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The Unique Trafficking Pattern of *Salmonella typhimurium*-Containing Phagosomes in Murine Macrophages Is Independent of the Mechanism of Bacterial Entry

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Although it has been known for some time that *Salmonella typhimurium* is able to survive and even replicate in the normally bactericidal environment of the macrophage phagosome, the mechanisms by which this organism accomplishes this feat remain obscure. In this study, a murine macrophage cell line and confocal immunofluorescence microscopy were used to more thoroughly define the specific nature of phagosomes containing latex beads or wild-type *S. typhimurium* (viable or heat-killed organisms). Live *S. typhimurium* organisms were observed to reside in phagosomes that diverge from the degradative pathway of the macrophage. These compartments contain lysosomal glycoproteins and lysosomal acid phosphatase, endocytic markers delivered to vacuoles by mannose 6-phosphate receptor-independent mechanisms, but are devoid of the mannose 6-phosphate receptor and cathepsin L. In contrast, phagosomes containing latex beads or heat-killed organisms appeared to be processed along the degradative pathway of the host cell; these compartments colocalized not only with lysosomal glycoproteins and lysosomal acid phosphatases but also with mannose 6-phosphate receptors and cathepsin L. The uniqueness of the phagosome containing viable *S. typhimurium* was confirmed by the observation that these compartments, in comparison to phagosomes containing latex beads, do not readily interact with incoming endocytic traffic. Finally, we show that an isogenic, noninvasive mutant of *S. typhimurium*, BJ66, ends up in an intracellular compartment identical to the wild-type *S. typhimurium*-containing phagosome. Thus, modifications of the *Salmonella*-containing compartment occur independently of the mechanism of bacterial entry.

Macrophages play a critical role in a host's innate immune response. Residing in all tissues of the body, these cells protect the host by engulfing foreign bodies and sequestering them within phagosomes. Macrophages are equipped with several different mechanisms with which to destroy microorganisms within phagosomes, including toxic oxygen derivatives, reactive nitrogen intermediates, nutrient limitation, acidification of the compartment, and fusion of phagosomes with lysosomes that are rich in degradative enzymes (16). Despite this battery of defense mechanisms, several bacterial and parasitic species survive and even replicate within macrophage phagosomes. Pathogens that fall within this category include *Salmonella* spp., several mycobacteria, *Legionella pneumophila*, *Toxoplasma gondii*, *Coxiella burnetii*, *Chlamydia trachomatis*, *Leishmania* spp., and *Francisella tularensis*. These organisms have developed a variety of strategies by which to defend themselves from the assorted assaults of the macrophage. Several mycobacteria, *Legionella pneumophila*, *Chlamydia trachomatis*, and *T. gondii* reside in remodeled vacuoles which neither fuse with lysosomes nor acidify (7, 14, 15, 21, 27, 30–32, 34, 56–58, 60, 62). *Coxiella burnetti*, *Leishmania* spp., and *F. tularensis*, on the other hand, are capable of surviving in acidic phagolysosomes (2–4, 6, 8, 18, 27, 41, 50, 54).

Phagosomes destined to traffic along the degradative pathway of the host cell's endocytic network are thought to undergo a series of sequential biochemical modifications and vesicle fusion reactions (10, 17, 47, 48, 51, 52). In recent years, a number of markers specific for unique compartments within the endocytic pathway have been used to more thoroughly characterize the changes that occur during this process. As the phagosome matures, proteins which initially compose the newly formed compartment disappear and are replaced, first by proteins present in early endosomes, such as rab5, the transferrin receptor, and low-density lipoproteins, and later by proteins inherent to late endosomes/prelysosomes, including rab7, rab9, cation-independent (CI) or cation-dependent mannose 6-phosphate receptors (M6PRs), and lysosomal membrane glycoproteins (LAMPs). The rab proteins are small GTP-binding proteins that have been demonstrated to play a role in regulating membrane fusion events (9, 12, 24, 25), whereas the function of M6PRs is to deliver a subset of newly synthesized, soluble, lysosomal enzymes from the trans-Golgi network (TGN) to the prelysosomal compartments (37, 38). The role of LAMPs in vacuolar maturation is, at this time, still unclear. It has been suggested that these glycoproteins may protect vacuolar membranes from degradation by enzymes carried within the compartment. The prelysosome is transformed into a degradative compartment through the further incorporation and/or processing of lysosomal hydrolases such as cathepsins, lysosomal acid phosphatases (LAP), and β-glucuronidase.

Examination of the presence or absence of various endocytic markers in phagosomes containing replicating pathogens has substantially contributed to our current understanding of how and why organisms such as *Mycobacteria tuberculosis*, *Legionella pneumophila*, *Chlamydia trachomatis*, and *T. gondii* survive in this seemingly hostile environment (reviewed in refer-

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ence 23). Several laboratories have examined the attributes of *Salmonella*-containing phagosomes in macrophages, but, to date, a definitive characterization of these compartments has not emerged. Certain studies have shown that live *S. typhimurium* organisms, in contrast to heat- and UV-killed organisms or nonpathogenic *Escherichia coli*, inhibit phagosome-lysosome fusion (13, 33), while others investigations have indicated that *S. typhimurium*-containing vacuoles (STVs) acidify to a pH of 4.0 to 5.0 and are rich in some of the known lysosomal proteins (5, 53).

In this study, we examined the specific nature of *Salmonella*-containing phagosomes in RAW 264.7 macrophages, a murine macrophage cell line, and investigated whether the mechanism of bacterial entry plays a significant role in phagosome modification. Confocal immunofluorescence microscopy was used to characterize phagosomes containing wild-type *S. typhimurium* or the *S. typhimurium* mutant BJ66. BJ66, unlike the wild type, is unable to invade nonphagocytic cells and is believed to be taken up by macrophages via host cell-directed processes such as lectinophagocytosis (16) rather than through bacterium-induced membrane ruffling (35, 44). Our results indicate that viable *S. typhimurium* organisms, regardless of the mechanism of entry, reside in a unique compartment that clearly diverges from the degradative pathway of the macrophage host cell. In contrast, heat-killed bacteria and latex beads are unable to induce the formation of this specialized compartment and are instead processed through the normal endocytic degradation pathway.

MATERIALS AND METHODS

Reagents. The primary antibodies used were polyclonal rabbit anti-*S. typhimurium* (Denise Monack, Stanford University, Stanford, Calif.) (44), monoclonal mouse anti-*S. typhimurium* lipopolysaccharide (Bruce Stocker, Stanford University, Stanford, Calif.), monoclonal rat anti-LAMP 1 and affinity-purified rabbit anti-bovine liver CI-M6PR (David Russell, Washington University, St. Louis, Mo.) (54), goat anti-rat LAP (Masaru Himeno, Kyushu University, Fukuoka, Japan) (61), and affinity-purified rabbit anti-mouse cathepsin L (Gary Sahagian, Tufts University, Boston, Mass.) (49). The secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG), FITC-conjugated goat anti-mouse IgG, tetramethyl rhodamine isothiocyanate (TRITC)-conjugated rabbit anti-rat IgG, TRITC-conjugated goat anti-rabbit IgG, and TRITC-conjugated rabbit anti-goat IgG (all purchased from Sigma Chemical Co.). All antibody combinations used in this study were tested for cross-reactivity and found to have little or none. Diferric transferrin was prepared as previously described (63), except that 5 mg of transferrin was used in the reaction mixture. Rhodamine-conjugated transferrin was prepared by dissolving diferric transferrin in 100 ml of 50 mM sodium borate (pH 9.2) and dialyzing it against 10 mg of TRITC dissolved in the same buffer at room temperature. The solution was cooled to 4°C and stirred for 1 day in the dark. The dialysis bag was removed from the TRITC solution and dialyzed against six changes (15 liters each) of phosphate-buffered saline (PBS) at 4°C over the course of 3 days. Fixable Texas red dextran (molecular weight [MW] of 70,000) and TRITC dextran (MW of 10,000) were purchased from Molecular Probes.

Bacterial strains and growth conditions. The *S. typhimurium* strains used in this study were SL1344, a wild-type strain fully virulent for mice (29), and BJ66, an isogenic mutant strain of SL1344 containing an insertion in *orgA* (35). The bacteria were grown in Luria-Bertani broth (55) with aeration for 12 h, subcultured, and grown overnight as standing cultures at 37°C. These growth conditions have been found to provide optimal efficiency of *S. typhimurium* invasion into mammalian host cells (40). The bacteria were pelleted by centrifugation, resuspended in PBS, and used immediately. The LIVE/DEAD BacLight bacteria viability kit (Molecular Probes) was used to analyze bacterial viability. The initial inoculum was determined to be 96% viable. Heat-killed organisms were prepared by incubating bacteria at 65°C for 15 to 20 min. Opsonization of heat-killed organisms was accomplished by incubating the bacteria with normal mouse serum for 10 min prior to use.

