Successful invasion of *Trypanosoma cruzi* trypomastigotes is dependent on host cell actin cytoskeleton

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**Abstract**
Cellular invasion by *Trypanosoma cruzi* metacyclic trypomastigotes (MTs) or tissue culture trypomastigotes (TCTs) is a complex process involving host–parasite cellular and molecular interactions. Particularly, the involvement of host cell actin cytoskeleton during trypomastigote invasion is poorly investigated, and still, the results are controversial. In the present work, we compare side by side both trypomastigote forms and employ state-of-the-art live-cell imaging showing for the first time the dynamic mobilization of host cell actin cytoskeleton to MT and TCT invasion sites. Moreover, cytochalasin D, latrunculin B, and jasplakinolide-pretreated cells inhibited MT and TCT invasion. Furthermore, our results demonstrated that TCT invasion decreased in RhoA, Rac1, and Cdc-42 GTPase-depleted cells, whereas MT invasion decreased only in Cdc42- and RhoA-depleted cells. Interestingly, depletion of the three studied GTPases induced a scattered lysosomal distribution throughout the cytosol. These observations indicate that GTPase depletion is sufficient to impair parasite invasion despite the importance of lysosome spread in trypomastigote invasion. Together, our results demonstrate that the host cell actin cytoskeleton plays a direct role during TCT and MT invasion.

**KEYWORDS**
actin cytoskeleton, cell invasion, metacyclic trypomastigote, Rho GTPases, tissue culture trypomastigote, *Trypanosoma cruzi*
vacuole formation (Fernandes et al., 2011; Tardieux et al., 1992). A lysosomal-independent pathway for TCT invasion has also been proposed, where the parasites rely on a PI3K-dependent mechanism in which PIP3 accumulates in parasite interaction sites, culminating in membrane invagination and consequent parasite internalization (Burleigh, 2005; Cortez et al., 2016).

The actin cytoskeleton has essential functions in eukaryotic cells and is responsible for promoting internal vesicle traffic, stress fibers, signaling, cell division, and endocytosis (Spiering & Hodgson, 2011). During actin filament polymerization by the Arp2/3 complex, some nucleation-promoting factors are activated by a GTPase, primarily from the Rho family. Rho GTPases act in different ways in the cell, mainly regulating gene expression, membrane transport, cell adhesion, stress fiber formation, filopodia, and lamellipodia. Three Rho GTPases, Cdc42, RhoA, and Rac1, are mostly involved in actin cytoskeleton modulation (Nobes & Hall, 1999).

Few studies have evaluated actin cytoskeleton participation in trypanosomatid host cell invasion, and the available findings remain controversial. Studies on TCTs incubated with cytochalasin D- or jasplakinolide-treated mammalian cells reported a reduced trypomastigote invasion. In addition, the removal of these drugs from the medium restored trypomastigote infectivity (Rosestolato et al., 2002). In contrast, Ferreira et al. (2006) demonstrated that actin participation during MT invasion is strain-dependent. In addition, Mortara (1991) demonstrated that compared with extracellular amastigote invasion, trypomastigote invasion is less affected by host cell treatment with cytochalasin D. Furthermore, studies on macrophages have indicated that actin filaments participate in the trypomastigote invasion associated with PI3K signaling, which consequently activates a phagocytosis-like mechanism (De Souza, 2002). Although TCTs and MTs engage different pathways for internalization, the results presented here demonstrate that both forms use the host cell actin cytoskeleton during the invasion.

**EXPERIMENTAL PROCEDURES**

**Cell cultures**

HeLa (human uterine cervical epithelial cells) and Vero cells (monkey kidney epithelial cells green) (Institute Adolfo Lutz) were, respectively, used for assays and to maintain the *T. cruzi* cycle in vitro, as described previously (Procópio et al., 1998). The cells were grown in RPMI 1640, containing 10% fetal calf serum (FCS), and maintained at 37°C in a humid atmosphere with 5% CO2. HEK293T cells (human renal embryonic cells) were cultured in complete Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich). The TCTs were obtained from the supernatant of infected Vero cells cultured in RPMI medium, containing 2.5% FCS, at 37°C in a humid atmosphere with 5% CO2. The epimastigotes were grown in liver infusion tryptose medium (pH 7.2), supplemented with 10% FCS, at 27°C; the MTs were obtained after incubating the epimastigotes in Grace medium at 27°C and were purified on a diethylaminoethyl cellulose column, as previously described (Yoshida, 1983). *T. cruzi* from CL strain (*T. cruzi* VI (Zingales et al., 2009)) were used in all experiments.

