Cell, Vol. 123, 157–170, October 7, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2005.08.018

Quantitative and Dynamic Assessment of the Contribution of the ER to Phagosome Formation

Matters Arising

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Summary

Phagosomes were traditionally thought to originate from an invagination and scission of the plasma membrane to form a distinct intracellular vacuole. An alternative model implicating the endoplasmic reticulum (ER) as a major component of nascent and maturing phagosomes was recently proposed (Gagnon et al., 2002). To reconcile these seemingly disparate hypotheses, we used a combination of biochemical, fluorescence imaging, and electron microscopy techniques to quantitatively and dynamically assess the contribution of the plasmalemma and of the ER to phagosome formation and maturation. We could not verify even a transient physical continuity between the ER and the plasma membrane, nor were we able to detect a significant contribution of the ER to forming or maturing phagosomes in either macrophages or dendritic cells. Instead, our data indicate that the plasma membrane is the main constituent of nascent and newly formed phagosomes, which are progressively remodeled by fusion with endosomal and eventually lysosomal compartments as phagosomes mature into acidic, degradative organelles.

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Introduction

Phagocytosis of invading pathogens by neutrophils and macrophages is an essential component of the immune response. Engulfment is initiated by interaction of receptors on the surface of phagocytes with ligands on the target particles. Receptor activation triggers a complex signaling cascade that culminates with the extension of pseudopods and the ingestion of the particle into a membrane bound vacuole known as the phagosome (Greenberg and Grinstein, 2002; Silverstein et al., 1977). Newly formed phagosomes undergo acute remodeling by an orderly sequence of fusion events with organelles of the endocytic and biosynthetic pathways, while keeping their surface area nearly constant through concomitant fission of membrane vesicles (de Chastellier et al., 1995; Desjardins et al., 1994b; Pitt et al., 1992; Steinman et al., 1983). The net result of this process is an extensive restructuring of the membrane and contents of the phagosome (de Chastellier et al., 1995; Desjardins et al., 1994b; Lang et al., 1988) that is characterized by the acquisition of lysosomal enzymes, the ability to generate reactive oxygen intermediates, and luminal acidification (Tjelle et al., 2000; Vieira et al., 2002).

For nearly four decades, the dominant paradigm was that the membrane of nascent phagosomes originates primarily from the plasma membrane (Silverstein, 1977; Ulsamer et al., 1971). Indeed, the major glycoproteins of phagosomes were found to be largely identical to those found on the cell surface (de Chastellier et al., 1995, 1983; de Chastellier and Thilo, 2002; Muller et al., 1980b). Recently, however, an alternative model was proposed implicating the endoplasmic reticulum (ER) in phagosome formation. Using electron microscopy (EM) and glucose 6-phosphatase cytochemistry, Desjardins and colleagues (Gagnon et al., 2002) suggested that during phagocytosis, the ER is recruited to sites of particle ingestion, where it fuses with the plasma membrane. Fusion was proposed to establish continuity between the two compartments, creating an opening at the junction that allows the particle access to the lumen of the ER. Resealing of the plasma membrane was envisaged to complete the entry process (Desjardins, 2003; Gagnon et al., 2002). The concept of ER-mediated phagocytosis is appealing because it helps explain how phagocytes are able to internalize multiple large particles without a correspondingly large decrease in surface area. Even more provocatively, it provides a framework for understanding how antigens derived from internalized material might be presented by MHC class I molecules, a process known as antigen cross-presentation and most often associated with dendritic cells (DCs) (Guermonprez et al., 2003; Houde et al., 2003).

Until now, cross-presentation of most peptide antigens was thought to involve the egress of antigens from endocytic compartments (e.g., phagosomes) into the cytosol by an unspecified mechanism. Once in the cytosol, they would be digested by proteasomes and translocated into the ER by the ATP-dependent TAP1/TAP2 peptide transporter (Watts and Amigorena, 2001). Peptide loading onto MHC class I molecules would then take place in the ER lumen. By placing ER components in the phagosome, the new model allows for spatial coordination of the cross-presentation machinery. Although this scheme would not obviate the need for antigen egress into the cytosol, the Sec61 translocation channel, an ER protein, was proposed to fulfill this function (Desjardins, 2003; Wiertz et al., 1996).

The work of Gagnon et al. (2002) was based largely on a detailed proteomic analysis of isolated phagosomes containing latex beads, combined with static electron microscopic determinations. However, none of these data attempted to quantify the contribution of the ER to the membrane of phagosomes or define the kinetics of ER recruitment. Due to the profound implications of the new paradigm, we sought to characterize the role of the ER in phagocytosis by undertaking a quantitative assessment of the contribution of the plasma membrane, endocytic compartment, and the ER to the composition of the nascent and maturing vacuole.

Results

Contribution of the Plasma Membrane to Early Phagosomes

To quantify the contribution of individual compartments to the phagosomal membrane, we imaged phagocytosis in living cells labeled with organelle-specific probes. First, RAW264.7 macrophages stably expressing GPI-GFP as a marker of the plasma membrane were allowed to internalize IgG-coated latex beads (Figure 1A) or IgG-coated red cells (Figure S1 in the Supplemental Data available with this article online), and the distribution of the fluorescent marker was monitored by confocal microscopy. Live-cell imaging (Movie S1) revealed that GPI-GFP was readily detectable on the membrane of sealed phagosomes where it persisted for several minutes. A quantitative assessment of the density of GPI-GFP on the phagosomal membrane, relative to its density at the plasma membrane, was performed on fixed samples at defined time points after completion of phagocytosis, using image analysis. Note that fixation dissipates phagosomal proton gradients, obviating pH-induced changes of GFP fluorescence. Representative images are illustrated in Figure 1A, and a summary of 200 independent measurements is presented in Figure 1C. Phagosomal sealing was defined as the time when fluorescence formed a continuous ring around the particles and was confirmed by exclusion of antibodies or of FM4-64 added extracellularly. The density of GPI-GFP, which is comparable at the phagocytic cup to the rest of the plasma membrane, decreased only modestly ($\sim$25%) over the first 2 min following phagocytosis. The probe disappeared gradually from the phagosomes at longer times (Figures 1A and 1C). The presence of GPI-YFP in the phagosomal membrane was confirmed by immunoelectron microscopy (immuno-EM) using anti-GFP antibodies (Figure 1D). Quantification of the density of gold particles indicated that the phagosomal membrane retained $\approx$50% of the GPI-YFP 5 min after particle ingestion (Figure 1E), in good agreement with Figure 1C.

Since GPI-GFP may associate with specialized lipid microdomains on the plasma membrane, which might be segregated during Fc receptor-mediated phagocytosis (Kwiatkowska et al., 2003), we repeated these experiments using a second plasmalemmal marker. Macrophages were transfected with YFP-GT46, a chimeric construct that is targeted to the plasma membrane, yet is excluded from lipid microdomains (Kenworthy et al., 2004). The behavior of YFP-GT46 was indistinguishable both qualitatively and quantitatively from GPI-GFP (Figures 1B and 1C). These observations imply that components derived from the plasma membrane comprise a sizable fraction of the early phagosomal membrane, setting an upper limit to the contribution of the ER.

Contribution of the ER to Early Phagosomes

To quantify the contribution of the ER to phagosome biogenesis, we examined various markers of the ER during the course of particle engulfment. RAW macrophages expressing either luminal (GFP-KDEL) or integral membrane markers (Sec61α-GFP) of the ER were allowed to internalize beads. To ascertain the location of the phagosomal membrane the cells were also labeled with either FM4-64 or with fluorophore-conjugated B subunit of cholera toxin (CTxB), a ligand of exofacial gangliosides. Remarkably, even after repeated imaging of living cells by spinning disk or scanning confocal fluorescence microscopy (Figure 2; Movie S2), we failed to detect colocalization of ER markers with the limiting membrane of the phagosome. This impression was validated by quantitative analysis of colocalization (Figures 2A and 2B), yielding small or negative Pearson's coefficients, indicative of no significant ($p > 0.10$) overlap of ER and phagosome markers. Similar quantitative analyses in phagosomes from over 50 cells in four experiments yielded equally insignificant Pearson's coefficients for several combinations of phagosomal and ER markers (Table S1). These findings were not the consequence of overexpression of ER markers, as immunostaining for an endogenous ER component (protein disulfide isomerase [PDI]) produced similar negative results (Table S1). The observation that the ER is not a major component of the phagosome membrane was not limited to RAW cells, as identical experiments performed in J774 murine macrophages and murine DCs yielded virtually identical results. Quantitative colocalization analysis of GFP-KDEL with either CTxB (Figures S2A and S2C) or FM4-64 (Figure S2B) yielded insignificant Pearson's coefficients (Table S1).

The ability to detect colocalization was verified by staining the phagosomal membrane with FM4-64 in RAW cells transfected with either GPI-YFP (Figure 2C) or with a GFP-tagged version of the human FcγRIIA receptor, both of which localized primarily to the plasma membrane and the early phagosomal vacuole. In addition, transferrin receptors were found to colocalize with newly formed phagosomes (Figure 2D). Quantitation of

