

Lysosomal Fusion Is Essential for the Retention of *Trypanosoma cruzi* Inside Host Cells

Luciana O. Andrade¹ and Norma W. Andrews^{1,2}

¹Section of Microbial Pathogenesis and ²Department of Cell Biology, Boyer Center for Molecular Medicine, New Haven, CT 06536

Abstract

Trypomastigotes, the highly motile infective forms of *Trypanosoma cruzi*, are capable of infecting several cell types. Invasion occurs either by direct recruitment and fusion of lysosomes at the plasma membrane, or through invagination of the plasma membrane followed by intracellular fusion with lysosomes. The lysosome-like parasitophorous vacuole is subsequently disrupted, releasing the parasites for replication in the cytosol. The role of this early residence within lysosomes in the intracellular cycle of *T. cruzi* has remained unclear. For several other cytosolic pathogens, survival inside host cells depends on an early escape from phagosomes before lysosomal fusion. Here, we show that when lysosome-mediated *T. cruzi* invasion is blocked through phosphoinositide 3-kinase inhibition, a significant fraction of the internalized parasites are not subsequently retained inside host cells for a productive infection. A direct correlation was observed between the lysosomal fusion rates after invasion and the intracellular retention of trypomastigotes. Thus, formation of a parasitophorous vacuole with lysosomal properties is essential for preventing these highly motile parasites from exiting host cells and for allowing completion of the intracellular life cycle.

Key words: trypomastigote • invasion • intracellular • fusion • lysosome

Introduction

Chagas' disease is a serious debilitating disease that affects millions of people in Latin America. It is caused by infection with the protozoan parasite *Trypanosoma cruzi*, which is present in several vertebrate wild animals and is transmitted by reduviid insects that inhabit human dwellings (1). The infective forms of the parasite, trypomastigotes, are highly motile and capable of invading several different cell types. After invasion, trypomastigotes escape from the vacuole into the cytosol, where they differentiate into amastigotes and replicate (2).

Studies of the mechanism used by trypomastigotes to invade nonphagocytic cells have revealed two distinct strategies. The first one involves the induction of Ca²⁺ signaling through IP₃ generation, recruitment and fusion of host cell lysosomes at the parasite invasion site, and formation of a parasitophorous vacuole with lysosomal properties (3–5). A second pathway of entry involving the plasma membrane was suggested by morphological studies (6) and was recently

characterized after inhibition of early lysosomal recruitment and fusion by phosphoinositide (PI) 3-kinase inhibitors (7). In cells treated with wortmannin or Ly294002, the lysosomal entry pathway is abolished, but a reduced level of invasion is still detected. These remaining parasites enter cells enveloped in vacuoles containing plasma membrane markers, by a mechanism independent of host cell actin polymerization. This population of parasitophorous vacuoles derived from the plasma membrane also gradually acquires lysosomal markers, through a unique maturation process that is not blocked with PI 3-kinase inhibitors (7).

After entry into host cells, *T. cruzi* disrupts the lysosome-like parasitophorous vacuole through the action of a low pH-dependent lytic protein, and escapes into the cytosol (8–11). During this transition, the parasites differentiate into amastigotes, which are morphologically and antigenically distinct from the invasive trypomastigotes and are capable of replicating in the cytosol (2). Thus, *T. cruzi* trypomastigotes can survive lysosomal fusion, an event that may actually facilitate their escape from the vacuole and differentiation

The online version of this article contains supplemental material.

Address correspondence to Norma W. Andrews, Section of Microbial Pathogenesis and Dept. of Cell Biology, Boyer Center for Molecular Medicine, Yale University School of Medicine, 295 Congress Ave., New Haven, CT 06510. Phone: (203) 737-2410; Fax: (203) 737-2630; email: norma.andrews@yale.edu

Abbreviations used in this paper: CHO, Chinese hamster ovary; DAPI, 4',6-diamidino-2'-phenylindole; MEF, murine embryonic fibroblast; MOI, multiplicity of infection; PI, phosphoinositide.

into replicative amastigotes (10, 12, 13). In this respect, *T. cruzi* differs markedly from other intracellular pathogens such as *Listeria monocytogenes* and *Shigella flexneri*, which also disrupt the phagosome after cell entry and replicate free in the cytosol. *L. monocytogenes* disrupts phagosomes shortly after internalization, through the secretion of a low pH-active pore-forming toxin, LLO (14, 15). When LLO is mutated, *L. monocytogenes* cannot escape from the phagosome or grow intracellularly (16). Similar observations were made with *S. flexneri* IpaB mutants (17). These examples have contributed to the view that avoiding lysosomal fusion is advantageous for the survival of pathogens that are adapted for growth in the cytosol. The fact that *T. cruzi* resides for a significant period inside lysosomes before escaping into the cytosol raises questions regarding the role of lysosomal fusion in the life cycle. We set out to investigate this issue, taking advantage of the identification of a plasma membrane-mediated invasion pathway that does not involve early lysosome recruitment and fusion (7). It was conceivable that avoiding lysosomes during the initial entry steps would favor intracellular survival, and lead to more vigorous intracellular growth. Surprisingly, we found that lysosomal fusion is critical for the intracellular retention and subsequent replication of *T. cruzi*. When early interaction with lysosomes is inhibited, trypomastigote entry is reversible and does not lead to productive infections. In contrast, under normal conditions, the great majority of internalized trypomastigotes survives the rapid fusion with lysosomes, and subsequently replicates in the cytosol.

Materials and Methods

Antibodies and Reagents. Anti-mouse and hamster Lamp1 mAbs (1D4B and UC1) were obtained from the Developmental Studies Hybridoma Bank. The anti-rat Lamp1 mAb LY1C6 was a gift from I. Mellman (Yale University, New Haven, CT). Anti-*T. cruzi* polyclonal antibodies were generated by immunizing a rabbit with *T. cruzi* trypomastigotes, and anti-Ssp-4 mAbs as described previously (18). Anti-PI 3-kinase p85 polyclonal antibody was obtained from Upstate Biotechnology. Bovine brain PIs, wortmannin, Ly294002, and cytochalasin D were obtained from Sigma-Aldrich, protein G-agarose was obtained from Invitrogen, and [³²P]-γ-ATP (3,000 Ci/mmol, 10 mCi/ml) was obtained from Amersham Biosciences.