Bacterial infection of murine macrophages. RAW 264.7 cells (ATCC T1B71), a murine macrophage-like cell line, were grown in Dulbecco minimal essential medium (DMEM) containing 10% fetal calf serum (FCS) and 1 mM glutamine. Bone marrow-derived macrophages were prepared by culturing bone marrow cells isolated from BALB/c mice femurs in DMEM containing 20% FCS, 10% L-cell-conditioned medium, 1 mM glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml for 5 days. Prior to infection, the medium was replaced with DMEM containing 10% FCS and 1 mM glutamine. Macrophages were seeded on coverslips in 24-well tissue culture dishes at approximately 3.0×10^5 cells/coverslip and incubated overnight in 5% carbon dioxide at 37°C. The cells were infected for 10 or 20 min with SL1344 or BJ66 at a multiplicity of infection (MOI) of 1 bacterium to one macrophage or 10 bacteria to one macrophage, respectively. Alternatively, fluorescent latex beads were added to the cells for 10 to 20 min (at a ratio of 10 to 1). Following infection, the macrophages were washed extensively with PBS to remove extracellular bacteria and incubated for 30 min or 10 h in medium containing gentamicin (100 μg/ml for the first 2 h and 10 μg/ml for the remainder of the experiment). Finally, the infected macrophages were washed with PBS and prepared for confocal immunofluorescence microscopy as described below.

Fluorescence actin staining assays of infected RAW 264.7 macrophages. Wild-type SL1344 or BJ66 was added to monolayers of RAW 264.7 macrophages at an MOI of 50:1. After the bacteria were centrifuged onto the monolayers, the cells were immediately washed with PBS, fixed, and permeabilized. Bacteria were labeled by incubating the fixed cells primarily with rabbit anti-*S. typhimurium* and secondarily with FITC-conjugated goat anti-rabbit IgG. Filamentous actin was labeled with rhodamine phalloidin as previously described (19). Fluorescence microscopy was used to assess bacterium-induced rearrangements of host cell actin.

Uptake of endocytic tracers by infected macrophages. Rhodamine-conjugated transferrin (5 μg/ml) was added to macrophages either during the infection period or at various times postinfection. The cells were incubated with the transferrin for 30 min, washed, and prepared for confocal immunofluorescence microscopy. Receptor-mediated uptake of rhodamine-conjugated transferrin was confirmed by out-competing the uptake of the labeled endocytic tracer with a 10-fold excess of unlabeled ligand. Texas red dextran (2 μg/ml) was added to macrophages that had been infected for 30 min or 10 h. The cells were incubated for 30 min, washed, and incubated an additional 0 to 1.5 h at 37°C. Finally, the samples were prepared for confocal immunofluorescence microscopy.

Confocal immunofluorescence microscopy. Infected cells were fixed for 10 min with 4% formaldehyde in PBS, permeabilized for 10 min with 0.1% saponin in PBS, and then blocked with a solution of 0.1% saponin in PBS containing 2% bovine serum albumin (BSA). Next, the cells were incubated for 30 to 60 min with primary antibodies and then with secondary antibodies diluted in the saponin-BSA-PBS solution. Finally, the coverslips were washed and mounted in 90% glycerol in PBS containing 1 mg of phenylenediamine per ml. The samples were analyzed with a Bio-Rad (Hercules, Calif.) model MRC1000 laser confocal microscope, and images of the fluorescein (488-nm excitation and 510-nm emission) and rhodamine or Texas red (568-nm excitation; 590-nm emission) fluorescence were collected by using Bio-Rad COMOS software. Bio-Rad Confocal Assistant software was utilized to format and then merge the green and red images. Finally, the percentage of bacterium- or bead-containing phagosomes that colocalized with a given marker was calculated by analyzing 50 phagosomes from at least five random fields per time point. The percentages are based on the numbers derived from two or three experiments.

RESULTS

Phagosomes containing wild-type *S. typhimurium* diverge from the macrophage degradative endocytic pathway. We began our studies by comparing the intracellular trafficking pattern of live *S. typhimurium* with that of latex beads. Bacteria or latex beads were added to RAW 264.7 macrophages for 20 min, washed extensively, and then incubated with gentamicin to kill extracellular bacteria. We wished to examine phagosomal characteristics shortly after the compartment had formed and well after the phagosome had trafficked through the host cell's endocytic pathway. To this end, the cells were incubated for 30 min or 10 h before immunofluorescence microscopy. The samples were probed with antibodies specific for one of the following prelysosomal or lysosomal markers: CI-M6PR, LAMP 1, LAP, or cathepsin L. The role of CI-M6PRs is to deliver a subset of lysosomal enzymes from the TGN to late endocytic compartments; cathepsin L falls within this subset of M6PR-dependent enzymes. LAMP 1 and LAP, on the other hand, are delivered to endocytic compartments independent of M6PR. Colocalization of CI-M6PR, LAMP 1, LAP, or cathepsin L with phagosomes containing either *S. typhimurium* or latex beads was assessed by confocal microscopy.

Our results indicate that latex beads, as expected, progress along the macrophage's degradative endocytic pathway. At 30 min postinfection, only a small percentage of bead-containing phagosomes colocalized with any of the markers tested, but by 10 h postinfection the majority of these phagosomes were

TABLE 1. Percentage of phagosomes that colocalize with a given marker

Marker	% Colocalization at the indicated time postinfection in cells containing[a]:					
	Latex beads		*S. typhimurium* SL1344		*S. typhimurium* BJ66	
	30 min	10 h	30 min	10 h	30 min	10 h
Man-6PR	4 ± 2	82 ± 3	16 ± 1	24 ± 4	20 ± 4	22 ± 4
LAMP 1	22 ± 2	90 ± 5	97 ± 3	90 ± 5	84 ± 4	89 ± 3
LAP	±	++	++	++	++	++
Cathepsin L	11 ± 3	83 ± 2	17 ± 2	24 ± 4	ND	33 ± 6

[a] The results are means ± standard deviations in percentages except for LAP, the results for which are expressed as the degree of colocalization (++, strong; ±, weak).

positive for M6PR, LAMP 1, LAP, and cathepsin L (Table 1; Fig. 1E to H). Thus, ligands delivered by both M6PR-dependent and -independent mechanisms could be detected in over 80% of mature bead-containing compartments. In sharp contrast, phagosomes containing wild-type *S. typhimurium* dramatically diverged from this trafficking pattern (Table 1). To begin with, LAMP 1 and LAP were detected in over 90% of bacterium-containing phagosomes at 30 min postinfection (Fig. 1A and B and Fig. 2A and B). Only 22% of phagosomes containing latex beads were positive for these same markers at the corresponding time point, indicating that the LAMP 1 and LAP were delivered to these two types of compartments at significantly different rates. Moreover, the majority of STVs differed from bead-containing phagosomes in that STVs, although positive for LAMP 1 and LAP, did not acquire M6PR at any time analyzed (Fig. 3A). Correspondingly, cathepsin L could be detected in, at most, only 24% of the bacterium-laden phagosomes, even at 10 h postinfection (Fig. 3E). These findings indicate that the majority of STVs circumvent trafficking along the macrophage's degradative pathway but are, nevertheless, accessible to the delivery of LAMP 1 and LAP.

The experiments described above were performed with RAW 264.7 cells, an established macrophage cell line. The recent findings of Oh et. al. have suggested that in primary macrophages, phagosomes containing *S. typhimurium* undergo rapid and complete fusion with macrophage lysosomes (45). Hence, it seemed possible that the type and possibly the activation state of the infected macrophages could confer different STV trafficking patterns. In order to test this possibility, bone marrow-derived macrophages were collected and infected with live *S. typhimurium*. Colocalization of STVs with LAMP 1, M6PR, and cathepsin L was subsequently examined. Each experiment was conducted in triplicate and repeated a total of three times. Analysis of infected bone marrow-derived macrophages indicated, once again, that *S. typhimurium* resides in a modified compartment; approximately 65% of STVs colocalized with LAMP 1 by 30 min postinfection, but the majority of phagosomes (greater than 70%) acquired neither M6PR nor cathepsin L, even at 10 h postinfection (data not shown). Thus, the type of macrophage being used did not appear to dramatically affect the trafficking pattern of *Salmonella*-containing phagosomes. In all further experiments, we therefore confined our observations to the RAW 264.7 macrophage cell line.

***S. typhimurium*-containing phagosomes do not readily interact with incoming endocytic traffic.** To assess the interaction of phagosomes containing either the latex beads or *S. typhimurium* with incoming endocytic traffic, RAW 264.7 macrophages were infected for 30 min or 10 h and then incubated with rhodamine-labeled transferrin or fixable Texas red dextran for 30 min.

