Fusion, Fission, and Secretion During Phagocytosis

Phagocytosis is essential for the elimination of pathogens and for clearance of apoptotic bodies. The ingestion process entails extensive remodeling of the cellular membranes, particularly when large and/or multiple particles are engulfed. The membrane fusion and fission events that accompany phagocytosis are described. The coordinated sequence of membrane trafficking events required for phagocytosis involves multiple organelles and also serves other cellular functions, such as cytokine secretion.

Cells of the innate immune system remove invading microorganisms and other foreign particles by phagocytosis. Phagocytosis is also used for the ongoing clearance of apoptotic bodies from senescent or remodeling tissues. The engulfment of apoptotic or potentially noxious particles by phagocytes is a summation of carefully orchestrated events beginning with receptor engagement, pseudopod extension, “zippering” of the membrane around the target, and finally scission of the nascent vacuole or phagosome from the plasmalemma. As illustrated in **FIGURE 1**, phagocytosis involves a large expanse of membrane to encircle the prey. Because the patch of the plasma membrane that engages the particle is ultimately internalized, the surface area of the cells has the potential to decrease during phagocytosis, especially when multiple and/or large particles such as apoptotic bodies are engulfed by a single cell (**FIGURE 2**). Indeed, phagocytes have an astounding appetite, being capable of internalizing so many particles that the area of the vacuoles formed can be comparable to that of the cell’s own surface! Yet, video microscopy reveals that the cell shape and size are remarkably conserved. It has been suggested that, because the surface of phagocytes is corrugated, “unwrinkling” may suffice to maintain the cell size when a sizable area is internalized (23). This would enable the phagocyte to retain its volume while decreasing its area by smoothing it. However, at least in the case of macrophages, quantitative spectroscopic and electrophysiological measurements have demonstrated that the surface area of the cell in fact increases following phagocytosis (22, 26). Elastic lateral stretching of the bilayer could conceivably account for the increased surface area. However, both synthetic pure-lipid bilayers and biological membranes can tolerate only a maximum of 4% stretch before their structural integrity is compromised (24). This small contribution cannot account for the changes observed experimentally during phagocytosis of even a few mid-size particles (e.g., **FIGURE 2B**). The inescapable conclusion is, therefore, that the expanse of the surface membrane internalized during engulfment is replaced by membranes derived from an intracellular reservoir.

The fusion of endomembrane vesicles with the plasma membrane is, of course, not unprecedented. Although regulated exocytosis is most often regarded as a means of delivering soluble cargo to the extracellular milieu, a variety of instances have been documented where insertion of intrinsic membrane components and the accompanying extra surface area are the primary objectives. Osteoclasts, specialized cells designed for bone remodelling, gain their ability to form large, acidic resorptive lacunae by the targeted exocytosis of late endosomes bearing V-ATPases to areas of the plasma membrane in contact with the bone matrix (38). Also, in cells that are not professional phagocytes, exocytosis is known to be stimulated by invading pathogens. *Trypanosoma cruzi*, an obligate intracellular parasite, gains entry into host cells by a process that requires fusion of lysosomes with the plasma membrane at the site of contact (2). Finally, all cells have the need to patch their plasma membrane, using endomembranes, during abscission in cytokinesis (29). The objectives of this review is to briefly summarize the evidence supporting the occurrence of focal exocytosis during phagocytosis, to discuss the potential endomembrane compartments involved, and to describe a recently appreciated aspect of the phenomenon, namely the opportunity this affords to simultaneously secrete cytokines that accompany exocytosis during the inflammatory response.

**Evidence for Focal Exocytosis During Phagocytosis**

The notion that the plasma membrane needs to be restored following phagocytosis was first introduced 35 years ago by Web and Cohn (46). These authors noted that surface-specific markers were depleted from phagocytes following particle uptake and were subsequently replenished. The replenishment occurred during a comparatively long period of time, and its sensitivity to protein and RNA synthesis...
inhibitors served to implicate biosynthetic pathways (46).