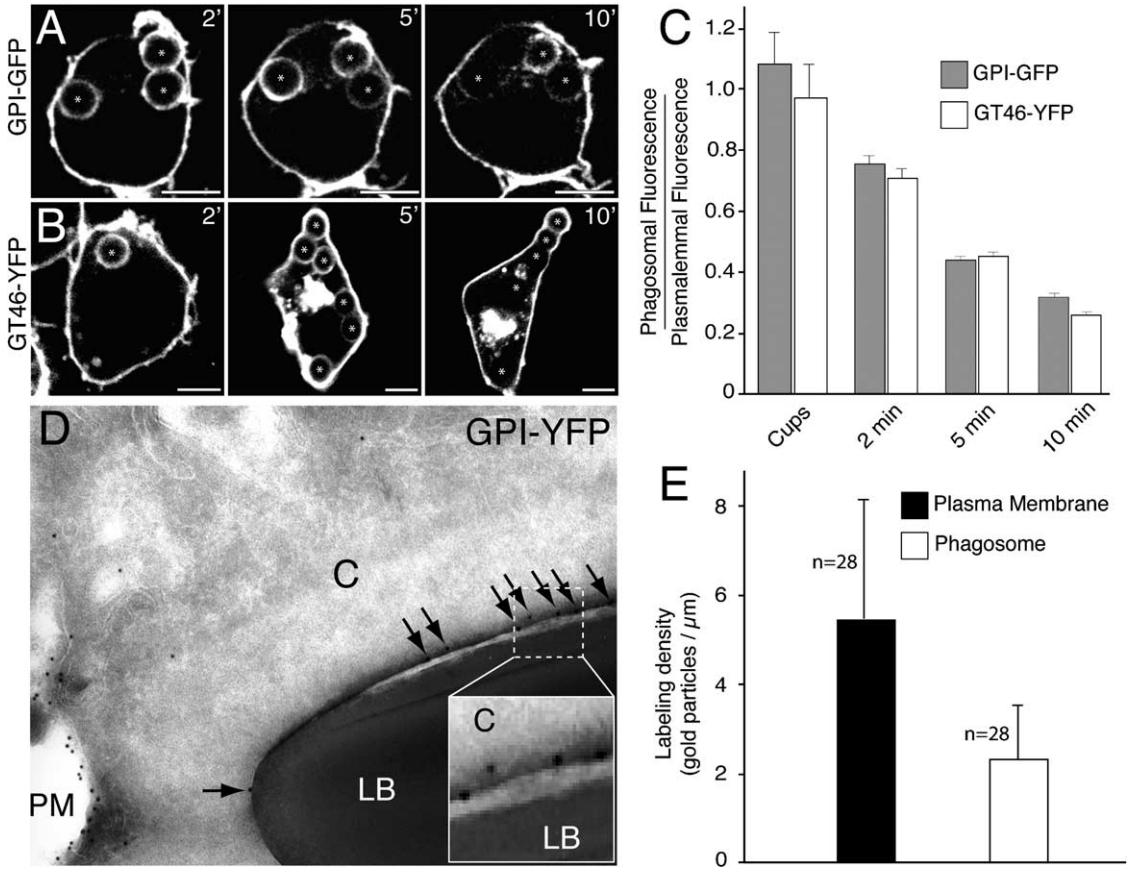

Figure 1. Contribution of the Plasma Membrane to Phagosome Formation

Confocal microscopy was used to monitor the distribution of the plasmalemmal markers GPI-GFP (A) and YFP-GT46 (B) in RAW cells. Images were acquired 2, 5, and 10 min after initiation of latex bead (3 μm) engulfment.

(A and B) Representative images. Scale bars represent 5 μm.

(C) Quantitation of >200 phagosomes (asterisks). Data in (C) were normalized to the fluorescence intensity of the surface membrane of the same cell. Relative fluorescence intensity of unsealed phagocytic cup or phagosomes at various times after sealing shown.

(D) Immunogold localization of GPI-YFP following phagocytosis. Arrows indicate location of gold particles. PM = plasma membrane ; LB = Latex bead ; C = Cytosol.

(E) Quantification of the density of gold particles per μm on the plasma and phagosomal membranes.

Data in (C) and (E) are the means ± SE of the number of cells (n).

these and other data is summarized in Table S1, which demonstrates that plasmalemmal and early endosomal markers were readily detectable in early (2–5 min) phagosomes, while ER-derived components were not.

A closer inspection of the relationship between the ER and the phagosomal membrane was performed by immuno-EM. Cryosectioned samples of RAW and J774 macrophages that had previously ingested latex beads for 5 min were labeled with antibodies to either the luminal or cytosolic domain of calnexin, an ER-resident protein. Despite the fact that the ER cisternae were extensively labeled, calnexin could not be detected on the phagosomal membrane (Figures 3 and S3). Quantitative analysis of multiple sections revealed that the density of gold particles per unit of phagosomal membrane was insignificantly small, comparable to the gold associated nonspecifically with extracellular beads that were not ingested or organelles not expected to contain calnexin (Figure 3B). Similar results were obtained in J774 cells using antibodies to another endogenous ER marker, PDI (Figure 3D).

The sensitivity of immunofluorescence or immunogold labeling may be insufficient to detect a comparatively scarce component of phagosomes. For this reason, immunocytochemical staining for the ER marker glucose 6-phosphatase (G6Pase), as used by Gagnon et al. (2002), was employed as a third approach to assess the presence of ER in phagosomes. As illustrated in Figures 4A–4D, reaction products that appear as electron-dense deposits were readily detected on the nuclear membrane and in branching tubules indicative of the ER in all cells analyzed. That the method is sufficiently sensitive to detect phagosomes that fuse with the ER was shown in macrophages that had ingested *Brucella abortus.* This bacterium reroutes the normal maturation of the phagosome, dictating its fusion with components of the ER-to-Golgi intermediate compartment (Celli et al., 2003). As demonstrated recently, G6Pase activity

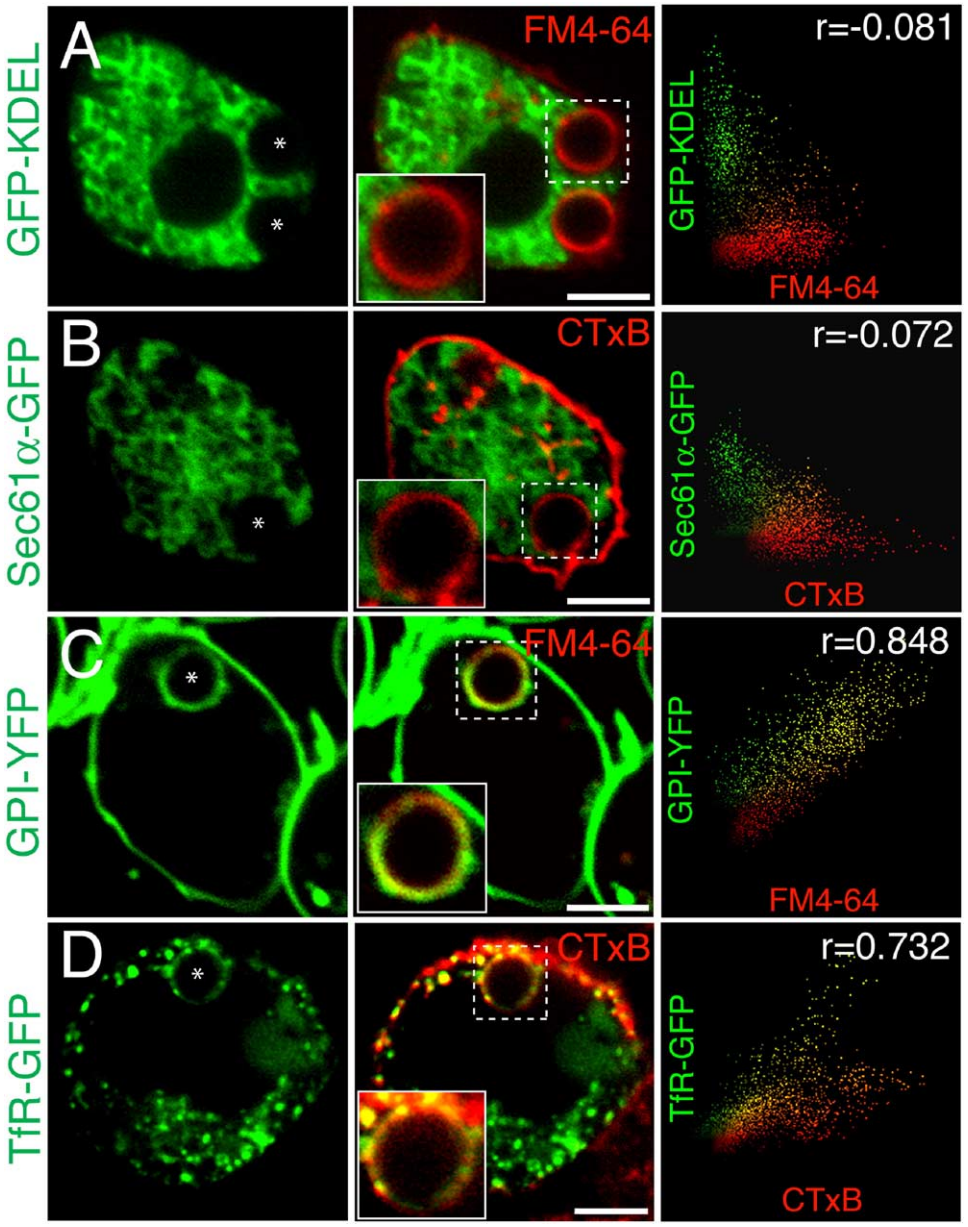

Figure 2. Assessment of the Contribution of ER to Phagosomes by Fluorescence Microscopy

RAW cells expressing GFP-tagged organellar markers were allowed to internalize IgG-opsonized beads. In (A) and (C), FM4-64 was present at the time of engulfment to label the phagosomal vacuole. In (B) and (D), the plasma membrane was prestained with Alexa 555-conjugated B subunit of CTxB. Images were acquired 2–5 min after particle engulfment.

(A) Comparison of GFP-KDEL (green) and FM4-64 (red).

(B) Sec61α-GFP (green) and CTxB (red).

(C) GPI-YFP (green) and FM4-64 (red).

(D) Transferrin receptor-GFP (TfR-GFP; green) and CTxB (red). Phagosomes are indicated by asterisks. Middle panels show a magnified version of the indicated phagosome. The rightmost panels are scatter plots of green *vs.* red fluorescence of individual pixels in the area shown in insets. Colocalization Pearson's coefficients (r) are shown for each set of markers.