Transferrin has been shown to be delivered to early endosomes via the transferrin receptor. The ligand dissociates from the receptor as the endosome acidifies and, in contrast with ligands that are delivered to lysosomes, recycles to the plasma membrane where it is released to the extracellular milieu (36, 39). As shown in Table 1, 62% of phagosomes containing latex beads readily fused with transferrin at early time points (Fig. 4B) and, as expected, became fusion incompetent as they progressed along the endocytic pathway. In comparison, STVs were, for the most part, inaccessible to incoming transferrin at early or late time points. We were able to detect transferrin in only 14% of STVs at the 30-min time point (Fig. 4A). It was formally possible that STVs transiently pass through an early endosome stage in which they are competent for fusion with transferrin and that, by assaying them at 30 min postinfection, we had overlooked this stage. To test this possibility, we performed two assays. In the first, macrophages were infected in the presence of the labeled transferrin, and in the second the cells were infected for 10 min, washed, and immediately incubated with the marker. Following 10, 20, or 30 min of incubation, the cells were assayed for colocalization of phagosomes with the labeled transferrin. We did not detect significant levels of transferrin within the bacterium-containing phagosomes with either of these protocols (data not shown), confirming the observation that STVs do not interact with the early endosome recycling pathway.

In contrast to transferrin, dextran is taken up by host cells through fluid-phase endocytosis. This marker segregates from early recycling endosomes, progresses along the cell's endocytic pathway, and is finally degraded in lysosomes (28, 52). The dextran trafficking results were similar to those obtained with transferrin in that phagosomes containing latex beads interacted with endocytosed markers but STVs remained inaccessible to incoming endocytic traffic (Table 2). At early time points postinfection, dextran was observed to traffic into 80% of phagosomes containing latex beads (Fig. 4D). At 10 h postinfection, only 9% of these phagosomes were accessible to this marker (Fig. 4F). This latter finding was surprising in that the endocytic marker profile of latex bead-containing phagosomes (see above) indicates that these compartments are lysosomal-like at this late time point. Thus, we expected a portion of the dextran routed along the degradative pathway to also end up in this compartment. It was possible that the 30-min dextran incubation period was not sufficient to allow for complete trafficking of the marker through the endocytic pathway into terminal degradative compartments. We therefore modified the experiment in that the infected cells were incubated with the dextran for 30 min, washed, and then placed at 37°C for an additional 1.5 h to allow for more complete trafficking of the marker. With this protocol, the percentage of the vacuoles containing latex beads colocalizing with dextran increased to 54% (Fig. 4H). Thus, by 10 h postinfection, vacuoles containing latex beads had trafficked through the early compartments of the endocytic pathway. Incoming dextran, in turn, required longer periods of time (several hours versus 30 min) to reach these terminal compartments.

Analysis of STV colocalization with internalized dextran indicated, once again, that phagosomes containing *S. typhimurium* do not readily interact with endocytic traffic (Table 2). Dextran could be detected in only 2% of STVs at 30 min postinfection (Fig. 4C). Bacterium-containing phagosomes analyzed at 10 h postinfection were only slightly more accessible to the marker in that approximately 8% of the STVs fused with incoming dextran (Fig. 4E). This percentage did not signifi-

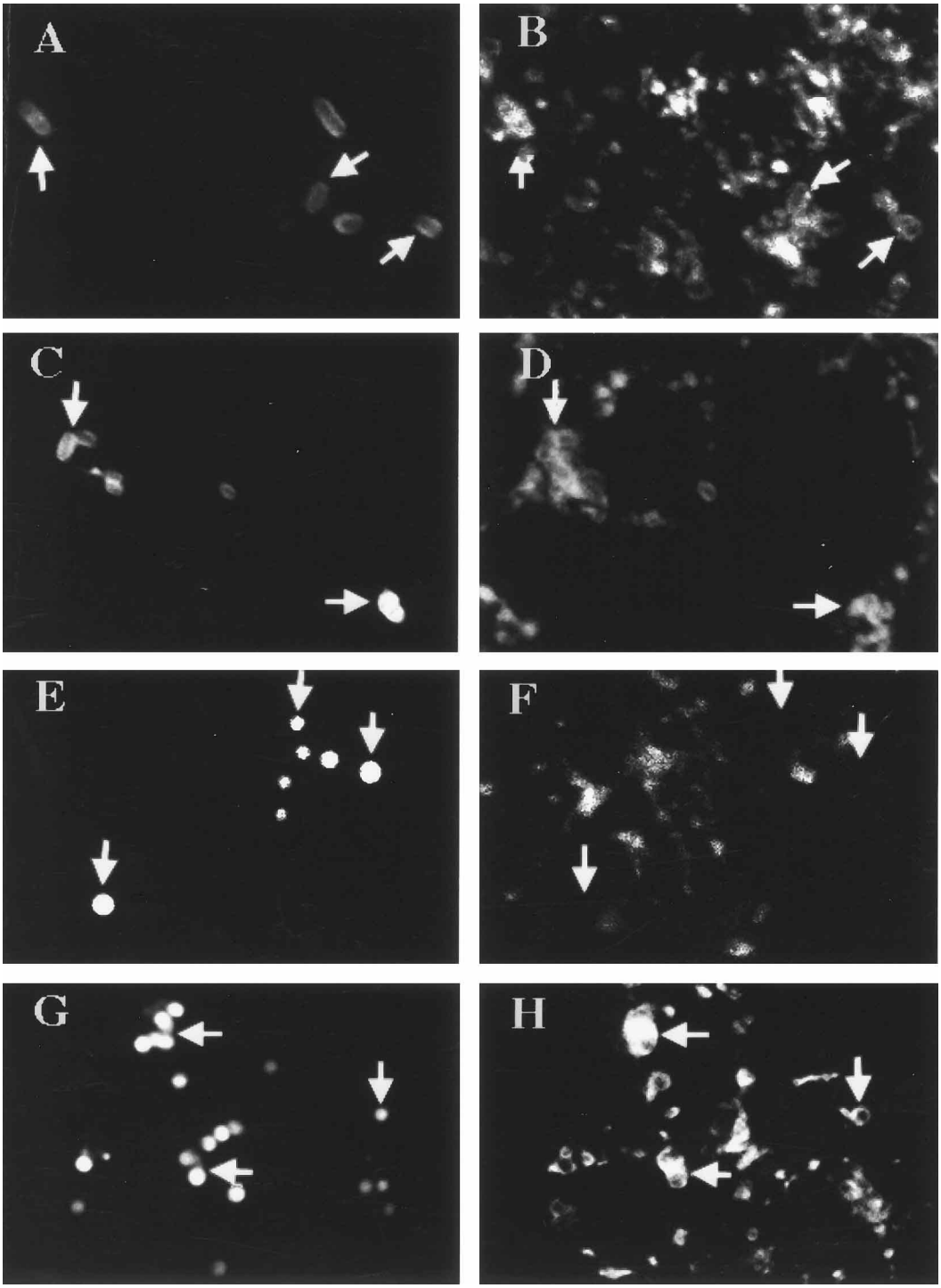

FIG. 1. STVs colocalize with LAMP 1 as early as 30 min postinfection. Infected RAW 264.7 macrophages were fixed, permeabilized, and blocked with BSA solution. The samples were incubated first with rabbit anti-*S. typhimurium* and rat anti-LAMP 1, followed by FITC-conjugated goat anti-rabbit IgG and, finally, TRITC-conjugated rabbit anti-rat IgG. Each antibody incubation lasted 60 min and was followed by several washes. (A and B) Labeled intracellular wild-type *S. typhimurium* at 30 min postinfection and distribution of LAMP 1 in the same cell, respectively. Arrows mark colocalization of bacteria with LAMP 1. (C and D) Labeled intracellular BJ66 at 30 min postinfection and distribution of LAMP 1 in the same cell, respectively. Arrows mark colocalization of BJ66 with LAMP 1. (E and F) Fluorescent latex beads at 30 min postinfection and distribution of LAMP 1 in the same cell, respectively. Arrows mark the location of the beads within the cells. Colocalization of the marker and beads is not detectable. (G and H) Fluorescent latex beads at 10 h postinfection and distribution of LAMP 1 in the same cell. Colocalization of fluorescent beads with LAMP 1 is detectable at late time points.

