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Responsive microtubule dynamics promote cell invasion by *Trypanosoma cruzi*

Kevin M. Tyler,[†] George W. G. Luxton, Derek A. Applewhite, Sean C. Murphy and David M. Engman*
Departments of Microbiology-Immunology and Pathology, Northwestern University Feinberg School of Medicine, 303 East Chicago Avenue, Chicago, IL 60611, USA.

Summary

The American trypanosome, *Trypanosoma cruzi*, can invade non-phagocytic cell types by a G-protein-mediated, calcium-dependent mechanism, in which the cell's natural puncture repair mechanism is usurped in order to recruit lysosomes to the parasite/host cell junction or 'parasite synapse.' The fusion of lysosomes necessary for construction of the nascent parasitophorous vacuole is achieved by directed trafficking along microtubules. We demonstrate altered host cell microtubule dynamics during the initial stages of the entry process involving *de novo* microtubule polymerization from the cytoplasmic face of the parasite synapse which appears to serve as a secondary microtubule organizing centre. The net result of these dynamic changes to the host cell's microtubule cytoskeleton is the development of the necessary infrastructure for transport of lysosomes to the parasite synapse.

Introduction

According to the World Health Organization (WHO, 2002) approximately 18 million people in 18 countries of Latin America are infected with *Trypanosoma cruzi*, the haemoflagellate protozoan parasite that causes Chagas disease. There are some 300 000 new infections annually. Most frequently, ill health and death ensue from a chronic inflammation of the myocardium and this makes *T. cruzi* the world's foremost infectious cardiomyopathy. Currently, 13 000 deaths per year are ascribed to Chagas disease but the chronic nature of the disease leads to the loss of some 649 000 disability adjusted life-years annually, a huge burden to the development of the affected economies (Tanowitz *et al*., 2002).

As part of a complex digenetic life cycle involving insect and vertebrate hosts (Tyler and Engman, 2001; Tyler *et al*., 2002), *T. cruzi* invades nucleated mammalian cells by a novel microtubule-mediated mechanism (Burleigh and Woolsey, 2002; Yoshida, 2002). The trypomastigote form of the parasite enters the cell, escapes the vacuole via which it entered, and differentiates into the non-flagellated amastigote form of the parasite in the host cell cytoplasm. An initial step in the invasion process involves the induction of a calcium flux in the host cell via the action of a parasite-derived calcium agonist, which is generated by the action of a parasite oligopeptidase (Caler *et al*., 1998). The calcium flux drives cytoskeletal rearrangement and lysosome recruitment to the cell surface, juxtaposed to the parasite. We will hereafter refer to this juxtaposition of parasite and host cell membranes as the 'parasite synapse', by analogy to the neuronal and immunologic synapses, sites of cell to cell contact where regulated exocytosis occurs. Host cell signalling during *T. cruzi* invasion occurs via the synaptotagmin VII pathway (Caler *et al*., 2001), essentially serving to usurp the machinery involved in puncture repair and deliver lysosomes to the synapse where membrane fusion is likely to be mediated by the SNARE machinery (Rao *et al*., 2004). Indeed, the current paradigm for wound repair, the 'membrane patch hypothesis' (Reddy *et al*., 2001) was first derived from experiments investigating *T. cruzi* invasion (Tan and Andrews, 2002). Interestingly, recent work demonstrates that both lysosome fusion (Andrade and Andrews, 2004) and host actin polymerization (Woolsey and Burleigh, 2004) are requisite for parasite retention.

We reasoned that, because lysosomes are rapidly and directionally transported to the parasite synapse along the microtubule cytoskeleton, host cell microtubule dynamics must be stimulated by parasite contact and microtubules must either be recruited to, or polymerized *de novo* at, the parasite synapse. In these studies, we use a cell line expressing α-tubulin fused to green fluorescent protein (GFP) to visualize host cytoskeletal responses to parasitization in real time, employing fluorescence microscopy and captured by time-lapse videomicroscopy. Additional immunofluorescence microscopy and drug treatments were used to confirm microtu-

Received 23 January, 2005; revised 2 June, 2005; accepted 2 June, 2005. *For correspondence. E-mail d-engman@northwestern.edu; Tel. (+1) 312 503 1288; Fax (+1) 312 503 1265. [†]Present address: School of Medicine, University of East Anglia, Norwich, Norfolk, UK.

bule polarity and begin to dissect the cytoskeletal components involved.

Results

It has been clearly demonstrated in somatic cell types that lysosomes are transported to the parasite synapse in a microtubule-dependent manner. Several previous studies have visualized this lysosomal migration and the importance of microtubules in both lysosomal transport and *T. cruzi* cell invasion has been well established by use of drug studies (Tardieux *et al*., 1992; Rodriguez *et al*., 1996). To determine how lysosomes target the parasite synapse, components of the microtubule cytoskeleton were visualized. Previous studies have established that tachyzoites of the sporozoan *Toxoplasma gondii*, which enter the cell powered by their own motility and facilitated by their own secretions, have no discernible interaction with the host cytoskeleton during cell invasion (Morisaki *et al*., 1995; Dobrowolski and Sibley, 1997). *T. gondii* is of similar size *to T. cruzi* and, like *T. cruzi*, invades most nucleated cell types in a period of less than 30 min, making it a good control organism for our investigation. We utilized confluent layers of the heart-derived myoblast cell line H9C2, a non-professional phagocyte line which supports proliferation of both *T. cruzi* and *T. gondii*. Initially, we visualized the cell's microtubules using an α-tubulin-specific antibody, which visualizes all microtubules, host and parasite. At time points between 15 min and 30 min after washing, invading parasites were clearly seen in close association with high densities of the host microtubules (Fig. 1A). This 'nest' of microtubules was never observed in association with *T. gondii*.

Clear visualization of the parasite's interaction with host microtubules was difficult using the tubulin-specific antibody, both because of the large numbers of host microtubules in this cell type and because of difficulty distinguishing where parasite microtubules end and host microtubules begin. We therefore utilized the rat monoclonal antibody YL1/2, which binds specifically to tyrosinated microtubules. This subset of microtubules is predominantly composed of newly polymerized/dynamic microtubules and is absent from the non-dividing trypomastigote stage of the parasite. We reasoned that, if lysosomal trafficking resulted from recently polymerized microtubules associating with the parasite synapse and vacuole, then these microtubules would be highlighted with YL1/2. This was indeed the case, and parasites encased in lysosome-associated membrane glycoprotein 1 precursor (LAMP-1) positive vacuoles were seen to occupy 'hubs' of tyrosinated microtubules (Fig. 1B). LAMP-1 marks a subset of cellular vesicles including lysosomes and late endosomes. Interestingly, LAMP-1 staining vesicles in proximity to the parasite and the vacuole itself clearly lined up along the tyrosinated microtubules, consistent with their being trafficked to the vacuole in this manner.

Although the tyrosinated microtubules arising from the parasitophorous vacuole frequently had the appearance of a radial array, it was not clear whether the array was nucleated by the vacuole or whether the vacuole was acting to 'capture' the microtubules. We therefore elected to investigate how tubulin initially becomes associated with parasite synapse during cell invasion. To do so, we utilized PtK2 cells stably transfected with a construct expressing a GFP-α-tubulin chimera. Under ultraviolet illumination, flashes of fluorescence as well as bright fixed points associated with invasion were apparent. Microscopy revealed that, as parasites come in contact with the cell membrane, GFP fluorescence appeared to immediately concentrate at the contact point (Fig. 2 – 0, 5 and 10 s). This accumulation of fluorescence persisted as long as the parasite was present. When the parasite made an unproductive interaction with the cell, as seen in this figure, the concentration of fluorescence was transient, apparently diffusing from the point of contact once contact with the parasite was lost (Fig. 2 – 15 s).

