Cellular Microbiology (2006) **8**(7), 1106–1120 doi:10.1111/j.1462-5822.2006.00695.x
First published online 3 March 2006

Role of caveolae in *Leishmania chagasi* phagocytosis and intracellular survival in macrophages

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Summary

Caveolae are membrane microdomains enriched in cholesterol, ganglioside M1 (GM1) and caveolin-1. We explored whether caveolae facilitate the entry of *Leishmania chagasi* into murine macrophages. Transient depletion of macrophage membrane cholesterol by 1 h exposure to methyl-β-cyclodextrin (MβCD) impaired the phagocytosis of non-opsonized and serum-opsonized virulent *L. chagasi*. In contrast, MβCD did not affect the phagocytosis of opsonized attenuated *L. chagasi*. As early as 5 min after phagocytosis, virulent *L. chagasi* colocalized with the caveolae markers GM1 and caveolin-1, and colocalization continued for over 48 h. We explored the kinetics of lysosome fusion. Whereas fluorescent-labelled dextran entered macrophage lysosomes by 30 min after addition, localization of *L. chagasi* in lysosomes was delayed for 24–48 h after phagocytosis. However, after transient depletion of cholesterol from macrophage membrane with MβCD, the proportion of *L. chagasi*-containing phagosomes that fused with lysosomes increased significantly. Furthermore, intracellular replication was impaired in parasites entering after transient cholesterol depletion, even though lipid microdomains were restored by 4 h after treatment. These observations suggest that virulent *L. chagasi* localize in caveolae during phagocytosis by host macrophages, and that cholesterol-containing macrophage membrane domains, such as caveolae, target parasites to a pathway that promotes delay of lysosome fusion and intracellular survival.

Introduction

Leishmania spp. are parasitic protozoa with two life stages. The extracellular promastigote is found in the gut of the sand fly vector, and the obligate intracellular amastigote resides in mononuclear phagocytes such as macrophages in a mammalian host. While feeding on a mammal, the sand fly inoculates promastigotes into a pool of blood in the skin. Promastigotes are subsequently phagocytosed by resident mononuclear phagocytes, whereupon they convert to obligate intracellular amastigotes. Thereafter amastigotes are the only form of the parasite found in the mammalian host (Bogdan *et al*., 2000; Handman and Bullen, 2002).

A remarkable feature of the *Leishmania* spp. infections is the parasite's ability to survive and replicate inside mammalian macrophages, a hostile environment that is lethal to many microbes (Blackwell, 1985). It has become evident that ligation of different host cell receptors during phagocytosis results in activation of distinct signalling pathways. For example, Fc-receptor ligation triggers an oxidative response, whereas independent ligation of complement receptor 3 (CR3) does not lead to NADPH oxidase activation (Aderem and Underhill, 1999; Chimini and Chavrier, 2000). As such, the pathway utilized by a pathogen during entry into a host cell might determine the ultimate fate of the microbe. The *Leishmania* sp. are taken up by macrophages through facilitated receptor-mediated phagocytosis. Receptors that can participate in internalization of Leishmania promastigotes include CR3, CR1, the mannose receptor, the fibronectin receptor and Fc receptors (Blackwell, 1985; Mosser and Edelson, 1985; Wyler *et al*., 1985; Wilson and Pearson, 1988; da Silva *et al*., 1989; Rosenthal *et al*., 1996). The full implications of parasite entry through different receptor pathways for parasite survival have not been delineated.

Macrophages can internalize particles or microorganisms through caveolae or through clathrin coated pits. Caveolae are lipid-containing membrane microdomains that appear as flask-shaped or tubular invaginations in the plasma membrane, characterized by the presence of the proteins caveolin-1, -2, or -3 (Kenworthy, 2002). Similar to other lipid rafts, they are enriched in cholesterol, glycanphosphatidylinositol (GPI) anchored membrane molecules, and glycosphingolipids such as ganglioside M1 (GM1). At least some of the above-mentioned receptors (e.g. CR3) are localized in caveolae (Harris *et al*., 2002a; Kenworthy, 2002). Pathogens that are taken up via caveolae-mediated phagocytosis include FimH-expressing *Escherichia coli*, Simian Virus 40 (SV-40), Group A strep-

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tococci and *Brucella abortus*. In some cases entry through caveolae targets virulent microorganisms to compartments that avoid fusion with lysosomes, thereby enhancing microbial survival (Pelkmans *et al*., 2001; Shin and Abraham, 2001a; Watarai *et al*., 2002). Agents that disrupt caveolae [filipin, nystatin, methyl-β-cyclodextrin (MβCD)] inhibit internalization of FimH-expressing *E. coli* and other microorganisms that enter through caveolae (Shin *et al*., 2000; Baorto, 2002).

Previous work in our laboratory demonstrated that infection with *Leishmania chagasi* increases the abundance of transcripts encoding caveolin-1 and caveolin-3, as well as caveolin-3 protein levels in BALB/c macrophages (see figure 5 in Rodriguez *et al*., 2004). The fact that caveolin expression is increased in response to leishmania phagocytosis could indicate that parasite infection has triggered a feedback mechanism leading to increased caveolin synthesis, perhaps by utilizing and depleting surface caveolae. As such, we hypothesized that caveolae play a role in *L. chagasi* entry and intracellular survival in mammalian macrophages. Our data suggested not only that Leishmania promastigotes are internalized through caveolin-1 and GM1-lined lipid enriched compartments, but also that forcing parasites to enter through a non-lipid raft pathway delivers them into a compartment that readily fuses with lysosomes. We hypothesize that entry through caveolae may contribute to a delay in lysosome fusion, and as such enhance parasite survival in the host cell.

Results

Phagocytosis of L. chagasi *is dependent on macrophage cholesterol*

We previously reported that the steady state abundance of mRNAs encoding caveolin-1, caveolin-3 and dynamin-2 increased 6.9-fold, 5.0-fold and 3.3-fold, respectively, 4 h after infection of BALB/c bone marrow-derived macrophages with *L. chagasi* (Rodriguez *et al*., 2004). Because *L. chagasi* infection increases the steady state abundance of transcripts encoding these caveolae-associated proteins, we questioned whether *L. chagasi* promastigotes are taken up through macrophage caveolae. Caveolae are enriched in cholesterol, GM1, GPI-anchored proteins and signalling molecules (Kiss *et al*., 2002; Lin and Rikihisa, 2003). As such, we began by depleting cholesterol from macrophage cell membranes with MβCD. MβCD should disrupt all membrane lipid rafts including caveolae, and consequently impair caveolae-mediated endocytosis (Shin *et al*., 2000; Naroeni and Porte, 2002; Watarai *et al*., 2002; Lin and Rikihisa, 2003; Duncan *et al*., 2004). A preliminary dose-response experiment showed that macrophages treated for 1 h with 5, 12, or 25 mM MβCD retained a level of viability similar to control-untreated macrophages, measured by both exclusion of trypan blue and by macrophage survival for 96 h in culture. We performed all subsequent experiments with 5 or 10 mM MβCD for 1 h (Garner *et al*., 2002; Rhode *et al*., 2003).

Methyl-β-cyclodextrin chelates cholesterol without interfering with cholesterol synthesis. As such, membrane cholesterol should be depleted at the end of MβCD treatment, but quickly replaced after removal of the compound from living cells and suspending in serum-containing medium which is enriched in cholesterol (Brown and London, 1998). To illustrate the effects of cholesterol depletion on lipid rafts and caveolae, macrophages were treated with 10 mM MβCD for 1 h, rinsed and fixed immediately or suspended in serum containing medium without MβCD and allowed to recover for 4 or 24 h before studying. Negative control macrophages were either left untreated or simultaneously treated with 10 mM MβCD complexed to 200 μg ml^{-1} water-soluble cholesterol to prevent cholesterol depletion. Macrophages were stained with filipin to detect cholesterol, or with fluorescently conjugated cholera toxin B (CTX-B) which binds the GM1 found in lipid rafts.

Untreated macrophages displayed discrete filipin and CTX-B staining on the plasma membrane suggesting membranes are enriched in both cholesterol- and GM1-containing domains (Fig. 1). In contrast, MβCD treatment resulted in a smeared CTX-B staining while abrogating filipin staining to the macrophage membrane. The 'smeared' CTX-B staining could be due to the disruption of lipid rafts and hence GM1 redistribution. A similar loss of surface staining after MβCD treatment was observed for caveolin-1 staining (data not shown). Intracellular staining with filipin is suggestive of an intracellular cholesterol pool, and this remained unchanged by MβCD treatment. Furthermore, untreated and MβCD + cholesterol conditions displayed intact surface staining with anti-caveolin-1 antibody, but MβCD treatment resulted in a reduction of caveolin-1 staining to undetectable levels (data not shown). After 4 h of recovery from MβCD with exogenous cholesterol addition, the patterns of filipin, CTX-B and caveolin-1 staining were restored to or more intense than control. These results suggest that MβCD treatment transiently disrupts lipid rafts, of which caveolae are a subset, on the macrophage membrane.