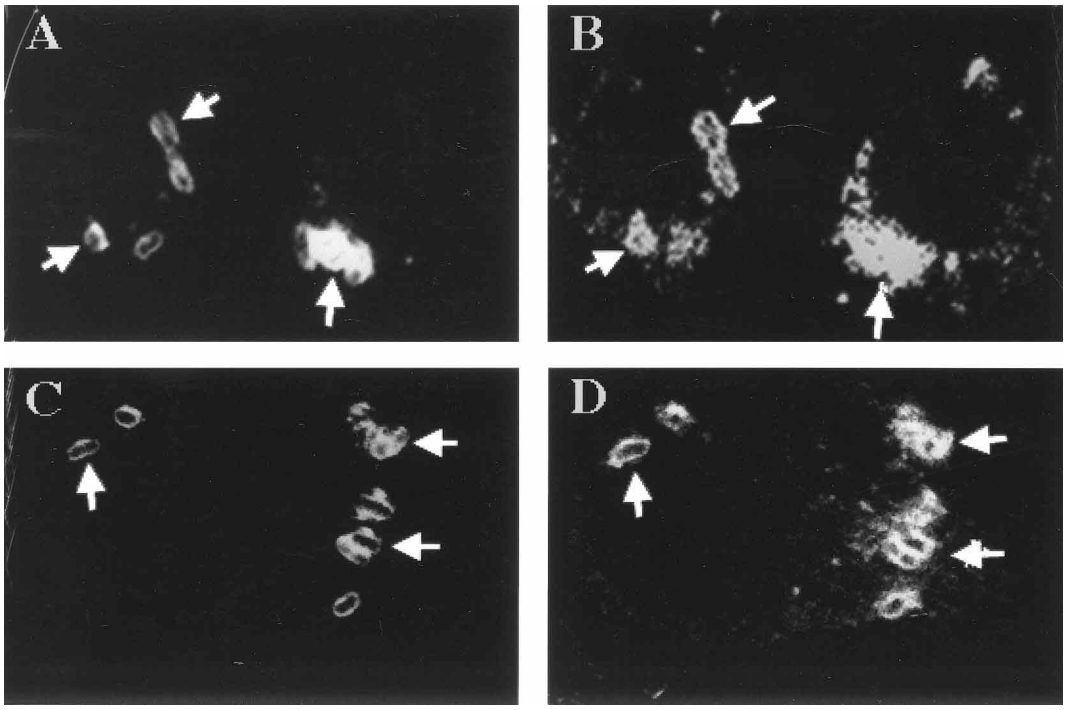

FIG. 2. STVs colocalize with LAP as early as 30 min postinfection. Infected RAW 264.7 macrophages were fixed, permeabilized, and blocked with BSA solution. The samples were incubated first with mouse anti-*S. typhimurium* LPS and goat anti-LAP, followed by TRITC-conjugated rabbit anti-goat IgG and, finally, FITC-conjugated goat anti-mouse IgG. Each antibody incubation lasted 60 min and was followed by several washes. (A and B) Labeled intracellular wild-type *S. typhimurium* at 30 min postinfection and distribution of LAP in the same cell, respectively. Arrows mark colocalization of bacteria with LAP. (C and D) Labeled intracellular BJ66 at 30 min postinfection and distribution of LAP in the same cell, respectively. Arrows mark colocalization of BJ66 with LAP.

cantly increase when the dextran was chased through the endocytic pathway for an additional 1.5 h as described above (Fig. 4G).

Recent studies have indicated that movement of fluid-phase solute probes through endocytic compartments may vary according to the size of the probe and that uniform mixtures of fluorescent dextrans with different sizes segregate by size into distinct lysosomes (11). We therefore tested whether the observed lack of fusion of STVs with endocytosed dextran was related to the size of the probe being used in these studies (MW of 70,000). RAW 264.7 macrophages infected with *S. typhimurium* were incubated in medium containing a low-MW fluorescent dextran (MW of 10,000), and colocalization of the dextran with STVs was quantitated according to the procedures described above. Less than 15% of *S. typhimurium*-containing phagosomes fused with the incoming, low-MW fluorescent dextran at any time point analyzed (data not shown), indicating that STV fusion with incoming dextran does not vary with the size of the dextran being used.

The method of entry does not influence the intracellular trafficking pattern of *S. typhimurium*. Our data indicate that the majority of phagosomes containing *S. typhimurium* circumvent trafficking along the macrophage's degradative pathway and, although accessible to the delivery of LAMP 1 and LAP, do not readily interact with other compartments of the endocytic pathway including incoming traffic. The question of how this organism selectively interacts with some endocytic compartments but avoids fusion with others arises. We wanted to investigate whether the route of bacterial entry into the macrophage is responsible for the unique trafficking pattern of STVs. To that end, we compared the trafficking pattern of wild-type *S. typhimurium* SL1344 with that of a noninvasive *S. typhimurium* mutant, BJ66. Wild-type organisms have been shown to enter host cells by a ruffling mechanism (20, 46). BJ66 is unable to induce its uptake into human epithelial cells (35) and is taken up by murine macrophages 6 to 10 times less efficiently than the wild type (44). Furthermore, uptake of BJ66 by RAW 264.7 macrophages is not accompanied by host cell membrane ruffling, as demonstrated in Fig. 5. Thus, BJ66 is thought to be internalized by macrophages through host cell-directed mechanisms, such as lectinophagocytosis, as opposed to pathogen-induced entry mechanisms.

Analysis of the endocytic marker profile of phagosomes containing BJ66 indicated that these phagosomes traffic through the host cell in the same manner as those containing wild-type organisms (Table 1). At 30 min postinfection, 84% of phagosomes containing the mutant *S. typhimurium* colocalized with LAMP 1 (Fig. 1C and D) and were positive for LAP (Fig. 2C and D), whereas M6PR could be detected in only 20% of these compartments. At 10 h postinfection the percentage of M6PR-positive BJ66-containing phagosomes did not significantly increase (Fig. 3B). Finally, the majority of phagosomes containing BJ66 did not colocalize with cathepsin L (Fig. 3F) and remained largely inaccessible to incoming transferrin or dextran (Table 2) for the duration of the experiment. These results suggest that the membrane ruffling mechanism of entry which enables *S. typhimurium* to invade epithelial cells and increases bacterial uptake into macrophages does not play an obvious role in determining the subsequent intracellular fate of this pathogen.

Bacterial viability is essential for modification of the *Salmonella*-containing phagosome. The goal of our final set of experiments was to investigate whether viability of the bacteria influenced the intracellular trafficking pattern of STVs. Mac-