Scale bars represent 5 μm.

was clearly detectable in phagosomes 24 hr after ingestion of *B. abortus* (Figure 4A). In contrast, phagosomal cups (Figure S4A) or phagosomes studied 15 or 30 min after uptake of latex beads were unlabeled (Figures 4 and S4B). Similar negative results were obtained in J774 cells (Figures 4F and S4) and in primary, bone marrow-derived murine macrophages (Figure 4E), whether the beads were or not IgG-opsonized (Figures 4E and 4F) and regardless of whether their surface was hydrophobic (Figure 4C) or hydrophilic (Figure 4D). No significant phagosomal staining was observed using either small (1 μm) or large (3 μm) latex beads (Figure

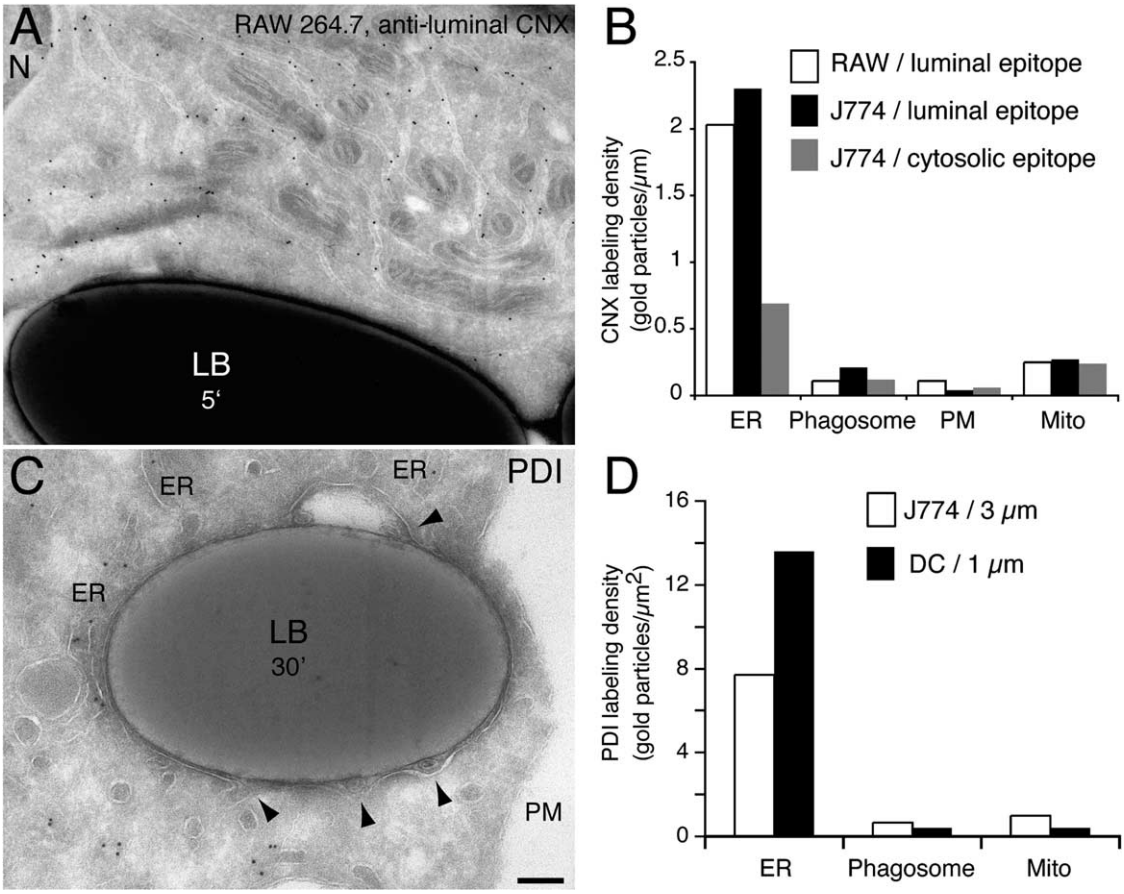

Figure 3. Immunogold EM of Phagosomes in Macrophages

(A) Immunogold localization of calnexin (CNX) in RAW cells containing latex beads. An antibody recognizing the luminal domain of calnexin was used.

(B) Quantification of CNX in different organelles of RAW and J774 cells using antibodies directed to the luminal domain or cytosolic domain of CNX.

(C) Immunogold localization of PDI in human DCs following uptake of latex beads (LB). Arrowheads: sites of apparent membrane fusion.

(D) Summary of quantitation of gold particles for the indicated structures in J774 and human DCs using the method of Mayhew et al. (2002). PM = plasma membrane; N = nucleus; LB = Latex bead.

4E), indicating that particle size is not a critical determinant of ER association with the phagosome. Moreover, negative results were also obtained when *Mycobacterium avium* was used as prey (Figures 4B and 4E). Jointly, these findings suggest that the ER is not a normal component of the phagosome, though it can be actively recruited by effectors of certain bacteria to preclude phagolysosome formation. Accordingly, G6Pase activity was not detected in phagosomes containing *B. abortus* mutants lacking VirB, the virulence factor that directs fusion with the ER (Celli et al., 2003).

Contribution of ER to Early Phagosomes in Dendritic Cells

We next examined the ER during phagocytosis in murine bone marrow-derived DCs by live-cell imaging. GFP-KDEL was expressed by retroviral infection (Chow et al., 2002), while the plasma membrane was visualized using CTxB (Figure S2C). As found for RAW macrophages, the early phagosomal membrane in immature DCs was rich in the plasma membrane marker, but contained no detectable ER marker. Quantitative colocalization analysis of over 20 phagosomes yielded a Pearson's coefficient of 0.065 ($p > 0.10$; Table S1), indicating no significant overlap. Furthermore, EM analysis of immunogold labeled samples confirmed the absence of the ER marker from phagosomes, while lgp120/LampI, a late endosomal/lysosomal marker, was readily detected on late phagosomes (data not shown). Therefore, the ER did not appear to be a major contributor to the formation of early phagosomes in mouse DCs.

We also examined the contribution of the ER in DCs derived from human monocytes. The distribution of PDI was visualized by immuno-EM in cells that had ingested latex beads. As shown in Figure 3C, abundant labeling of typical ER elements was observed, while the label associated with phagosomes was negligible and, after quantitation, indistinguishable from the background (Figure 3D). The PDI content of phagosomes

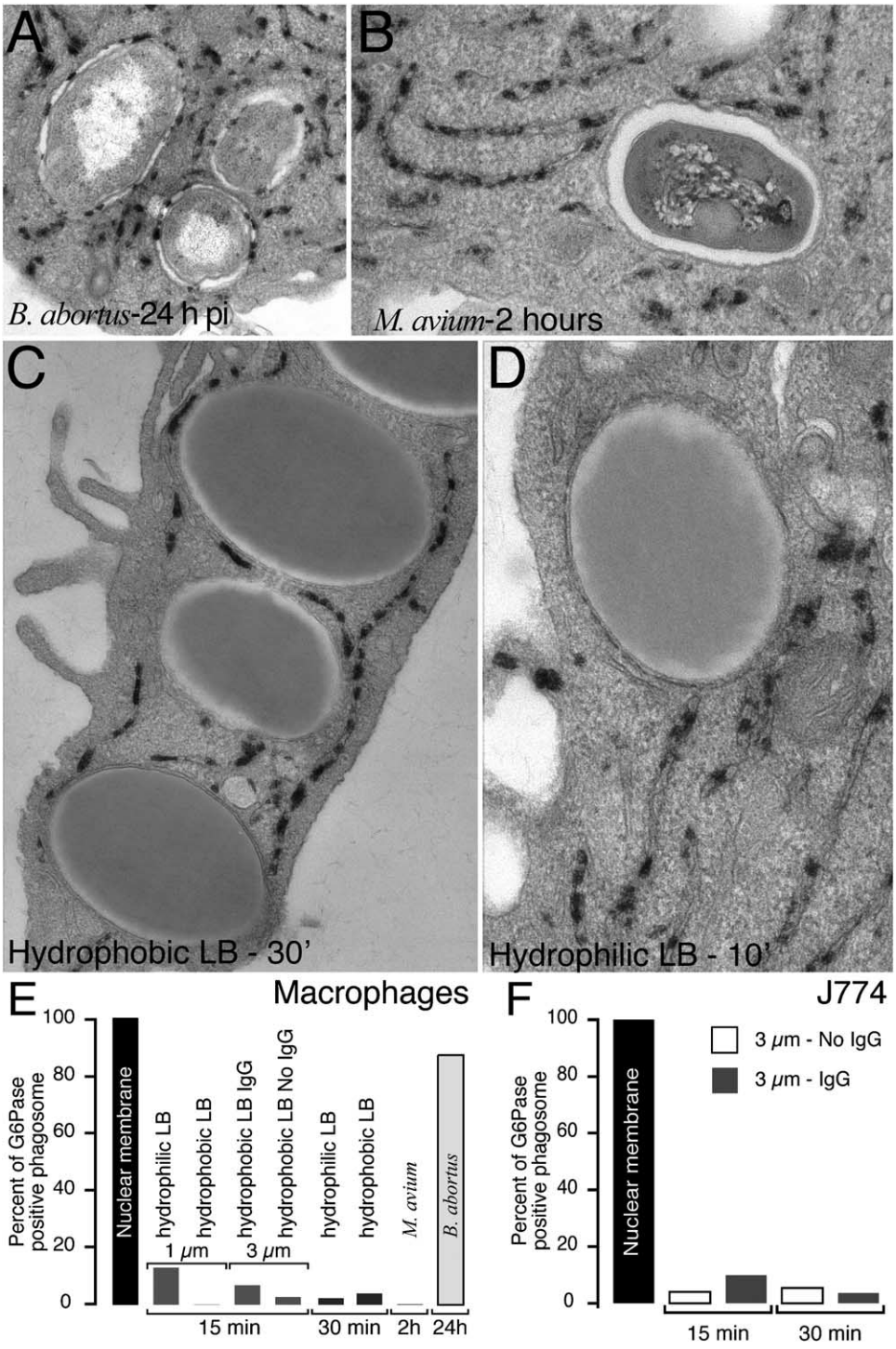

Figure 4. G6Pase Staining in Macrophages

Bone marrow-derived macrophages (A–E) or J774 cells (F) were allowed to internalize particles, fixed and stained for G6Pase activity.

(A) Macrophage containing *Brucella abortus*, fixed 24 hr after infection.

(B) Macrophage containing *Mycobacterium avium* fixed after 2 hr of uptake.

(C) Macrophage containing hydrophobic latex beads (LB), fixed after 30 min.

(D) Macrophage containing hydrophilic (carboxylated) latex beads, fixed after 10 min.

(E) Quantitation of G6Pase activity; 30–50 phagosomes counted per condition.

(F) Quantification of G6Pase in J774 cells.