More recently, the advent of modern biophysical methods enabled investigators to re-analyze the phenomenon with greater temporal resolution. Greenberg and colleagues (12) used the dye FM1-43, a solvothermic membrane indicator, to estimate the surface area of macrophages undertaking “frustrated phagocytosis,” an abortive form of phagocytosis where overambitious macrophages try to engulf a flat surface coated with an opsonic ligand. Remarkably, an increase of over 20% of the original surface area of the cells was recorded in these studies. The change was blocked by inhibitors of phosphatidylinositol 3-kinase, an enzyme critical to endocytic membrane traffic, suggesting involvement of endomembrane fusion events. Of note, the increased binding of FM1-43 is not a peculiarity of the frustrated phagocytosis model, since it was subsequently replicated in cells that had performed bona fide phagocytosis of yeast particles that were fully ingested (22).

The surface area of phagocytes was studied with even finer temporal resolution by Palfrey and colleagues (36), who used an electrophysiological approach. These investigators monitored the electrical capacitance, a reliable index of the plasmalemmal area, of macrophages patched in the whole cell configuration. These elegant studies revealed that stepwise increases in capacitance, indicative of exocytic events, occurred when phagocytosis was induced.

Jointly, these studies provided convincing indications that endomembranes are stimulated to fuse with the surface membrane during the course of particle

FIGURE 1. Membrane engagement during phagocytosis
Scanning electron micrograph showing phagocytosis of beads. An expanse of membrane protrudes from the cell surface, forming the phagocytic cup, which initially surrounds and ultimately engulfs particles.

FIGURE 2. Surface area changes during phagocytosis
A: comparison of the size of phagocytes and their prey (latex beads). To simplify the calculations of surface area, macrophages were assumed to be spherical and smooth, i.e., without any corrugations. R and r denote the radius of the macrophage and latex beads, respectively. The fractional surface area internalized (FI) is dependent on the number of latex beads taken up and is denoted by n. B–C: RAW 264.7 macrophages were exposed to IgG-opsonized latex beads of 3.87-μm (B) or 8.1-μm (C) diameter, and images were acquired at regular intervals. The FI was calculated at the end of the experiment and found to be 0.18 and 0.16 for B and C, respectively. Internalized beads are indicated by an asterisk. Size bar = 10 μm.
ingestion. In accordance with this conclusion, interference with the fusogenic machinery of the cells was found to impair phagocytosis. Cleavage of defined SNARE proteins by injection of tetanus neurotoxin reduced the phagocytic efficiency, and comparable results were also obtained when a dominant negative form of NSF (N-ethylmaleimide-sensitive factor) was expressed (9).

**The Source of Endomembranes**

What is the origin of the endomembranes that fuse with the surface membrane to compensate for the area internalized during phagocytosis? An unnervingly large number of different intracellular compartments have been implicated in the process, including elements from the endocytic and secretory pathways (FIGURE 4). This surprising multiplicity of candidates may be attributable to the variety of experimental systems utilized but may also be an indication that several sources need to be tapped to offset the large area internalized.

Several subcompartments of the endocytic pathway feature most prominently among the sources of endomembrane. This consensus was reached on the basis of the appearance of organelle-specific markers at sites of ingestion before or at the time of phagosome sealing. These markers feature prominently Rab GTPases, which direct the traffic of vesicles, and SNARE proteins, which mediate their fusion. Because phagocytosis was found earlier to be sensitive to tetanus and botulinum B toxins, the involvement of the R-SNAREs was suspected (22). Accordingly, GFP-tagged VAMP3 was found to be recruited to nascent phagocytic cups in CHO cells that were rendered phagocytic by transfection with Fcγ receptors (5). Such engineered phagocytes were used to circumvent the difficult task of transfecting myeloid cells. This technical limitation was subsequently overcome, and two other groups independently confirmed that, in macrophages, VAMP3 is also recruited to forming phagosomes (7, 34) (see also **FIGURE 3**). This SNARE is found almost exclusively in recycling endosomes, and the importance of this compartment for the phagocytosis of some particles (but not others) was documented using VAMP3 knockout mice. Bone marrow-derived macrophages obtained from VAMP3-null animals have a reduced ability to ingest zymosan particles, whereas they take up complement or IgG-opsonized particles normally (1).