Cells and Parasites. Mouse murine embryonic fibroblasts (MEFs) were prepared from day 13.5 mouse embryos (19) and maintained in culture in high glucose DMEM (GIBCO BRL) supplemented with 10% FBS, 1% penicillin-streptomycin, and 2 mM glutamine. Rat L6E9 and Chinese hamster ovary (CHO) cells were maintained in DMEM and α-MEM (GIBCO BRL), respectively, containing 10% FBS, 1% penicillin-streptomycin and 2 mM glutamine. Bone marrow-derived macrophages were isolated from the femurs of 8–10-wk-old female BALB/c mice (20) and cultured on 12-mm-diameter glass coverslips in six-well tissue-culture plates at a density of 2.5×10^5 macrophages per well in 2 ml macrophage medium (RPMI 1640 containing 30% L-fibroblast culture supernatant and 20% FBS).

Tissue culture trypomastigotes from the *T. cruzi* Y strain were maintained in infected LLC-MK2 monolayers and purified as described previously (3).

Cell Treatments. MEF, CHO, or L6E9 cells were plated at 0.63×10^5 cells/ml in media containing 10% FBS on 6-cm plastic tissue culture dishes containing 12-mm round coverslips and grown for 48 h at 37°C in a humidified atmosphere containing 5% CO₂. 50 nM wortmannin or 50 μM Ly294002 was added to cells for 30 min and 10 μM cytochalasin D was added for 15 min. Both cell treatments were performed immediately before infection. In the case of Ly294002, the drug was kept in the medium throughout the experiment.

***T. cruzi* Invasion Assay.** Infection of MEFs with purified trypomastigotes was performed for 15 min at 37°C at a multiplicity of infection (MOI) of 50 (or 100 in Ly294002 experiments). Infection of CHO or L6E9 cells was performed for 30 min at an MOI of 100. Drug-treated cells were rinsed with infection media (DME 10% FBS) before incubation with parasites. In some experiments, *T. cruzi* trypomastigotes were pretreated with 50 nM wortmannin for 30 min at 37°C before incubation with mammalian cells. Immediately after infection, cells were washed five times with PBS to remove extracellular parasites, and either fixed in 4% (wt/vol) paraformaldehyde/PBS overnight at 4°C or reincubated with media for different times before fixation with paraformaldehyde. Macrophages were activated by treatment with 50 U/ml IFNγ and 50 ng/ml LPS for 24 h, infected with *T. cruzi* trypomastigotes at 37°C for 1 h at an MOI of 50, washed, and reincubated at 37°C for 24 h. After fixation, coverslips with attached cells were washed three times in PBS, incubated for 20 min with PBS containing 2% BSA, and processed for an inside/outside immunofluorescence invasion assay as described previously (3). At least 300 cells were analyzed per coverslip in triplicate.

Immunofluorescence and Video Microscopy. After extracellular parasite staining, cells were permeabilized with PBS/BSA containing either 0.5% (MEFs), 0.2% (L6E9 cells), or 0.1% saponin (CHO cells) for 20 min and incubated with a 1:50 dilution of hybridoma supernatants producing the appropriate anti-Lamp1 antibodies (mouse, rat, or hamster) or 5 μg/ml of the anti-Ssp-4 monoclonal antibody 2C2 (18) in PBS/BSA/saponin for 45 min. After several washes with PBS/BSA/saponin, coverslips were incubated for 30 min with a 1:250 dilution of the appropriate FITC-conjugated affinity-purified goat IgG antibody (Molecular Probes), mounted, and examined on a Zeiss Axiovert 135 microscope equipped with a Hamamatsu Orca II cooled CCD camera controlled by Metamorph Software (Universal Imaging Corp.). Time lapse images (2 frames/s) of trypomastigote invasion were acquired with the same system on a heated microscope stage.

PI 3-Kinase Activity Assay. To assay PI 3-kinase activity in cell lysates, 9-cm dishes containing confluent MEFs were treated or not with wortmannin as described before, washed with PBS, and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EGTA, 1 mM Na₃VO₄, 1 mM NaF, 0.5 mM PMSF, 0.7 μM pepstatin A, 0.1 mM chymostatin, 1.5 mM p-aminobenzamidine, 1 mM ε-aminobenzoic acid, 0.8 μM aprotinin, and 0.1 mM leupeptin) at different time points. Cell lysates containing 400 μg of protein were incubated with 5 μg of anti-PI 3-kinase p85 antibodies for 1 h at 4°C under agitation, followed by addition of 50 μl protein G-agarose and an additional incubation for 1 h. Immunoprecipitated proteins were washed twice in RIPA buffer and divided into two fractions. One was resuspended in 30 μl SDS-PAGE loading buffer and the other was washed once and resuspended in 50 μl of kinase assay buffer (25 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, and 120 mM NaCl). As a negative control, RIPA buffer with no cell lysate was submitted to the same process. The kinase assay was performed as described previously (21) with some modifications. 15 μg of bovine brain PIs,

100 μ M of cold ATP, and 10 μ Ci of [32 P]- γ -ATP were added to the immunoprecipitated PI 3-kinase. The samples were incubated at 37°C for 20 min, and the reaction was stopped by adding 300 μ l HCl 1N and 400 μ l of 20 μ g/ml PI in CHCl_3 . The organic phase was transferred to a new tube, dried out, resuspended in 10 μ l CHCl_3 , and resolved by TLC followed by autoradiography.

Online Supplemental Material. Video 1 depicts time-lapse microscopy of the *T. cruzi* invasion process and the initial period of intracellular residence. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20041408/DC1>.

Results

PI 3-Kinase-independent Invasion Leads to a Gradual Loss in Internalized Parasites, a Process That Is Inversely Correlated with the Acquisition of Lysosomal Markers. When host cells are treated with the PI 3-kinase inhibitor wortmannin, the lysosome-mediated *T. cruzi* entry pathway is abolished and the remaining invasion events detected appear to be mediated exclusively by invagination of the plasma membrane (7). To study the influence of this alternative, wortmannin-resistant invasion pathway in the survival of *T. cruzi* inside host cells, we investigated the fate of parasites after invasion of MEFs. Confirming previous papers (7), pretreatment of

MEFs with wortmannin inhibited trypomastigote entry (Fig. 1 A, 0 h) and the reduced number of parasites still able to infect cells under these conditions was not detected inside Lamp1 positive compartments shortly after invasion (Fig. 1 B, 0 h).