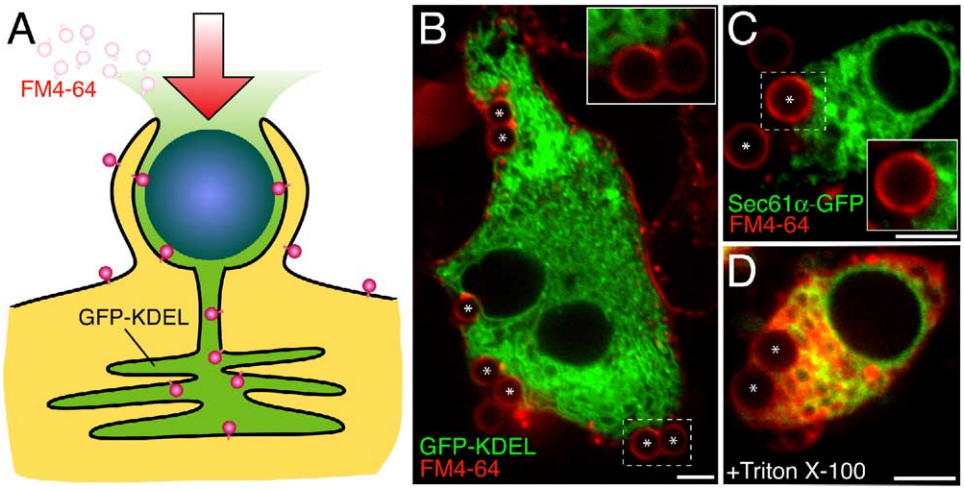

Figure 5. Monitoring Accessibility of Extracellular Probes to the ER during Phagosome Formation

(A) Schematic representation of the experimental protocol. FM4-64 was present at the time of particle ingestion to monitor the continuity of the phagosomal membrane with the ER. RAW cells expressing GFP-tagged markers were used to visualize the ER.

(B) FM4-64 (red), GFP-KDEL (green).

(C) FM4-64 (red), Sec61α-GFP (green).

(D) Cells transfected with Sec61α-GFP (green) were allowed to internalize latex beads and were gently permeabilized using 0.01% Triton X-100 before addition of FM4-64. (B)–(D) are representative of ≥3 experiments each. Scale bars represent 5 μm.

remained insignificant regardless of the duration of the phagocytosis pulse (0–30 min). By contrast, fusion of PDI-negative tubulovesicular structures was commonly seen (arrow Figure 3C), and immunogold-labeled transferrin receptors were also readily observed in phagosomes (data not shown).

Is the Forming Phagosome Ever Continuous with the ER?

Experiments were designed to determine if the ER even transiently fused with forming phagosomes, perhaps failing to deliver the bulk of its contents. For this purpose, we turned to the dye FM4-64, which should diffuse into the ER membrane even if fusion created only a small or evanescent fusion pore (see Figure 5A). Macrophages stably transfected with GFP-KDEL were exposed to opsonized beads in the presence of a high concentration (10 μM) of the dye, which was kept in the medium during particle uptake to increase its opportunity to access the ER. A representative experiment is illustrated in Figure 5B, which demonstrates that, while FM4-64 stained the plasma membrane and reached all aspects of the phagosomal membrane, it was never found to enter the ER.

Conceivably, fusion of the ER with the phagocytic cup during particle internalization may have been too transient to allow detectable amounts of FM4-64 to enter its lumen. Indeed, Gagnon et al. (2002) found that in normal cells, sites of apparent continuity between the ER and the phagocytic cup were difficult to observe. These authors found that arresting phagocytosis with wortmannin was able to stabilize the apparent connections, facilitating their visualization by EM. We therefore tested the effects of the inositide kinase inhibitor on the distribution of FM4-64. In agreement with earlier reports (Cox et al., 1999; Ninomiya et al., 1994), we found that wortmannin halted phagocytosis following extension of incipient pseudopods. FM4-64 was readily able to reach the base of such aborted phagosomal cups, implying that diffusion of the dye into the cleft is not restricted. Nevertheless, despite the sustained arrest of phagocytosis, the dye never reached any aspect of the ER (Figure S5). We repeated these experiments using Sec61α-GFP, an ER integral membrane protein. As shown in Figure 5C, neither did Sec61α-GFP enter the phagosome, nor did FM4-64 reach the ER. That FM4-64 could partition detectably into the ER was shown by gently permeabilizing the cells with 0.01% Triton X-100 (Figure 5D).

The inability to detect FM4-64 in the ER following phagocytosis might conceivably reflect an unsuspected subcompartmentalization in which only a specialized and small region of the ER engages in phagosomal fusion. Such as possibility would require that the ER itself be discontinuous, which is inconsistent with previous observations in nonphagocytic cells (Lippincott-Schwartz et al., 2003; Nehls et al., 2000). To assess the continuity of the macrophage ER during phagocytosis, we monitored the distribution of GFP-KDEL fluorescence after repeated photobleaching of one half of a cell (Figure S6). The ER behaved like a single continuous structure, as there was no detectable residual GFP-KDEL fluorescence in or around the phagosome formed in the unbleached area (asterisk, Movie S3). Therefore, any FM4-64 entering through the putative fusion pore should have access to ER elements spread throughout the cytoplasm. Because this was not observed even in cells treated with wortmannin, it is unlikely that physical continuity between the phagosomal cup and the ER was ever established.

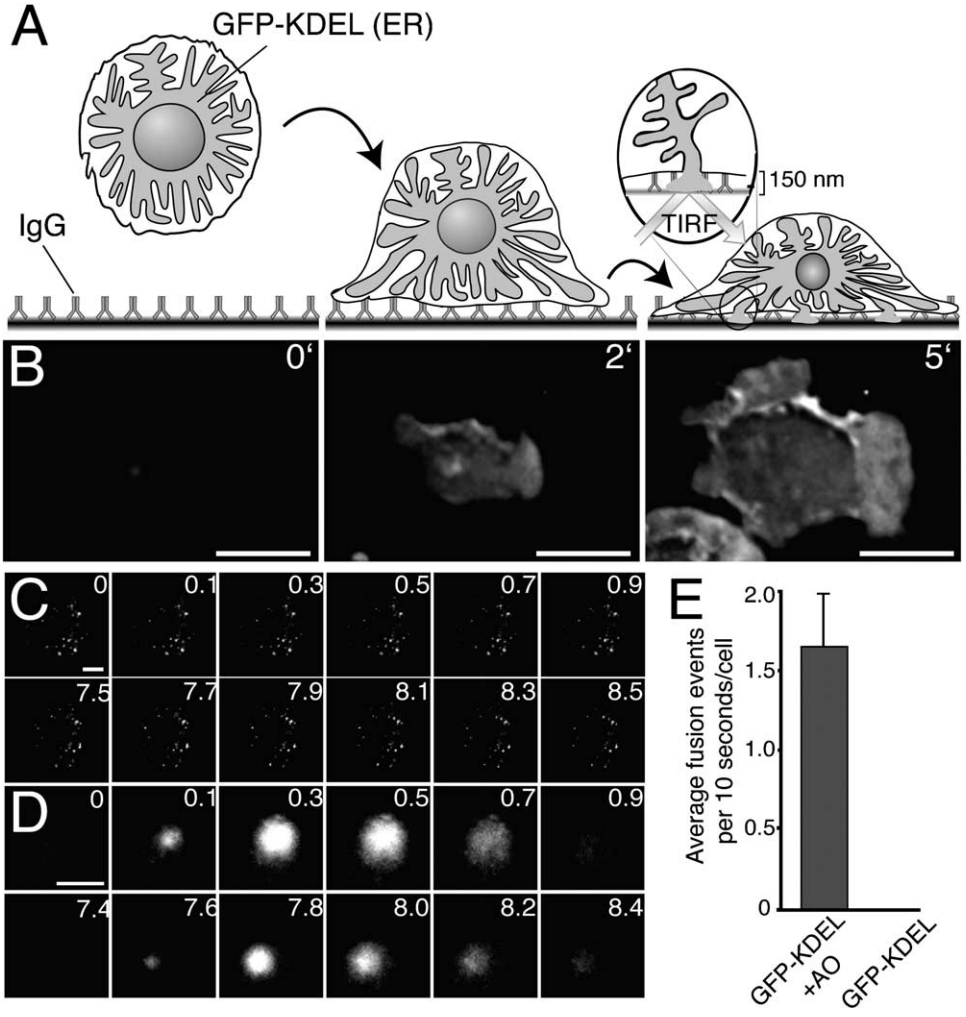

Figure 6. Assessment of ER Fusion Using TIR-FM

(A) Schematic representation of experimental protocol. Suspended macrophages expressing GFP-KDEL sedimented onto IgG-coated coverslips to induce sustained frustrated phagocytosis at a defined focal plane. Release of fluorescent probes is monitored by TIR-FM.

(B) Visualization of GPI-YFP over time (in min).

(C) Time-lapse sequence of a cell expressing GFP-KDEL acquired by TIR-FM during spreading.

(D) Time-lapse sequence of acridine orange in a spreading cell acquired by TIR-FM. The left-most panels in (C) and (D) show a whole cell, whereas the subsequent panels are of higher magnification. Numbers in (C) and (D) are time in min after acquisition was initiated.

(E) Quantitation of organelle fusion events in >40 cells during frustrated phagocytosis. Left bar: cells expressing GFP-KDEL that were also stained with acridine orange (AO). Right bar: cells expressing GFP-KDEL only (bar is not visible, mean = 0). Scale bars in (B) and (C) represent 5 μm and in (D), they represent 0.5 μm.