Using the above depletion method, we examined whether lipid rafts/caveolae are necessary for leishmania phagocytosis. BALB/c macrophages were pretreated for 1 h without or with MβCD to transiently diminish membrane cholesterol, and then infected with either virulent or attenuated (L5 strain) *L. chagasi* for 30 min. Intracellular parasites were enumerated microscopically. These quantitative results showed that pretreatment with MβCD

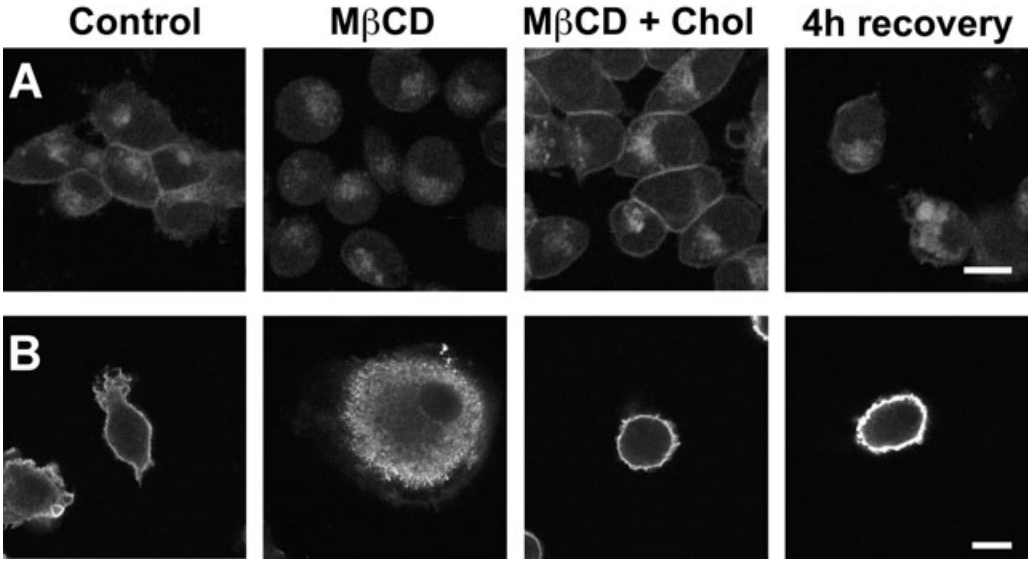

Fig. 1. MβCD affects the structure of macrophage lipid rafts. Macrophages were either left untreated or pretreated with 10 mM MβCD, or 10 mM MβCD + 200 μg ml^{-1} water-soluble cholesterol for 1 h in serum-free medium. Cells in the first three columns were fixed and stained immediately, and cells in the last column (4 h recovery) were incubated in 10 mM MβCD and allowed to recover in the serum-containing medium RP-10 for 4 h before fixing. Cholesterol and GM1 were assayed by filipin and CTX-B staining, panels A and B respectively. Scale bar: 10 μm.

decreased the initial uptake of serum-opsonized virulent *L. chagasi*, but did not significantly affect the phagocytosis of opsonized attenuated L5 parasites (Fig. 2A). In contrast, macrophages that were allowed to recover in cholesterol (serum)-containing medium for either 4 or 24 h after MβCD treatment were able to phagocytose virulent *L. chagasi* at a similar level as untreated macrophages (Fig. 2B). The level of phagocytosis of attenuated parasites was similar to virulent parasite uptake by cholesterol-depleted macrophages, leading us to hypothesize that the cholesterol-dependent pathway might account for the increased uptake of virulent parasites. Nonetheless, when the phagocytosis of virulent and attenuated was similar (Fig. 3), MβCD still inhibited phagocytosis of opsonized virulent but not opsonized attenuated parasites.

To determine whether cholesterol depletion diminishes phagocytosis of all particles, we assessed the internalization of non-virulent *E. coli* DH5α by MβCD-pretreated or -untreated macrophages. There was no significant difference between the phagocytosis of non-virulent *E. coli* by MβCD-treated or -untreated macrophages (Fig. 2B), similar to published results with the J774A.1 cell line (Pucadyil *et al*., 2004). This differs from the uptake of virulent, FimH-positive *E. coli*, which are taken up through mast cells caveolae (Shin *et al*., 2000; Shin and Abraham, 2001b).

We used cytochalasin D, an inhibitor of actin polymerization, to determine whether the decreased parasite entry seen in MβCD-treated macrophages was due to inhibition of attachment or a decrease in phagocytosis (Wilson and Hardin, 1988). The former finding would imply that receptors must cluster in lipid raft microdomains before parasites can efficiently attach to macrophages, whereas the latter would implicate a requirement for lipid raft domains for parasite internalization once receptor ligation had occurred. The data showed that attachment of promastigotes to MβCD-treated macrophages occurred at a similar level as untreated macrophages, even though MβCD consistently decreased parasite entry (Fig. 2C). These results suggest that intact lipid rafts are not necessary for promastigote attachment to macrophages, but that intact lipid rafts are needed for the optimal phagocytosis of promastigotes.

Requirement for opsonization

Opsonization with either complete serum or polyclonal murine antibodies has been shown to render some pathogens insensitive to cholesterol depletion with MβCD, presumably by causing entry through an alternate cholesterol-independent pathway (Shin *et al*., 2000; Naroeni and Porte, 2002; Pucadyil *et al*., 2004). Furthermore, opsonization has been shown to alter the signalling intermediates that are activated during *Leishmania amazonensis* internalization by Chinese hamster ovary cells (Morehead *et al*., 2002). To investigate whether there is a role for opsonization in cholesterol-dependent *L. chagasi* phagocytosis, MβCD-pretreated or -untreated macrophages were infected with non-opsonized parasites, or with parasites that had been opsonized with 5% A/J serum that is deficient in complement protein 5 (C5), to avoid com-

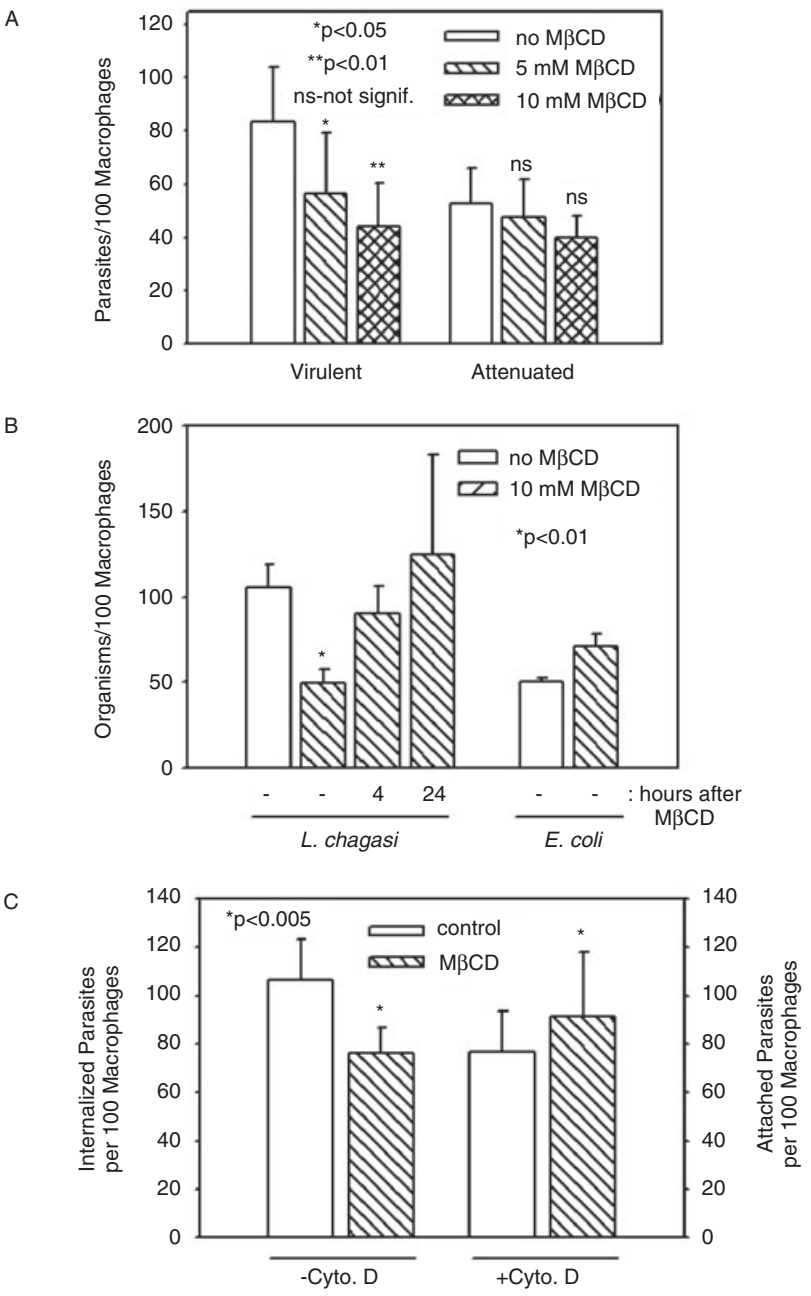

Fig. 2. *L. chagasi* infection of MβCD-treated macrophages.
A. MβCD decreases entry of virulent, but not attenuated *L. chagasi*. Macrophages were pretreated with 5 or 10 mM MβCD, and then infected with either attenuated L5 or virulent *L. chagasi*. After 30 min the ratio of parasites to macrophages was quantified microscopically. Shown is the mean ± SE for four replicate experiments, each with conditions performed in triplicate.
B. The MβCD effect on phagocytosis is reversible. Macrophages were pretreated with or without 10 mM MβCD. Conditions marked with '–' were immediately infected with either *L. chagasi* promastigotes (5:1 moi) or with *E. coli* DH5α (20:1 moi). Other macrophages were allowed a recovery period of 4 or 24 h in serum-containing medium prior to infection with 5:1 promastigotes. Infections were allowed to proceed for 30 min after which macrophages were fixed and stained. Shown is the mean ± SD parasite load of one representative out of a total of three experiments, each with triplicate conditions.
C. MβCD decreases *L. chagasi* internalization without affecting attachment to macrophages. Macrophages were preincubated with or without 10 mM MβCD, and in some conditions 10 μg ml^{-1} of cytocholasin D was added 30 min before and maintained throughout the infection. After a 30 min infection, parasite loads were quantified microscopically, and represented as either parasites internalized per 100 macrophages in cells without cytochalasin, or parasites attached per 100 macrophages (MP) in cells pretreated with cytochalasin D. Shown is the mean ± SE of three experiments with triplicate conditions.

plement-mediated lysis. Because opsonization enhances promastigote uptake, we normalized the initial phagocytosis level by infecting macrophages with 5:1 opsonized or with 10:1 non-opsonized promastigotes.