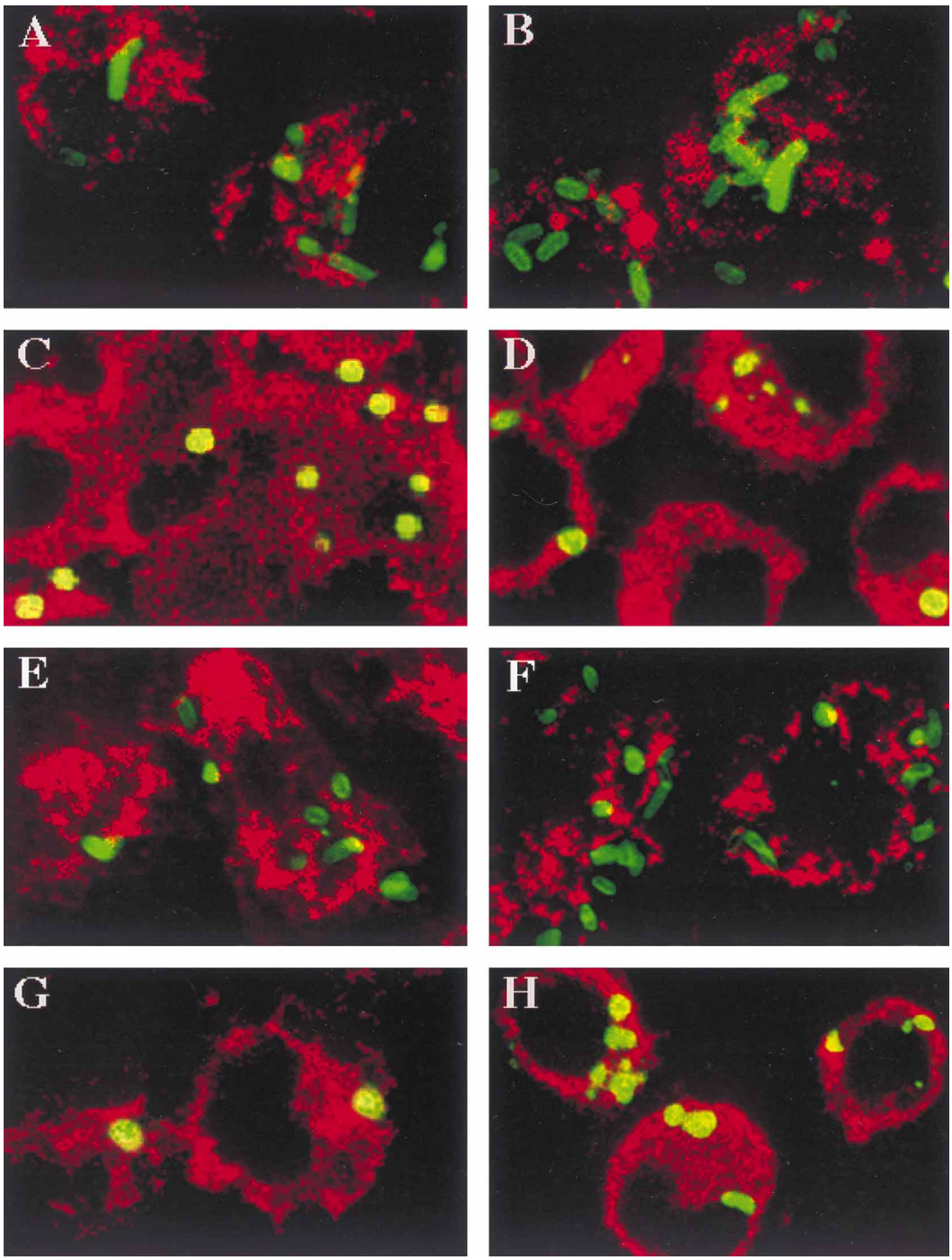

FIG. 3. Colocalization of latex beads and *S. typhimurium* with CI-M6PR and cathepsin L. Infected RAW 264.7 macrophages were fixed, permeabilized, and blocked with BSA solution. (A to D) The samples were incubated with mouse anti-*S. typhimurium* LPS and rabbit anti-CI-M6PR for 60 min, washed, and then incubated with TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG for an additional 60 min. (E to H) The samples were incubated with rabbit anti-cathepsin L and mouse anti-*S. typhimurium* LPS for 60 min, washed, and incubated with TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG for an additional 60 min. Viable *S. typhimurium* organisms, wild type (A and E) or mutant BJ66 (B and F), do not colocalize with M6PR (A and B) or cathepsin L (E and F) at 10 h postinfection. In contrast, latex beads (C and G) and heat-killed bacteria (D and H) do reside in compartments that are positive for M6PR (C and D) and cathepsin L (G and H) at late time points. Fluorescent latex beads and FITC-labeled bacteria that colocalize with M6PR or cathepsin L appear more yellow than green.

rophages were infected with opsonized, heat-killed organisms, and colocalization of the phagosomes with the various endocytic markers was examined. Not surprisingly, phagosomes containing heat-killed organisms appeared to progress along the degradative pathway. In fact, very few intact bacteria could be detected 10 h after infection. For this reason, colocalization of phagosomes containing heat-killed organisms with endocytic markers was quantitated at 30 min and 2 h postinfection.

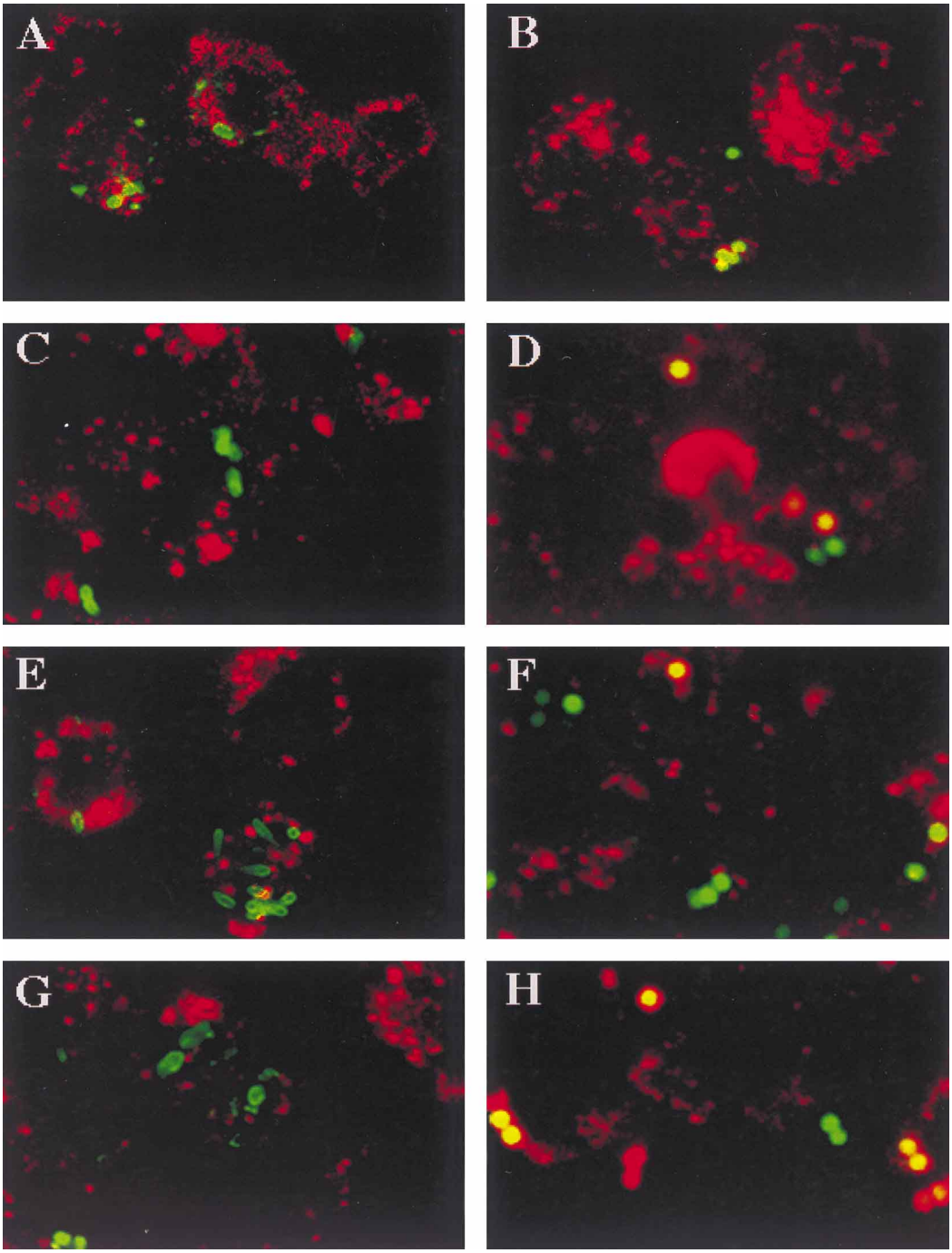

FIG. 4. Colocalization of latex beads and *S. typhimurium* with incoming endocytic traffic. (A and B) RAW 264.7 macrophages were infected, placed at 37°C for 30 min, and then incubated with rhodamine-labeled transferrin for an additional 30 min. All samples were fixed. Cells infected with *S. typhimurium* were subsequently permeabilized, incubated with rabbit anti-*S. typhimurium* for 30 min, washed, and incubated with FITC-conjugated goat anti-rabbit IgG for an additional 30 min. Phagosomes containing viable, wild-type *S. typhimurium* (A) do not interact with incoming transferrin at 30 min postinfection, whereas the majority of bead-containing phagosomes (B) do contain the ligand at this same time point. Colocalized beads appear yellow. (C to H) Macrophages were infected, placed at 37°C for either 30 min or 10 h, and then incubated with Texas red dextran for 30 min (B to E) or 2 h (F and G). At 30 min postinfection, phagosomes containing the viable wild-type *S. typhimurium* (C) do not interact with incoming dextran whereas the marker can be detected in phagosomes containing latex beads (D). At 10 h postinfection, neither STVs (E) nor phagosomes containing latex beads (F) are readily accessible to dextran that is chased into the cell for 30 min. If the marker is allowed 2 h to traffic through the endocytic pathway of the host cell, the dextran does traffic into the bead-containing phagosomes (H) whereas STVs (G) remain inaccessible to the incoming traffic. Colocalized beads appear yellow.

As early as 30 min postinfection, 74% ± 2% (mean ± standard deviation) of the phagosomes were positive for LAMP 1 and 72 ± 5% colocalized with cathepsin L. By 2 h postinfection, these numbers increased to 89 ± 5% and 82 ± 4%, respectively. Acquisition of M6PR (Fig. 3D) corresponded with the observed increase in cathepsin L colocalization (Fig. 3H). Thus, in contrast to that observed for viable organisms, cathepsin L was being delivered to phagosomes containing heat-killed

TABLE 2. Percentage of phagosomes that fuse with incoming endocytic traffic

Labeled marker	% Colocalization at indicated time postinfection in cells containing[a]:					
	Latex beads		*S. typhimurium* SL1344		*S. typhimurium* BJ66	
	30 min	10 h	30 min	10 h	30 min	10 h
Transferrin	62 ± 5	8 ± 4	14 ± 3	24 ± 2	20 ± 7	24 ± 4
Dextran	80 ± 5	9 ± 3 (54 ± 6)	3 ± 1	8 ± 3 (16 ± 4)	15 ± 4	9 ± 3 (16 ± 5)

[a] At 30 min or 10 h postinfection, macrophages were incubated with the labeled marker for 30 min and then analyzed immediately. Numbers in parentheses are the results from experiments in which infected cells were incubated with the labeled marker for 30 min, washed, and then incubated for an additional 1.5 h before analysis.