The ER Does Not Fuse with the Plasma Membrane during "Frustrated Phagocytosis"

We next used a biophysical approach to assess whether the contents of the ER are released to the medium through the connection proposed to form with nascent phagosomes. Due to the ephemeral nature of the connection and the unpredictable site of phagocytic events in cells ingesting small particles, we imaged fusion during "frustrated phagocytosis" (Figure 6A). Abortive phagosomal "cups" can be induced by plating macrophages on IgG-coated coverslips, which engage Fc receptors at the attached surface (Marshall et al., 2001; Silverstein et al., 1977). These surrogate cups are sustained for long periods and form at a predetermined focal plane, facilitating analysis by total internal reflection fluorescence microscopy (TIR-FM). The suitability of this model was verified using cells expressing the GPI-YFP. As shown in Figure 6B, cell spreading was clearly evident and sustained for many minutes. To assess the appearance of a connecting pore between the ER and the frustrated phagocytic cup, cells expressing GFP-KDEL were allowed to interact with the opsonized surface. No fusion events, defined by the sudden appearance of a radially diffusing cloud of fluorescence (Fix et al., 2004), were detected (Figure 6C; Movie S4). However, fusion events could be readily observed when endosomes/lysosomes were loaded with acridine orange (Figure 6D; Movie S5), consistent with earlier reports (Braun et al., 2004; de Chastellier and Thilo, 1997; Niedergang and Chavrier, 2004). Quantitation of fusion

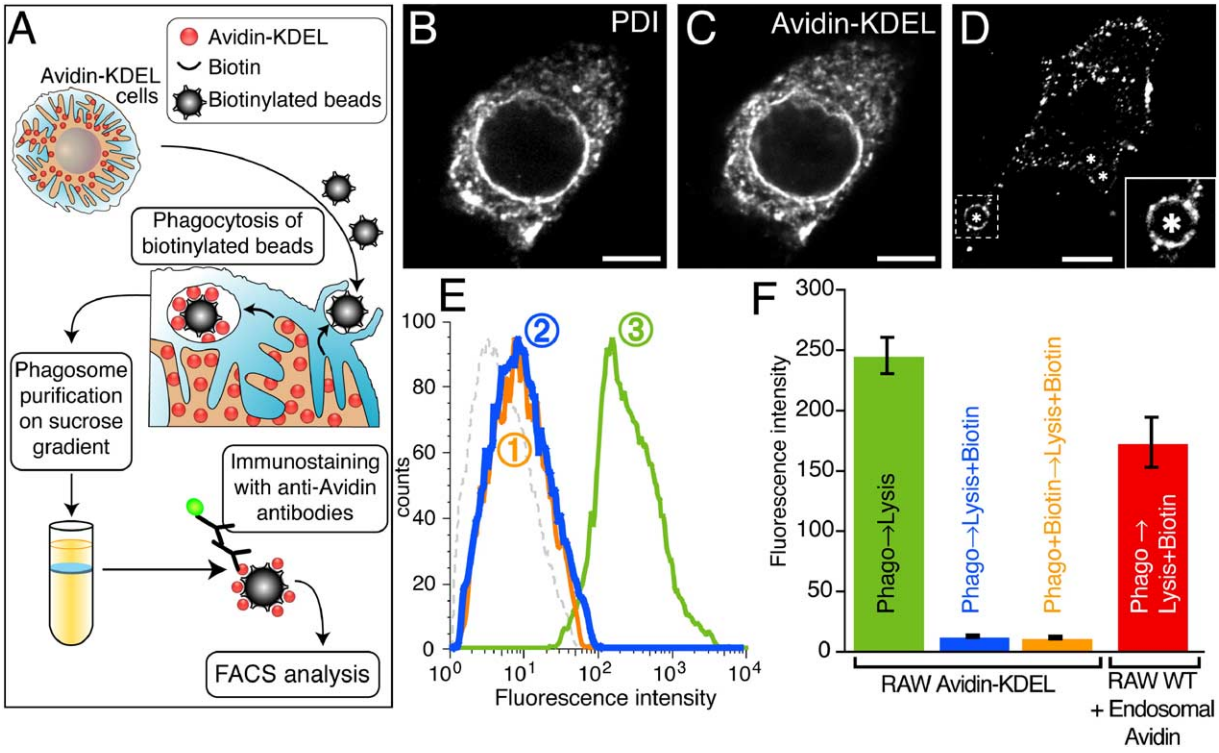

Figure 7. Assessment of ER Fusion by Fractionation and Flow Cytometry

(A) Experimental protocol: RAW cells stably expressing an ER-targeted form of avidin (Avidin-KDEL) internalized IgG-opsonized biotinylated beads. After 15 min, the cells were homogenized and phagosomes purified on a sucrose gradient. Beads were permeabilized and immunostained with anti-avidin and secondary antibodies. The amount of avidin-KDEL bound to the biotinylated beads was quantified by flow cytometry.

(B) Distribution of PDI.

(C) Distribution of avidin-KDEL in cell shown in (B), revealed with anti-avidin antibodies.

(D) Distribution of avidin internalized by fluid-phase uptake. Cells preincubated with soluble avidin (50 μg/ml, 20 min at 37°C) were allowed to engulf IgG-coated beads for 15 min. Asterisk = phagosome, magnified in inset. Scale bars in (B)–(D) represent 5 μm.

(E) Typical flow cytometry profiles from phagosomes prepared as in (A), with the following modifications. Profile 1: negative control. The beads were added to avidin-KDEL cells in the presence of excess (10 mM) free biotin. Excess biotin was also present during lysis to prevent binding of avidin released upon ER disruption. Profile 2: experimental sample. Beads were added to avidin-KDEL cells in the absence of free biotin to allow interaction with avidin during maturation. Excess biotin was added during lysis to prevent binding of avidin released upon ER disruption. Profile 3: positive control. Beads added to avidin-KDEL cells, which were then lysed with Triton X-100 to allow interaction of avidin with the beads.

(F) Summary of flow cytometric quantitations. Mean fluorescence from experiments like that in (E). To assess endosomal fusion with phagosomes, cells were allowed to take up avidin as in (D), processed as described for profile 2, and normalized according to the amount of total intracellular avidin. Data are the means ± SE of ≥4 experiments of each type.

events (Figure 6E) indicated that between one to two fusion events involving endosomes/lysosomes could be detected every 10 s, while in the same cells fusion of ER elements was never observed.

Biochemical Assessment of ER and Phagosome Interaction

To address the possibility of intermittent ER-phagosome fusion during maturation, we determined the delivery of a soluble ER marker to the lumen of formed phagosomes using a biochemical method that would record and integrate any such interactions over time. The experimental design and rationale are depicted in Figure 7A. Briefly, macrophages were stably transfected with avidin-KDEL, a soluble marker of the ER, which was shown to be properly targeted to PDI-containing ER elements (Figures 7B and 7C). The cells were next allowed to ingest biotinylated beads, homogenized, and phagosomes were isolated by sucrose gradient centrifugation. These were then washed several times in PBS containing Triton X-100 to permeabilize their membranes and eliminate any adherent contaminants. Finally, the association of avidin with the beads was monitored by immunostaining with anti-avidin antibodies using flow cytometry.

The sensitivity of the system was first tested by lysing the cells with detergent following phagocytosis, thereby releasing the avidin-KDEL into the medium and allowing its unrestricted interaction with the biotinylated beads. Such beads displayed readily detectable fluorescence, indicating that they had bound avidin (curve 3; Figures 7E and 7F). Next, the specificity of this association was evaluated by adding an excess soluble biotin at the time of bead ingestion, as well as during

lysis. Only background levels of fluorescence were detected under these conditions (curve 1; Figures 7E and 7F). Finally, we assessed if beads that had undergone phagocytosis gained access to avidin trapped in the ER by allowing bead ingestion in the absence of biotin. Curve 2 in Figure 7E and the blue bar in Figure 7F indicated that no avidin was detectable on the beads. Similar negative results were obtained when phagosomes were allowed to mature for up to 4 hr.

We used an analogous approach to assay the fusion of endosomes with phagosomes. Endosomes of RAW macrophages were loaded with avidin by fluid phase pinocytosis (Figure 7D). When such preloaded cells ingested biotinylated latex beads, avidin was consistently detected in the immediate vicinity of the internalized particles (Figure 7D). That the probe had in fact reached the lumen of phagosomes was verified using the purification and flow cytometry approach described above. Endosomal avidin associated with the internalized biotinylated beads was readily detectable (Figure 7F), despite the fact that the total amount of cellular avidin trapped in endosomes was substantially lower than that trapped in the ER in the preceding experiments.

Use of pH-Sensitive Proteins to Probe Association of Phagosomes with the ER

One of the defining characteristics of normal phagosomes is their ability to undergo rapid acidification shortly after formation. Any components of the ER delivered to the lumen of the phagosomes would therefore experience an acute change in pH. We took advantage of the pH sensitivity of GFP and its derivatives (Tsien, 1998) to assess the fusion of phagosomes with the ER by an independent approach. Macrophages stably expressing the exofacial plasma membrane marker GPI-YFP, or the luminal ER marker GFP-KDEL, were allowed to internalize latex beads. The location of these probes with respect to the lumen of the phagosome can be defined by monitoring the fluorescence changes induced by rapidly dissipating the acidic pH of phagosomes. This can be simply accomplished by acute addition of permeant weak bases, like NH_3. As illustrated in Figures S7A and S7B, the emission of GPI-YFP located in endosomes and phagosomes increased markedly upon addition of NH_3, indicating their presence in an acidic compartment. Ratio imaging of the fluorescence before and after addition of NH_3 yielded a 1.5- to 2.5-fold increase in emission intensity (Figures S7C and S7D).

By contrast, addition of NH_3 had no effect on the fluorescence of GFP-KDEL either in the immediate vicinity of phagosomes or elsewhere in the cell (Figures S7E–S7H). The failure to see fluorescence changes cannot be attributed to insensitivity of GFP-KDEL to pH, since manipulation of the intracellular pH between 5.5 and 7.5 caused pronounced changes in emission (Figures S7I–S7K). These findings are consistent with earlier conclusions that the ER is not acidic (Wu et al., 2001, 2000) and failed to give any indication of fusion of phagosomes with the ER.

Quantitative Assessment of the Contribution of Endosomes to the Phagosomal Membrane

While we were unable to detect a significant contribution of the ER to the membrane of phagosomes, we nevertheless found a comparatively rapid disappearance of plasmalemmal components from the vacuole, such that only ≈30% could be accounted for by plasmalemmal components after 10 min (Figure 1). We therefore sought to determine the source of membrane involved in this remodeling. Because the classical phagocytosis and maturation model postulates the involvement of endosomes (Lang et al., 1988; Pitt et al., 1992), we devised a method for the quantitation of the fraction of the phagosomal area contributed by endosomal membranes. We selectively labeled endosomes by incubating macrophages at 37°C with FM4-64 for 15 min prior to phagocytosis (Figure 8B), followed by selective removal of remaining plasma membrane FM4-64 by repeated washing in medium devoid of dye. Subsequent addition of opsonized particles revealed that FM4-64 was rapidly delivered to the phagosomal membrane, verifying the occurrence of fusion between endosomes and phagosomes (Figure 8C). The area of endosomal membrane delivered to phagosomes was next approximated by comparing the intensity of phagosomal FM4-64 fluorescence with plasma membrane fluorescence of the same cells incubated with FM4-64 at 4°C (Figure 8A). These calculations suggested that the contribution of endosomes to the phagosomal membrane increased between 2–10 min, from ~40% to ~60%. When added to the fraction contributed by the remaining plasma membrane, the endosomal contribution accounts for most of the phagosomal area and sets a very modest upper limit to any possible contribution by the ER.