Similar to the above-mentioned reports, serum opsonization of L5 attenuated parasites rendered them insensitive to MβCD cholesterol depletion (Fig. 3A). A possible explanation is that opsonization with cross-reacting antibody could channel these parasites to undergo phagocytosis via Fc-receptors in clathrin coated pits, thereby avoiding caveolae. Interestingly, non-opsonized L5 attenuated parasites were still sensitive to MβCD-mediated cholesterol depletion. Regardless of the initial phagocytosis levels, attenuated L5 parasites failed to survive at later time points of infection.

In contrast to attenuated parasites, MβCD pretreatment decreased phagocytosis of virulent *L. chagasi* both in the opsonized and the non-opsonized state (Fig. 3B). There was a marked reduction in parasite load 24 h after infection in all conditions studied. However, 48–72 h after infection, virulent parasites replicated in control macrophages whereas parasites failed to replicate in MβCD-pretreated

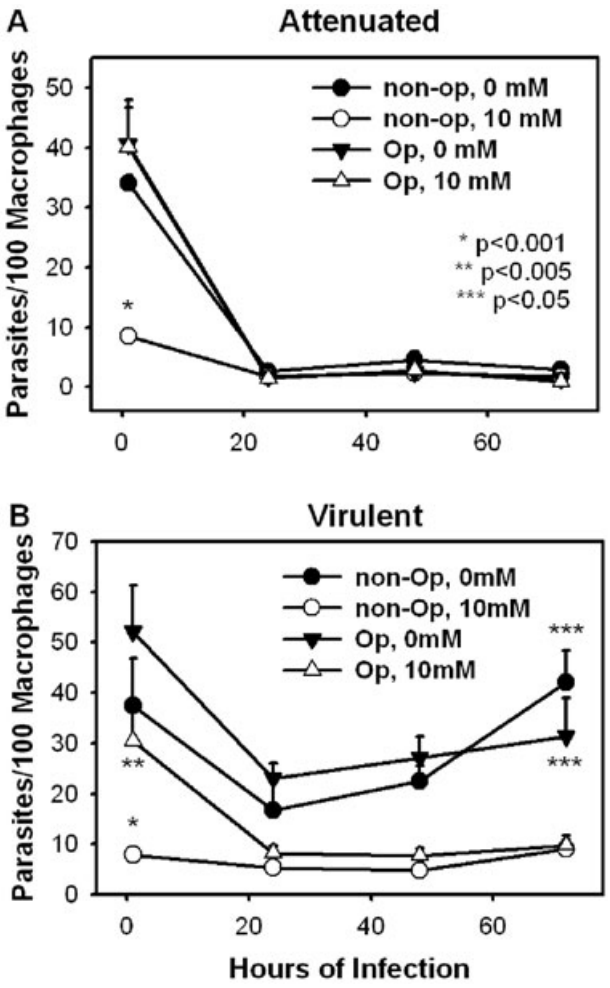

Fig. 3. Entry and survival of virulent *L. chagasi* in macrophages is cholesterol dependent. Macrophages were either untreated (closed symbols) or pretreated for 1 h with 10 mM MβCD (open symbols). Attenuated L5 (A) or virulent (B) *L. chagasi* promastigotes were either non-opsonized or opsonized with A/J (C5-deficient) mouse serum. Macrophages were infected with non-opsonized (10:1 moi) or opsonized parasites (5:1 moi) for 30 min before removing extracellular parasites. At the appropriate time points, samples were collected and infection levels were quantified using light microscopy. Values represent the mean ± SE for three experiments, each with triplicate conditions. Statistical analysis (*t*-test) at time 0 compares parasite entry in macrophages without or with MβCD. *P*-values at 72 h compare the numbers of intracellular parasites at the 24 versus the 72 h time point (i.e. intracellular replication).

macrophages. This occurred despite the fact that lipid rafts should begin to re-form by 4 h after phagocytosis (see Fig. 1). Thus, transient cholesterol depletion with MβCD at the time of phagocytosis affected not only the initial phagocytosis of virulent parasites, but also their ability to replicate intracellularly.

Inhibition of L. chagasi *phagocytosis with cholera toxin*

Cholera toxin B binds plasma membrane GM1 at a 5:1 molar ratio, thereby blocking access to membrane lipid rafts (Lencer and Tsai, 2003). Hence, CTX-B serves as a marker for all lipid rafts, of which caveolae are a subset (Shin *et al*., 2000; Naroeni and Porte, 2002; Lin and Rikihisa, 2003). To determine whether this alternate means of blocking interactions with lipid rafts would alter *L. chagasi* uptake, murine macrophages were pretreated for 30 min with increasing concentrations of CTX-B and then infected at a 5:1 ratio with opsonized *L. chagasi* or latex beads. CTX-B pretreatment decreased phagocytosis of *L. chagasi* but did not affect phagocytosis of latex beads (Fig. 4A). Interestingly, CTX-B could not fully inhibit *L. chagasi* phagocytosis, suggesting either that parasites bind lipid rafts/caveolae more efficiently than CTX-B, or that some parasites enter through a non-CTX-B inhibitable pathway.

Although cholera toxin consistently inhibited initial phagocytosis, the subsequent rate of intracellular parasite replication was parallel in CTX-B-treated versus control macrophages (Fig. 4B). Given the biochemical differences between MβCD and CTX-B, this result is not unexpected. Although CTX-B initially binds to GM1 in lipid rafts, its subsequent entry into cells occurs largely through clathrin-dependent endocytosis (Lencer and Tsai, 2003; Hansen *et al*., 2005). Unlike MβCD which temporary disassembles lipid rafts, the inhibition of promastigote internalization due to CTX-B could occur by steric inhibition of lipid rafts or temporary internalization of a subset, but not by actual disruption of caveolae. Those parasites that do enter in the presence of CTX-B therefore could do so through effective competition for intact caveolae and could therefore replicate normally. A requirement for binding to lipid rafts has been described for other molecules that enter cells through a clathrin-dependent mechanism including anthrax toxin and the B cell receptor (BCR) (Stoddart *et al*., 2002; Abrami *et al*., 2003). The actual molecule to which the parasite binds (e.g. surface receptors such as CR3) could be contained within intact caveolae.

Confocal microscopy

Membrane-associated lipid rafts that are enriched in both cholesterol and GM1 would include caveolae as well as other lipid rafts (Kiss *et al*., 2002; Lin and Rikihisa, 2003). The abilities of MβCD and CTX-B to inhibit parasite entry therefore do not discriminate between the involvement of caveolae versus other lipid rafts in *L. chagasi* phagocytosis. We utilized confocal microscopy to detect caveolin-1, the main protein component of caveolae in murine bone marrow macrophages, to assess the possible involvement of caveolae in promastigote phagocytosis (Harris *et al*., 2002b).

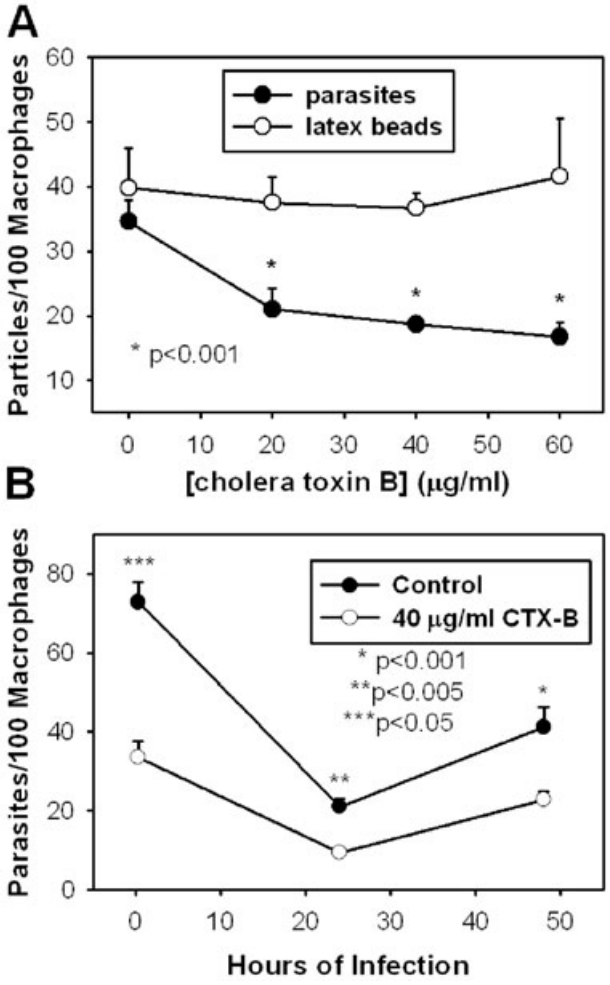

Fig. 4. A. Cholera Toxin- B (CTX-B) decreases *L. chagasi* phagocytosis. Macrophages were incubated with the indicated concentrations of CTX-B 30 min before infection with *L. chagasi* promastigotes (5:1 moi) or with 0.75 μm latex beads (10:1 moi). After an additional 20 min incubation, cells were fixed and stained. Parasite loads were quantified microscopically. Values represent the mean ± SD in one representative out of four repeat experiments, each with triplicate conditions.
B. CTX-B does not affect *L. chagasi* replication. Macrophages pretreated with CTX-B or non-treated controls were infected with opsonized *L. chagasi*. After an additional 20 min incubation, extracellular parasites were removed and infected cells were maintained for 24–48 h. Parasite loads were quantified microscopically. Values represent the mean ± SE of three repeats experiments with triplicate conditions.