**Lentiviral transduction and establishment of depleted HeLa cell lineages**

HeLa cell lines, stably expressing the interfering RNA sequences for Cdc42, RhoA, and Rac1, used in this project had previously been established in our laboratory (Bonfim-Melo, et al., 2018). The sequences obtained commercially (Sigma–Aldrich) are presented below, and the lentiviruses were produced according to the protocol described (Bonfim-Melo et al., 2015). A suspension of 5 × 10^6 HEK293T cells was seeded in 10 cm dishes with complete DMEM. After 24 h, the cells were transduced by calcium phosphate precipitation with a 15 μg shRNAi vector (pLKO.1), 10 μg viral protein (pDR8.9), and 5 μg VSV-G envelope (pCI.VSVG) per dish. After 6 h, the cells were incubated with 15% glycerol in phosphate-buffered saline (PBS) for 2 min, washed twice with PBS, and incubated with a complete medium. After 24 and 48 h of transfection, the culture supernatant was filtered through a 0.45 μm pore membrane and stored at −80°C. For lentiviral transduction, 5 × 10^5 HeLa cells were seeded in a 6-well plate and 1 ml lentivirus supernatant was added to cells with 8 μg/ml polybrene® (Sigma–Aldrich) after 24 h. The supernatant was completely replaced 24 h post-transduction and shRNAi-expressing cells were selected using increasing puromycin concentrations (from 0.2 to 10 μg/ml) in the following 2 weeks. Protein depletion was evaluated using standard western blotting protocols. The primary antibodies used were anti-Rac1 mouse mAb (clone 23A8; Millipore), anti-RhoA rabbit pAb (R9404, Sigma–Aldrich), anti-Cdc42 mouse pAb (EPR15620; Abcam), and anti-β-actin mouse mAb (3700; Cell Signaling), while the secondary antibodies used were goat anti-rabbit IgG-peroxidase (A6154; Sigma–Aldrich) and anti-mouse IgG-peroxidase (A4416; Sigma–Aldrich). Chemiluminescence reaction was performed using the ECL Prime western blotting detection kit (Amersham Biosciences) and assessed on an Alliance 2.7 photodocumenter (UVitec). pLKO1 plasmids
containing the shRNAi oligos, purchased from Sigma–Aldrich, were as follows: Cdc42/NM_001791 (TRCN0000299931; CCGGCCTGATATCCTACACAACAAACTCGAGTTTGTTGTAGGATATCGTAAATGTCAAGACAGT AGGGTTTGTG), Rac1/NM_006908 (TRCN0000318375; CCGGCCCTACTGTCTTTGACAATTACTCGAGTAATTGTCAAAGACAGTGAGGGTTTTTG), RhoA/NM_001664 (TRCN0000047711; CCGGTGGAAAGACATGCTTGCTCACTCGAGATGAGCAAGCATGTCTTTCCATTTTTG), and Scramble/Scr (SHC001; CCGGCAACAGATGAGAGACACCAACTTCAGTTTGTCTTTTG). The lentivirus-packing plasmids pdR8.9 and pCI.VSVG have been previously used by our group (Bonfim-Melo et al., 2015, 2018).

Invasion assays

For assays with depleted HeLa cells, $1.5 \times 10^5$ cells/well were plated in a 24-well plate and incubated at 37°C. After 24 h, MTs or TCTs were added (MOI 25) and the plates were incubated for 2 h at 37°C and 5% CO₂. The coverslips were then stained with Giemsa. Intracellular parasites were counted in 500 cells/coverglass, in quadruplicate.