Surprisingly, when wortmannin-treated cells were examined a few hours after infection, the number of internalized parasites was further reduced when compared with untreated cells (Fig. 1 A, 5 h). The few remaining intracellular parasites at these later time points were all located in Lamp1 positive vacuoles, similar to what is observed in untreated cells (Fig. 1 B, 5 h). A time course in which cells were examined at increasing intervals after infection revealed that the progressive loss of intracellular parasites caused by wortmannin treatment is fast, being already detected 15–30 min after infection. In contrast, in untreated MEFs, a constant number of intracellular parasites was detected from 15 to 60 min after infection (Fig. 1 C). In these cells, most parasitophorous vacuoles acquired Lamp1 rapidly; most likely in this case there is a contribution of the early recruitment of lysosomes that occurs during the invasion process (Fig. 1 D). In wortmannin-treated cells, on the other hand, there was a gradual increase in the percentage

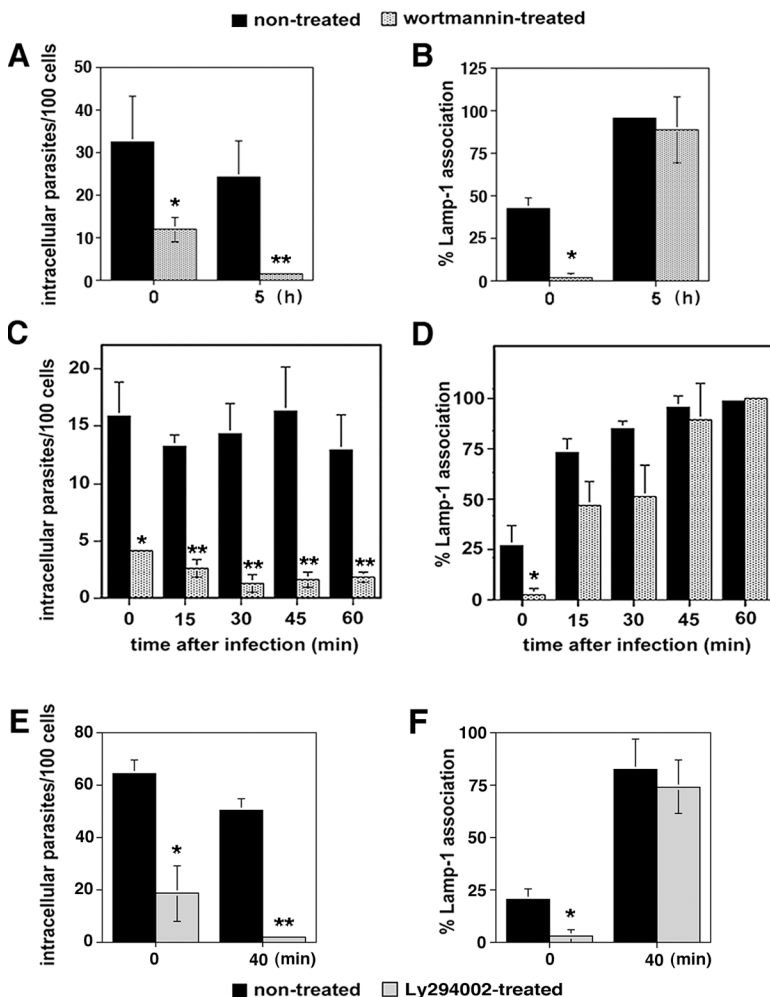


Figure 1. The loss of intracellular *T. cruzi* after inhibition of lysosome-mediated entry is inversely correlated with the kinetics of intracellular lysosomal fusion. MEFs were pretreated or not with wortmannin or Ly294002, infected with *T. cruzi*, fixed or further incubated for the indicated periods of time, and processed for immunofluorescent detection of intracellular parasites. (A) Intracellular parasites at 0 or 5 h after infection of cells pretreated or not with wortmannin. (B) Percentage of intracellular parasites associated with the lysosomal marker Lamp1 at 0 and 5 h after infection of cells pretreated or not with wortmannin. (C) The number of intracellular parasites at increasing periods after infection. (D) Percentage of intracellular parasites associated with Lamp1 at increasing periods after infection. (E) Intracellular parasites at 0 or 40 min after infection of cells treated or not with Ly294002. (F) Percentage of intracellular parasites associated with the lysosomal marker Lamp1 at 0 and 40 min after infection of cells treated or not with Ly294002. The data in A–F correspond to the mean of triplicates \pm SD. Asterisks indicate statistically significant differences ($P < 0.05$, Student's *t* test) between wortmannin-treated and untreated cells at the 0 h time point (*) or between wortmannin-treated cells at 0 h and at other time points (**).

of vacuoles positive for Lamp1, reaching 100% at ~45 min after infection (Fig. 1 D). Thus, the decrease in intracellular parasites observed after PI 3-kinase inhibition was inversely proportional to the rate of lysosomal fusion to the parasitophorous vacuoles. Parasite loss was no longer observed once all parasites were inside Lamp1 positive compartments, suggesting that lysosomal fusion plays an important role in retaining trypomastigotes inside host cells for a productive infection. Very similar results were obtained in experiments in which the MEFs were treated with a different PI-3 kinase inhibitor, Ly294002 (Fig. 1, E and F).

To rule out the possibility that residual wortmannin remaining in the dish after host cell treatment was adversely affecting the parasites, *T. cruzi* trypomastigotes were preincubated with the drug for 30 min, washed, and used to infect MEFs. No change in the total number of intracellular parasites was observed, up to 2 h after infection (Fig. 2 A). These results indicate that the loss of intracellular parasites is likely to result from an effect of wortmannin on host cells, and not on the parasites. However, wortmannin treatment does not cause toxic effects leading to MEF loss because the cells remained well spread and attached to the coverslips for at least 4 h after treatment (Fig. 2 B).

The reduction in intracellular parasites after treatment with wortmannin was not exclusive of MEFs; the same was seen when two cell lines, L6E9 rat myoblasts and CHO cells, were submitted to the same treatment and infection protocol and assayed for trypomastigote persistence inside cells. In both cases, pretreatment of cells with wortmannin not only reduced infectivity, but also decreased the number of intracellular parasites over time (Fig. 2, C and D). Wortmannin treatment also nearly abolished the number of parasites associated with Lamp1 positive compartments shortly after infection (Fig. 2, E and F, 0 h). Alternately, most of the remaining intracellular parasites at 1 h after infection were detected inside lysosomes (Fig. 2, E and F, 1 h). Thus, in several cell types, when lysosome-mediated entry is abolished, the only parasites detected intracellularly after longer periods of incubation were the ones within vacuoles that had subsequently fused with lysosomes. This finding suggested that lysosomal fusion is required to retain *T. cruzi* trypomastigotes inside host cells.