BALB/c macrophages were infected with opsonized *L. chagasi* promastigotes for times ranging from 5 min to 48 h. Confocal microscopic examination showed clusters of caveolin-1 colocalizing with *L. chagasi* as early as 5 min after infection. Figure 5A shows that caveolin-1 colocalizes with intracellular parasites, but not with the extracellular promastigote. Furthermore, control stains (not shown) consistently showed that antibody to caveolin-1 did not bind to *L. chagasi* promastigotes in the absence of macrophages.

Colocalization of caveolin-1 and GFP-promastigotes was observed up to 48 h after invasion of macrophages (Fig. 5B and C). Similar results were obtained with non-opsonized virulent *L. chagasi* (data not shown).

Confocal microscopy was used to further confirm the association of GM1, a marker of all lipid rafts including caveolae, with phagocytosed parasites. Thirty minutes after infection, clusters of GM1 and caveolin-1 colocalized with *L. chagasi* (Fig. 6A). In contrast, phagocytosis of fluorescent latex beads failed to cluster either caveolin-1 or GM1 (Fig. 6C). Intracellular parasites remained in a compartment surrounded by caveolin-1 and GM1 for at least 24 h after infection (Fig. 6B).

Delayed fusion of L. chagasi*-containing caveolae with lysosomes*

It has been suggested that caveolae-mediated entry of viral or bacterial pathogens is a means by which pathogens are targeted toward an endocytic pathway that avoids fusion with lysosomes (Pelkmans *et al*., 2001; Watarai *et al*., 2002; Rhode *et al*., 2003). Using confocal microscopy, we examined whether caveolae-mediated phagocytosis affects the rate at which *L. chagasi*-containing phagosomes fused with lysosomes. Lysosomes were labelled by pre-loading macrophages with low molecular weight (Mr 10 000) dextran conjugated to tetramethyl rhodamine isothiocyanate (TRITC), followed by a chase period to allow dextran to traffic through the endosomal system to lysosomes. GFP-labelled *L. chagasi* promastigotes were added at time = 0 and fusion with lysosomes was investigated at time points between 30 min and 48 h after phagocytosis. As a control substance targeted toward lysosome fusion, some macrophages were exposed at time = 0 to fluorescein-conjugated low molecular weight dextran, which would traffic to the same lysosomal compartment as pre-loaded TRITC-dextran.

Thirty minutes after addition, control FITC-dextran colocalized with pre-loaded dextran and remained colocalized. FITC-dextran failed to cause clustering of caveolin-1 (Fig. 7A). In contrast, *L. chagasi* colocalized with caveolin-1 clusters but failed to colocalize with the pre-loaded dextran for several hours after infection (Fig. 7B).

An antibody to LAMP-1 was used to verify TRITC-dextran localization in lysosomes, and to mark lysosomes after *L. chagasi* phagocytosis. LAMP-1 is found in lysosomes and late endosomes. CSFE-labelled *L. chagasi* promastigotes were added at time = 0 and fusion with lysosomes was investigated at time points between 30 min and 48 h after phagocytosis. Co-stain with LAMP-1 verified that both pre-loaded dextran and the control dextran stimulus localized in lysosomes (a 5 h example is

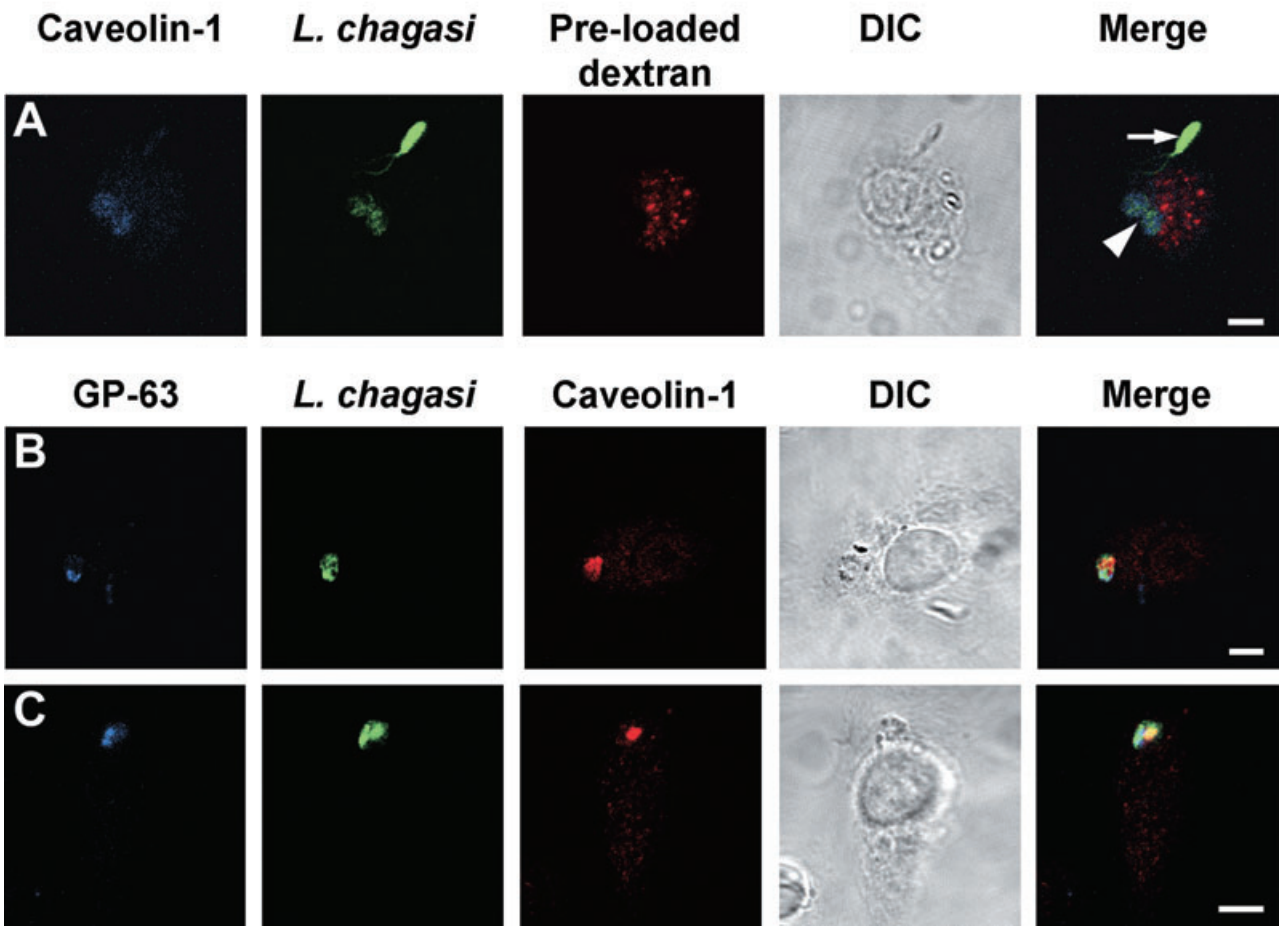

Fig. 5. Internalized *L. chagasi* promastigotes colocalize with caveolin-1.
A. Blue: caveolin-1; green: CFSE-stained *L. chagasi*; red: pre-loaded dextran. The endosome/lysosome pathway was marked by pre-loading macrophages with TRITC-dextran. As early as 5 min after infection, caveolin-1 colocalized with intracellular *L. chagasi*. Shown is a 1 h time point in which caveolin-1 colocalized with two intracellular parasites (arrowhead), but not with the extracellular parasite (arrow). Similar results were observed with non-opsonized parasites (data not shown).
B and C. Two examples are shown of parasites colocalizing with an intracellular cluster of caveolin-1. Blue: the parasite surface glycoprotein GP-63, green: CFSE-labelled *L. chagasi*, red: caveolin-1. Colocalization of blue and green produces turquoise; colocalization of green and red produces yellow. Parasites in rows B and C are smaller than parasites in row A because micrographs were taken 48 h after internalization, after the conversion from the larger promastigote to the smaller intracellular amastigote has occurred. The small region of blue staining in B likely corresponds to a part of a lysed parasite which has lost its CFSE stain. Panels are representative of more than 20 repeat experiments. DIC, differentiation interference contrast; scale bar: 5 μm.

shown in Fig. 8A). In contrast, *L. chagasi* failed to localize with pre-loaded dextran or with LAMP-1 at time points ranging from 30 min to more than 10 h after phagocytosis (a 5 h example is shown in Fig. 8B). Twenty-four hours after infection some but not all parasites colocalized, whereas by 48 h most or all parasites were in compartments that had fused with lysosomes according to TRITC-dextran and LAMP-1 staining (24 h shown in Fig. 8C and D). Similar results were obtained in three replicate assays. To further examine the difference in lysosome fusion kinetics, *L. chagasi* and TRITC-dextran were simultaneously added to macrophages. Two hours after addition, TRITC-dextran readily colocalized with LAMP-1 whereas *L. chagasi* does not (Fig. 8E). The data indicate that virulent promastigotes are targeted toward an intracellular pathway that delays fusion with lysosomes for 24–48 h after phagocytosis, but does not prevent fusion.