To determine whether the observed rapid recruitment of tubulin is isotype-specific, we stained cells 5 min after exposure to parasites in order to catch the parasites as they entered the host cells. We found both tyrosinated α-tubulin and γ-tubulin associated with nascent parasitophorous vacuoles. However, the distributions of these tubulins were somewhat different, with the γ-tubulin more evenly coating the vacuole (Fig. 3A) while the tyrosinated tubulin appeared most concentrated at the leading edge (Fig. 3B), giving a characteristic 'crescent moon' appearance. In comparison, at this time point there was no visible interaction of cytoskeletal components with *T. gondii* (Fig. 3E–M).

We considered whether the recruitment of tubulins was dependent upon their polymerization. Parasites were allowed to invade host cells for 15 min in the presence of colchicine, making use of the observations that trypanosome tubulins are resistant to depolymerization by this drug (Filho *et al*., 1978) and that microtubule depolymerization is not completely effective at inhibiting cell invasion (Rodriguez *et al*., 1996). Colchicine was then washed out of test wells but maintained during washes of controls. Cells were incubated at room temperature for 15 min to allow microtubule repolymerization to occur. Images of parasitophorous vacuoles in test and control wells were then compared. In the presence of colchicine, both tyrosinated α-tubulin (Fig. 4A) and γ-tubulin (Fig. 4B) were recruited to the parasitophorous vacuole but, as expected, there was no sign of microtubules associated with the structure. By comparison, at room temperature 15 min after colchicine was washed out,

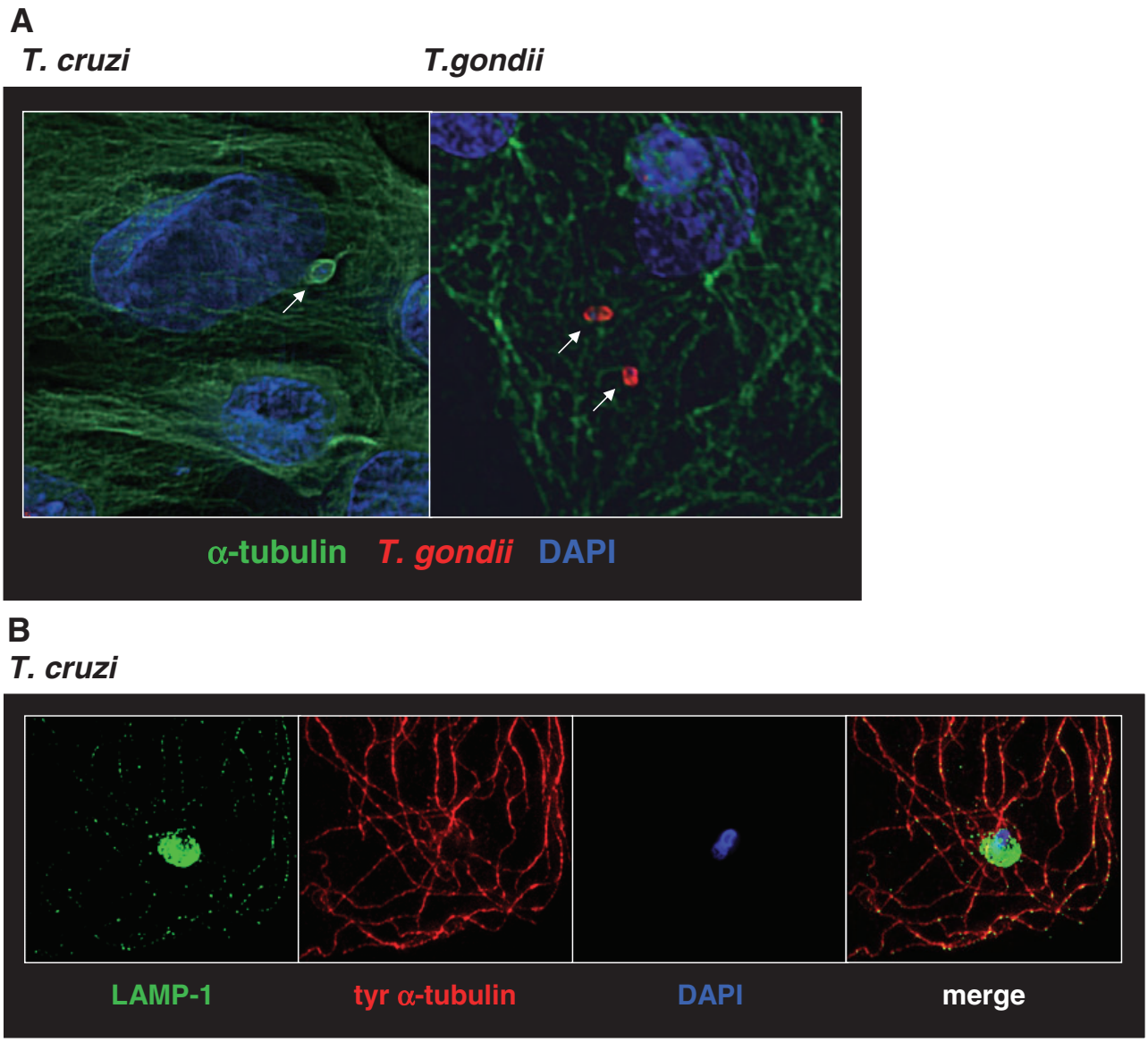


Fig. 1. Association of host microtubules with invading *T. cruzi*. Confluent cardiac-derived myoblasts were incubated with *T. cruzi* trypomastigotes or *T. gondii* tachyzoites for 15 min.
A. Shortly after invasion, microtubules can be seen in close association with *T. cruzi* but not *T. gondii*.
B. When lysosomes (LAMP-1), newly formed microtubules (tyrosinated α-tubulin) and *T. cruzi* (DAPI) are visualized at the same time point, the LAMP-1-positive parasitophorous vacuole can be seen to occupy the hub of a nest of microtubules. Images are 2-dimensional representations of a 3-dimensional capture set. See *Supplementary material* Movie S1 for a volume view of the 3-dimensional capture set.

short microtubules staining for tyrosinated α-tubulin were clearly visible, radiating from the parasitophorous vacuole (Fig. 4E).

The presence of γ-tubulin at the surface of the parasitophorous vacuole and the apparent ability of the vacuole to seed a radial array of short microtubules led us to postulate that first the parasite synapse, and later the parasitophorous vacuole, might be able to act as a microtubule organizing centre (MTOC). We utilized dynamitin overexpression to test this hypothesis. Overexpression of dynamitin disrupts dynactin, and hence the dynein cargo carrying complex, leading to the redistribution of lysosomes and other organelles to the plus ends of microtubules predominantly at the cell periphery (Burkhardt *et al.*, 1997). Superficially, because dynamitin expression puts lysosomes into close proximity to the invading parasite, it should promote uptake of the parasite, as has been described for pH-dependent displacement of lysosomes to the periphery (Tardieux *et al.*, 1992). If microtubules arise from the parasite synapse, as our data suggested, the microtubule plus ends would be away from the parasites and lysosomes would be unable to reach the para-