Giemsa staining

The coverslips used for Giemsa staining were washed six times with PBS to remove noninternalized parasites, fixed with Bouin (Sigma–Aldrich) for 5 min, washed five times with PBS, stained with Giemsa (1:4 in tap water) for 1 h, and sequentially dehydrated in acetone, followed by a graded series of acetone:xylol (9:1, 7:3, and 3:7) and finally xylol. This technique allows the optic distinction between intracellular parasites, which are surrounded by a halo. The coverslips were then mounted on slides with Entellan (Merck) and analyzed using optical microscopy, as described previously (Ferreira et al., 2017).

For assays with drugs that impair actin cytoskeleton dynamics, $1.5 \times 10^5$ HeLa cells were plated per well in a 24-well plate. After 24 h, the cells were treated with $20 \mu M$ cytochalasin D (Kustermans et al., 2005; Legrand-Poels et al., 2007; Lin et al., 2018), $4 \mu M$ latrunculin B (Ferreira et al., 2006), and $0.1 \mu M$ jasplakinolide (Lin et al., 2018; Rosestolato et al., 2002), (Sigma–Aldrich). Cell viability tests showed that the treatment did not cause a significant change in cell viability (revealed by morphology recovery after drug removal). After 1 h of treatment, cells were washed thrice with PBS to remove the drugs and incubated with MTs and TCTs (MOI 25) for 2 h at 37°C and 5% CO₂. Giemsa staining protocol was followed. Intracellular parasites were counted in 500 cells/coverglass in triplicate.

Immunofluorescence

Cells on the coverslips were gently washed with PBS, fixed with 3% paraformaldehyde and 0.2% glutaraldehyde (0.1% or 0.2% of glutaraldehyde combined with 3%–4% of paraformaldehyde has been shown to improve the fixation since it fixes faster and more completely, consequently generating more preserved cell structures Richter et al., 2018; Tanaka et al., 2010) for 15 min at room temperature (22–25°C), and stained with 4,6-diamidino-2-phenylindole (DAPI, #1306, Sigma–Aldrich), phalloidin-TRITC (Sigma–Aldrich) and the respective specific PGN-Saponin-diluted antibodies for each experiment (Bonfim-Melo et al., 2015), for 1 h. For lysosome localization mouse mAb anti-lamp2 antibody (H4B4, Thermo) was diluted 1:100 and subsequently reacted with Alexa Fluor-488 anti-mouse IgG (Invitrogen) 1:200 as a secondary antibody. The coverslips were mounted on slides with 1 mM glycerol-p-phenylenediamine (Sigma–Aldrich) buffer before evaluation using epifluorescence (BX51; Olympus) or confocal (TCS SP5 II Tandem Scanner; Leica) microscopes. Lysosomal spread index was performed by measuring the lysosome signal distributed through-out the cytoplasm subtracted from the perinuclear region on ImageJ using the Freehand selection tool followed by a Measurement tool. GraphPad Prism was used for statistical analysis.

Actin recruitment assays

For the time-lapse assay, HeLa cells were plated in Hi-Q4 (ibidi) plates for 24 h and transfected with 2 µg LifeAct-GFP and 6 µl FuGene HD according to the manufacturer’s instructions. The next day, the medium was replaced and 48 or 72 h post-transfection, the cells were incubated with Lysotracker (Invitrogen) (for labeling and tracking acidic organelles in live cells) for 1 h. Then, MTs or TCTs were added (MOI 25) and interactions were evaluated using time-lapse confocal microscopy. The cells were incubated at 37°C with 5% CO₂ in a humid atmosphere. Five focal planes were acquired (0.7-µm thickness and 2-µm z-step), with 3–4 min intervals, using 63X and 1.40 N.A. objective. At
least four fields were evaluated in two independent experiments. Confocal images were processed and rendered with IMARIS 7.0.0 (Bitplane) and evaluation of actin and lysosome dynamics during parasite interaction was performed with ImageJ. Twenty-two events were analyzed in two independent experiments.

For actin recruitment assays with fixed cells, 10^5 HeLa cells per well were plated on coverslips in 24-well plates. After 24 h, MTs and TCTs were added to the wells (MOI 25) for 1 h, after which the coverslips were subjected to the immunofluorescence protocol. Invading parasites were counted and actin recruitment in each interaction was evaluated (100 interactions per coverslip). Representative images were acquired with confocal microscopy and edited using the IMARIS 7.0.0 (Bitplane) software.