In contrast to the above results with virulent promastigotes, attenuated parasites entered compartments that underwent rapid fusion with lysosomes (Fig. 9). Five hours after infection, the extent of lysosome fusion was determined by counting the number of parasites colocalizing with TRITC-dextran and/or LAMP-1. We assessed lysosome-parasite colocalization for 140 attenuated and 110 virulent parasites. Whereas 87% (122) of attenuated parasites colocalized with lysosomal markers, only 43% (48) of virulent parasites colocalized with lysosomal markers during the same period of time ($P < 0.001$, chi square).

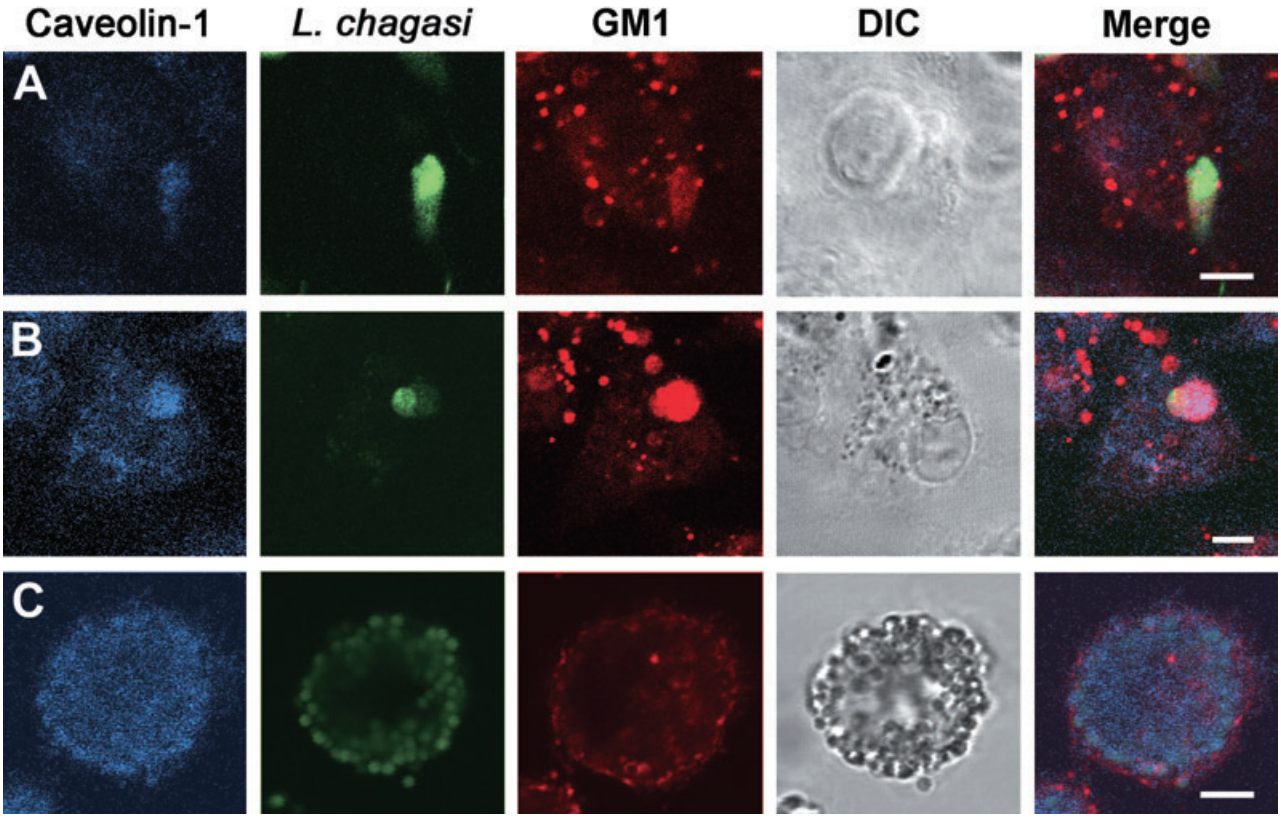

Fig. 6. Phagocytosis of *L. chagasi* induces clustering and colocalization of caveolin-1 and GM1 with parasites. Macrophages were infected with CFSE-stained *L. chagasi* promastigotes and stained with Alexa Fluor 555-conjugated CTX- B (red) to identify GM1, as well as antibody to caveolin-1 (blue).
A and B. Stains were performed (A) 30 min and (B) 24 h after infection.
C. In contrast, internalization of 0.75 μm fluorescent latex beads did not induce clustering of either CTX-B or caveolin-1. Micrographs are representative of three repeat experiments. DIC, differentiation interference contrast; scale bar: 5 μm.

Thus, the delay in fusion appears to be a function of parasite virulence.

Because disrupting lipid rafts with MβCD interfered with the intracellular replication of virulent parasites (see Fig. 3B), we hypothesized that entry through caveolae might target parasites to a pathway that enhances their intracellular survival. As such, we disrupted lipid rafts with MβCD and assessed colocalization with lysosomal markers. Five hours after phagocytosis, many more parasitophorous vacuoles were observed to colocalize with lysosomal markers in cells that were pretreated with MβCD than control-untreated cells (Fig. 9C). We assessed lysosome-parasite colocalization for 557 parasites in control, and 542 parasites in MβCD-treated macrophages. In the absence of MβCD, 119 parasites (21.4%) colocalized with pre-loaded dextran and LAMP-1. In contrast, after pretreatment with 10 mM MβCD, 273 parasites (50.4%) were found to colocalize with lysosomal markers ($P < 0.001$, chi square). Thus, *L. chagasi* was 2.4 times more likely to fuse with an endosomal/lysosomal compartment after transient, partial cholesterol depletion with MβCD.

Discussion

The route through which a phagocytic cell takes up a microbe can direct the subsequent intracellular trafficking, and consequently the intracellular fate of the microorganism (Pelkmans *et al*., 2001; Duncan *et al*., 2002; Manes *et al*., 2003). Although there have been many studies of membrane receptors ligated by the *Leishmania* sp. promastigotes (Mosser and Edelson, 1985; Brittingham *et al*., 1995; 1999; Kima *et al*., 2000), the purpose of the current study was to define the membrane structures that initiate internalization of the parasite. Localization of receptors such as CR3, CR1 or mannose receptors that facilitated parasite ligation within these structures might be essential for trafficking to the proper location after phagocytosis. Macrophages internalize particles or microorganisms through either caveolae or clathrin-coated pits. Previous