That recycling endosomes are indeed critical to the early stages of phagocytosis is also suggested by the effects of mutant forms of Rab11. Like VAMP3, Rab11 is a normal resident of recycling endosomes (45), where it is thought to control traffic toward the surface. Predictably, Cox et al. (11) detected Rab11 on nascent phagosomes. Moreover they also found that mutant alleles that prevent the normal cycling of Rab11 between its GDP- and GTP-bound states reduced the ability of macrophages to engulf particles.

The traffic of recycling vesicles to the membrane is also controlled by Arf6 (14, 45), which prompted two different studies analyzing the role of this GTPase in phagocytosis. In one, mutants of Arf6 that alter its cycling were reported to depress the phagocytic efficiency (48). A subsequent study by Niedergang et al. (35) not only confirmed these findings but in addition demonstrated that inhibitory forms of Arf6 prevent the focal delivery of VAMP3 to the phagocytic cup and block pseudopod extension.

Other endosomal membranes are also recruited to the phagocytic cup. Evidence exists for the recruitment of both late endosomes and lysosomes to sites of phagocytosis. VAMP7, a SNARE that localizes to late endosomes and lysosomes (27), was also detected in nascent phagosomes, where it appears shortly after the delivery of VAMP3 (7). The appearance of VAMP7 is functionally significant, since its depletion reduced not only the exocytosis of late endocytic vesicles but also the extension of pseudopods and, as a consequence, the phagocytic efficiency.

The fusion of VAMP7-containing vesicles is regulated by synaptotagmin VII, a ubiquitously expressed calcium-sensing protein that controls the secretion of lysosomes (32). Recent observations in mice lacking synaptotagmin VII support of a role for late endocytic/lysosomal vesicles in the formation of phagosomes. Macrophages from such animals displayed an impaired ability to engulf particles, a defect that was modest when few small particles were ingested but became more apparent as the phagocytic load increased (13). In view of these observations, it is tempting to propose that
The delivery of endocytic membranes is graded and occurs on demand, with recycling endosomes delivered when only a smaller area of the plasmalemma is internalized and later compartments being summoned only when compensation for a larger area is required.

In addition to membranes derived from the endosomal pathway, some specialized secretory organelles present in phagocytes can contribute to phagosome formation. Neutrophils contain a number of specialized secretory granules and vesicles that are seemingly unique to this cell type. Three types of secretory granules are formed and stored during neutrophil ontogeny: the primary (or azurophilic), secondary (or specific), and tertiary (or gelatinase) granules. All of these granule types, plus small secretory vesicles, can be mobilized, to varying extents, during neutrophil activation (47). Two of these, the primary and secondary granules, have been shown to fuse with the plasma membrane during phagocytosis (42). The primary granules, which are lysosomal in nature, are secreted focally at the phagocytic cup and likely supply membrane to the forming phagosome (41).

The Endoplasmic Reticulum
Controversy

The data reviewed thus far clearly point to a significant contribution of the plasma membrane and endosomes to the nascent phagosome membrane. In recent years, the endoplasmic reticulum (ER) was invoked as an alternative source of internal membranes to forming and early phagosomes. Based on proteomic analyses, in combination with electron microscopy and glucose-6-phosphatase cytochemistry, Gagnon et al. (19) proposed that during particle engulfment the ER fuses with the plasma membrane at the base of the phagocytic cup. The establishment of continuity between these two membranes was proposed to establish a pathway whereby the target particle could “slide” into the lumen of the ER. Scission of the ER and resealing of the plasmalemma was envisaged to complete the phagocytic event.

Independent evidence in support of this model was provided by targeting the SNAP proteins of the ER. In one report, trapping antibodies to ERS24/Sec22b inside macrophages depressed the efficiency of phagocytosis (6). A related study re-evaluated the role of ERS24/Sec22b and additionally analyzed two other SNAP proteins of the ER: syntaxin 18 and D12. Interference with the function of syntaxin 18 and D12 produced a modest reduction in the phagocytic activity, whereas impairment of ERS24/Sec22b had no discernible effect (25). The source of the apparent discrepancy between these studies is not readily apparent.