S. typhimurium. To ensure that the degradative trafficking pattern of heat-killed organisms was not a result of opsonization, parallel experiments in which unopsonized, heat-killed bacteria were added to the macrophages were conducted. Although fewer bacteria were internalized, the observed trafficking pattern was the same as that observed for serum-coated, nonviable organisms, verifying that lack of bacterial viability rather than opsonization was responsible for the phagosomal processing pattern (data not shown). These results clearly indicate that bacterial viability is essential for the unique trafficking pattern of phagosomes containing *S. typhimurium.*

DISCUSSION

The present study provides evidence that phagosomes containing wild-type *S. typhimurium* diverge from the degradative pathway of the macrophage host cell. This conclusion is based on an analysis of the endocytic trafficking patterns of phagosomes containing latex beads, heat-killed organisms, or viable *S. typhimurium*. Phagosomes containing latex beads and heat-killed organisms exhibit a trafficking pattern that is suggestive of fusion with degradative lysosomal compartments. These phagosomes become positive for not only LAMP 1 and LAP but also CI-M6PR and cathepsin L. Historically, mature lysosomal compartments have been operationally defined as LAMP-containing, M6PR-negative structures (26). M6PRs are believed to dissociate from their ligands in a prelysosomal compartment and subsequently to recycle to the TGN. This historical definition of mature lysosomal compartments is based on immunocytochemical analyses of uninfected eucaryotic cells. Our results indicate that phagosomes containing latex beads continue to stain positive for M6PR even at 10 postinfection. One possible conclusion from this data is that phagosomes containing latex beads are detained in a prelysosomal compartment. It is formally possible that the shear bulk of a latex bead prevents the compartment from being fully processed by the macrophage. Alternatively, we suggest that mature degradative compartments are not necessarily devoid

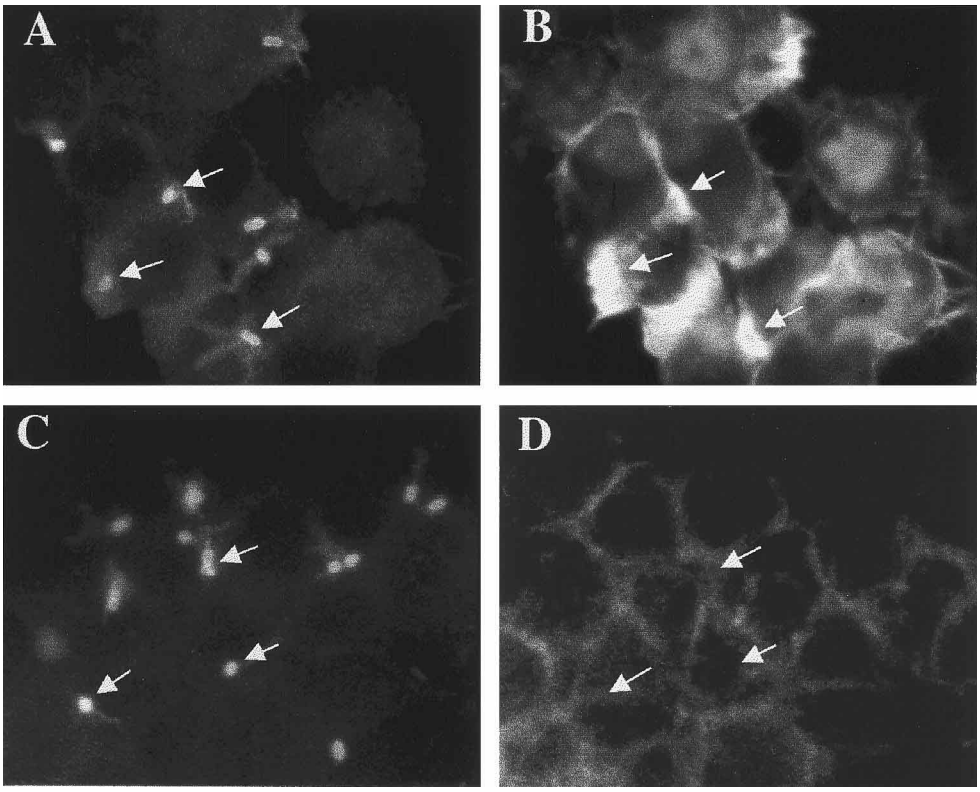

FIG. 5. Fluorescence actin staining assay of RAW 264.7 macrophages infected with wild-type SL1344 (A and B) or the isogenic mutant BJ66 (C and D). Representative images of labeled bacteria (A and C) and rhodamine-phalloidin staining for filamentous actin (B and D) are shown. Arrows mark the location of specific bacteria within the infected cells. Unlike wild-type SL1344, BJ66 does not induce polymerization of actin filaments during entry into the host cell.

of M6PRs. Support for this hypothesis comes from several other trafficking studies. Racoosin and Swanson have shown that the levels of CI-M6PR staining of maturing micropinosomes continue to rise even as the compartments fuse with prelabeled lysosomes (52). Additional studies by Pitt et al. have indicated that mature phagosomes containing antibody-coated *Staphylococcus aureus* maintain high levels of M6PR even as they continue to acquire LAMP-1, β-glucuronidase, and cathepsin D (48). Finally, our own results indicate that at 2 h postinfection, 84% of phagosomes containing heat-killed organisms stain positive for M6PR (data not shown) yet appear to be lysosomal like (i.e., contain active degradative enzymes) since the nonviable bacteria are clearly being digested. Thus, we propose that phagolysosomes do not recycle the M6PR as efficiently their vacuolar counterparts, perhaps, in part, due to the size of the ingested particle.

In contrast to phagosomes containing latex beads or heat-killed organisms, vacuoles containing viable bacteria colocalize only with those markers delivered by M6PR-independent mechanisms, LAMP 1 and LAP. These molecules can be detected in STVs as early as 30 min postinfection. Ligands delivered through M6PR-dependent mechanisms, such as cathepsin L, and the CI-M6PR itself cannot be detected in the majority of STVs even at 10 h postinfection. Thus, viable *S. typhimurium* organisms, in contrast to heat-killed organisms and latex beads, divert degradative processing of the phagosome within which they reside in murine macrophages. Although the majority of STVs do appear to adhere to this modified pattern of phagosome trafficking within the macrophage, a certain degree of heterogeneity is evident. By 10 h postinfection, macrophages are filled with replicating bacteria; nevertheless, approximately 25% of the STVs analyzed at this time point colocalize with cathepsin. This 25% could represent vacuoles that have, for whatever reason, successfully trafficked along the host cell's degradative pathway. On the other hand, recent work by Sturgill-Koxzycki et al. indicates that *Mycobacterium*-containing phagosomes acquire cathepsin D with kinetics that resemble the acquisition of LAMP 1 but that the cathepsin D remains in an immature form (59). Whether the cathepsin staining of 25% of the *Salmonella*-containing phagosomes is indeed indicative of actual fusion with mature lysosomes or merely the incorporation of an immature form of the enzyme cannot be determined by confocal microscopy. An answer to this question awaits the isolation of bacterium-containing phagosomes for Western blot analysis.

The immunofluorescence microscopy observations presented in this study and the subsequent characterization of the endocytic position of the STV in the macrophage host cell extend the findings of two other laboratories that have previously reported the inhibition of macrophage phagosome-lysosome fusion by *S. typhimurium*. Experiments conducted by Ishibashi and Arai indicate that viable *S. typhimurium* organisms, in contrast to UV-killed organisms or organisms treated with bacterial protein synthesis inhibitors, resist fusion with lysosomes preloaded with acridine orange or sulforhodamine (33). Various lipopolysaccharide (LPS) mutants of *S. typhimurium* were shown to block lysosomal fusion as efficiently as their wild-type counterparts. Buchmeier and Heffron similarly demonstrated that viable *S. typhimurium* organisms, in comparison to heat-killed organisms and avirulent *E. coli*, fail to fuse with secondary lysosomes preloaded with thorium dioxide (13). These investigators verified that inhibition of fusion is not affected by opsonization of the organisms with normal mouse serum and confirmed the observation that a complete LPS molecule is not essential for this unique trafficking pattern of viable *S. typhimurium.*

The findings reported here are also in agreement with the observations of Garcia-del Portillo and Finlay, who have defined the trafficking pattern of STVs in epithelial cells (22). These investigators have shown that *Salmonella*-containing vacuoles, although positive for LAMPs, bypass compartments containing M6PRs, even when the host cells are treated with lysosomotrophic, weak bases which block transit of M6PRs from late endosomes to the TGN. They have further confirmed that *Salmonella*-containing vacuoles are functionally separated from the normal endocytic pathway of the epithelial cell and that these compartments avoid fusion with lysosomes.