Statistical analysis

Results are presented as mean ± SD. Each point represents a replicate from independent duplicate, triplicate, or quadruplicate experiments. Analyses were performed using ANOVA with Tukey's multiple comparison test using the GraphPad Prism 7.0® software.

**FIGURE 1** Trypomastigotes selectively recruit host cell actin cytoskeleton to membrane adhesion site. HeLa cells were incubated with (A) metacyclic trypomastigotes (MTs) or (C) tissue culture trypomastigotes (TCTs) fixed with paraformaldehyde and stained with 4,6-diamidino-2-phenylindole (DAPI; nucleic acid marker) and phalloidin-TRITC (filamentous actin marker). Yellow and blue arrows show the adhered MTs and actin recruitment by the parasite, respectively. 3D: three-dimensional surface renderization showing recruitment or not of actin to the parasite invasion site. Bar: 5 μm. (B, D) F-actin recruitment quantification in at least 100 parasites per replicate (three independent experiments, in triplicate)
RESULTS AND DISCUSSION

Actin participates in MT and TCT invasion

To elucidate the role of the actin cytoskeleton during trypomastigote invasion, we first incubated HeLa cells with TCTs and MTs for 1 h and then fixed the cells to observe the F-actin using fluorescent microscopy. Interestingly, approximately half of the interactions displayed actin recruitment to the invasion sites of both forms (Figure 1A–D). However, confocal live-cell imaging experiments, using HeLa cells transfected with Lifeact-GFP, revealed that both MT and TCT were internalized only after actin recruitment (Figure 2A,B). Furthermore, Figure 2C,D indicate adherent

**FIGURE 2**  F-actin recruitment is required for trypomastigote internalization. HeLa cells were transfected with the Plasmid Lifeact-GFP (green) and incubated with Lysotracker (red) for visualization of actin and lysosome dynamics, using confocal live-cell imaging, during interaction with MTs and TCTs. (A and B) Interaction of MT and TCT with the host cell actin cytoskeleton results in successful parasite internalization. All observed internalizations were followed by actin recruitment. (C and D) No actin recruitment to trypomastigote invasion site results in parasite detachment from the host cell membrane. Particularly for MTs, lysosomes are recruited to the adherent parasites regardless of actin recruitment without internalization (C). Arrowheads indicate the parasites. Bar = 10 μm. Images were edited and processed using Imaris (Bitplane) and ImageJ (NIH open source).
parasites without actin recruitments, which are consequently not internalized. The interactions shown in Figure 2 can be found on Videos S1–S4. Video S1 shows actin recruitment and MT internalization, and Video S2 shows the opposite, with no actin recruitment and consequently no internalization. Videos S3 and S4 show the same pattern in TCTs (internalization with actin recruitment and no internalization with no actin recruitment, respectively).

Thus, parasites can adhere to the cells, as observed by lysosome recruitment (MT); however, internalization is completed only after F-actin recruitment.

Additionally, with live-cell imaging we were able to elucidate temporal aspects of actin and lysosome dynamics during trypomastigote invasion. For MTs, lysosome accumulation occurs before actin recruitment and the contrary occurs for TCTs. Both actin and lysosomes remain recruited until TCT and MT internalization. Table 1 shows the average time of actin and lysosomes dynamic during trypomastigote invasion. The fact that MTs recruit lysosomes before F-actin suggests the higher dependency of lysosomal recruitment from MTs than TCTs for invasion. In addition, during live-cell experiments, we observed parasites that are not internalized but still temporarily accumulate actin. Therefore, we could not find any temporal pattern in these interactions. Comparable results were obtained during the invasion of *T. cruzi* extracellular amastigotes. In previous works from our group, we demonstrated that the depletion of the ERM proteins or the here studied GTPases (Bonfim-Melo et al., 2018; Ferreira et al., 2017) leads to a significantly delayed internalization but the actin dynamics and recruitment to invasion site was still preserved displaying same features as WT cells. We conclude that invasion of trypomastigotes is dependent on an efficient actin cytoskeleton signaling cascade that may not be triggered in all interactions.