Parasite Loss Is Not Due to Intracellular Death, and Also Occurs after Disruption of the Host Cell Actin Cytoskeleton. A close examination of the 4',6-diamidino-2'-phenylindole (DAPI)-stained nuclei and kinetoplasts of intracellular trypomastigotes did not provide any evidence that the reduction in parasite numbers observed in wortmannin-treated cells was due to intracellular killing. At several time points after infection, from 15 to 60 min, intracellular trypomastigotes detected inside cells showed no signs of nuclear degradation (Fig. 3 A), a feature that can be readily detected when trypomastigotes are killed in activated macrophages (Fig. 3 B). In addition, the detection of partially internalized trypomastigotes (detected by a partial staining pattern with anti-*T. cruzi* antibodies) was significantly more frequent in wortmannin-treated cells (Fig. 3, C–G). These findings suggested that blocking lysosome-mediated

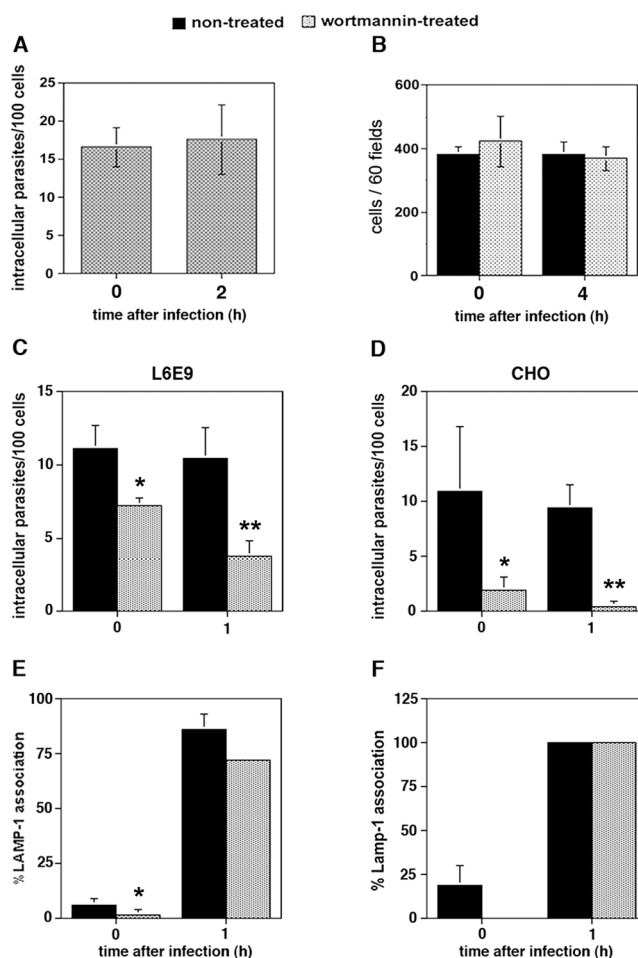


Figure 2. (A) Treatment of *T. cruzi* with a PI 3-kinase inhibitor does not affect the number of intracellular parasites over time. Trypomastigotes were pretreated with wortmannin before infection of MEFs, and the number of internalized parasites was determined at 0 and 2 h after infection. (B) Inhibition of MEF PI 3-kinase does not lead to MEF loss. MEFs were pretreated or not with wortmannin, infected with *T. cruzi*, fixed or further incubated for 4 h, and stained with DAPI, and the total number of MEFs in 60 microscopic fields (100 \times) was determined. (C–F) Loss of parasites after wortmannin treatment is also observed in other cell types. L6E9 (C and E) and CHO cells (D and F) were pretreated or not with wortmannin and infected with *T. cruzi*, and the number of internalized parasites (C and E) as well as the percentage of those parasites associated with Lamp1 (D and F) at 1 h after infection were determined. The data in A–F correspond to the mean of triplicates \pm SD. Asterisks indicate statistically significant differences ($P < 0.05$, Student's *t* test) between wortmannin-treated and untreated cells at the 0 h time point (*) or between wortmannin-treated cells at 0 h and at other time points (**).

entry leads to a reversal of the internalization process, with a fraction of the recently internalized parasites subsequently exiting the host cells. This possibility was reinforced by the fact that trypomastigotes remain actively motile for several minutes after entering cells (Video 1, available at <http://www.jem.org/cgi/content/full/jem.20041408/DC1>).

We reasoned that if trypomastigote-containing parasitophorous vacuoles were able to fuse with the plasma membrane releasing the parasites, disruption of the cortical actin cytoskeleton might modulate this process. We examined this

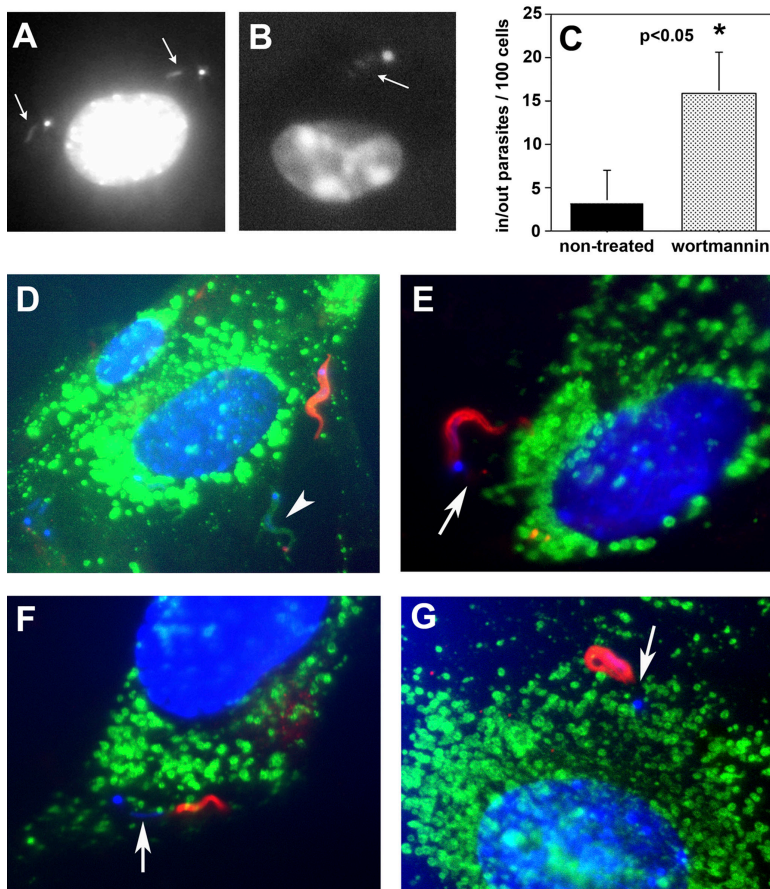


Figure 3. PI 3-kinase inhibition does not cause intracellular death, but leads to reversal of *T. cruzi* invasion. (A) Intact trypomastigotes inside a MEF 1 h after the cells were pre-treated with wortmannin. The arrows point to the normal nuclear morphology. (B) Intracellularly degraded trypomastigote 24 h after infection of a macrophage activated with IFN γ /LPS. The arrow points to remnants of the parasite's nucleus; the altered, enlarged kinetoplast is adjacent. (C) The number of partially internalized trypomastigotes after infection of MEFs treated or not with wortmannin. The data correspond to the mean of triplicates \pm SD. (D) Untreated MEFs with one extracellularly attached trypomastigote (red) and one completely internalized trypomastigote (arrow-head, green). (E, F, and G) Wortmannin-treated MEFs with partially internalized trypomastigotes (the arrows point to internalized portions, not stained by the extracellularly added anti-*T. cruzi* antibody). Lysosomes were stained with anti-Lamp1 antibodies (green), extracellular trypomastigotes with anti-*T. cruzi* antibodies (red), and MEF and trypomastigote DNA with DAPI (blue).