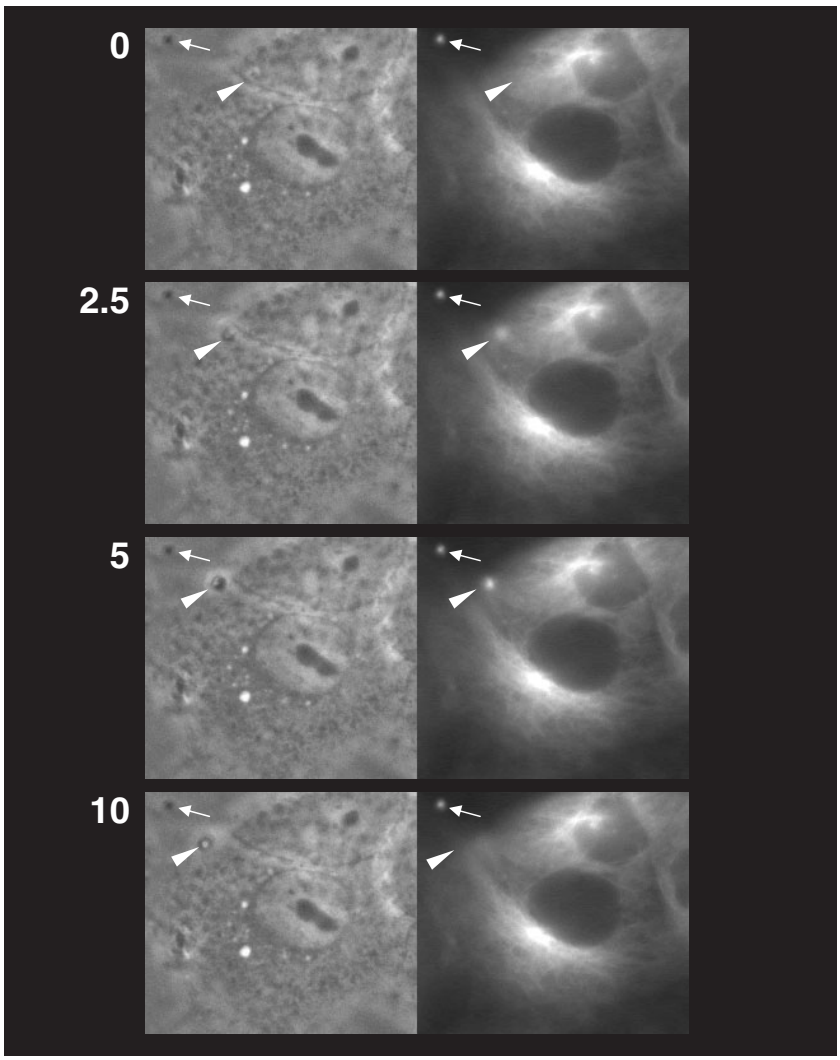


Fig. 2. α-Tubulin concentrates rapidly at parasite synapse. PtK_2 cells stably expressing GFP-α-tubulin were incubated with *T. cruzi* trypomastigotes and the cells were viewed by fluorescence videomicroscopy. Images captured at 0, 2.5, 5 and 10 s after initial parasite contact are shown here. Within seconds of parasite contact with the cell, GFP-α-tubulin accumulated at the point of contact (2.5, 5), but disseminated (10) in this transient/non-productive interaction. Phase contrast (left) and fluorescence (right) images are shown and arrowheads mark the parasite. A stable vacuole in an adjacent cell is marked with an arrow in each image. See *Supplementary material* Movie S2 for a videomicrograph showing several non-productive contacts.

site synapse, presumably inhibiting parasite invasion in dynamitin-overexpressing cells.

We transfected subconfluent cells with dynamitin, which led to lysosome transport to the periphery, as predicted (Fig. 5A, arrowheads). Dynamitin staining was used to identify transfected cells (Fig. 5D), which were compared with untransfected cells on the same coverslips for number of parasites invading per cell. These counts were compared with similar counts made on cells transfected with the same plasmid expressing GFP alone as a marker of transfection. While cells transfected with the sham plasmid did show some evidence of inhibition of cell invasion, perhaps because of the action of lipofectamine on membrane integrity, the degree of inhibition of cell invasion by the dynamitin plasmid was significantly higher than the inhibition by the sham transfectants (Fig. 5H). This experiment supports the idea that the minus ends of the microtubules are subtended by the parasitophorous vacuole, which acts as a new MTOC in the cell.

In analogous systems for regulated exocytosis, such as cytotoxic T lymphocyte killing and neutrophil phagocytosis, lysosomes direction towards the cell-cell contact site (synapse) is accompanied by movement of the centrosome itself. We considered this possibility by first interrogating fixed cells for the relative positions of parasite and host MTOC, 30 min after invasion. By this time some parasites had escaped from their vacuoles into the cytoplasm. The cell shown in Fig. 6 is an example. Here, a parasite appears to have escaped from its parasitophorous vacuole (Fig. 6A) and the host MTOC (Fig. 6B) abuts a hole in the microtubule cytoskeleton that is likely to be the remains of the vacuole. Note that the parasite is not stained by the GTU-88 monoclonal antibody, which does not cross-react with parasite γ-tubulin. This suggests that it may be the vacuole, rather than the parasite, towards which MTOC migration occurs. To test whether the MTOC may, in fact, migrate towards the invading parasite or its vacuole, we sought to determine whether par-