A contribution of the ER to phagosome formation and maturation is attractive in several respects. First, as the largest single intracellular compartment, the ER can potentially provide enormous amounts of membranes to satisfy the need for entrapment of multiple large particles. Second, by delivering foreign particles to the ER, this mode of phagocytosis could favor antigen cross-presentation (the presentation on class I histocompatibility complexes of antigens internalized by endocytosis). However, because antigens require proteasomal degradation before class I presentation, direct exposure to the ER lumen would not simplify the loading procedure; the antigens would purportedly be retrotranslocated across the ER membrane to the cytosol, where proteasomal degradation would ostensibly occur, followed by re-uptake of the resulting peptides into the ER by the TAP transporter.

However attractive, the notion that the phagosome is composed largely of ER-derived membranes is
inconsistent with a plethora of earlier biochemical and immunostaining data and is seemingly incompatible with at least some of the established physical attributes of phagosomes. In dendritic cells and macrophages, the lumen of the nascent phagosome becomes acidic shortly after sealing. This acidification has been attributed to the inward pumping of protons by V-ATPases, which are thought to be acquired through fusion with endosomes. The acidification becomes more accentuated as phagosomes age, and this correlates with the graded acidification of the compartments of the endocytic pathway that fuse sequentially with maturing phagosomes. It is difficult to envisage how acidification would develop in a phagosome composed largely of ER, which is believed to be devoid of V-ATPases and is inherently permeable to protons (37).

“A very obvious possibility is that dynamin and amphiphysin are required for fission of vesicles from the endosomal compartment...”

Research in our laboratory sought to reconcile the classical model of phagocytosis with the paradigm of ER-mediated entry. To this end, we compared the contribution of the ER and of endosomes to the formation of nascent phagosomes by a variety of quantitative methods, including biochemical, immunological, fluorescence imaging, and electron microscopy techniques. Not one of these approaches supported the fusion of the ER with plasma membrane during phagocytosis or a contribution of the ER to phagosome maturation (43). Instead, the limiting membrane of phagosomes was confirmed to derive largely from the plasma membrane, becoming subsequently modified through a series of fusion reactions with subcompartments of the endocytic pathway. In view of the existing evidence, we conclude that the contribution of the ER to the phagosomal membrane is at best small and favor the canonical concept that phagocytosis and maturation involve primarily the plasma membrane and endocytic organelles.

**Exocytosis and the Secretion of Cytokines During Phagosome Formation**

Using the endocytic pathway, particularly the recycling endosomes, as a source of membrane for phagosome formation has an added bonus feature. Recycling endosomes are a key station for the delivery of pro-inflammatory cytokines in the secretory pathway, en route to the cell surface after being synthesized in the ER and transported through the Golgi complex (31, 34). When VAMP3-positive membrane from recycling endosomes is being delivered to the surface for phagocytic cup formation, it simultaneously carries cargo such as the cytokine TNF-α. VAMP3-containing vesicles are delivered to the phagocytic cup where the cognate SNAREs (syntaxin 4 and SNAP-23) promote fusion. VAMP3-positive recycling compartment en route to the plasma membrane, where the soluble form of the cytokine is released by proteolysis. During phagosome formation, the stimulated exocytosis of recycling endosomes delivers VAMP3-containing vesicles to sites where the cognate SNAREs (syntaxin 4 and SNAP-23) promote fusion. The cytokine precursor is also inserted into the phagocytic cup, where the specific protease TACE cleaves and releases soluble TNF-α (34). This focal secretion process is selective, however, in that IL-6, another pro-inflammatory cytokine that also traffics via recycling endosomes, is not focally delivered to the phagocytic cup (31); indeed, neither are transferrin receptors, the quintessential markers of recycling endosomes, which are not delivered preferentially to the phagocytic cup (31) but are instead recruited to the phagosome shortly after sealing (15, 16, 39). These events imply that sorting within the recycling endosomes ensures that membrane and cargo can be selectively deployed for phagocytosis and concomitant secretion without compromising other endosomal functions. Although the mechanisms for this sorting are not yet understood, cholesterol-rich lipid rafts in phagocytic membranes may be involved (30).