possibility by treating MEFs or CHO cells with cytochalasin D, exposing them to a short infection period with *T. cruzi* trypomastigotes, and quantifying the number of intracellular parasites immediately, or after 1 h of further incubation. Cytochalasin D treatment did not significantly alter the infection levels of MEFs (Fig. 4 A), but in CHO cells this treatment stimulated *T. cruzi* entry by almost threefold in relation to untreated cells (Fig. 4 C) as reported previously (3). Interestingly, similar to what is observed after wortmannin treatment, most of the parasites that invaded cytochalasin D-treated cells were not found in Lamp1 positive vacuoles immediately after invasion (Fig. 4, B and D, 0 h). Moreover, a marked reduction in the number of internalized parasites was observed in the cytochalasin D-treated cells after the additional incubation period. At 1 h after infection, 60–70% less intracellular parasites were detected and, at this time, all were inside Lamp1 positive compartments (Fig. 4, B–D, 1 h). Consistent with the aforementioned data for wortmannin treatment (Fig. 1, A and C), in untreated cells no differences were seen in the number of intracellular parasites over time (Fig. 4, A and C). Thus, disruption of the cortical actin cytoskeleton appears to promote trypomastigote invasion by facilitating invagination of the plasma membrane, a pathway that favors their subsequent exit from cells.

In the Absence of Lysosomal Fusion, T. cruzi Invasion Is Reversible. The aforementioned results suggest that in the absence of lysosomal fusion, *T. cruzi* trypomastigotes that enter

cells can subsequently return to the extracellular medium. If this hypothesis is correct, and if trypomastigotes remained viable after exiting cells, new invasion events should occur with time. This was investigated by infecting wortmannin-pretreated MEFs, washing out the extracellular parasites, and reincubating the cells for increasing periods of time (up to 24 h). In untreated cells, the number of intracellular parasites at each time point remained constant throughout the 24-h period (Fig. 5 A). This is consistent with the fact that *T. cruzi* does not start replicating intracellularly until \sim 24 h after invasion (22), and confirms that the extracellular trypomastigotes were effectively removed, not leading to a significant number of later invasion events. The pattern of association with lysosomal markers in untreated cells was also as predicted; the number of parasites in Lamp1 positive vacuoles increased initially and gradually decreased as a consequence of the ability of trypomastigotes to disrupt the parasitophorous vacuole (Fig. 5 B and reference 23). These results also show that in primary fibroblasts, *T. cruzi* resides in a lysosomal compartment for a significantly longer period when compared with cultured cell lines (10).

When the lysosome-mediated invasion pathway was blocked with wortmannin, the loss in intracellular parasites occurring during the first hour of incubation was gradually reversed (Fig. 5 A). This was not a consequence of host cell loss because the MEF numbers per coverslip remained constant throughout the experiment (passage three MEFs do

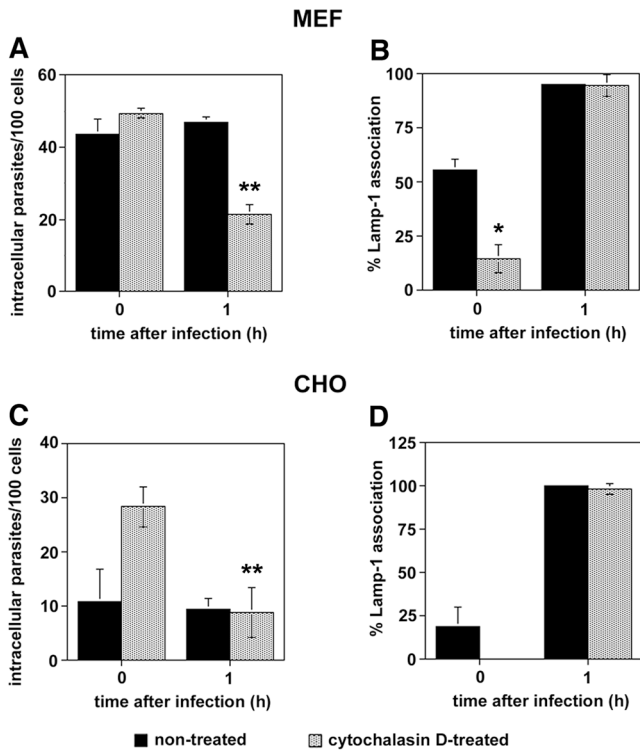


Figure 4. Disruption of the cortical actin cytoskeleton also leads to reversal of *T. cruzi* invasion. (A) The number of intracellular parasites in cytochalasin D-treated MEFs at 0 and 1 h after infection. (B) Percentage of intracellular parasites associated with Lamp1 in cytochalasin D-treated MEFs. (C) The number of intracellular parasites in cytochalasin D-treated CHO cells. (D) Percentage of Lamp1-associated parasites in cytochalasin D-treated CHO cells. Cells were pretreated or not with cytochalasin D, infected with *T. cruzi*, fixed or incubated for the indicated periods of time, and processed for immunofluorescence and detection of intracellular parasites. The data in A–D correspond to the mean of triplicates \pm SD. Asterisks indicate statistically significant differences ($P < 0.05$, Student's *t* test) between wortmannin-treated and untreated cells at the 0 h time point (*) or between wortmannin-treated cells at 0 h and at other time points (**).

not replicate within 24 h) (Fig. 5 C). The increased number of intracellular parasites observed over time after wortmannin treatment was clearly due to recent infection by extracellular trypomastigotes. Thus, parasites entering wortmannin-treated cells can exit back into the extracellular medium and subsequently reinvade cells. This conclusion is based on the fact that a significant fraction of the intracellular parasites detected at the later time points colocalized with Lamp1 (Fig. 6, B–D), retained the trypomastigote morphology (Fig. 6 D), and did not express the amastigote-specific surface antigen Ssp-4 (Fig. 6 F and references 18, 24). The newly invaded trypomastigotes detected in cells pretreated with wortmannin could have reentered through plasma membrane invagination followed by lysosomal fusion or directly through the lysosome-mediated pathway. Our finding that, after 24 h, MEFs recover from wortmannin treatment reinforces the latter possibility (Fig. 5 D).