The conclusions derived from the studies described above as well as our own converge on the central theme that live *S. typhimurium* is capable of diverting the endocytic processing of the epithelial or macrophage compartment in which it resides. In contrast, the recent findings of Oh et al. suggest that *S. typhimurium*-containing phagosomes undergo rapid and complete fusion with macrophages. We examined the possibility that the observed differences in this latter work and our own might be due to the fact that our experiments were conducted with a cultivated macrophage cell line whereas Oh and coworkers used primary macrophages. There are striking differences between such cells; primary macrophages, for example, are far more susceptible to *Salmonella*-induced apoptosis and up to 80% of these cells are killed within 3 h after bacterial entry (44). It was this reason, in part, that led us to employ a cultivated cell line which suffers only about 20% programmed cell death following *Salmonella* entry. Nevertheless, our results indicate the phagosomes containing *S. typhimurium* follow essentially similar trafficking patterns within primary or cultivated macrophages. It is possible that the use of different technical procedures may be responsible for the observed experimental discrepancies between the work reported here and that of Oh et al. Recent evidence suggests that different methods of fixation and permeabilization, especially protocols which include an alcohol fixation step, can produce conflicting immunofluorescence colocalization results (42). Unlike Oh et al., we do not rely on a methanol extraction step during preparation of samples but feel confident that this omission does not prohibit one from detecting colocalization of phagosomes with markers such as cathepsin L, since we were able to clearly detect this marker in phagosomes containing heat-killed bacteria or latex beads. We have also used a different means of analyzing and quantitating colocalization events. Whereas Oh et al. relied on fluorescence microscopy for their analysis, we have completed our studies using immunofluorescence confocal microscopy. As noted earlier, we found about 25% of intracellular *S. typhimurium* organisms within compartments that stain positive for cathepsin or M6PR. This finding could represent the presence of two populations of *Salmonella* within macrophages, as postulated by Abshire and Neidhardt (1), in which one population follows the expected degradative pathway of lysosomal fusion while the other represents the end result of a *Salmonella* strategy for intracellular replication and survival. We did not expect *Salmonella* intracellular persistence to be an all-or-none phenomenon. In any event, the work reported here and by others (13, 22, 33) is consistent with the view that a substantial majority of intracellular *Salmonella* organisms reside in a unique cellular compartment that does not fuse with functional lysosomes. Our studies have further confirmed that the capacity to influence the outcome of intracellular trafficking of the phagosomal compartment is dependent on the viability of the salmonellae at the onset of infection.

Very little is known about how microorganisms orchestrate the remodeling of phagosomal compartments to prevent fusion with lysosomes. For *T. gondii*, a parasite previously demon-

strated to reside within a modified macrophage phagosomal compartment, the method of parasite entry appears to play a significant role in the subsequent processing of the phagosome. Hence, opsonized parasites, in contrast to live organisms, are taken up into phagosomes which fuse with mature lysosomes (34). The observations of Buchmeier and Heffron indicate that opsonization of live *S. typhimurium* does not block inhibition of fusion with lysosomes (13). However, it is unclear if this treatment actually alters the method of *Salmonella* entry since opsonized organisms are still able to induce dramatic membrane ruffling in RAW 264.7 macrophages (43). To investigate further whether the method of entry plays a role in modification of STVs, we examined the trafficking patterns of BJ66, a mutant that is believed to be taken up by host cell-directed processes such as lectinophagocytosis rather than through bacterium-induced membrane ruffling. Our results indicate that the trafficking pattern of BJ66 is no different from that of its wild-type counterpart; it acquires LAMP 1 and LAP but does not colocalize with CI-M6PR or cathepsin L. Furthermore, BJ66 has recently been shown to be fully capable of replicating within macrophages (44). Thus, remodeling of *Salmonella*-containing compartments does not seem to depend so much on the method of pathogen uptake as it does on subsequent bacterial activities.

The results reported here indicate that phagosomes containing *S. typhimurium* do indeed diverge from the normal endocytic pathway of the host cell. How the bacterium accomplishes this feat is unknown. Future investigations into the bacterial products involved, the host cell processes modified, and the regulation of these events will, most certainly, contribute to our understanding of bacterium-host cell interactions.

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REFERENCES

1. **Abshire, K. Z., and F. C. Neidhardt.** 1993. Growth rate paradox of *Salmonella typhimurium* within host macrophages. J. Bacteriol. **175:**3744–3748.
2. **Akporiaye, E. T., J. D. Rowatt, A. A. Aragon, and O. G. Baca.** 1983. Lysosomal response of a murine macrophage-like cell line persistently infected with *Coxiella burnetii*. Infect. Immun. **40:**1155–1162.
3. **Alexander, J.** 1981. *Leishmania mexicana*: inhibition and stimulation of phagosome-lysosome fusion in infected macrophages. Exp. Parasitol. **52:**261–270.
4. **Alexander, J., and K. Vickerman.** 1975. Fusion of host cell secondary lysosomes with the parasitophorous vacuoles of *Leishmania mexicana*-infected macrophages. J. Protozol. **22:**502–508.
5. **Alpuche-Aranda, C. M., J. A. Swanson, W. P. Loomis, and S. I. Miller.** 1992. *Salmonella typhimurium* activates virulence gene transcription within acidified macrophage phagosomes. Proc. Natl. Acad. Sci. USA **89:**10079–10083.
6. **Antoine, J.-C., E. Prina, C. Jouanne, and P. Bongrand.** 1990. Parasitophorous vacuoles of *Leishmania amazonensis*-infected macrophages maintain an acidic pH. Infect. Immun. **58:**770–787.
7. **Armstrong, J. A., and P. D. Hart.** 1971. Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. J. Exp. Med. **134:**713–740.
8. **Baca, O. G., Y.-P. Li, and H. Kumar.** 1994. Survival of the Q fever agent *Coxiella burnetii* in the phagolysosome. Trends Microbiol. **2:**476–480.
9. **Balch, W. E.** 1990. Small GTP-binding proteins in vesicular transport. Trends Biochem. Sci. **15:**473–477.
10. **Berón, W., C. Alvarez-Dominguez, L. Mayorga, and P. D. Stahl.** 1995. Membrane trafficking along the phagocytic pathway. Trends Cell Biol. **5:**100–104.
11. **Berthiaume, E. P., C. Medina, and J. A. Swanson.** 1995. Molecular size-fractionation during endocytosis in macrophages. J. Cell Biol. **129:**989–998.
12. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature **349:**117–127.
13. **Buchmeier, N. A., and F. Heffron.** 1991. Inhibition of macrophage phagosome-lysosome fusion by *Salmonella typhimurium*. Infect. Immun. **59:**2232–2238.
14. **Crowle, A. J., R. Dahl, E. Ross, and M. H. May.** 1991. Evidence that vesicles containing living, virulent *Mycobacterium tuberculosis* and *Mycobacterium avium* in cultured human macrophages are not acidic. Infect. Immun. **59:**1823–1831.
15. **de Chastellier, C., C. Fréhel, C. Offredo, and E. Skamene.** 1993. Implication of phagosome-lysosome fusion in restriction of *Mycobacterium avium* growth in bone marrow macrophages from genetically resistant mice. Infect. Immun. **61:**3775–3784.
16. **Densen, P., and G. L. Mandell.** 1995. Granulocytic phagocytes, p. 78–101. *In* G. L. Mandell, R. G. Douglas, and J. E. Bennett (ed.), Principles and practice of infectious diseases, 4th ed., vol. 1. Churchill Livingstone, Inc., New York, N.Y.
17. **Desjardins, M., L. A. Huber, R. G. Parton, and G. Griffiths.** 1994. Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. J. Cell Biol. **124:**677–688.
18. **Fortier, A. H., D. A. Leiby, R. B. Narayanan, E. Asafoadjei, R. M. Crawford, C. A. Nacy, and M. S. Meltzer.** 1995. Growth of *Francisella tularensis* LVS in macrophages: the acidic intracellular compartment provides essential iron required for growth. Infect. Immun. **63:**1478–1483.
19. **Francis, C. L., A. E. Jerse, J. B. Kaper, and S. Falkow.** 1991. Characterization of interactions of enteropathogenic *Escherichia coli* O127:H6 with mammalian cells in vitro. J. Infect. Dis. **164:**693–703.
20. **Francis, C. L., M. N. Starnbach, and S. Falkow.** 1992. Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with *Salmonella typhimurium* grown under low-oxygen conditions. Mol. Microbiol. **6:**3077–3087.
21. **Frehel, C., C. de Chastellier, T. Lang, and N. Rastogi.** 1986. Evidence for inhibition of fusion of lysosomal and prelysosomal compartments with phagosomes in macrophages infected with pathogenic *Mycobacterium avium*. Infect. Immun. **52:**252–262.
22. **Garcia-del Portillo, F., and B. B. Finlay.** 1995. Targeting of *Salmonella typhimurium* to vesicles containing lysosomal membrane glycoproteins bypasses compartments with mannose 6-phosphate receptors. J. Cell Biol. **129:**81–97.
23. **Garcia-del Portillo, F., and B. B. Finlay.** 1995. The varied lifestyles of intracellular pathogens within eukaryotic vacuolar compartments. Trends Microbiol. **3:**373–380.
24. **Gorvel, J. P., P. Chavier, M. Zerial, and J. Gruenberg.** 1991. rab5 controls early endosome fusion in vitro. Cell **64:**915–925.
25. **Goud, B., and M. McCaffrey.** 1991. Small GTP-binding proteins and their role in transport. Curr. Opin. Cell Biol. **3:**626–633.
26. **Griffiths, G., R. Matteoni, R. Back, and B. Hoflack.** 1990. Characterization of the cation-independent mannose 6-phosphate receptor-enriched prelysosomal compartment in NRK cells. J. Cell Sci. **95:**441–461.
27. **Heinzen, R. A., M. A. Scidmore, D. D. Rockey, and T. Hackstadt.** 1996. Differential interaction with endocytic and exocytic pathways distinguish parasitophorous vacuoles of *Coxiella burnetti* and *Chlamydia trachomatis*. Infect. Immun. **64:**796–809.
28. **Hewlett, L. J., A. R. Prescott, and C. Watts.** 1994. The coated pit and macropinocytic pathways serve distinct endosome populations. J. Cell Biol. **124:**689–703.
29. **Hoiseth, S. K., and B. A. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature **291:**238–239.
30. **Horwitz, M. A.** 1983. Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila*) in human monocytes. J. Exp. Med. **158:**1319–1331.
31. **Horwitz, M. A.** 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. **158:**2108–2126.
32. **Horwitz, M. A., and F. R. Maxfield.** 1984. *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. J. Cell Biol. **99:**1936–1943.
33. **Ishibashi, Y., and T. Arai.** 1990. Specific inhibition of phagosome-lysosome fusion in murine macrophages mediated by *Salmonella typhimurium* infection. FEMS Microbiol. Immunol. **64:**35–44.
34. **Joiner, K. A., S. A. Fuhrman, H. M. Miettinen, L. H. Kasper, and I. Mellman.** 1990. *Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. Science **249:**641–646.
35. **Jones, B. D., and S. Falkow.** 1994. Identification and characterization of a *Salmonella typhimurium* oxygen-regulated gene required for bacterial internalization. Infect. Immun. **62:**3745–3752.
36. **Klausner, R. D., J. V. Renswoude, G. Ashwell, C. Kempf, N. A. Schechter, A. Dean, and K. R. Bridges.** 1983. Receptor-mediated endocytosis of transferrin in K562 cells. J. Biol. Chem. **258:**4715–4724.
37. **Kornfield, S.** 1992. Structure and function of the mannose 6-phosphate/insulin-like growth factor receptors. Annu. Rev. Biochem. **61:**307–330.
38. **Kornfield, S., and I. Mellman.** 1989. The biogenesis of lysosomes. Annu. Rev. Cell Biol. **5:**483–525.
39. **Lamb, J. E., F. Ray, J. H. Ward, J. P. Kushner, and J. Kaplan.** 1983. Internalization and subcellular localization of transferrin and transferrin receptors in HeLa cells. J. Biol. Chem. **258:**8751–8758.