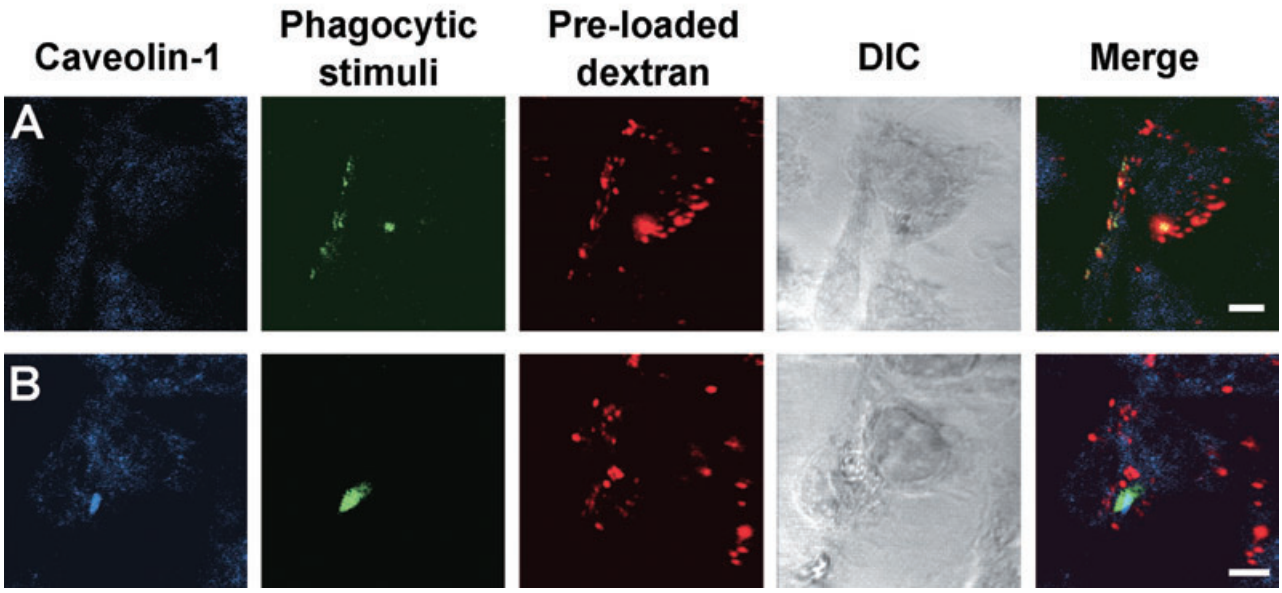

Fig. 7. *L. chagasi* colocalization with caveolin-1 delays lysosome fusion. Bone marrow macrophages were pre-labelled with 10 kDa TRITC-dextran followed by a chase without dextran, to mark the lysosomal compartment. This was followed by addition of (A) fluorescein-labelled dextran as a positive control or (B) GFP-labelled promastigotes. Confocal microscopy was used to assess colocalization of markers at serial time points. These figures show stains 5 h after the addition of dextran (A) or parasites (B). Colocalization of (green) fluorescein-dextran with (red) TRITC-dextran results in a yellow colour in A. Colocalization of GFP-parasites with caveolin-1 results in a light blue colour in B. Micrographs are representative of three identical repeats. DIC, differentiation interference contrast; scale bar: 5 μm.

work from our laboratory demonstrated that phagocytosis of *L. chagasi* by macrophages stimulates an increase in the abundance of caveolin-1 and -3 transcripts and protein (Rodriguez *et al*., 2004). This observation prompted us to investigate whether caveolae play a role in the phagocytosis of *L. chagasi* by macrophages.

Like other membrane rafts, caveolae are specialized membrane microdomains enriched in cholesterol, glycosphingolipids, GPI-anchored membrane molecules and GM1. Caveolae are additionally lined with caveolins-1, -2 and/or -3, and they contain some transmembrane receptors and lipid-modified signalling molecules such as G proteins (Harris *et al*., 2002a; Pelkmans and Helenius, 2002). Surface receptors that associate with caveolae include CR3, scavenger receptors, TNRF1, CD14, CD4, CD45 and CD55 (Shin and Abraham, 2001a; Harris *et al*., 2002a). Caveolae function as sites of signal transduction, and they bind free cholesterol through scaffolding domains, resulting in trafficking of cholesterol between intracellular compartments and the cell membrane (Gargalovic and Dory, 2003).

Using confocal microscopy, we observed that leishmania parasites enter compartments that are lined with caveolin-1 and GM1, and remained in these compartments for at least 24–48 h after infection. Pre-incubation of macrophages with the cholesterol chelating agent MβCD resulted in transient depletion of membrane cholesterol and disruption of caveolin/G1-containing lipid rafts. MβCD treatment inhibited both the initial phagocytosis of *L. chagasi* promastigotes and their ability to replicate intracellularly in murine macrophages, even though MβCD was removed and serum was added at the time of phagocytosis, resulting in re-formation of lipid rafts by 4 h. These data could be explained if lipid raft depletion caused parasites to enter through an alternate pathway, targeting them along an intracellular path in which their survival was impaired. The fact that lipid raft depletion also significantly increased early fusion of parasitophorous vacuoles with compartments containing features of lysosomes (i.e. low Mr dextran and LAMP-1) suggests that entry through the lipid raft/caveolae-mediated pathway might be necessary for promastigote survival.

In contrast to virulent promastigotes, opsonized attenuated *L. chagasi* or non-virulent *E. coli* DH5α apparently entered through alternate pathways that were unaffected by MβCD treatment. Furthermore, depletion of cholesterol with MβCD decreased the phagocytosis of virulent parasites to the same low level as that of attenuated parasites. Although not proven, these observations are consistent with the existence of two pathways for promastigote internalization, one of which requires intact membrane cholesterol enriched domains and leads to parasite survival, and the other which allows phagocytosis through non-cholesterol rich compartment and leads to parasite death.

The state of opsonization influences whether cholesterol is required for phagocytosis of several pathogens

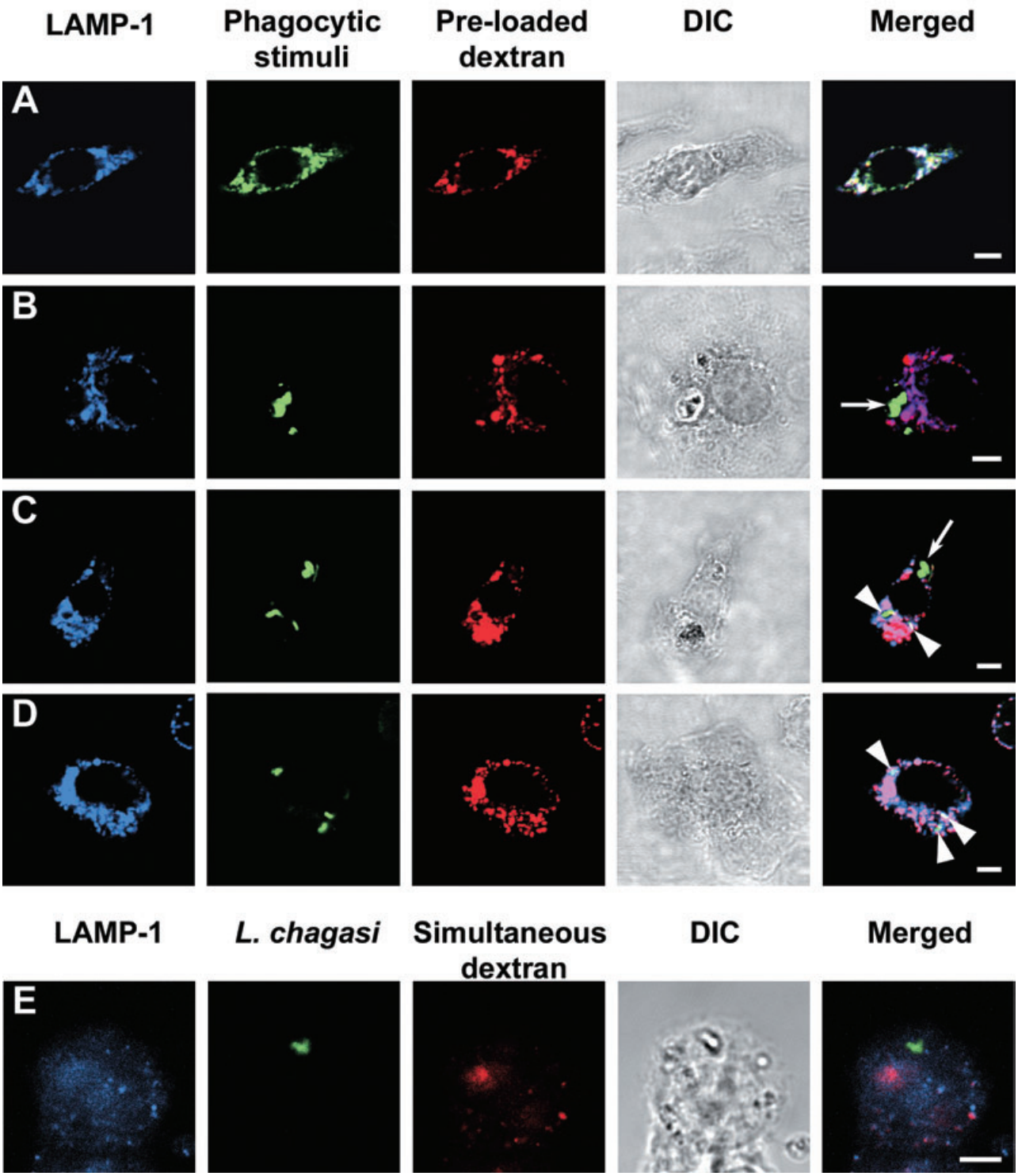

Fig. 8. LAMP-1 staining and the kinetics of phagosome–lysosome fusion.
A. Control macrophages were pre-labelled with TRITC-dextran and exposed to FITC-dextran at time 0. After 30 min (not shown) to 5 h (shown), lysosome colocalization was verified with LAMP-1 staining. The blue + green + red stains produce a white colour in the merged panel.
B–D. CFSE-labelled promastigotes (green) underwent phagocytosis by macrophages that had been pre-labelled with TRITC-dextran (red). Macrophage lysosomes were labelled with LAMP-1 (blue). Colocalization of blue + red stains produced a pink colour. Row B shows a 5 h infection, whereas rows C and D show two examples of 24 h infections. Cells in B, C and D contain between one and three phagocytosed parasites, which can extend in and out of the plane of focus. Arrows point to non-fused and arrowheads to fused parasites.
E. Simultaneous addition of TRITC-dextran and *L. chagasi* resulted in lysosomal colocalization of dextran producing a pink colour, but not of parasites which remained green (shown 2 h after infection). DIC, differentiation interference contrast; scale bar: 5 μm.