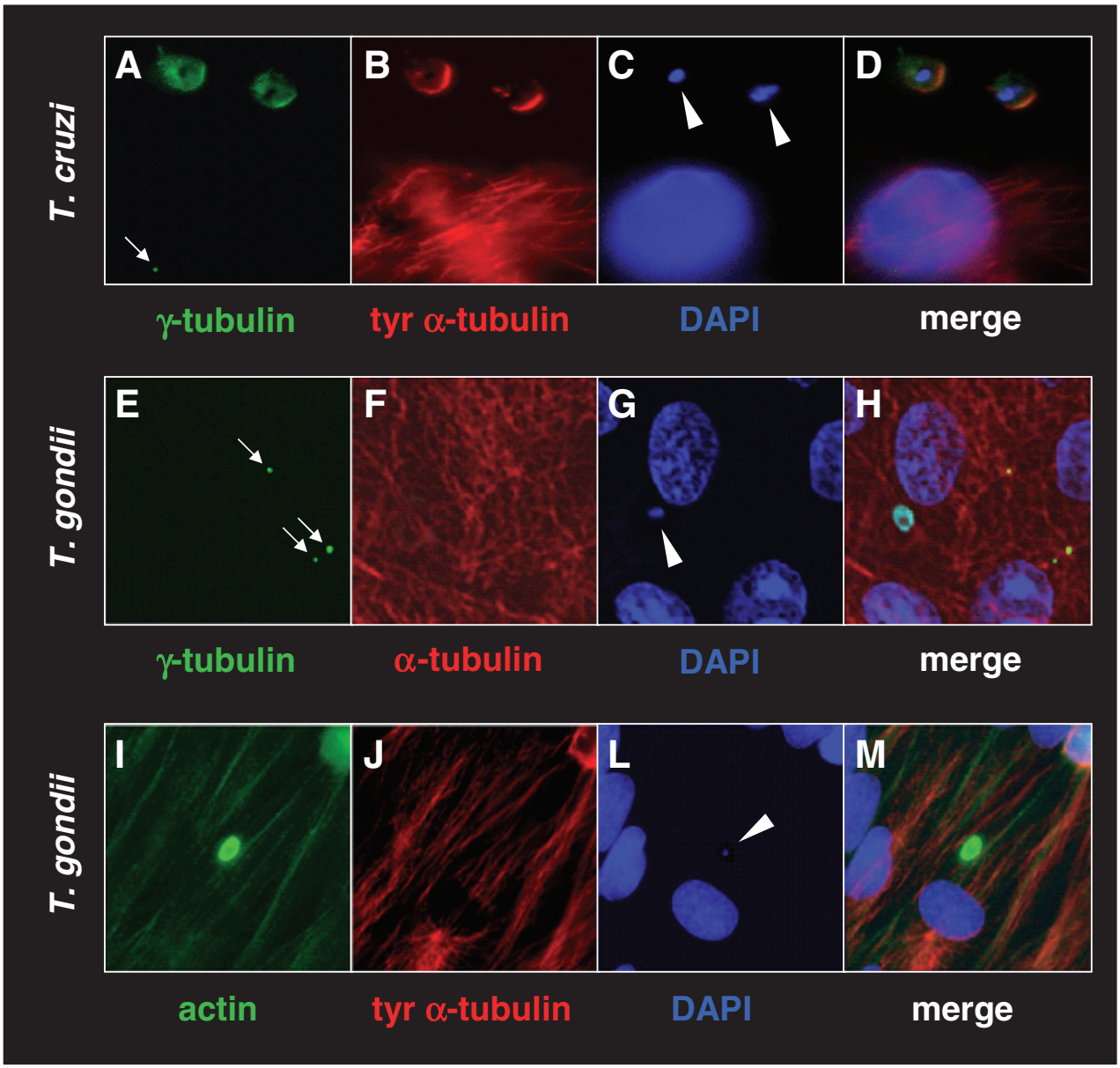


Fig. 3. The *T. cruzi* parasitophorous vacuole is coated by tubulins. Cardiac-derived myoblasts were infected with *T. cruzi* (A–D) or *T. gondii* (E–M) and cells were analysed by immunofluorescence microscopy at 5 min post infection.
A–D. Both γ-tubulin and tyrosinated α-tubulin are recruited to the parasitophorous vacuole early during *T. cruzi* invasion, but their specific localizations are distinct, with γ-tubulin coating the vacuole (A) and tyrosinated α-tubulin concentrated at one margin (B).
E–M. *T. gondii* shows no interaction with γ-tubulin (E), α-tubulin (F), actin (I) or tyrosinated α-tubulin (J). Parasite and host cell DNA can be seen in the DAPI images (C, G, L; also marked by arrowheads), and *T. gondii* was additionally visualized by staining with a TRITC-conjugated secondary antibody (not shown separately) and overlaid on panels E–G to create the merged image (H). The actin-specific antibody also stains *T. gondii* actin strongly (Cintra and De Souza, 1985) (I), and host MTOCs identified by γ-tubulin staining are marked by arrows (A, E). Tachyzoites that have recently invaded appear to nestle among the microfilaments and new microtubules (H, M) but, in contrast to *T. cruzi* (D), no concentration of tubulins or actin is found in the immediate proximity of these parasites.

asites were closer to the centrosome than would be expected by random distribution.

Cells were examined for evidence of MTOC migration towards the invading parasite. Two methods of analysis were performed on the same sets of cells. The first method involved quadrant analysis, and tested the frequency with which MTOC and parasite are located in the same quadrant of the cell. A frequency higher than 25% would suggest an association between parasite and host MTOC. In the second method, the mean angle formed between the centre of the cell, the parasite and the MTOC was measured. This mean angle would be

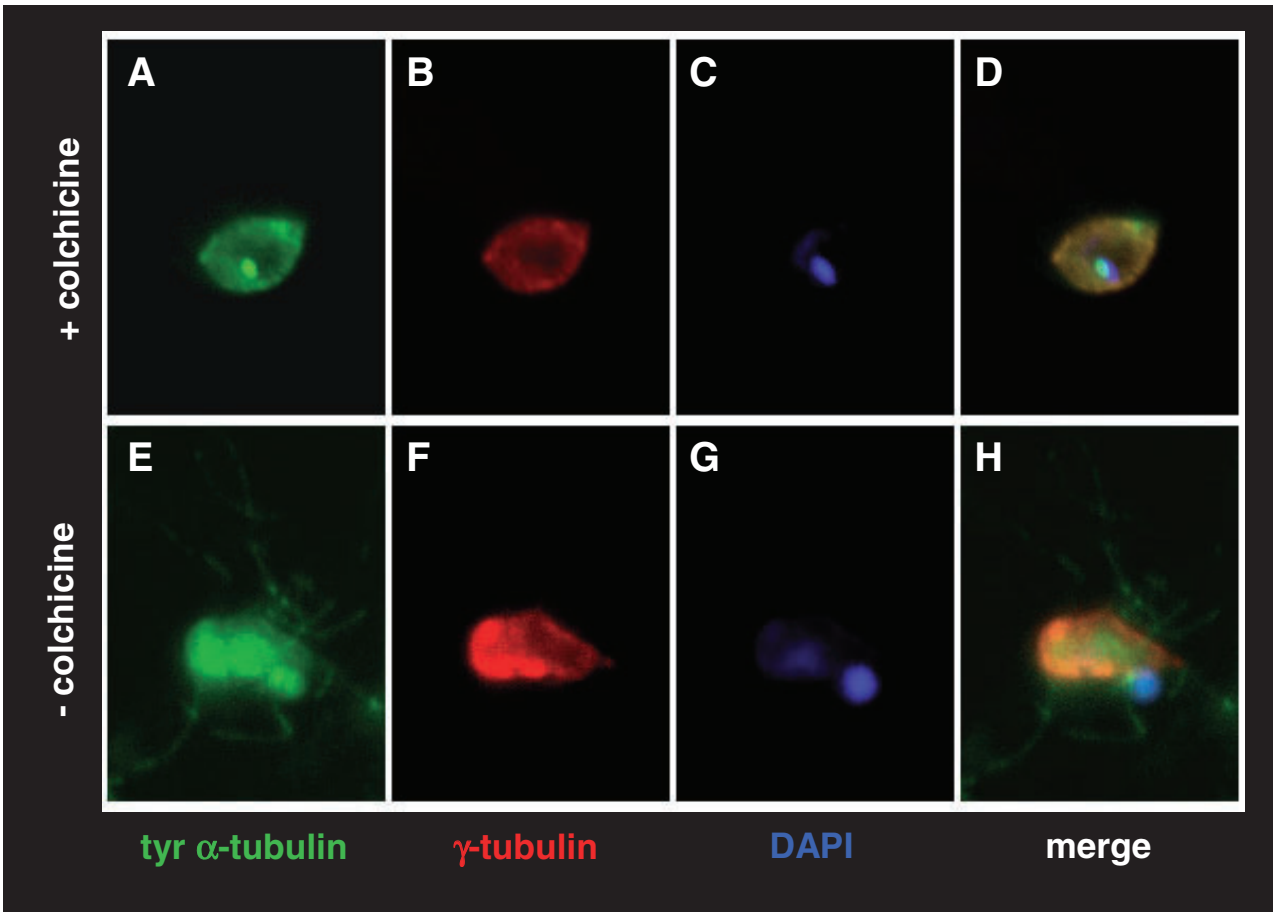


Fig. 4. New microtubules are synthesized at the parasitophorous vacuole. Parasites were allowed to proceed through the initial stages of invasion in the presence of colchicine. Tyrosinated α-tubulin (A, E) and γ-tubulin (B, F) were observed to concentrate around the vacuole, marked by the presence of DAPI-labelled parasite DNA (C, G). Washing of the cells and incubation with colchicine-free medium permitted elongation of short tyrosinated microtubules from the vacuole (E).

expected to be 90° if the localization of parasite and MTOC are random with respect to each other, with a lower mean angle supporting the idea of centrosome repositioning towards the parasite. The second method is more sensitive but, like the first, is flawed by the fact that the cells in question are far from circular and the expected angle of 90° may be an overestimate. To overcome this we utilized *T. gondii* as a control, because it invades the same cells but does not interact with the host cytoskeleton during invasion (Morisaki *et al.*, 1995 and Fig. 1). Results of both tests revealed significant association of *T. cruzi* with the host MTOC, suggesting some degree of centrosome migration towards the invading trypanosome.