In wortmannin-treated cells, many parasites negative for Ssp-4 expression (trypomastigotes and intermediate forms)

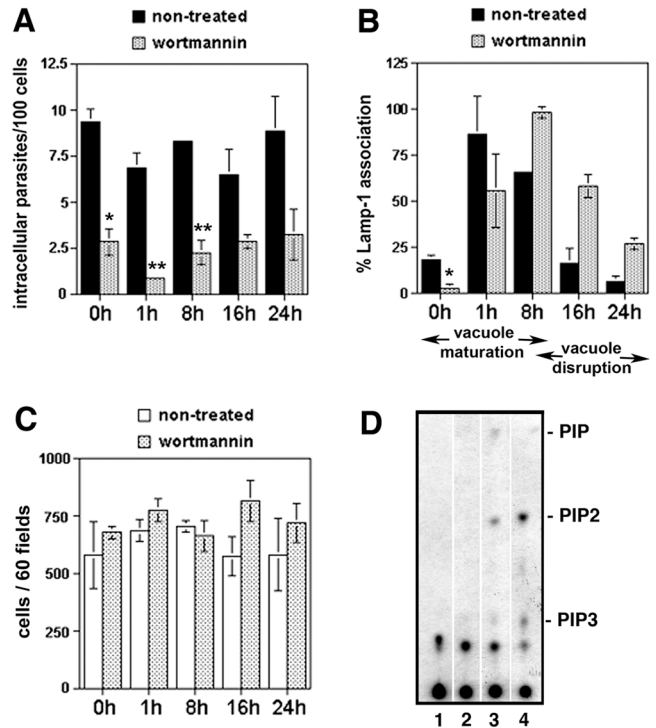


Figure 5. In the absence of lysosomal fusion, trypomastigotes exit host cells and reinfest at later time points. (A) Intracellular parasites at increasing periods after infection. (B) Percentage of intracellular parasites associated with Lamp1 at increasing periods after infection. MEFs were pretreated or not with wortmannin, infected with *T. cruzi*, and washed, and the number of intracellular parasites was determined. (C) The total number of MEFs in 60 microscopic fields (100 \times) was determined for the same experiment shown in A and B. The data in A–C correspond to the mean of triplicates \pm SD. Asterisks indicate statistically significant differences ($P < 0.05$, Student's *t* test) between wortmannin-treated and untreated cells at the 0 h time point (*) or between wortmannin-treated cells at 0 h and at other time points (**). (D) PI 3-kinase activity in untreated or wortmannin-treated MEFs. (1) Negative control (no cell lysate). (2) MEF lysates immediately after wortmannin treatment. (3) MEF lysates 24 h after wortmannin treatment. (4) MEF lysates not treated with wortmannin. White lines indicate that intervening lanes have been spliced out.

were observed side-by-side with a few Lamp1 negative amastigotes, which are easily recognizable by the markedly distinct kinetoplast morphology (Fig. 6, B and F) and expression of Ssp-4 (Fig. 6 F). In contrast, in control cells, most intracellular parasites were free in the cytoplasm (as indicated by the lack of Lamp1 staining), showed the typical amastigote morphology (round cell body and bar shaped kinetoplast) (Fig. 6 A), and expressed the amastigote-specific surface antigen Ssp-4 (Fig. 6 E). Given that the trypomastigote–amastigote transition only occurs after the lysosome-like parasitophorous vacuole is disrupted, the presence of Ssp-4 positive amastigotes clearly reflects invasion events that occurred several hours before. The total number of intracellular parasites remained approximately constant throughout this critical transition, from lysosome-resident trypomastigotes to cytosol-resident amastigotes (Fig. 5 A), indicating that the great majority of internalized *T. cruzi* resists lysosomal degradation.

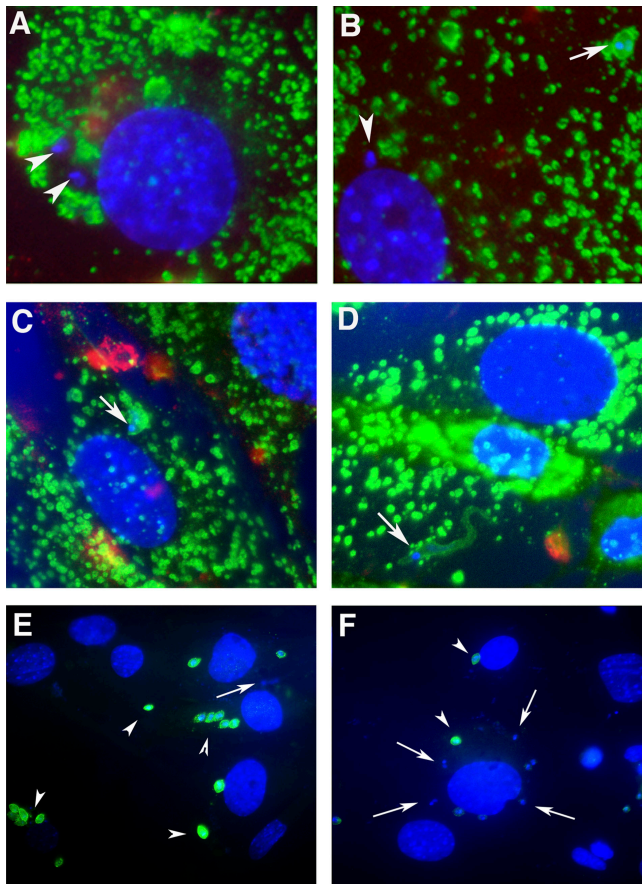


Figure 6. Intracellular parasites found at later time points in wortmannin-treated cells correspond to recent reinternalization events. MEFs were pretreated or not with wortmannin, infected with *T. cruzi*, washed to remove extracellular parasites, reincubated for 24 h, and processed for immunofluorescence. (A) After 24 h, only cytoplasmic amastigotes are detected in untreated cells (arrowheads). (B–D) In cells pretreated with wortmannin, recently internalized trypomastigotes inside Lamp1 positive vacuoles are also present (arrows). Lysosomes were stained with anti-Lamp1 antibodies (green), extracellular trypomastigotes with anti-*T. cruzi* antibodies (red), and MEF and *T. cruzi* DNA with DAPI (blue). (E) After 24 h, all intracellular parasites in untreated cells express the amastigote-specific surface antigen Ssp-4 (arrowheads). (F) In cells pretreated with wortmannin, most intracellular parasites still do not express Ssp-4, confirming that they have recently entered the cells (arrows). Staining with the Ssp-4-specific mAbs is shown (green), as is MEF and *T. cruzi* DNA staining with DAPI (blue).