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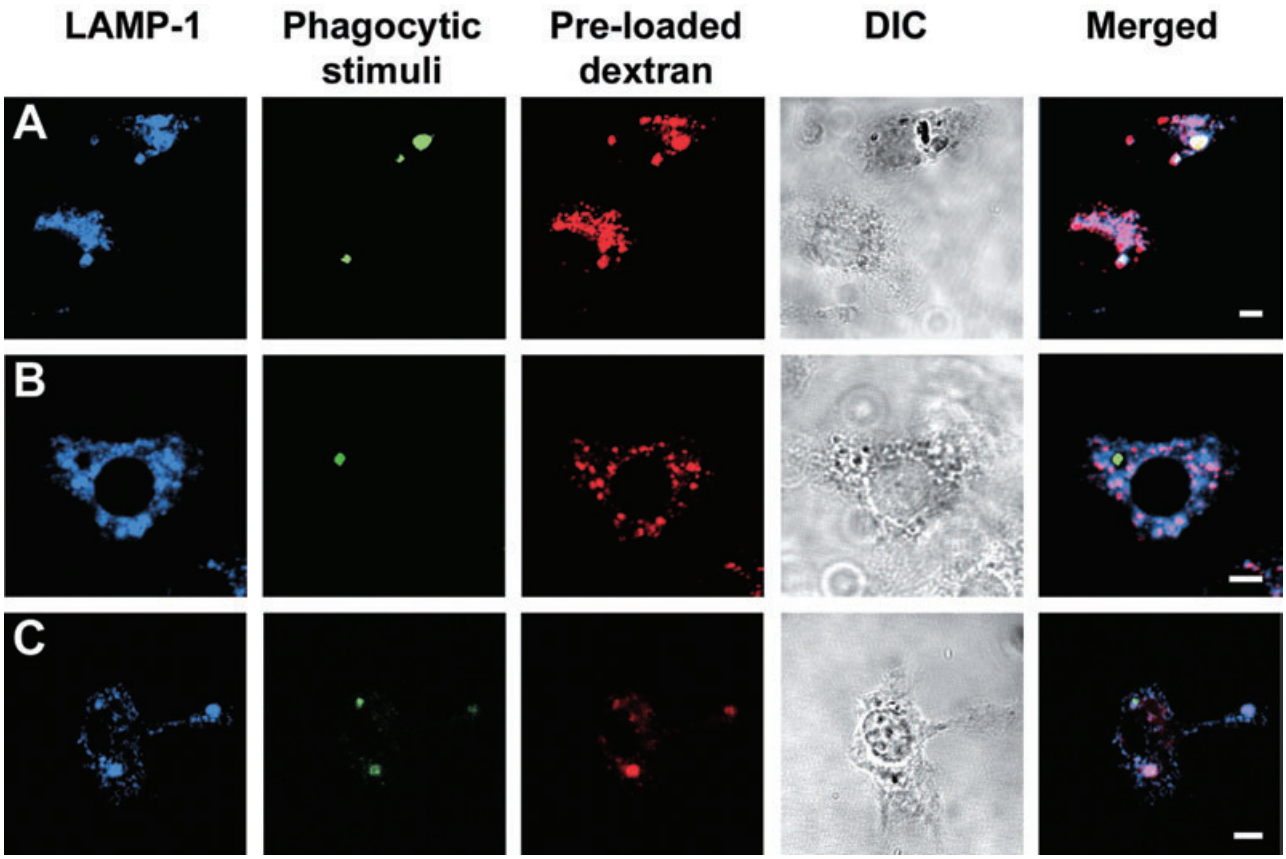

Fig. 9. Contrast between the kinetics of lysosome fusion in macrophages containing attenuated versus virulent parasites.
A and B. After pre-loading the endosome-lysosome pathway overnight with 10 kDa TRITC-dextran, macrophages were infected with either CFSE-labelled attenuated (A) or CFSE-labelled virulent (B) parasites. Five hours later, samples were fixed and stained with a blue LAMP-1 marker. Blue LAMP-1 colocalizes with red dextran to generate pink, whereas blue LAMP-1, red dextran and green parasites colocalizing generate white.
C. MβCD treatment accelerates fusion of lysosomes with virulent parasites. Macrophages were incubated without or with 10 mM MβCD for 1 h followed by infection with CFSE (green)-stained virulent parasites for 1, 3 or 5 h. Staining of the lysosomal compartment and quantification of lysosome fusion was performed as in the top two panels. Micrographs are representative of five repeat experiments. DIC, differentiation interference contrast; scale bar: 5 μm.

(Shin *et al*., 2000; Naroeni and Porte, 2002; Pucadyil *et al*., 2004). However, we found that depletion of macrophage cholesterol with MβCD inhibited the phagocytosis of virulent *L. chagasi* promastigotes regardless of whether the parasites were opsonized or not. This is in contrast with attenuated parasites, whose uptake in their non-opsonized state was inhibited by MβCD cholesterol depletion, but for which opsonization abrogated the apparent cholesterol dependence. These results suggest that the pathway of phagocytosis differs between virulent and attenuated parasites, and that virulent parasites utilize a cholesterol-dependent pathway even in the presence of serum whereas attenuated parasites do not. One possible explanation is that attenuated parasites could be opsonized with cross-reactive antibodies, allowing Fc-receptor-mediated phagocytosis through clathrin-coated pits. In contrast, virulent parasites, which display higher levels of surface molecules that promote C3 deposition [GP63 and lipophosphoglycan (LPG)], might become complement-opsonized efficiently through either exogenous complement or complement components produced locally at the macrophage surface, enabling phagocytosis through the complement receptor CR3 localized in caveolae (Russell, 1987; Brittingham *et al*., 1995; Shin and Abraham, 2001a; Harris *et al*., 2002a). Our observations are reminiscent of reports that C3 opsonization enhances the survival of *L. major* in macrophages (Mosser and Edelson, 1987).

Pucadyil and coworkers showed that depletion of cholesterol from the macrophage-like cell line J774 inhibited the phagocytosis of *Leishmania donovani* (Pucadyil *et al*., 2004). In the current manuscript we extend these observations, first, to document the specific localization of *L. chagasi* in caveolae, a subset of lipid rafts, during phagocytosis. Second, the current data suggest that entry through cholesterol-containing lipid rafts influences sub-

sequent intracellular events, i.e. fusion with lysosomes and intracellular survival. Third, Pucadyil *et al*. reported that serum opsonization of their *L. donovani* isolate rendered parasites insensitive to MβCD-mediated cholesterol depletion, but we found this to be the case with attenuated but not virulent promastigotes. It is possible the *L. donovani* isolate used by Pucadyil was attenuated by our definition, as it was not passed through animals. Alternatively, differences between Pucadyil's findings and ours could be due to the study of different *Leishmania* species (although we have found that *L. donovani* isolates colocalize with caveoloin-1 in data not shown), or the use of a cell line (J774) by Pucadyil *et al*. as opposed to our study in primary bone marrow macrophages. Indeed, J774 cells do not express caveolin-1, but they do express caveolin-2 in their Golgi (Matveev *et al*., 1999; Gargalovic and Dory, 2001). The *Leishmania* species are inoculated by sand flies into a pool of blood where they are likely to be opsonized, and our observation that cholesterol is important for phagocytosis of virulent *L. chagasi* even in the presence of serum is consistent with a role for cholesterol in phagocytosis of parasites *in vivo*.

Cell membrane caveolae mediate the uptake of several non-protozoan pathogens. FimH-expressing *E. coli* and SV-40 are each internalized via a receptor localized in caveolae (CD48 or MHC I respectively) (Shin *et al*., 2000; Pelkmans *et al*., 2001). Other microbes that are internalized through host cell caveolae include Polyoma virus, Echovirus 1, RSV, *Chlamydia trachomatis* and *Ehrlichia chaffeensis* (Harris *et al*., 2002a; Naroeni and Porte, 2002; Pelkmans and Helenius, 2002). SV-40 enters through caveolae and subsequently traffic to caveosomes and the smooth ER, avoiding fusion with lysosomes (Pelkmans *et al*., 2001). There is evidence that caveolin-containing vesicles can fuse with early endosomes and undergo subsequent acidification. These endosomes are positive for endosomal markers including the small GTPase Rab5 (Pelkmans *et al*., 2004; Parton, 2005). As such, caveolae can be transit compartments that direct molecules to specific locations in the cell.

An association between microbial virulence and entry through caveolae has been suggested in the case of SV-40, polyoma virus, Echovirus 1, RSV, *C. trachomatis, E. chaffeensis,* Group A streptococci and *Mycobacterium bovis* BCG (Harris *et al*., 2002a; Pelkmans and Helenius, 2002). However, a role for caveolae in cellular entry by protozoa has not been previously demonstrated. It is known that intracellular *Plasmodium falciparum* colocalize with lipid and protein components in erythrocyte detergent resistant microdomains, but no direct role for host cell caveolae in parasite uptake has been shown (Lauer *et al*., 2000). *Toxoplasma gondii* colocalize with cholesterol and GPI-anchored proteins (Sca-1 and CD55) during the first 5 min after invasion of fibroblasts, but they fail to colocalize with caveolin-1. Furthermore, studies with CTX-B and caveolin-1 overexpressing cells indicate that *T. gondii* do not require caveolae for fibroblast entry, and they actually exclude lipid rafts components from the parasitophorous vacuole after the first hour of infection (Mordue *et al*., 1999; Coppens and Joiner, 2003). The current report is the first to demonstrate a role for caveolae in the entry and survival of a parasitic protozoan.