Because of the potential for misinterpretation of fixed cell microscopy on an asynchronous process such as cell invasion, and the potential weakness of the *T. gondii* control, we looked for evidence of MTOC migration in a GFP-α-tubulin expressing line during *T. cruzi* infection. We were unable to visualize significant movement of the MTOC towards the parasite synapse in most cells. However, in a few in which the microtubule cytoskeleton was largely broken down during mitosis, quite rapid movement of the host MTOC towards the parasite and away from its usual perinuclear location was observed over a 10 min period (Fig. 7).

Discussion

The manner by which an intracellular microorganism exploits endogenous cellular machineries for its uptake is often surprising and illuminating. The role of membrane ruffling mediated by the actin cytoskeleton in the uptake of bacteria by somatic cell types is increasingly well understood mechanistically and appears to be quite widespread (Le Clainche and Drubin, 2004). A few bacteria have also been described that utilize a microtubule-dependent mode of entry. *Campylobacter jejuni* is the best characterized and appears to initially bind to membrane protrusions formed by disruption of actin and extension of localized

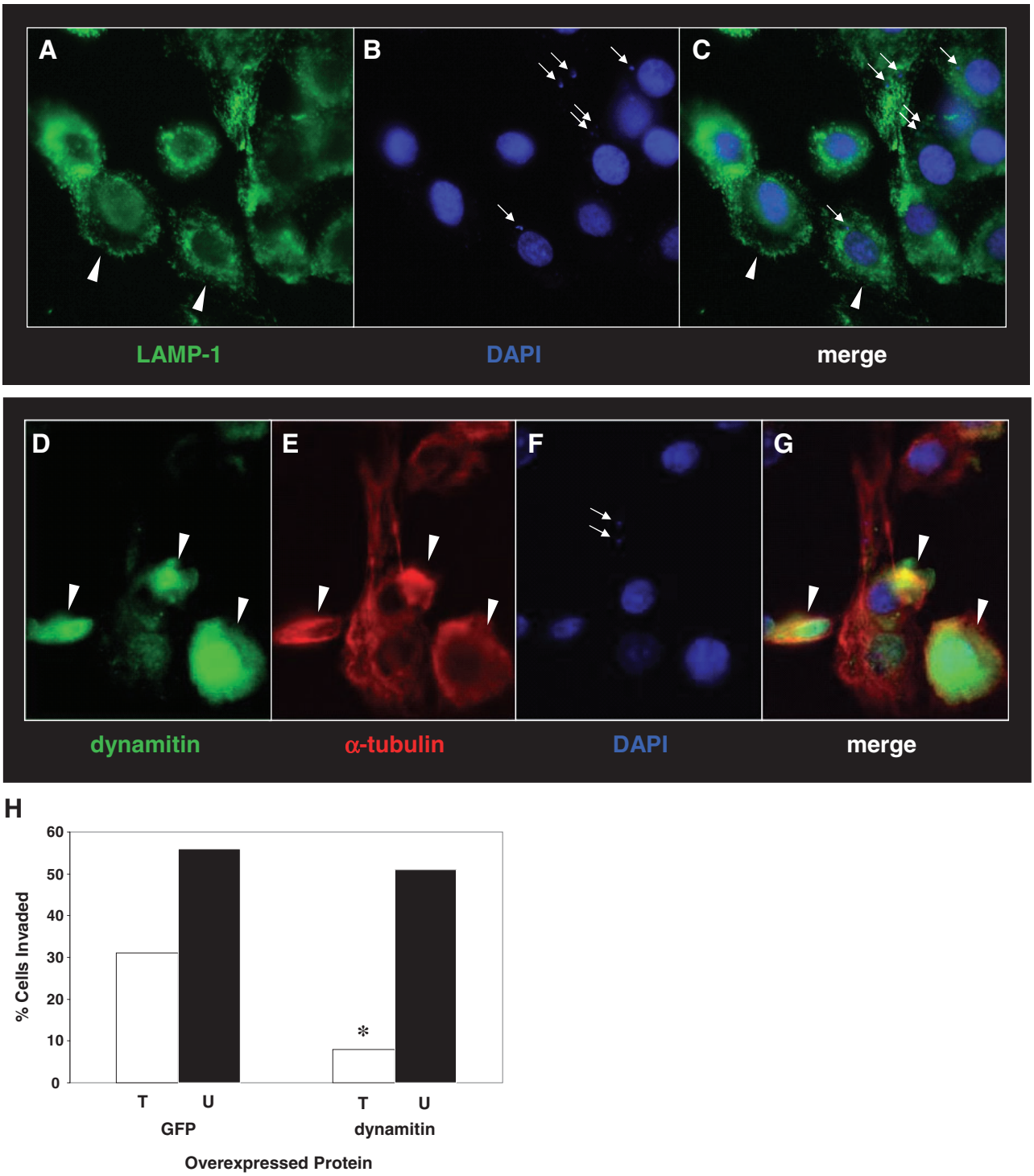
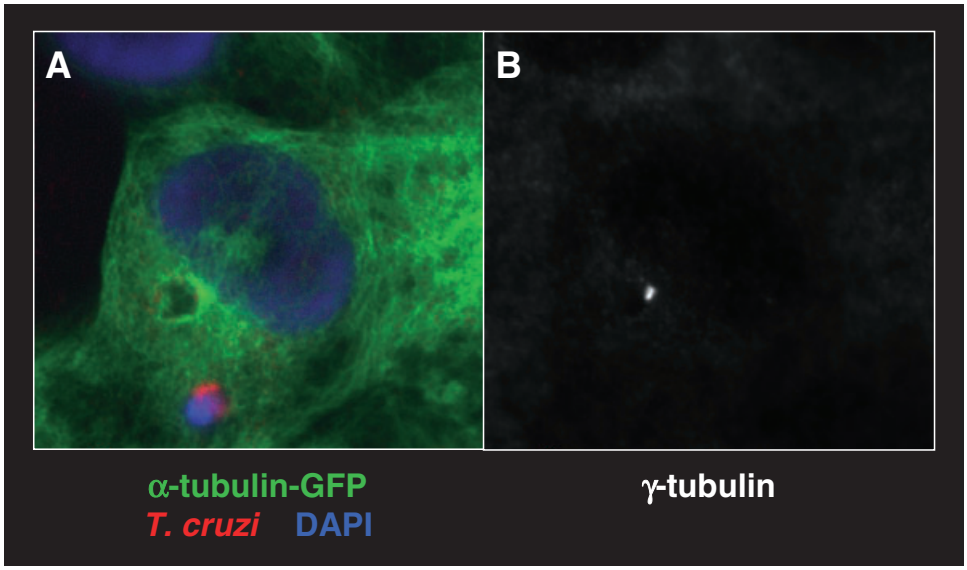


Fig. 5. Overexpression of dynamitin inhibits parasite invasion. Lysosomes (LAMP-1) move to the periphery in some cells transiently expressing dynamitin (A, arrowheads). Those cells with peripheral lysosomal distribution were seldom parasitized (B, parasites stained with DAPI marked by arrows). To pursue this observation, dynamitin transfectants were analysed for expression of dynamitin (D) and α-tubulin (E). Dynamitin expressors (D, E, arrowheads) and non-expressors (E, dynamitin negative, α-tubulin positive) from the same slides were compared with regard to parasite invasion (F, parasites stained with DAPI marked by arrows). One hundred cells were analysed. Cells expressing dynamitin showed an 80% reduction in parasitization compared with non-expressors. A lesser difference was seen when cells were transfected with a construct expressing GFP (not shown). Quantification of these results (H) indicated that the reduction in infection mediated by dynamitin overexpression was significantly greater than that observed of the GFP control (*$P < 0.05$ by χ^2 test).