Discussion

In this paper, we identified a novel role for the early fusion with host cell lysosomes seen during host cell invasion by *T. cruzi*. Trypomastigotes enter host cells by two mechanisms, both of which involve an early interaction with host cell lysosomes. The first mechanism to be characterized is mediated by a direct fusion of lysosomes with the plasma membrane at the parasite's attachment site, a process that gradually gives rise to the parasitophorous vacuole (3). Recently, a second mechanism was revealed after the lysosome-mediated entry pathway was abolished with PI 3-kinase inhibitors (7). The remaining trypomastigote invasion observed in wortmannin-treated cells appears to in-

volve invagination of the plasma membrane, without participation of the host cell actin cytoskeleton. Parasitophorous vacuoles formed by this alternative mechanism initially contain plasma membrane markers and rapidly mature by fusing with lysosomes (7). Thus, both mechanisms used by *T. cruzi* to enter host cells involve early fusion of lysosomes with the parasitophorous vacuole.

The role of early lysosomal fusion in the parasite's intracellular cycle was unclear, in particular because most pathogens that subsequently replicate free in the cytosol are known to avoid lysosomal fusion during invasion. Our findings have clarified this issue by showing that fusion with lysosomes is critical for retaining trypomastigotes inside host cells. This unexpected role of lysosomes was revealed through an investigation of the intracellular fate of parasites in wortmannin-treated cells. Precise quantification of intracellular parasites allowed us to determine that trypomastigotes can exit host cells shortly after invasion, and that the gradual fusion of lysosomes with plasma membrane-derived parasitophorous vacuoles counteracts this exit process.

In addition to PI 3-kinase inhibition, we found that disruption of the host cell actin cytoskeleton also favors trypomastigote entry through a pathway that does not initially involve lysosome recruitment. The enhancement in *T. cruzi* invasion observed in some cell types after cytochalasin D treatment was originally attributed to a facilitation in the docking and fusion of lysosomes with the plasma membrane (3). However, subsequent studies raised the possibility that actin depolymerization might favor invasion through plasma membrane invagination (6, 7). Our results and those of a recent paper (25) support this view because disruption of the actin cytoskeleton leads to invasion events that generate Lamp1 negative parasitophorous vacuoles. Here, we show that these invasion events are also reversible, as indicated by the rapid loss of parasites from cytochalasin D-treated infected cells. Interestingly, numerous plasma membrane-enveloped trypomastigotes protruding from cytochalasin D-treated cells have been observed (25), confirming the increased capacity for plasma membrane deformation after disruption of the cortical cytoskeleton. The protrusion events may have been generated directly during invasion because partial staining of parasites with extracellularly added antibody was observed (25). In the absence of a cortical cytoskeleton barrier, another possibility is that completely internalized trypomastigotes can return to the cell surface and protrude, becoming enveloped by two layers of membrane: one from the original invasion vacuole and one from the evaginated plasma membrane. Thus, cytochalasin D-induced protrusion events are not likely to correspond to an early step in the release of trypomastigotes capable of reinfecting other cells, as observed in our work.

The ability of *T. cruzi* trypomastigotes to exit cells when lysosomal fusion is delayed is consistent with our frequent observation of partially internalized parasites in wortmannin-treated cells. It is conceivable that parasitophorous vacuoles formed by membrane invagination, in the absence of direct lysosomal involvement, may not always pinch off from the plasma membrane intracellularly. In this case, for

reversal of the process, trypomastigotes would just have to slide back out of the cells. However, because a significant number of the intracellular parasites totally exclude the extracellularly added antibody, it is also possible that the vacuole separates completely from the plasma membrane, in which case reversal would require a fusion event. We confirmed that trypomastigotes originally located intracellularly can be released back into the medium, by performing reinfection experiments. *T. cruzi* only starts replicating after the invasive trypomastigotes escape from vacuoles into the cytosol (10, 23) and differentiate into amastigotes (22), events which occur ~24 h after invasion. Therefore, a short infection period followed by extensive washes to remove extracellular parasites allows a precise follow-up of the intracellular differentiation process and of recent internalization events occurring within this period. In untreated cells, we found that practically all trypomastigotes that initially enter cells escape from the vacuole and differentiate into amastigotes. In contrast, when the lysosome-mediated entry pathway is inhibited, fewer amastigotes are detected, and several recent trypomastigote invasion events are seen. This was evident through the detection of Lamp1 on parasitophorous vacuoles, and the lack of expression of amastigote-specific markers by the parasites. Because extracellular trypomastigotes in these experiments were effectively removed several hours before, the recent invasion events detected must have been derived from trypomastigotes originally resident inside cells, which were released back into the medium.

Together, our data indicate that *T. cruzi* trypomastigotes are capable of entering host cells through invagination of the plasma membrane, forming a “reversible” vacuole that can release the parasites back into the extracellular medium. Our results also indicate that this reversible entry process can only occur before the parasitophorous vacuole fuses with lysosomes. Thus, lysosomal fusion, either during entry (3) or shortly after entry (7, 25), is a critical event for completion of the *T. cruzi* intracellular cycle, and the establishment of a productive infection. This finding is consistent with the remarkable resistance of *T. cruzi* to the lysosomal environment, with practically 100% of the internalized trypomastigotes surviving inside cells for the subsequent 24 h. Although other intracellular protozoa such as *Toxoplasma gondii* (26) lose their active motility after host cell invasion, *T. cruzi* trypomastigotes retain their vigorous undulating movements intracellularly (Video 1). The attachment of lysosomes to microtubules through molecular motors (27, 28) may explain why fusion of lysosomes with *T. cruzi*-containing vacuoles is so effective in retaining these highly motile parasites inside host cells.

We are very grateful to G. Di Paolo, L. Lucast, T. Itoh, and P. De Camilli for help with PI 3-kinase assays, M. Rioult for video microscopy, J. Wilson and D. Zamboni for helpful suggestions and critical reading of the paper, and H. Tan for excellent technical assistance.

This work was supported by National Institutes of Health grant no. AI34867 (to N.W. Andrews).

The authors have no conflicting financial interests.