Leishmania donovani mutants deficient in the surface glycolipid LPG are unable to delay phagosome–lysosome fusion (Desjardins and Descoteaux, 1997; Dermine *et al*., 2000). This delay involves selective exclusion of Rab7 from the leishmania parasitophorous vacuole (Duclos *et al*., 2000). Amastigotes, which lack LPG, reside in 'fusogenic' phagolysosomes resembling lysosomes (Alexander and Russell, 1992). In our study attenuated parasites, which lack the highly virulent 'metacyclic' form of LPG (Miller *et al*., 2000), were insensitive to MβCD depletion. Furthermore, fusion of lysosomes with virulent parasite vacuoles was delayed but not prevented. Indeed, the timing of parasitophorous vacuole–lysosome fusion corresponded approximately to the time when one would expect *Leishmania* sp. to convert to the amastigote stage, a form that lacks LPG and that is relatively resistant to killing by toxic oxidants (data not shown, M. Wilson and M. Miller). Data reported in the current study suggest that the initial entry of virulent promastigotes into macrophages through caveolae is necessary to target promastigotes toward this late- or non-fusogenic pathway. We hypothesize that the caveolae-associated pathway of entry is important for promoting survival of parasite infections initiated by the infectious promastigote stage of the organism.

Experimental procedures

Parasites

A Brazilian strain of *L. chagasi* (MHOM/BR/00/1669) is maintained by serial passage in male Syrian hamsters and used within 3 weeks for experiments (Wilson and Pearson, 1986). Promastigotes are grown in hemoflagellate-modified minimal essential medium (HOMEM), and used in the stationary phase of growth (Berens *et al*., 1976). The attenuated L5 is a clonal line derived from the original *L. chagasi* parasite that has been serially passaged in liquid culture for more than 10 years. L5 exhibits reduced infection levels in murine hosts, and reduced expression of key surface antigens such as GP63 and GP46, compared with virulent (recent) parasite isolates (Roberts *et al*., 1995). Stable *L. chagasi* transfectants expressing GFP contain the pXG-GFP plasmid were maintained in 1000 μg of G418 ml^{-1} pXG-GFP was kindly provided by Steven Beverley, Ph.D., Washington University, St Louis (Ha *et al*., 1996). Stationary phase promastigotes were opsonized in 5% fresh serum from C5-deficient A/J mice in Hank's Balanced Salt Solution (Gibco) for 30 min at 37°C, and rinsed by centrifugation at 4°C immediately before experiments. Viability of opsonized parasites was assayed by exclusion of

Trypan blue, and was consistently over 90% for both opsonized and non-opsonized promastigotes.

Bone marrow macrophages

Bone marrow cells from BALB/c mouse femurs were cultured at 37°C, 5% CO_2 in RP-10 [10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U of penicillin ml^{-1}, and 50 μg of streptomycin ml^{-1} in RPMI (Gibco, Carlsbad, CA)] containing 20% cell culture supernatant from L929 cells (American Tissue Type Collection, Manassas, VA) as a source of macrophage colony-stimulating factor. After 7–9 days, differentiated adherent macrophages were detached from the plate with 2.5 mg trypsin ml^{-1} plus 1 mM EDTA (Gibco) (Coligan *et al*., 1991). Macrophages of 5×10^5 were allowed to adhere to coverslips in 24-well plates, and infected with promastigotes at a multiplicity of infection (moi) of 5:1. The infection was synchronized by centrifugation (3 min, 330 *g*, 4°C), followed by warming to 37°C. Infected macrophages were incubated in 5% CO_2, 37°C. Extracellular parasites were removed by rinsing twice with PBS after 30 min or less of infection. Cells were incubated in fresh RP-10 until the appropriate time points.

Confocal microscopy

Macrophages (5×10^5) on 12 mm coverslips were infected with GFP-expressing promastigotes or promastigotes stained by incubating for 30 min with carboxy-fluorescein diacetate succinimidyl ester (CFSE; H. K. Chang and M. E. Wilson, manuscript in preparation). CFSE diffuses into the cytoplasm and in living cells, it is converted to a fluorescent state by cellular esterases. Latex beads (Sigma) or 10 000 Mr fluorescein-labelled dextran (Molecular Probes) were used as controls for phagocytosis or pinocytosis through the endocytic or lysosomal pathways respectively. To pre-label macrophage lysosomes, macrophages were incubated in 0.8 μg of Mr 10 000 TRITC-conjugated dextran ml^{-1} (Molecular Probes) overnight, rinsed, and chased in RP-10 medium without dextran for an additional 30 min before infection. Infected or dextran-laden cells were fixed in 2% paraformaldehyde (30 min; EMS, Hatfield, PA), permeabilized in 0.2% Triton X-100 (15 min), incubated in 50 mM glycine (15 min), and blocked in 5% non-fat dry milk/PBS (30 min). Macrophages were incubated with primary antibodies overnight at 4°C, rinsed, and incubated with 1:200 secondary antibodies at room temperature for 1 h. After rinsing in PBS and mounting with Vectashield H-1000 (Vector Labs, Burlingame, CA), slides were examined on a Zeiss 510 laser confocal microscope. Images were captured using the laser scanning microscope (LSM) 510 version 3.2 software. Confocal optical sections were analysed using the LSM 5 image browser. All microscopy studies were performed at the University of Iowa Central Microscopy Research Facility.

Antibodies and dilutions were rabbit anticaveolin-1 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:35, sheep anti-GP63 [generated from purified *L. chagasi* GP63 (Yao *et al*., 2002)] at 1:500, and goat anti-LAMP-1 (Santa Cruz) at 1:50. Secondary antibodies were Alexa Fluor 568 (red) goat anti-rabbit IgG or Alexa Fluor 647 (blue) goat anti-rabbit for caveolin-1, Alexa Fluor 488 (green) donkey anti-sheep for GP-63, and Alexa Fluor 647 (blue) donkey anti-goat for LAMP-1. As a primary stain for ganglioside GM1, CTX-B Alexa 555 (red) was used at 20 μg ml^{-1}. DNA was stained with TOPRO-3 (Molecular Probes) at a 1:3000 dilution.

Methyl-β-cyclodextrin-treated macrophages

Macrophages on glass coverslips were rinsed with PBS to remove residual serum from RP-10, and replaced with RPMI. Macrophages were left untreated, or incubated in 10 mM MβCD (Sigma) alone or with 200 μg ml^{-1} water-soluble cholesterol (Sigma) at 37°C for 1 h. After treatment cells were rinsed with PBS, and either used immediately or incubated in fresh RP-10 containing serum, in which they were allowed to recover at 37°C for 4 or 24 h prior to fixation with 3% paraformaldehyde. After fixing, cells were rinsed with PBS, incubated with 50 mM glycine (15 min), and then stained with either 300 μg ml^{-1} filipin III (Sigma) 1 h, or with 0.1 ng ml^{-1} CTX-B Alexa 555 (15 min). Samples were PBS rinsed and mounted with Vectashield H-1000 and examined in a Bio-Rad Radiance 2100 MP multiphoton microscope. For filipin the iris was completely open and the Mai Tai laser was at 10% and tuned to 800 nm. For CTX-B the Helium/Neon laser was set at 60%. Microscope parameters were kept the same during examination of all four samples.

Infection in MβCD-treated macrophages

Macrophages on glass coverslips were incubated in 5 or 10 mM MβCD at 37°C for 1 h as described above. After rinsing with PBS, macrophages were infected with attenuated L5 or virulent *L. chagasi* promastigotes that had either been opsonized (5:1 ratio) or not opsonized (10:1 ratio) in RP-10. Alternatively, macrophages were infected with 20:1 *E. coli* log phase ($OD_{600} = 0.08$) DH5α that had been grown in antibiotic-free Luria–Bertani broth, fixed in 2% paraformaldehyde and rinsed extensively in PBS. After 30 min extracellular microorganisms were removed by rinsing. Cultures were maintained at 37°C, 5% CO_2 for up to 96 h. At each time point coverslips were blow dried and stained with Diff Quik (Wright-Giemsa). The number of intracellular parasites or bacteria was determined by light microscopy.

Inhibition of L. chagasi *phagocytosis by cholera toxin-B*

Macrophages on coverslips were incubated with 0, 40, 80 or 120 μg ml^{-1} of CTX-B (Sigma) at 37°C for 30 min, rinsed twice with PBS, and infected synchronously with either *L. chagasi* promastigotes or 0.75 μm latex beads for 20 min prior to staining. The proportion of parasite-infected or latex bead-containing macrophages was determined microscopically.

Statistical analyses

Statistical analyses were performed using *T*-test, paired *T*-test, or chi square as implemented in Sigma Stat (Jandel Scientific) or Excel software.

Acknowledgements

The authors have no conflicting financial interests. We are grateful to Dr Kenneth Moore and the staff from the University of Iowa Central Microscopy facility for training and help with microscopy. We particularly thank Jian Shao, Thomas Moninger and Dr Chantal Allamargot of the microscopy facility. We are also thankful to Paul Reimann and Dennis Dunnwald for help in preparing illustrations. This work was supported in part by a Merit Review grant

from the Department of Veteran's Affairs (MEW), by National Institutes of Health Grants R01 AI045540 (MEW), RO1 AI048822 (MEW) and R01 AI032135 (MEW). N.E.R. was supported on NIH training Grants T32 AI07511 and AI07343.

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