolysosome formation, a question barely addressable using other approaches and of potentially high therapeutic interest.

### ***In vitro* fusion advantage II: ‘isolated place of action’**

Vergne et al. (190) investigated the role of mycobacterial phosphoinositol mannoside (PIM) in membrane trafficking using an *in vitro* assay between streptavidin-bead phagosomes and biotinylated peroxidase as an endosomal label and found a small but reproducible increase in fusion activity in the presence of PIM. The same was seen using an endosome–endosome *in vitro* assay with externally added PIM. Similarly, the interference of various bacterial ‘effector’ proteins, some of which divert membrane trafficking (191), can be more precisely defined and dissected than with *in vivo* tools alone. Third, concanamycin A, an inhibitor of vacuole-type ATPase (vATPases), inhibits phagolysosome formation *in vitro* to some extent (22). However, it is still unclear whether this inhibition is directly because of collapse of the phagosome pH gradient. Alternatively, concanamycin A could inhibit fusion with the phagosome of a vATPase-positive vesicle that delivers cargo into phagosomes, which is required for further maturation of

these phagosomes. Which of these scenarios is true could be analysed *in vitro* by testing the fusion of purified phagosomes with purified endosomes in presence of concanamycin A. These three examples underscore the utility of *in vitro* assays to directly study the influence of isolated compounds on fusion of distinct subcellular organelles without having to consider side-effects of such compounds on other elements of the cell.

Analysis of *in vitro* fusion assays showed that, in keeping with observations of the endocytic pathway, the avidity to fuse with endocytic organelles decreases as phagosomes mature (19, 50, 192). LBP are active in *in vitro* fusion assays when they are 1–4 h of age or more than 24 h, but are little active when harvested at 8–12 h into the infection. Interesting questions to be addressed now include those which SNAREs, Rabs and other proteins participate in different steps of phagosome maturation.

While most efforts in the field are to understand trafficking of endocytic compartments into phagosomes, some groups study trafficking of recyclable material out of phagosomes. That such trafficking exists *in vivo* is clear from the studies of the kinetics of acquisition by and loss from the phagosome of transmembrane proteins (17, 18, 193) and from the kinetics of appearance of phagosomal proteins on the plasma membrane (194). That vesicle fission from phagosomes does in fact occur is further supported by the observation that the size of a phagosome usually remains constant, despite fusion events with other endocytic organelles. The coat protein I apparatus (for vesicle formation in the Golgi network) may play an important role in recycling from the phagosome (195). Leiva et al. (196) reported that recycling from phagosomes can also be reconstituted in a semi-intact cell system using phagosomes containing *Staphylococcus* and that Rab11 is required, which hints to a role of the endocytic recycling system.

### ***In vitro* fusion advantage III: ‘removal of steric hindrance’**

Another advantage of cell-free fusion systems is that transport barriers within cells may be removed *in vitro*, e.g. cytoskeletal elements, which inhibit two otherwise fusion-competent membranes to meet and fuse. Very few data on any *in vitro* fusion reaction are so far available on this aspect.

## Macrophages Are Not Alone

If all these intracellular pathogens are so good at undermining our phagocyte defence, how can our bodies still eradicate them? Two major answers are (i) our immune system does not only use isolated macrophages to fight its battle but also uses much more potently bactericidal neutrophils (which, however, do not significantly contribute to antigen presentation) and (ii) the adapted immune defense becomes more important as the infection progresses and it supports the innate response. Release of

proinflammatory cytokines such as IFN- $\gamma$  by T cells, natural killer cells and mononuclear cells influence the fates of microorganisms and phagosomes and can, in some cases, normalize aberrant phagosome trafficking (93,197).

Interferon- $\gamma$ -induced factors such as the p47 family of GTPases can influence phagosome fate, e.g. by promoting autophagy [*Mycobacterium* (198)] or by aiding in the destruction of phagosome membranes, followed by pathogen eradication [*Toxoplasma* (164)]. Activation of macrophages also induces the production of superoxide radicals and nitric oxide synthesis. Nitric oxide can, particularly in context with the production of superoxide radicals and hydrogen peroxide, be a very powerful killer by forming peroxynitrite (199). Recent studies also suggest an additional role for reactive oxygen radicals in modulating phagosome pH and proteolytic activities in opposite ways to aid in antigen presentation (by dendritic cells) and innate killing activities (in neutrophils), respectively (200). Hence, we can effectively defend ourselves against intracellular pathogens because almost all pathogens are eventually killed in activated macrophages and neutrophils from healthy donors!

### **A Bright Future for Phagosomes**

The phagosome is a hot spot of defense when infection with bacteria or protozoa occurs. This is the place where the microbes are killed and digested, and this is where the presentation of antigen through MHC II starts. This is where, particularly upon macrophage activation, many bactericidal effector proteins are acting and where the immune cell concentrates its microbicidal machinery. Many groups are currently looking into these various aspects of phagocyte biology and progress is steady. However, no comprehensive phagosome proteome or lipidome has been published for any pathogen-containing vacuole. There is an amazing diversity of compartments that intracellular pathogens inhabit, and they seem to use a stunning number of different factors to establish these compartments. Understanding how phagocytes and their phagosomes co-operate not only in these pathological but also in 'normal' situations and how an immune activation often can normalize pathological phagosome biogenesis or turn a phagosome from a hospitable into a killing environment is a very active field of investigation. To gain a molecular understanding of this amazing organelle will certainly help to better fight infectious diseases. There is still a lot to be discovered: as an example, the identity of membrane fusion factors that close the phagocytic cup is completely unknown!

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