Discussion

The ability of macrophages and dendritic cells to eliminate invading microorganisms and to process their antigens for presentation relies on the dynamic remodeling of the membrane and contents of their phagosomes. Most of the functions attributed to phagosomes were thought to result from a maturation process involving sequential fusion to endosomes and lysosomes. Recently, however, an alternate model was proposed by Gagnon et al. (2002), invoking fusion of the forming phagosomes with the ER. This model is supported by earlier observations that *Dictyostelium* mutants lacking calreticulin and calnexin have reduced phagocytosis (Muller-Taubenberger et al., 2001) and is also consistent with recent findings of Becker et al. (2005) that impairment of the function of ERS24/Sec22b, a SNARE protein of the ER, reduced the engulfment of 3 μm (but not 0.8 μm) particles by macrophages. The possibility that the ER fused with the plasma membrane during phagocytosis had immense appeal in the context of antigen cross-presentation. As a result, several recent reports focusing on the capabilities of phagosomes to process antigen for MHC class I presentation emphasized the ER-mediated phagocytosis model (Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003). Finally, the ER has also come

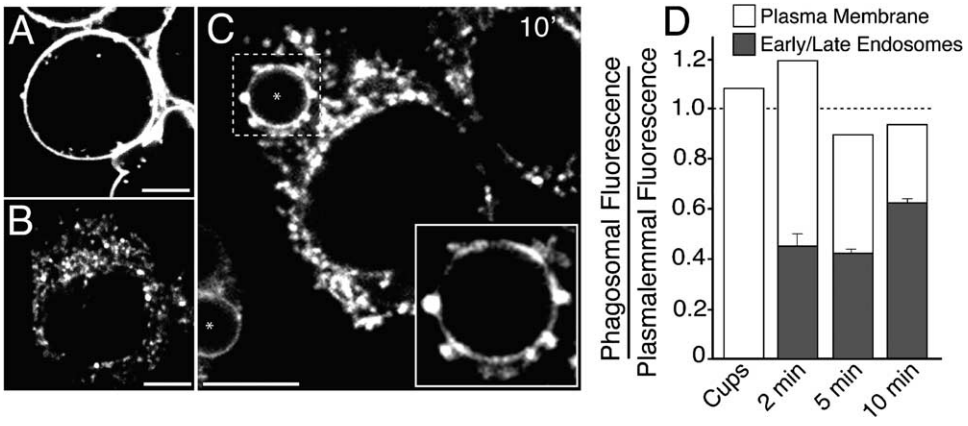

Figure 8. Quantitation of Endosomal Contribution to the Phagosome Membrane

(A) Labeling of the plasma membrane of RAW cells with FM4-64.

(B) Labeling of endosomes with FM4-64 following incubation with dye for 15 min at 37°C and subsequent removal of the plasmalemmal FM4-64 by washing.

(C) Endosomal fusion with phagosomes, assessed after labeling with FM4-64 as in (B), followed by ingestion of beads for 10 min. Scale bars in (A)–(C) represent 5 μm.

(D) Mean phagosomal FM4-64 fluorescence/pixel measured as in (C) is expressed relative to the plasmalemmal FM4-64 fluorescence, measured in the same cells as in (A). Normalized fluorescence intensities are illustrated (dark bars). Data are the means of 40 experiments. For reference, the contribution of the plasma membrane to the phagosomes (open bars) is replotted from Figure 1.

to be viewed as providing a quantitatively important reservoir of membrane inserted for the internalization of large particles (Brode and Macary, 2004; Desjardins, 2003; Niedergang and Chavrier, 2004).

On the other hand, several considerations make it difficult to conceive that the ER would fuse directly with the plasma membrane during particle uptake. A major contribution of the ER to the phagosomal membrane is difficult to reconcile with the well-established rapid acidification of the phagosomal lumen. The membrane of the ER is devoid of active proton-pumping V-ATPases and is extremely leaky to proton equivalents (Paroutis et al., 2004; Wu et al., 2001). It was also unclear how the proposed connection of the ER with the base of the forming phagosome would alter the ability of the ER to retain calcium, or nascent and misfolded secretory proteins contained within its lumen.

This led us to reexamine the role of the ER during phagocytosis. We devised six independent and quantitative methods to explore if the ER provides a substantial contribution to phagosomes in different cell types from two species. Our experiments failed to produce any evidence in support of fusion of the ER with either nascent or maturing phagosomes. It is noteworthy that we used phagocytic targets of various kinds that were of similar size those employed by Gagnon et al. (2002) and that we tested primary and immortalized macrophages, as well as dendritic cells. Therefore, neither difference in particle size or cell type can readily account for the apparent discrepancies. By contrast, we could easily document the presence of plasmalemmal remnants in the early phagosomes as well as the fusion of endosomes/lysosomes by several different approaches. The progressive replacement of plasma membrane components by endosomal membranes was verified in experiments like that of Movie S6. The rapid and gradual insertion of membranes of the endocytic pathway, which are endowed with V-ATPases and relatively tight to protons is eminently consistent with the development of phagosomal acidification.

The original hypothesis of ER-mediated phagocytosis stemmed from biochemical determinations of the protein composition of purified phagosomes (Desjardins et al., 1994a; Garin et al., 2001). As in any other biochemical purification procedure, contaminants are an inevitable component of the final preparation. Owing to its abundance, the ER is the most likely source of contamination of particulate preparations, and may account for the observed presence of the ER proteins in immunoblots and mass-spectroscopy analyses of phagosomes. Accordingly, treatment of "purified" phagosomes with ATP led to a 5- to 10-fold decrease in the content of ER markers and a concomitant enrichment for cathepsin D, a *bona fide* marker of phagolysosomes (Gotthardt et al., 2002). These observations imply that biochemical detection of ER markers on phagosomal preparations is not necessarily evidence that the phagosome membrane is made up largely, or even partly, of ER.

Although contamination by ER may account for some of the biochemical observations, it cannot explain the evidence obtained by Gagnon et al. (2002) using EM. The striking observation of products of G6Pase in close association with the phagosome membrane appears to lend strong support to the theory of ER-phagosome fusion. These findings contrast with our inability to detect markers of the ER within the phagosome by EM (Figures 3, 4, S3, and S4). Cytochemical reactions such as the cerium capture used for G6Pase determinations are often more sensitive than immunogold, possibly explaining the differential results. However, such reactions are not nearly as specific as immuno-EM and may reflect a minor nonspecific contribution of other enzymes, particularly in tissues with low G6Pase activity. The hydrolysis of glucose 6-phosphate can also be cat-

alyzed by other enzymes, including alkaline and acid phosphatases, which are enriched in the plasma membrane and lysosomes, respectively (Matsubara et al., 1999; Nichols et al., 1984; Ulsamer et al., 1971). In our experiments, alkaline phosphatase activity at the plasma membrane was not detectable, and reaction product due to lysosomal acid phosphatase was rarely seen. Under these conditions, we consistently failed to detect G6Pase activity in phagosomes formed by ingestion of different types of particles, while clear and reproducible staining of the nuclear membrane and of the ER was obtained routinely (Figures 4 and S4). Therefore, our observations are more consistent with previous studies reporting low G6Pase activity in phagosomal membranes, while finding a 10-fold enrichment of plasmalemmal NADPH oxidase and 5′-nucleotidase activity (Bellavite et al., 1982; Berton et al., 1982).

While our quantitative determination of the composition of the phagosomal membrane leaves little room for ER components, it does not rule out fusion of phagosomes with a small amount of ER, or the possibility that the phagosome communicates with the ER by alternative pathways of vesicular traffic. Even a small fractional contribution of the ER to the membrane of the phagosome is compatible with its proposed role in cross-presentation by MHC class I (Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003). Alternatively, antigen-containing vesicles may pinch off the phagosome and undergo retrograde transport to the Golgi complex and ultimately the ER, as has been shown for material that is internalized by endocytosis (Miesenbock and Rothman, 1995; Sandvig and van Deurs, 2002). In this regard, it is noteworthy that components of the COPI coatomer system that mediates retrograde transport from Golgi to the ER are also found on endosomes (Aniento et al., 1996; Daro et al., 1997; Whitney et al., 1995) and can affect the maturation of both endosomes and phagosomes. Material internalized by caveolar endocytosis can be delivered more directly to the ER, possibly bypassing the Golgi complex (Pelkmans et al., 2001), and a similar route may apply to vesicles derived from phagosomes, which appear to contain caveolar components (Dermine et al., 2001). Moreover, selected components of the ER could be delivered to the maturing phagosome via the *trans*-Golgi network in a manner akin to the delivery of newly synthesized proteins to prelysosomes. Lastly, cross-presentation can also occur by the novel process described recently by Neijssen et al. (2005), whereby peptides are delivered by other cells to the cytosol of antigen-presenting cells via gap junctions.

Our observation that the contribution of the plasma membrane to phagosome formation decreases over time, together with the notion that the total surface area of macrophages remains constant (Hackam et al., 1998), or even increases (Holevinsky and Nelson, 1998), during particle engulfment, is suggestive of a mechanism for the recycling of plasmalemmal components back to the cell surface following phagosome formation.

The concept of phagosome membrane recycling back to the cell surface is not without precedent, as studies by Muller et al. and by de Chastellier et al. demonstrated that early phagosomes efficiently recycled [^{125}I]-labeled or [^{3}H]galactose-labeled glycoconjugates back to the cell surface (de Chastellier et al., 1983; Muller et al., 1980a).