C

	N	% in Same Quadrant	*P* value by χ^2	Mean Relative Position	SD	*P* value by T-test
Expected		**25**		**90°**		
T. gondii	**100**	**31**	***P* = 0.1659[a]**	**68.29°**	**44.8**	***P* < 0.001[a]**
T. cruzi	**100**	**39**	***P* = 0.0012[a]**	**53.63°**	**42.6**	***P* < 0.001[a]**
			***P* = 0.0837[b]**			***P* = 0.0187[b]**

[a] versus expected

[b] versus *T. gondii*

Fig. 6. Parasite invasion is associated with repositioning of the centrosome.
A and B. An 'empty structure', which may be the remnant of the parasitophorous vacuole after the parasite escapes into the cytoplasm (A), is often located nearby the MTOC, marked by staining with γ-tubulin (B).
C. Analysis of 100 similar cells showed that the frequency with which the *T. cruzi* parasitophorous vacuole is located in the same quadrant as the MTOC is significantly higher than expected by random distribution, while colocalization of the *T. gondii* vacuole and MTOC is not. Determination of the mean angular distribution of vacuole and MTOC likewise revealed a non-random distribution although, in this case, the mean angular position of the *T. gondii* vacuole was also non-random. However, the angular association of the *T. cruzi* vacuole with the MTOC was significantly less random than that of the *T. gondii* vacuole.

microtubules (Hu and Kopecko, 1999). In comparison, *T. cruzi* enters some cell types by a microtubule-dependent mechanism which actually involves delivery of membrane, in the form of lysosomes, to the parasite synapse in what is effectively a regulated lysosomal exocytosis (Caler *et al.*, 2001). Indeed, this calcium-dependent process, which has apparently been subverted by *T. cruzi*, appears to be the one responsible for 'patch repair' of torn or punctured membranes (Kima *et al.*, 2000; Reddy *et al.*, 2001).

Another well-characterized process that involves the calcium regulated delivery of specialized lysosomes to a cell-cell synapse, and also is microtubule-dependent, is cytotoxic T cell killing (Kupfer *et al.*, 1983; Kuhn and Poenie, 2002; Tapper *et al.*, 2002). In this process, the MTOC migrates close to the immunologic synapse for efficient and safe delivery of the cytotoxic granules, which are believed to be specialized types of lysosome. We wondered whether the MTOC would behave similarly during *T. cruzi* invasion, or whether another mechanism participated in the delivery of lysosomes to the parasite synapse.

We initiated this investigation with the simple idea that, as lysosomes trafficked to the parasite synapse in a microtubule-dependent manner, microtubule dynamics might be affected and that these dynamic changes could be visualized by a mixture of fixed cell microscopy and live cell imaging. In agreement with others, we found an association of *T. cruzi* with host microtubules (Carvalho *et al.*, 1999) and were able to visualize this association with increased clarity using deconvolution microscopy. We also chose the antibody YL1/2 to visualize only the dynamic

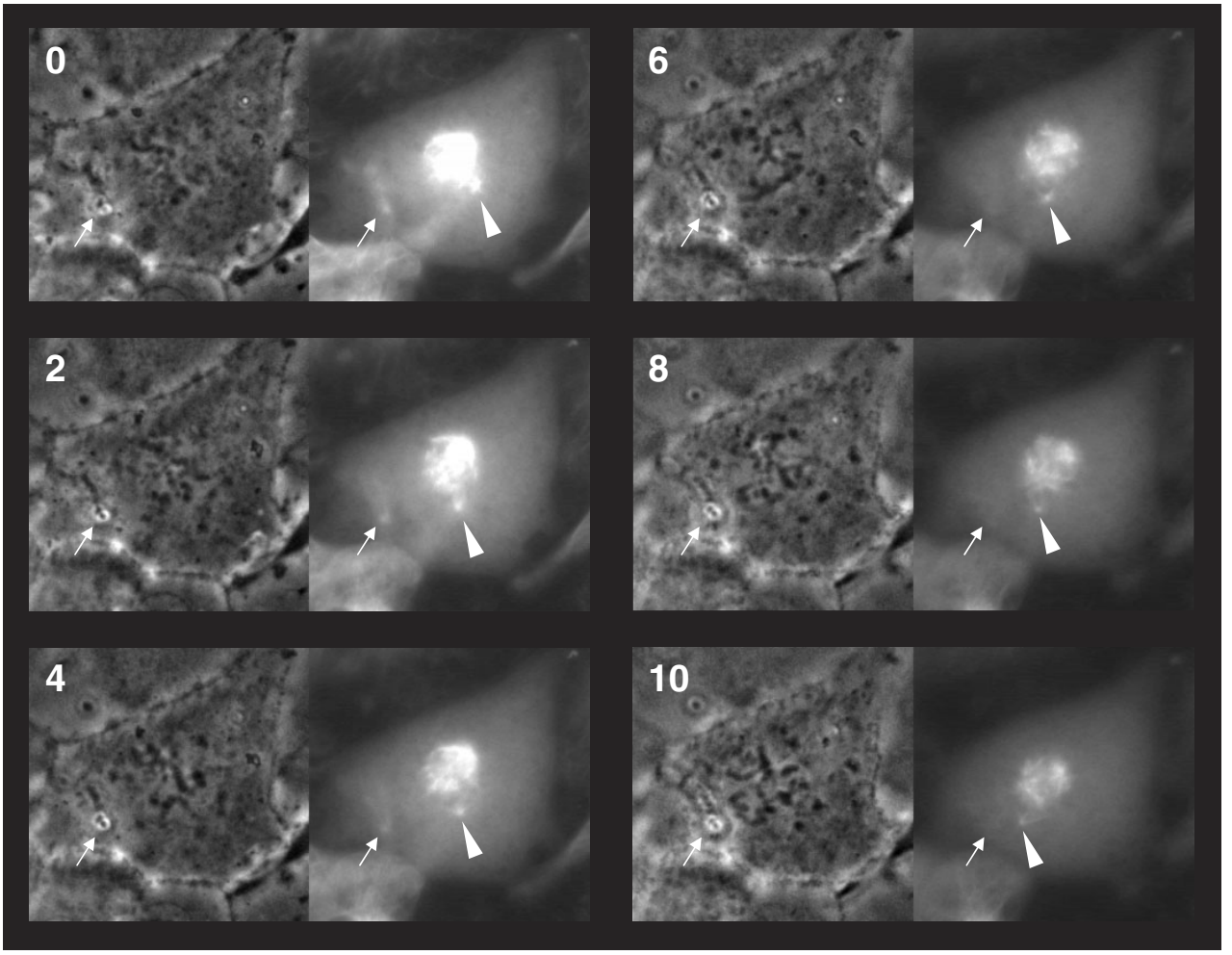


Fig. 7. Temporal repositioning of the centrosome during *T. cruzi* invasion of a mitotic cell. *T. cruzi* infection of a mitotic PtK_2 cell expressing GFP-α-tubulin led to migration of a host cell MTOC (arrowhead) towards the position of the parasite (arrow) over a 10 min period. No such movement was observation in uninfected cells over a similar time period. See *Supplementary material* Movie S3 for a videomicrograph showing the movement of the host MTOC.

subset of host tyrosinated microtubules without interference from the rest of the host and parasite microtubules.