Submitted: 13 July 2004

Accepted: 8 September 2004

References

1. Chagas, C. 1909. Nova trypanozomiaz humana. *Mem. Inst. Oswaldo Cruz.* 1:11–80.
2. Brener, Z. 1973. Biology of *Trypanosoma cruzi*. *Annu. Rev. Microbiol.* 27:347–383.
3. Tardieux, I., P. Webster, J. Ravesloot, W. Boron, J.A. Lunn, J.E. Heuser, and N.W. Andrews. 1992. Lysosome recruitment and fusion are early events required for trypanosome invasion of mammalian cells. *Cell.* 71:1117–1130.
4. Rodriguez, A., M.G. Rioult, A. Ora, and N.W. Andrews. 1995. A trypanosome-soluble factor induces IP3 formation, intracellular Ca²⁺ mobilization and microfilament rearrangement in host cells. *J. Cell Biol.* 129:1263–1273.
5. Rodriguez, A., E. Samoff, M.G. Rioult, A. Chung, and N.W. Andrews. 1996. Host cell invasion by trypanosomes requires lysosomes and microtubule/kinesin-mediated transport. *J. Cell Biol.* 134:349–362.
6. Schenkman, S., and R.A. Mortara. 1992. HeLa cells extend and internalize pseudopodia during active invasion by *Trypanosoma cruzi* trypomastigotes. *J. Cell Sci.* 101:895–905.
7. Woolsey, A.M., L. Sunwoo, C.A. Petersen, S.M. Brachmann, L.C. Cantley, and B.A. Burleigh. 2003. Novel PI 3-kinase-dependent mechanisms of trypanosome invasion and vacuole maturation. *J. Cell Sci.* 116:3611–3622.
8. Tanowitz, H., M. Wittner, Y. Kress, and B. Bloom. 1975. Studies of in vitro infection by *Trypanosoma cruzi*. I. Ultrastructural studies on the invasion of macrophages and L cells. *Am. Trop. Med. Hyg.* 24:25–33.
9. Andrews, N.W., and M.B. Whitlow. 1989. Secretion by *Trypanosoma cruzi* of a hemolysin active at low pH. *Mol. Biochem. Parasitol.* 33:249–256.
10. Ley, V., E.S. Robbins, V. Nussenzweig, and N.W. Andrews. 1990. The exit of *Trypanosoma cruzi* from the phagosome is inhibited by raising the pH of acidic compartments. *J. Exp. Med.* 171:401–413.
11. Andrews, N.W., C.K. Abrams, S.L. Slatin, and G. Griffiths. 1990. A *T. cruzi*-secreted protein immunologically related to the complement component C9: evidence for membrane pore-forming activity at low pH. *Cell.* 61:1277–1287.
12. Kress, Y., B.R. Bloom, M. Wittner, A. Rowen, and H. Tanowitz. 1975. Resistance of *Trypanosoma cruzi* to killing by macrophages. *Nature.* 257:394–396.
13. Tomlinson, S., F. Vandekerckhove, U. Frevert, and V. Nussenzweig. 1995. The induction of *Trypanosoma cruzi* trypomastigote to amastigote transformation by low pH. *Parasitology.* 110:547–554.
14. Beauregard, K.E., K.D. Lee, R.J. Collier, and J.A. Swanson. 1997. pH-dependent perforation of macrophage phagosomes by listeriolysin O from *Listeria monocytogenes*. *J. Exp. Med.* 186:1159–1163.
15. Glomski, I.J., M.M. Gedde, A.W. Tsang, J.A. Swanson, and D.A. Portnoy. 2002. The *Listeria monocytogenes* hemolysin has an acidic pH optimum to compartmentalize activity and prevent damage to infected host cells. *J. Cell Biol.* 156:1029–1038.
16. Portnoy, D.A., V. Auerbuch, and I.J. Glomski. 2002. The cell biology of *Listeria monocytogenes* infection: the intersection of bacterial pathogenesis and cell-mediated immunity. *J. Cell Biol.* 158:409–414.

17. High, N., J. Mounier, M.C. Prevost, and P.J. Sansonetti. 1992. IpaB of *Shigella flexneri* causes entry into epithelial cells and escape from the phagocytic vacuole. *EMBO J.* 11:1991–1999.
18. Andrews, N.W., K.S. Hong, E.S. Robbins, and V. Nussenzweig. 1987. Stage-specific surface antigens expressed during the morphogenesis of vertebrate forms of *Trypanosoma cruzi*. *Exp. Parasitol.* 64:474–484.
19. Tournier, C., P. Hess, D.D. Yang, J. Xu, T.K. Turner, A. Nimnual, D. Bar-Sagi, S.N. Jones, R.A. Flavell, and R.J. Davis. 2000. Requirement of JNK for stress-induced activation of the cytochrome *c*-mediated death pathway. *Science.* 288:870–874.
20. Coers, J., C. Monahan, and C.R. Roy. 1999. Modulation of phagosome biogenesis by *Legionella pneumophila* creates an organelle permissive for intracellular growth. *Nat. Cell Biol.* 1:451–453.
21. Todorov, A.G., M. Einicker-Lamas, S.L. de Castro, M.M. Oliveira, and A. Guilherme. 2000. Activation of host cell phosphatidylinositol 3-kinases by *Trypanosoma cruzi* infection. *J. Biol. Chem.* 275:32182–32186.
22. Dvorak, J.A. 1975. New in vitro approach to quantitation of *Trypanosoma cruzi* vertebrate cell interactions. In *New Approaches in American Trypanosomiasis Research* (PAHO Scientific Publication, vol. 318. 109–120.
23. Hall, B.F., P. Webster, A.K. Ma, K.A. Joiner, and N.W. Andrews. 1992. Desialylation of lysosomal membrane glycoproteins by *Trypanosoma cruzi*: a role for the surface neuraminidase in facilitating parasite entry into the host cell cytoplasm. *J. Exp. Med.* 176:313–325.
24. Andrews, N.W., E.S. Robbins, V. Ley, K.S. Hong, and V. Nussenzweig. 1988. Developmentally regulated, phospholipase C-mediated release of the major surface glycoprotein of amastigotes of *Trypanosoma cruzi*. *J. Exp. Med.* 167:300–314.
25. Woolsey, A.M., and B.A. Burleigh. 2004. Host cell actin polymerization is required for cellular retention of *Trypanosoma cruzi* and early association with endosomal/lysosomal compartments. *Cell. Microbiol.* 6:829–838.
26. Gaskins, E., S. Gilk, N. DeVore, T. Mann, G. Ward, and C. Beckers. 2004. Identification of the membrane receptor of a class XIV myosin in *Toxoplasma gondii*. *J. Cell Biol.* 165:383–393.
27. Matteoni, R., and T. Kreis. 1987. Translocation and clustering of endosomes and lysosomes depends on microtubules. *J. Cell Biol.* 105:1253–1265.
28. Schroer, T.A., and M.P. Sheetz. 1991. Functions of microtubule-based motors. *Annu. Rev. Physiol.* 53:629–652.