It is important to note that the contribution of individual cellular compartments to forming phagosomes is likely to vary with particle size (Cox et al., 1999). In the case of Fc receptors and particles of 1–3 μm in diameter, such as those used in our studies, the contribution of the plasma membrane and endosomes to the forming and maturing phagosome appears indisputable. However, other compartments, possibly including the ER, may play a more important role for phagosomes of other sizes or those induced by different receptors (Cox et al., 1999; Henry et al., 2004). Therefore, the involvement of the ER in phagosome formation and maturation cannot be discounted, but direct demonstration that ER-to-phagosome fusion occurs and that it is required for phagocytosis must be established on a case-by-case basis.

Experimental Procedures

Cell Culture and Transfection

RAW264.7 and J774 macrophages obtained from ATCC were cultured in DMEM with 10% heat-inactivated fetal calf serum. Bone-marrow-derived mouse macrophages and DCs were obtained and cultured as in (de Chastellier et al., 1995; Chow et al., 2002). RAW cells stably transfected with GPI-GFP, GPI-YFP, GFP-KDEL, or avidin-KDEL were selected using G418. Clones stably transfected with avidin-KDEL were identified by immunostaining, using anti-avidin D antibody (Vector; 1:1000 dilution). Retroviral infection was used for expression of GFP-KDEL in J774 macrophages and GFP-KDEL and lgp120-GFP in DCs, as described by Chow et al. (2002).

Phagocytosis

Polystyrene beads (Bangs; 3.1 μm) were opsonized with 1 mg/ml human IgG (Sigma) in PBS for 1 hr at room temperature. To induce phagocytosis, opsonized beads were added to cells and adherence synchronized by centrifugation for 20 s at 200 g. Adherence to DC was induced by incubation with beads for 10 min at 4°C. After completion of phagocytosis at 37°C, extracellular beads were identified by incubation with 1:500 dilution of Cy3-conjugated anti-human antibody (Jackson). Where indicated, unopsonized hydrophilic or hydrophobic latex beads (1 or 3 μm; Sigma) or sheep red cells (MP Biomedicals) opsonized with rabbit anti-sheep IgG (1:50; ICN Biomedicals) were used. Bone-marrow-derived macrophages were incubated for 15 or 30 min at 37°C with the different types of beads or infected as previously described with *Mycobacterium avium* (de Chastellier et al., 1995) or *Brucella abortus* (Celli et al., 2003).

Labeling and Immunostaining

To label the plasma membrane, cells were incubated with 1 mg/ml of Alexa 555-conjugated B subunit of cholera toxin (Molecular Probes) for 2–5 min prior to phagocytosis. Alternatively, a 10 μM solution of FM4-64 (Molecular Probes) was present during phagocytosis. To label endosomes prior to phagocytosis, RAW cells were incubated with 2 μM FM4-64 in Hepes-buffered RPMI at 37°C for 15 min, followed by washing to remove residual plasmalemmal dye. Immunostaining of PDI was performed by incubating with a 1:500 dilution of monoclonal anti-PDI antibody (Stressgen), followed by Cy3-conjugated goat anti-mouse antibody (Jackson). Avidin-KDEL was detected using a 1:1000 dilution of a anti-avidin D antibody (Vector) followed by a Cy3-coupled anti-goat (Jackson). Fluorescence microscopy and image analysis were essentially as described (Marshall et al., 2001).

Frustrated Phagocytosis and TIR-FM

Detached RAW cells were allowed to spread onto IgG-opsonized glass coverslips and analyzed by TIR-FM using an Olympus micro-

scope IX-70 equipped with TIRF accessories. To monitor fusion of acidic organelles, macrophages were incubated with 5 μM acridine orange for 5 min and washed prior to deposition onto coverslips.

Immunogold Labeling, Immunocytochemistry, and EM

Preparation of RAW, J774 cells and murine DCs for immuno-EM, cryosectioning and immunolabeling was as described before (Folsch et al., 2001). Cells were fixed for 1 hr in 4% paraformaldehyde, followed by overnight fixation in 8% paraformaldehyde at 4°C. Human DCs were fixed with 2% paraformaldehyde and processed as described (Peters and Hunziker, 2001). Quantification of gold particles was according to Mayhew et al. (2002). Staining for G6Pase was performed as described by Griffiths et al. (1983) with the modifications of Celli et al. (2003).

Biochemical Assessment of ER-Phagosome Fusion

RAW cells stably expressing avidin-KDEL were plated onto 10 cm petri dishes and allowed to ingest biotin-coated polystyrene beads (3.0–3.9 μm) opsonized with 0.1 mg/ml IgG. Phagocytosis was terminated, and the cells were harvested with 1 ml of 0.25 M sucrose, 3 mM imidazole, (pH 7.4) and disrupted using a Dounce homogenizer. Unless otherwise indicated, excess biotin (10 mM) was present during and after homogenization to prevent binding of avidin released upon disruption of the ER. Phagosomes were isolated as described (Gagnon et al., 2002), permeabilized, and washed with 0.1% Triton X-100, and immunostained with anti-avidin followed by Alexa 488-conjugated secondary. Endosomes were loaded by fluid phase uptake of 1 mg/ml avidin for 20 min at 37°C.

Supplemental Data

Supplemental Data include seven figures, one table, Supplemental Experimental Procedures, and six movies and can be found with this article online at http://www.cell.com/cgi/content/full/123/1/157/DC1/.

Acknowledgments

We thank Dr. A. Kenworthy (Vanderbilt University, Nashville, Tennessee) for the YFP-GT46 cDNA; Dr. A.D. Schreiber (University of Pennsylvania, Pennsylvania) for the FcγRIIa-GFP cDNA, Dr. S. High (University of Manchester, Manchester, United Kingdom) for the Sec61-GFP cDNA, Dr. H. Ploegh (Harvard Medical School, Boston) for anti-PDI antibody, Drs. D. Williams (University of Toronto) and J. Bergeron (McGill University) for anti-calnexin antibodies. We thank Dr. M. Michalak (University of Alberta) for useful suggestions. N.T. is supported by the Heart and Stroke Foundation of Canada, P.P. by a Canadian Cystic Fibrosis Foundation studentship; M.T. by the Canadian Association of Gastroenterology, S.G. by the Canadian Institutes of Health Research; N.v.d.W. by the Netherlands Leprosy Relief and C.C. by the French Institut National de la Santé et de la Recherche Médicale (INSERM).

Received: April 8, 2005
Revised: June 29, 2005
Accepted: August 1, 2005
Published: October 6, 2005

References

Ackerman, A.L., Kyritsis, C., Tampe, R., and Cresswell, P. (2003). Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. Proc. Natl. Acad. Sci. USA *100*, 12889–12894.

Aniento, F., Gu, F., Parton, R.G., and Gruenberg, J. (1996). An endosomal beta COP is involved in the pH-dependent formation of transport vesicles destined for late endosomes. J. Cell Biol. *133*, 29–41.

Becker, T., Volchuk, A., and Rothman, J.E. (2005). Differential use of endoplasmic reticulum membrane for phagocytosis in J774 macrophages. Proc. Natl. Acad. Sci. USA *102*, 4022–4026.

Bellavite, P., Serra, M.C., Davoli, A., and Rossi, F. (1982). Selective enrichment of NADPH oxidase activity in phagosomes from guinea pig polymorphonuclear leukocytes. Inflammation *6*, 21–29.

Berton, G., Bellavite, P., de Nicola, G., Dri, P., and Rossi, F. (1982). Plasma membrane and phagosome localisation of the activated NADPH oxidase in elicited peritoneal macrophages of the guinea-pig. J. Pathol. *136*, 241–252.

Braun, V., Fraisier, V., Raposo, G., Hurbain, I., Sibarita, J.B., Chavrier, P., Galli, T., and Niedergang, F. (2004). TI-VAMP/VAMP7 is required for optimal phagocytosis of opsonised particles in macrophages. EMBO J. *23*, 4166–4176.

Brode, S., and Macary, P.A. (2004). Cross-presentation: dendritic cells and macrophages bite off more than they can chew! Immunology *112*, 345–351.

Celli, J., de Chastellier, C., Franchini, D.M., Pizarro-Cerda, J., Moreno, E., and Gorvel, J.P. (2003). Brucella evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J. Exp. Med. *198*, 545–556.

Chow, A., Toomre, D., Garrett, W., and Mellman, I. (2002). Dendritic cell maturation triggers retrograde MHC class II transport from lysosomes to the plasma membrane. Nature *418*, 988–994.

Cox, D., Tseng, C.C., Bjekic, G., and Greenberg, S. (1999). A requirement for phosphatidylinositol 3-kinase in pseudopod extension. J. Biol. Chem. *274*, 1240–1247.

Daro, E., Sheff, D., Gomez, M., Kreis, T., and Mellman, I. (1997). Inhibition of endosome function in CHO cells bearing a temperature-sensitive defect in the coatomer (COPI) component epsilon-COP. J. Cell Biol. *139*, 1747–1759.

de Chastellier, C., Lang, T., and Thilo, L. (1995). Phagocytic processing of the macrophage endoparasite, Mycobacterium avium, in comparison to phagosomes which contain Bacillus subtilis or latex beads. Eur. J. Cell Biol. *68*, 167–182.

de Chastellier, C., Ryter, A., and Thilo, L. (1983). Membrane shuttle between plasma membrane, phagosomes, and pinosomes in Dictyostelium discoideum amoeboid cells. Eur. J. Cell Biol. *30*, 233–243.

de Chastellier, C., and Thilo, L. (1997). Phagosome maturation and fusion with lysosomes in relation to surface property and size of the phagocytic particle. Eur. J. Cell Biol. *74*, 49–62.

de Chastellier, C., and Thilo, L. (2002). Pathogenic Mycobacterium avium remodels the phagosome membrane in macrophages within days after infection. Eur. J. Cell Biol. *81*, 17–25.

Dermine, J.F., Duclos, S., Garin, J., St-Louis, F., Rea, S., Parton, R.G., and Desjardins, M. (2001). Flotillin-1-enriched lipid raft domains accumulate on maturing phagosomes. J. Biol. Chem. *276*, 18507–18512.

Desjardins, M. (2003). ER-mediated phagocytosis: a new membrane for new functions. Nat. Rev. Immunol. *3*, 280–291.

Desjardins, M., Celis, J.E., van Meer, G., Dieplinger, H., Jahraus, A., Griffiths, G., and Huber, L.A. (1994a). Molecular characterization of phagosomes. J. Biol. Chem. *269*, 32194–32200.