The most striking finding was the rapid association of α-tubulin with the parasitophorous vacuole. The differing distributions of tyrosinated α-tubulin and γ-tubulin on the nascent parasitophorous vacuole is consistent with microtubule polymerization beginning at the cytoplasmic face of the synapse and being slower to occur in the membranes drawn in behind the parasite as the invagination deepens. How this association occurs and what drives it is not clear; however, we would speculate that it is contemporaneous with the localized calcium flux associated with parasite synapse formation and may even precede it. The concentration of lipid products of class I PI 3-kinases, PtdInsP3/PtdIns(3,4)P2 (Woolsey *et al.*, 2003) and G protein-coupled receptors (Croxford *et al.* accompanying article) at the parasite synapse suggests that lipid rafts concentrate at this location. Raft-dependent recruitment of membrane associated tubulins might serve as a mechanism both for the rapid concentration of tubulin observed and for initiation of parasite signalling. While the mechanisms by which the host cell membrane might be induced to act as an MTOC are not clear and have not been approached in this study, it is well known that increased calcium concentration can have the effect of inhibiting microtubule polymerization, while modulation of heterotrimeric G-protein activity has been proposed as a mechanism by which microtubule dynamics can be modulated (Popova and Rasenick, 2003). In future work it will be important to dissect the possible roles of microtubule associated proteins known to associate with the polar ends of the microtubules and which may regulate nucleation and polymerization in response to such secondary signals.

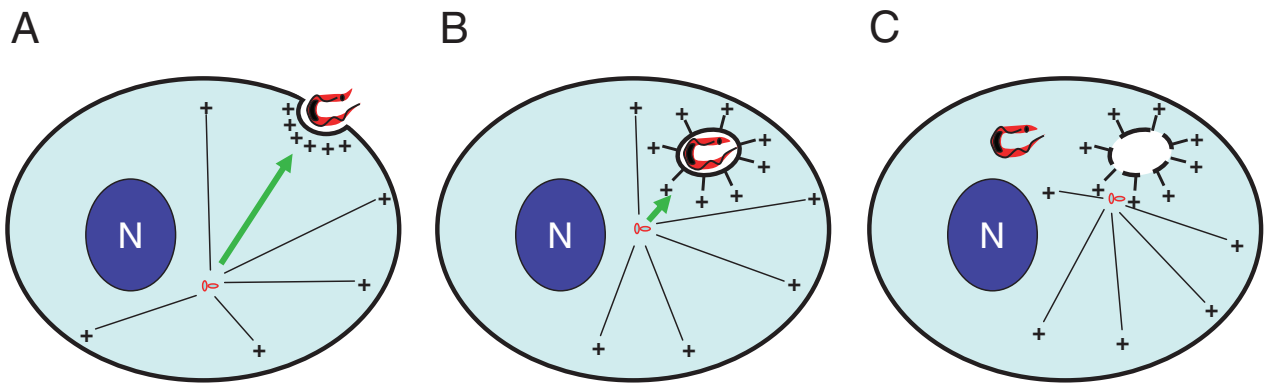


Fig. 8. Proposed microtubule dynamics during and after cell invasion by *T. cruzi* trypomastigotes.
A. Initially the parasite attaches to the cell forming a junction, the parasite synapse. Recruitment of γ-tubulin to the synapse promotes the seeding of microtubules as the invagination deepens and the parasite is eventually completely engulfed to form the parasitophorous vacuole. This vacuole now acts as a *de facto* secondary MTOC.
B and C. The cell's own MTOC, the centrosome, moves towards the parasitophorous vacuole, even after the parasite has escaped its vacuole into the cytoplasm (C).

Use of colchicine during invasion was possible because colcimids bind trypanosome microtubules with much lower affinity than host cell tubulins (reviewed in (Gull, 2002). Parasites can actually invade cells in a microtubule-independent fashion, invading even lightly fixed cells under their own power. We took advantage of these properties to create parasitophorous vacuoles in cells essentially devoid of tyrosinated microtubules and then to follow them during *de novo* microtubule polymerization. The appearance of arrays of short microtubules 15 min after washout of colchicine is strongly suggestive that new microtubules arise from the γ-tubulin-staining vacuole. To test this further we utilized the dynamitin construct to disrupt dynactin and consequently cytoplasmic dynein. A previous study showed that, if lysosomes were forced to the periphery of the cell by use of low pH which was then neutralized, trypanosome invasion was greatly facilitated (Tardieux *et al.*, 1992). Revealingly perhaps in that study, if the pH was not neutralized, invasion was not facilitated. We utilized dynamitin to similar effect, placing the lysosomes at the periphery of the cell. However, in contrast to the result in which pH was modulated, we saw inhibition rather than facilitation. This would be expected if the polarity of the microtubules arising from the parasite is from minus at the vacuole to plus in the cell interior, because the lysosomes would be unlikely to reach the parasitophorous vacuole in the presence of overexpressed dynamitin. Considered together with the rapid recruitment of tubulin to the parasite synapse and the crescent shaped distribution of tyrosinated α-tubulin and the presence of γ-tubulin in nascent parasitophorous vacuoles, our data support the notion that *T. cruzi* establishes its parasitophorous vacuole to be a novel MTOC, which is a critical first step in its microtubule-dependent invasion process.

Our initial hypothesis was that regulated lysosome exocytosis associated with *T. cruzi* cell invasion was directed in a manner analogous to cytotoxic T cell killing and would involve migration of the centrosome towards the parasite synapse. We found some evidence for migration of the centrosome towards the parasite in fixed cardiac myoblasts. We were unable to observe migration of the centrosome towards the parasitophorous vacuole in most GFP-α-tubulin-expressing PtK2 cells during a 10 min period. This may reflect the shortness of the time course. In longer time courses, many of the parasites begin to escape their vacuoles into the cytoplasm, complicating the interpretation. In mitotic cells, however, some movement of a host MTOC towards the parasite was seen, perhaps reflecting the freedom of the centrosome to move unencumbered by the radial array during this period of the cell cycle. We suggest that this may be an amplification of what is also a real effect in non-mitotic cells.

Based on the results described in this article we propose our current working hypothesis as a model of trypanosome cell invasion in which the centrosome repositions towards the parasitophorous vacuole, which itself is acting as a novel, secondary MTOC (Fig. 8). The result is to establish a bidirectional highway along which vesicles can traffic to and from the parasite immediately after cell invasion. It is not clear that such a model would be unique to trypanosomes. Recent reports of other prokaryote and eukaryote intracellular pathogens which utilize host microtubules during cell invasion, notably *C. jejuni* and *Paracoccidiodes brasiliensis*, indicate that this may be a mechanism of broader consequence in infection and immunity than to Chagas disease alone (Yoshida and Sasakawa, 2003; Mendes-Giannini *et al.*, 2004). While the directional exocytosis of lysosomes to the location of an invading pathogen has clearly evolved as an effective arm of the innate immune response, it would be surprising if

only one pathogen had coevolved effective mechanisms for its exploitation.