40. **Lee, C. A., and S. Falkow.** 1990. The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. Proc. Natl. Acad. Sci. USA **87:**4304–4308.
41. **Maurin, M., A. M. Benoliel, P. Bongrand, and D. Raoult.** 1992. Phagolysosomes of *Coxiella burnetii*-infected cell lines maintain an acidic pH during persistent infection. Infect. Immun. **60:**5013–5016.
42. **Mills, S., and B. B. Finlay.** Personal communication.
43. **Monack, D., and S. Falkow.** Unpublished observations.
44. **Monack, D. M., B. Raupach, A. E. Hromockyj, and S. Falkow.** 1996. *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. Proc. Natl. Acad. Sci. USA **93:**9833–9838.
45. **Oh, Y.-K., C. Alpuche-Aranda, E. Berthiaume, T. Jinks, S. I. Miller, and J. A. Swanson.** 1996. Rapid and complete fusion of macrophage lysosomes with phagosomes containing *Salmonella typhimurium*. Infect. Immun. **64:** 3877–3883.
46. **Pascopella, L., B. Raupach, N. Ghori, D. Monack, S. Falkow, and P. L. C. Small.** 1995. Host restriction phenotypes of *Salmonella typhi* and *Salmonella gallinarum*. Infect. Immun. **63:**4329–4335.
47. **Pitt, A., L. S. Mayorga, A. L. Schwartz, and P. Stahl.** 1992. Transport of phagosomal components to an endosomal compartment. J. Biol. Chem. **267:**126–132.
48. **Pitt, A., L. S. Mayorga, P. D. Stahl, and A. L. Schwartz.** 1992. Alterations in the protein composition of maturing phagosomes. J. Clin. Invest. **90:**1978–1983.
49. **Prence, E. M., J. Dong, and G. G. Sahagian.** 1990. Modulation of the transport of a lysosomal enzyme by PDGF. J. Cell Biol. **110:**319–326.
50. **Prina, E., J.-C. Antoine, B. Wiederanders, and H. Kirschke.** 1990. Localization and activity of various lysosomal proteases in *Leishmania amazonensis*-infected macrophages. Infect. Immun. **58:**1730–1737.
51. **Rabinowitz, S., H. Horstmann, S. Gordon, and G. Griffiths.** 1992. Immunocytochemical characterization of the endocytic and phagolysosomal compartments in peritoneal macrophages. J. Cell Biol. **116:**95–112.
52. **Racoosin, E. L., and J. A. Swanson.** 1993. Macropinosome maturation and fusion with tubular lysosomes in macrophages. J. Cell. Biol. **121:**1011–1020.
53. **Rathman, M., M. D. Sjaastad, and S. Falkow.** 1996. Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages. Infect. Immun. **64:**2765–2773.
54. **Russell, D. G., S. Xu, and P. Chakraborty.** 1992. Intracellular trafficking and the parasitophorous vacuole of *Leishmania mexicana*-infected macrophages. J. Cell Sci. **103:**1193–1210.
55. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
56. **Sibley, L. D., S. G. Franzblau, and J. L. Krahenbuhl.** 1987. Intracellular fate of *Mycobacterium leprae* in normal and activated mouse macrophages. Infect. Immun. **55:**680–685.
57. **Sibley, L. D., E. Weidner, and J. L. Krahenbuhl.** 1985. Phagosome acidification blocked by intracellular *Toxoplasma gondii*. Nature **315:**416–419.
58. **Sturgill-Koszycki, S., P. H. Schlesinger, P. Chakraborty, P. L. Haddix, H. L. Collins, A. K. Fok, R. D. Allen, S. L. Gluck, J. Heuser, and D. G. Russell.** 1994. Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. Science **263:**678–681.
59. **Sturgill-Koxzycki, S., U. E. Schaible, and D. G. Russell.** *Mycobacterium*-containing phagosomes are accessible to sorting endosomes and reflect a transitional state in normal phagosome biogenesis. EMBO J., in press.
60. **Swanson, M. S., and R. R. Isberg.** 1993. Formation of the *Legionella pneumophila* replicative phagosome. Infect. Agents Dis. **2:**224–226.
61. **Tanaka, Y., S. Yano, K. Furuno, T. Ishikawa, M. Himeno, and K. Kato.** 1990. Transport of acid phosphatase to lysosomes does not involve passage through the cell surface. Biochem. Biophys. Res. Commun. **170:**1067–1073.
62. **Xu, S., A. Cooper, S. Sturgill-Koszycki, T. van Heyningen, D. Chatterjee, I. Orme, P. Allen, and D. G. Russell.** 1994. Intracellular trafficking in *Mycobacterium tuberculosis* and *Mycobacterium avium*-infected macrophages. J. Immunol. **153:**2568–2578.
63. **Yamashiro, D. J., B. Tycko, S. R. Fluss, and F. R. Maxfield.** 1984. Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. Cell **37:**789–800.

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