Desjardins, M., Huber, L.A., Parton, R.G., and Griffiths, G. (1994b). Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. J. Cell Biol. *124*, 677–688.

Fix, M., Melia, T.J., Jaiswal, J.K., Rappoport, J.Z., You, D., Sollner, T.H., Rothman, J.E., and Simon, S.M. (2004). Imaging single membrane fusion events mediated by SNARE proteins. Proc. Natl. Acad. Sci. USA *101*, 7311–7316.

Folsch, H., Pypaert, M., Schu, P., and Mellman, I. (2001). Distribution and function of AP-1 clathrin adaptor complexes in polarized epithelial cells. J. Cell Biol. *152*, 595–606.

Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P.H., Steele-Mortimer, O., Paiement, J., Bergeron, J.J., and Desjardins, M. (2002). Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. Cell *110*, 119–131.

Garin, J., Diez, R., Kieffer, S., Dermine, J.F., Duclos, S., Gagnon, E., Sadoul, R., Rondeau, C., and Desjardins, M. (2001). The phagosome proteome: insight into phagosome functions. J. Cell Biol. *152*, 165–180.

Gotthardt, D., Warnatz, H.J., Henschel, O., Bruckert, F., Schleicher, M., and Soldati, T. (2002). High-resolution dissection of phagosome

maturation reveals distinct membrane trafficking phases. Mol. Biol. Cell *13*, 3508–3520.

Greenberg, S., and Grinstein, S. (2002). Phagocytosis and innate immunity. Curr. Opin. Immunol. *14*, 136–145.

Griffiths, G., Simons, K., Warren, G., and Tokuyasu, K.T. (1983). Immunoelectron microscopy using thin, frozen sections: application to studies of the intracellular transport of Semliki Forest virus spike glycoproteins. Methods Enzymol. *96*, 466–485.

Guermonprez, P., Saveanu, L., Kleijmeer, M., Davoust, J., Van Endert, P., and Amigorena, S. (2003). ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature *425*, 397–402.

Hackam, D.J., Rotstein, O.D., Sjolin, C., Schreiber, A.D., Trimble, W.S., and Grinstein, S. (1998). v-SNARE-dependent secretion is required for phagocytosis. Proc. Natl. Acad. Sci. USA *95*, 11691–11696.

Henry, R.M., Hoppe, A.D., Joshi, N., and Swanson, J.A. (2004). The uniformity of phagosome maturation in macrophages. J. Cell Biol. *164*, 185–194.

Holevinsky, K.O., and Nelson, D.J. (1998). Membrane capacitance changes associated with particle uptake during phagocytosis in macrophages. Biophys. J. *75*, 2577–2586.

Houde, M., Bertholet, S., Gagnon, E., Brunet, S., Goyette, G., Laplante, A., Princiotta, M.F., Thibault, P., Sacks, D., and Desjardins, M. (2003). Phagosomes are competent organelles for antigen cross-presentation. Nature *425*, 402–406.

Kenworthy, A.K., Nichols, B.J., Remmert, C.L., Hendrix, G.M., Kumar, M., Zimmerberg, J., and Lippincott-Schwartz, J. (2004). Dynamics of putative raft-associated proteins at the cell surface. J. Cell Biol. *165*, 735–746.

Kwiatkowska, K., Frey, J., and Sobota, A. (2003). Phosphorylation of FcgammaRIIA is required for the receptor-induced actin rearrangement and capping: the role of membrane rafts. J. Cell Sci. *116*, 537–550.

Lang, T., de Chastellier, C., Ryter, A., and Thilo, L. (1988). Endocytic membrane traffic with respect to phagosomes in macrophages infected with non-pathogenic bacteria: phagosomal membrane acquires the same composition as lysosomal membrane. Eur. J. Cell Biol. *46*, 39–50.

Lippincott-Schwartz, J., Altan-Bonnet, N., and Patterson, G.H. (2003). Photobleaching and photoactivation: following protein dynamics in living cells. Nat. Cell Biol. *46* (*Suppl*), S7–14.

Marshall, J.G., Booth, J.W., Stambolic, V., Mak, T., Balla, T., Schreiber, A.D., Meyer, T., and Grinstein, S. (2001). Restricted accumulation of phosphatidylinositol 3-kinase products in a plasmalemmal subdomain during Fc gamma receptor-mediated phagocytosis. J. Cell Biol. *153*, 1369–1380.

Matsubara, S., Yamada, T., Minakami, H., Watanabe, T., Takizawa, T., and Sato, I. (1999). Polymorphonuclear leukocytes in the fetal membranes are activated in patients with preterm delivery: ultrastructural and enzyme-histochemical evidence. Placenta *20*, 185–188.

Mayhew, T.M., Lucocq, J.M., and Griffiths, G. (2002). Relative labelling index: a novel stereological approach to test for non-random immunogold labelling of organelles and membranes on transmission electron microscopy thin sections. J. Microsc. *205*, 153–164.

Miesenbock, G., and Rothman, J.E. (1995). The capacity to retrieve escaped ER proteins extends to the trans-most cisterna of the Golgi stack. J. Cell Biol. *129*, 309–319.

Muller, W.A., Steinman, R.M., and Cohn, Z.A. (1980a). The membrane proteins of the vacuolar system I. Analysis of a novel method of intralysosomal iodination. J. Cell Biol. *86*, 292–303.

Muller, W.A., Steinman, R.M., and Cohn, Z.A. (1980b). The membrane proteins of the vacuolar system. II. Bidirectional flow between secondary lysosomes and plasma membrane. J. Cell Biol. *86*, 304–314.

Muller-Taubenberger, A., Lupas, A.N., Li, H., Ecke, M., Simmeth, E., and Gerisch, G. (2001). Calreticulin and calnexin in the endoplasmic reticulum are important for phagocytosis. EMBO J. *20*, 6772–6782.

Nehls, S., Snapp, E.L., Cole, N.B., Zaal, K.J., Kenworthy, A.K., Roberts, T.H., Ellenberg, J., Presley, J.F., Siggia, E., and Lippincott-Schwartz, J. (2000). Dynamics and retention of misfolded proteins in native ER membranes. Nat. Cell Biol. *2*, 288–295.

Neijssen, J., Herberts, C., Drijfhout, J.W., Reits, E., Janssen, L., and Neefjes, J. (2005). Cross-presentation by intercellular peptide transfer through gap junctions. Nature *434*, 83–88.

Nichols, B.A., Setzer, P.Y., and Bainton, D.F. (1984). Glucose 6-phosphatase as a cytochemical marker of endoplasmic reticulum in human leukocytes and platelets. J. Histochem. Cytochem. *32*, 165–171.

Niedergang, F., and Chavrier, P. (2004). Signaling and membrane dynamics during phagocytosis: many roads lead to the phagos (R)ome. Curr. Opin. Cell Biol. *16*, 422–428.

Ninomiya, N., Hazeki, K., Fukui, Y., Seya, T., Okada, T., Hazeki, O., and Ui, M. (1994). Involvement of phosphatidylinositol 3-kinase in Fc gamma receptor signaling. J. Biol. Chem. *269*, 22732–22737.

Paroutis, P., Touret, N., and Grinstein, S. (2004). The pH of the secretory pathway: measurement, determinants, and regulation. Physiology (Bethesda) *19*, 207–215.

Pelkmans, L., Kartenbeck, J., and Helenius, A. (2001). Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. Nat. Cell Biol. *3*, 473–483.

Peters, P.J., and Hunziker, W. (2001). Subcellular localization of Rab17 by cryo-immunogold electron microscopy in epithelial cells grown on polycarbonate filters. Methods Enzymol. *329*, 210–225.

Pitt, A., Mayorga, L.S., Stahl, P.D., and Schwartz, A.L. (1992). Alterations in the protein composition of maturing phagosomes. J. Clin. Invest. *90*, 1978–1983.

Sandvig, K., and van Deurs, B. (2002). Transport of protein toxins into cells: pathways used by ricin, cholera toxin and Shiga toxin. FEBS Lett. *529*, 49–53.

Silverstein, S.C. (1977). Endocytic uptake of particles by mononuclear phagocytes and the penetration of obligate intracellular parasites. Am. J. Trop. Med. Hyg. *26*, 161–169.

Silverstein, S.C., Steinman, R.M., and Cohn, Z.A. (1977). Endocytosis. Annu. Rev. Biochem. *46*, 669–722.

Steinman, R.M., Mellman, I.S., Muller, W.A., and Cohn, Z.A. (1983). Endocytosis and the recycling of plasma membrane. J. Cell Biol. *96*, 1–27.

Tjelle, T.E., Lovdal, T., and Berg, T. (2000). Phagosome dynamics and function. Bioessays *22*, 255–263.

Tsien, R.Y. (1998). The green fluorescent protein. Annu. Rev. Biochem. *67*, 509–544.

Ulsamer, A.G., Wright, P.L., Wetzel, M.G., and Korn, E.D. (1971). Plasma and phagosome membranes of Acanthamoeba castellanii. J. Cell Biol. *51*, 193–215.

Vieira, O.V., Botelho, R.J., and Grinstein, S. (2002). Phagosome maturation: aging gracefully. Biochem. J. *366*, 689–704.

Watts, C., and Amigorena, S. (2001). Phagocytosis and antigen presentation. Semin. Immunol. *13*, 373–379.

Whitney, J.A., Gomez, M., Sheff, D., Kreis, T.E., and Mellman, I. (1995). Cytoplasmic coat proteins involved in endosome function. Cell *83*, 703–713.

Wiertz, E.J., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T.R., Rapoport, T.A., and Ploegh, H.L. (1996). Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature *384*, 432–438.

Wu, M.M., Grabe, M., Adams, S., Tsien, R.Y., Moore, H.P., and Machen, T.E. (2001). Mechanisms of pH regulation in the regulated secretory pathway. J. Biol. Chem. *276*, 33027–33035.

Wu, M.M., Llopis, J., Adams, S., McCaffery, J.M., Kulomaa, M.S., Machen, T.E., Moore, H.P., and Tsien, R.Y. (2000). Organelle pH studies using targeted avidin and fluorescein-biotin. Chem. Biol. *7*, 197–209.