Experimental procedures

Cell lines, parasites and culture

PtK_2 cells stably transfected with GFP-α-tubulin were cultured in minimum essential medium supplemented with 1 mM sodium pyruvate, 10% FCS, 0.1 mM non-essential amino acid solution (Life Technologies, Grand Island, NY), and antibiotics. Cells were maintained in DME, 5% FCS, 200 μg ml^{-1} G418 (Life Technologies, Grand Island, NY), penicillin, and streptomycin in an atmosphere of 5% CO_2, at 37°C and passaged at confluence. The Brazil strain *T. cruzi* was used in all experiments because it is readily manipulated through its life cycle *in vitro* and causes well documented pathology in mice (Leon *et al*., 2001; Tyler and Engman, 2001). Epimastigotes were maintained by passage in LDNT medium, pH 7.2, as previously described (Tyler and Engman, 2000). Growth curves were obtained for triplicate cultures. To initiate cultures, cells were counted using a Neubauer haemocytometer and passaged to a density of 10^6 cells ml^{-1}. Cultures of rat cardiac-derived myoblasts (H9C2 cell line) were maintained in Dulbecco's minimal essential medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum. The tachyzoites of the RH strain of *T. gondii* used in these experiments were derived from human foreskin fibroblast cultures.

Antibodies

Monoclonal antibodies specific for tyrosinated α-tubulin (YL1/2), α-tubulin (TAT-1), LAMP-1, actin (AC-40), γ-tubulin (GTU-88) and p50 dynamitin (Pharmingen, San Diego, CA), a polyclonal rabbit serum specific for desmin, which crossreacts with the *T. cruzi* cell surface (Sigma-Aldrich, Louisville, KY), and pooled sera from individuals chronically infected with *T. cruzi* were used in this study. Secondary antibodies conjugated to Cy5 and Cy2 (Sigma-Aldrich, Louisville, KY) were used for two-colour fluorescence microscopy and a specific TRITC-conjugated goat anti-mouse IgG that reacts with *T. gondii* (Sigma-Aldrich, Louisville, KY) was used to visualize that organism.

Plasmids

The p50 dynamitin insert expressed from the cytomegalovirus promoter of the pCMV-Myc expression vector was utilized (Helfand *et al*., 2002). Enhanced GFP also expressed from the cytomegalovirus promoter in the commercially available plasmid pCMS-EGFP Vector (BD Biosciences, Franklin Lakes, NJ) was used as a control.

Transient transfection

For transient transfection, cells were grown on coverslips to 40% confluence. Lipofectamine™ (Life Technologies, Grand Island, NY) was used for transfection according to the manufacturer's instructions. Cells were incubated with a mixture of 1 μg of Qiagen-purified DNA and 6 μl of Lipofectamine™ in serum-free medium for 3 h, after which the mixture was replaced with standard culture medium. Cells were washed once with serum-free medium and incubated with the DNA/liposome mixture in serum-free medium for 6 h, at which time DNA was removed and replaced with complete medium. Cells were fixed for immunofluorescence microscopy 14 h later.

Infection, staining and microscopy

Cardiac myoblasts were grown on coverslips until subconfluent. Tissue culture derived trypomastigotes were then added in a ratio of 10 trypomastigotes per myoblast and incubated according to the requirements of the particular experiment. Primary antibodies were applied for 1 h in a humid chamber at room temperature, samples were washed three times in PBS for 5 min per wash, secondary antibodies were then applied and the process was repeated. Finally, 1 μg ml^{-1} 4′,6-diamidino-2-phenylindoledichydrochloride (DAPI, Boehringer Mannheim, Indianapolis, IN) was applied for 30 s and the sample was washed in distilled water before mounting under a cover slip using Permafluor (Immunotech, Marseille, France) and sealing with nail varnish. Microscopy was performed using an Olympus IX70 inverted system microscope (Olympus America, Melville, NY) and images were captured using the Deltavision Image Restoration and Deconvolution System (Applied Precision, Issaquah, WA).

Colchicine treatment

In some experiments colchicine (2 μg ml^{-1}; Sigma-Aldrich, Louisville, KY) in delipidated medium was added to the invasion culture. After 30 min, only a few microtubules could be detected by indirect immunofluorescence microscopy (data not shown). The medium was removed and the cells were washed five times with serum-free medium and allowed to recover in complete medium without colchicine. In control experiments, the washing and recovery steps were performed, but colchicine was maintained in all solutions.

Time-lapse fluorescence microscopy

Stably transfected PtK2 cells were grown on coverslips in multiwell culture dishes to 70% confluence in standard culture medium containing 10 mM HEPES, pH 7.0. These coverslips were inverted onto small pieces of 5 μm thick glass scattered on the surface of a microscope slide. Twenty-five microlitres of medium containing *T. cruzi* trypomastigotes were then allowed to enter the space between coverglass and slide by capillary action to create a chamber, which was then sealed with Vaseline/lanolin/paraffin at 1:1:1. The coverslips were placed on small glass feet (to prevent compression) on slides, sealed with a mixture of Vaseline, beeswax, and lanolin (1:1:1) and, 48–72 h later, the dish was mounted on an inverted microscope (Diaphot 300; Nikon, Melville, NY) equipped with a 40×, 0.6 NA, Nikon CFI Plan Fluor ELWD objective and a slow-scan cooled charge-coupled device (CCD) camera (Model CH350; Photometrics, Huntington Beach, CA). For GFP fusion protein imaging, fluorescent images were captured using a longpass filter set (Endow GFP; Chroma Technology Corporation, Rockingham, VT). Images were transferred to a computer workstation running MetaMorph® imaging software (Universal Imaging Corporation, Downingtown, PA). Images were collected at 2.5 s intervals for

5–10 min. During live cell imaging, the temperature was kept at 33 ± 1°C using flexible heaters (Omegalux, Omega Engineering Corporation, Stamford, CT) on the objective lens.

Quadrant and geometric analyses

Deltavision Softworx software (Applied Precision, Issaquah, WA) was utilized on the original captured images to define each cell as a two-dimensional polygon. The 'find centre of gravity' was utilized to define the geometric centre of each cell. The image generated was then saved in 8-bit TIFF format and opened in Adobe Photoshop. For the quadrant analysis, a simple cross comprised of a vertical and horizontal bar intersecting at 90° was then superimposed as a 'layer' onto each cell, with the intersect positioned at the previously defined centre. Record was then made of whether the host MTOC and parasite occupied the same quadrant. For the geometric analysis, the measure feature was utilized to determine the angle between the host MTOC and the parasitophorous vacuole subtended at the geometric centre of the cell. As the parasite was relatively large, the minimum and maximum measurements obtainable (either side of the parasite) were averaged.

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Supplementary material

The following supplementary material is available for this article online:

Movie S1. Three-dimensional representation of the association between lysosomes (green) and newly laid down microtubules (red) during formation of the *T. cruzi* parasitophorous vacuole. This supplemental file corresponds to Fig. 1B, with the DAPI removed for clarity.

Movie S2. Transient concentration of α-tubulin at the parasite synapse. Videomicroscopy shows intense and transient fluorescence at points of brief contact between *T. cruzi* and PtK_2 cells expressing GFP-α-tubulin. A parasitophorous vacuole in an adjacent cell is visible in the upper left corner of the field, which exhibits stable fluorescence. Phase contrast (left) and fluorescence (right) videos are shown. The video is best viewed by using keys to advance the frames rather than by pushing the play button. This supplemental file corresponds to Fig. 2.

Movie S3. Movement of a host MTOC towards the parasitophorous vacuole in a mitotic cell. Videomicroscopy of this mitotic, *T. cruzi*-infected PtK_2 expressing GFP-α-tubulin shows host MTOC movement towards the parasite. Phase contrast (left) and fluorescence (right) videos are shown. The parasitophorous vacuole is easily seen in the phase contrast video. The video is best viewed by using keys to advance the frames rather than by pushing the play button. This supplemental file corresponds to Fig. 7.