Thus, it can be inferred that actin plays a role in trypomastigote invasion. Several studies have previously given clues on the host cell membrane and actin recruitment, using fluorescence microscopy, but little has been concluded. Ferreira et al. (2006) demonstrated that actin recruitment by trypomastigotes is apparently strain-dependent since only the G strain presented actin recruitment to its invasion site (compared with the CL strain). Precisely, they probably observed the selective recruitment of actin, which was believed to be strain-dependent at that time. Instead, selective actin recruitment to the parasite invasion site occurred in the same strain in the same experiment, as presented here. In this study, we revealed the selective nature of actin recruitment for successful parasite internalization, using live-cell imaging coupled with F-actin probes. Furthermore, Mortara (1991) had previously described the selective fashion of actin recruitment to parasite attachment sites. In addition, scanning electron microscopy results of previous studies have revealed membrane projections toward interacting trypomastigotes, which may be indirectly associated with actin-driven mobilization (Nogueira & Cohn, 1976; Schenkman & Mortara, 1992).

To corroborate the involvement of actin filaments in trypomastigote invasion, we determined whether the use of cytochalasin D, latrunculin B, and jasplakinolide, drugs, which act on the actin cytoskeleton, impairs trypomastigote invasion (Lin et al., 2018; Osuna et al., 1993; Rosestolato et al., 2002). HeLa cells were pre-treated with the drugs, which reduced both MT and TCT invasion (Figure 3A,B). Although previous studies have reported the effect of some of these drugs on MT or TCT invasion (Ferreira et al., 2006; Mortara, 1991; Osuna et al., 1993; Rosestolato et al., 2002), we present, for the first time, the effect of all four drugs on both MT and TCT using the same system. In addition, we have previously demonstrated the selective fashion of actin recruitment to the parasite invasion site, with no actin recruitment and consequently no internalization. Videos S3 and S4 show the same pattern in TCTs. Both actin and lysosomes remain recruited until TCT and MT internalization. For MTs, lysosome accumulation occurs before actin recruitment and the contrary occurs for TCTs. Thus, parasites can adhere to the cells, as observed by lysosome recruitment (MT); however, internalization is completed only after F-actin recruitment.

### Table 1: Average time of actin and lysosomes dynamic during trypomastigote invasion

| Time (min) | Attachment | Actin recruitment | Lysosome accumulation | Invasion | Actin dispersal |
|------------|------------|-------------------|-----------------------|----------|-----------------|
| MT         | 0          | 4                 | 4                     | 140      | 144             |
| TCT        | 0          | 4                 | 24                    | 46       | 50              |

**Note:** Twenty-two events were analyzed in two independent experiments. For MTs, we observed that lysosomes recruitment takes place prior to actin recruitment. For TCTs, first we observe actin recruitment and then lysosomal accumulation. Both actin and lysosomes remain recruited until TCT and MT internalization. Noteworthy, the internalization time disposed here is useful to show the relation between invasion, actin, and lysosomes dynamics during the invasion. The total interaction time (attachment to invasion) observed here may not necessarily correspond to all different *Trypanosoma cruzi* strains and even for the different experimental conditions performed with CL strain (used in this study).
Participation of Rho GTPases during trypomastigote invasion

Previous studies have reported that Rho GTPases are key regulators of actin dynamics and subverted by diverse intracellular parasites (Spiering & Hodgson, 2011). Additionally, the participation of Rho GTPases during cell invasion by extracellular T. cruzi amastigotes has been reported (Bonfim-Melo, et al., 2018). Since we initially observed that actin participates in cell invasion by trypomastigotes, we investigated whether Cdc42, RhoA, and Rac1 GTPases participate in TCT and MT invasion. Hence, we established Cdc42, RhoA, or Rac1-depleted HeLa cells using lentiviral transduction and demonstrated that cell invasion by MTs was reduced by Cdc42 and RhoA, but not Rac1, depletion significantly inhibited MT invasion (Figure 4A). Depletion of all three Rho GTPases inhibited TCT invasion (Figure 4B). Figure S1 shows HeLa cell lines with shRNAi-knocked down Cdc-42, RhoA, or Rac1, as assessed with western blotting. In contrast, Rac1 plays a pivotal role in extracellular amastigote invasion, followed by a less prominent role of Cdc-42 and a nonsignificant part of RhoA (Bonfim-Melo et al., 2018). From these results, it can be concluded that regulating Rac1-driven lamellipodia formation (Spiering & Hodgson, 2011) is central to extracellular amastigote invasion, and actin dynamic disturbances driven by overall Rho GTPase depletion might impact its participation during trypomastigote invasion.