**Fission**

To complete the internalization of particles, the nascent phagosome must undergo fission from the plasma membrane to form an autonomous internal compartment. For many forms of endocytosis, including receptor-mediated endocytosis, rapid recycling of neurotransmitter vesicles, and internalization of caveolae, the protein dynamin is required for scission to occur (for review, see Ref. 33). In the case of phagocytosis, a role for dynamin has also been postulated. An initial study found that expression of dominant-negative mutants dynamin-2, the ubiquitously expressed dynamin isoform, greatly reduced the efficiency of phagocytosis (21). Moreover, the dynamin-recruiting protein amphiphysin was also found to be required for optimal phagocytosis (20). Yet, contrary to expectation, these studies found that phagocytosis was not arrested just before fission. Instead, when dynamin-2 was inhibited, the extension of membrane around the forming phagosome was precluded, halting phagocytosis at an earlier stage.

Two subsequent studies have attempted to clarify this apparent paradox. In a nutshell, what these studies concluded was that the main site of dynamin-2 action may not be at the plasma membrane but on internal membranes. Cells transfected with a
dynamin-1 dominant-negative mutant, which unlike dynamin-2 is found exclusively at the plasma membrane, did not display significant inhibition of phagocytosis despite extensive blockade of receptor-mediated endocytosis (44). In addition, electrophysiological determinations of plasma membrane capacitance such as those described above found that inhibition of dynamin-2 or amphiphysin blocked the focal delivery of endomembranes normally seen during phagocytosis (17). These authors also documented the fact that dynamin is present in the transferrin-positive compartment, presumably the recycling endosomes. Thus, although dynamin and amphiphysin are involved in the fission of plasma membrane-derived vesicles in a number of endocytic systems, their role in phagocytosis may be somewhat different. An obvious possibility is that dynamin and amphiphysin are required for fission of vesicles from the endosomal compartment, which are delivered to the phagocytic cup and favor pseudopod extension.

The actual proteins and processes involved in the fission of phagosomes from the plasma membrane thus remain unknown. Phagosome fission requires parts of the plasma membrane coming together from relatively long distances when compared with other forms of endocytosis. Such large displacements are generally mediated by actin and myosin, and there is evidence that myosins IC, II, V, IXb (18), and X (10) associate with forming phagosomes (3). Moreover, inhibition of myosin-dependent contractility prevented phagosome closure (4, 40), suggesting that a purse-string machinery that is engaged for engulfment (8). For all of these reasons, not all phagosomes are created equal, and extrapolation can be very misleading. Surely, new and unexpected features will be unveiled when systems other than Fcγ receptor-mediated phagocytosis are studied in detail.

Original work in the authors’ laboratories is supported by the Canadian Institutes of Health Research (CIHR), the Canadian Cystic Fibrosis Foundation (CCFF), the Heart and Stroke Foundation of Ontario, the National Health and Medical Research Council of Australia, and the National Institutes of Health. K. K. Huynh is the recipient of a CIHR Graduate Studentship. S. Grinstein is the current holder of the Pribilado Chair in Cell Biology.

Are All Phagosomes Created Equal?

Most of the data summarized in this review were obtained by studying Fcγ receptor-mediated phagocytosis. Although tempting, it would be simplistic to assume that the phenomena described for Fcγ receptors apply equally to other types of phagocytosis. It is becoming increasingly clear that the molecular processes underlying phagocytosis by different receptors can vary widely. The situation is compounded when, as is most likely the case in nature, multiple different receptor types are engaged simultaneously because an assortment of intrinsic phagocytic determinants and opsonins are exposed on the microbial surface. Moreover, even when a single type of receptor is engaged, the processes elicited during phagocytosis differ depending on the particle size. As an example, phagocytosis of large (>5 μm) IgG-coated particles is stringently dependent on phosphatidylinositol 3-kinase activity, whereas uptake of small particles is virtually independent of this enzyme (see Ref. 28 for review). These differences may relate to the amount of membrane that is required to encircle the target. Even the shape of the prey is now thought to influence the

References