Finally, owing to the importance of host cell lysosomal distribution in trypomastigote invasion (Cortez et al., 2016; Fernandes et al., 2011; Woolsey & Burleigh, 2004), we evaluated the lysosomal distribution in Cdc-42-, RhoA-, and Rac1-depleted cells, using confocal microscopy. Interestingly, our results demonstrated that depletion of the three
Figure 5  Rho GTPases, Cdc-42, RhoA, and Rac1 depletion increase lysosomal spread in HeLa cells. (A) HeLa cells stably depleted for Cdc-42, RhoA, or Rac1 were incubated, or not, with metacyclic or tissue culture trypomastigotes (arrows) and immunofluorescence using anti-lamp2 (specific lysosomal marker, green), 4,6-diamidino-2-phenylindole (DAPI, nuclei specific, blue) and kinetoplast and phalloidin-TRITC, red (F-actin specific). Lysosomal distribution (white arrows) was evaluated using confocal microscopy. Two experiments were performed in triplicate. Yellow arrows indicate parasites interacting with the host cell, better viewed in the inset items. Bar: 10 μm. (B) Lysosomes are significantly more spread in cells depleted to Cdc-42, RhoA, and Rac1 when compared to the control group (Scramble). Measurements of anti-lamp2 intensity show lysosomal spread index throughout the cytoplasm. The analyses were performed on ImageJ using the Measurement tool subtracting perinuclear lysosome fluorescent signal from lysosome signal throughout the cytoplasm. ± SD **p < 0.001 and ***p < 0.001. Statistical analysis was performed with ANOVA using Tukey's multiple comparison method.
studied Rho GTPases resulted in significantly increased lysosomal dispersion throughout the cytoplasm compared to that in the control group (scramble transduced group), which presents marked perinuclear lysosomal distribution (Figure 5A,B). In addition, incubating Rho GTPase-depleted cells with TCTs and MTs did not affect the already dispersed distribution of lysosomes in these cells. Intriguingly, trypomastigote, particularly MT, invasion is known to cause lysosomal spread (Cortez et al., 2016; Fernandes et al., 2011). Andrews (1993) suggested that lysosomal migration occurs only after cortical F-actin depolymerization. This is in accordance with the dispersed lysosomal distribution observed in Rho GTPase-depleted cells since Rho GTPase depletion might downregulate normal actin dynamics. Noteworthy, since we observed that GTPase depletion have less effect on inhibition of MT invasion (especially for Rac1 depletion in which we did not observe any effect) we may infer that the higher dependency of lysosome spread by MTs compared to TCTs (Burleigh, 2005; Cortez et al., 2016) might have counterbalanced the effect of GTPases depletion during MT invasion. Thus, we concluded that though the lysosomal spread is important, it does not superimpose the importance of functional actin (caused by Rho GTPase depletion in this study) for successful trypomastigote internalization.

Taken together the results presented here shed light on previous works and bring strong evidence of the participation of actin cytoskeleton during trypomastigote invasion. We highlight here the novelty of our results, in particular the study of TCT and MT side by side and the live-cell imaging experiments showing vividly the accumulation of actin to trypomastigotes invasion site. Further experiments should be conducted to explore the relationship between the established mechanisms for trypomastigote invasion and the participation of the host-cell actin cytoskeleton.

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CONFLICT OF INTERESTS
Authors declare no competing or financial interests.

AUTHOR CONTRIBUTIONS
Study conceptualization: R.A.M., E.R.F., and A.B.M. Study designing: E.R.F and A.B.M. Experimentation: B.S.B., E.R.F., and A.B.M. Result interpretation: E.R.F., A.B.M., R.A.M., and B.S.B. Manuscript writing: E.R.F. and B.S.B.

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