Monitoring of dynamin during the *Toxoplasma gondii* cell cycle

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Abstract

The obligate intracellular protozoan parasite *Toxoplasma gondii* actively invades virtually all warm-blooded nucleated cells. This process results in a non-fusogenic vacuole, inside which the parasites replicate continuously until egress signaling is triggered. In this work, we investigated the role of the large GTPase dynamin in the interaction of *T. gondii* with the host cell by using laser and electron microscopy during three key stages: invasion, development and egress. The detection of dynamin during invasion indicates the occurrence of endocytosis, while *T. gondii* egress appeared to be independent of dynamin participation. However, the presence of dynamin during *T. gondii* development suggests that this molecule plays undescribed roles in the tachyzoite's cell cycle.

1. Introduction

The apicomplexan parasite *Toxoplasma gondii*, which causes toxoplasmosis, is capable of infecting virtually all nucleated cells of warm-blooded hosts and is estimated to infect one-third of the world`s population. Host cell invasion relies on the active participation of the parasite and the concomitant generation of a non-fusogenic parasitophorous vacuole (PV), which is often translocated to the perinuclear region (Sibley 2004). After multiple replication

rounds, the parasites exit the PV, carrying remnants of the PV and ER membranes, before eventually crossing the host cell plasma membrane (Caldas et al 2010).

Recent studies demonstrated that the inhibition of the large GTPase dynamin successfully blocks internalization and/or infection for a variety of viruses (Abban et al 2008; Mues et al 2015; Piccini et al 2015) and *T. gondii* invasion (Caldas et al 2013). This GTPase is well known for pinching off endocytic vesicles from the plasma membrane and the trans-Golgi network by polymerizing to form a helix around the neck of budding vesicles, leading to membrane fission. The role of this GTPase has been extended to membrane remodeling, cytoskeleton regulation via direct interactions with actin, and participation in exocytosis (Arneson et al 2008; Mettlen et al 2009; Gu et al 2010; Ferguson and De Camilli 2012; Williams and Kim 2014).

These roles of dynamin led us to hypothesize that this GTPase participates not only in the disconnection of the PV from the host cell plasma membrane but also in the translocation of the PV to the perinuclear region of the infected cell. To test this hypothesis, we labeled dynamin during parasite invasion, development and egress, which are the three key steps of the *T. gondii* cellular cycle, and used confocal microscopy, super-resolution microscopy and cryo-immunomicroscopy techniques.

2. Materials and methods

2.1. Chemicals

The calcium ionophore A23817 was purchased from Sigma Chemical Company (St. Louis, MO, USA) was employed during egress assay. For this compound, viability tests with neutral red were performed, and no cytotoxic effect was observed with the concentrations and incubation time used.

2.2. Parasites and host cell culture

T. gondii tachyzoites of the RH wild-type strain were maintained in mice via intraperitoneal inoculation and harvested via peritoneal washing of mice infected for 2 to 3

days. The suspension was centrifuged at 1,000 × *g* for 10 min to remove cell debris and peritoneal leukocytes, and the number of parasites in the supernatant was quantified in a Neubauer chamber. The parasites were suspended in Dulbecco's modified Eagle's medium (DMEM). Swiss mice were bred at the animal facility of the Federal University of Rio de Janeiro. The experimental protocol was approved by the Instituto de Biofísica Carlos Chagas Filho's Ethics Committee for animal experimentation (Protocol n. IBCCF 096/097).

Macaca mulata monkey epithelial kidney cells (LLC-MK2) were maintained *in vitro* in DMEM supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. The cells were grown in 25 cm^2 plastic flasks or on round glass coverslips in 24-well plates.

2.3. In vitro infection

For the invasion assay, the tachyzoites of *T. gondii* were allowed to interact with LLC-MK2 cells, at ratios of 5:1 (parasites-host cell), initially for 15 min at 4°C followed by incubation at 37 ºC and 5% CO_2 for 10 min, when the samples were fixed. The intracellular development assay required 40–50 min of parasite-host cell incubation at 37 °C and 5% CO_2. After this time, the supernatant containing the free parasites was aspirated and replaced with fresh medium. The infection was allowed to proceed for 24 h, and the cells were then rinsed with DMEM and fixed. For the parasite egress assay, a 9 μM solution of the calcium ionophore A23817, diluted in serum-free DMEM, was added at this time and the monolayers were fixed after 5 min. Untreated infected monolayers of LLC-MK2 cells were used as negative controls.

2.4. Confocal microscopy

For immunofluorescence microscopy, the cells were seeded onto round coverslips and fixed with 4% formaldehyde in phosphate buffered saline (PBS), pH 7.2, for 20 min. Half of the samples were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. Pre-incubation was performed with 50 mM ammonium chloride and 3% BSA in PBS, pH 8.0, for 45 min to block the free aldehyde groups. The samples were then incubated with a primary anti-dynamin antibody (Invitrogen, Carlsbad, CA, USA) at a 1:100 dilution for 1 h, rinsed, and

incubated with a 1:400 dilution of the secondary goat anti-mouse IgG (H+L) antibody conjugated to AlexaFluor 488 (Invitrogen) at room temperature for 1 h. Actin was stained with phalloidin red (Sigma-Aldrich) at a dilution in PBS of 1:40 for 20 min in the dark. After rinsing with phosphate buffered saline PBS and mounting with prolong antifade (Vector Labs, Burlingame, CA, USA), the slides were visualized using a Zeiss 510 LSM microscope.

2.5. Super-resolution microscopy

For super-resolution microscopy, the infected cells were processed in a manner similar to that described for confocal microscopy, except additional care was taken at washing steps, gradually increasing in duration, as recently described by Whelan and Bell (2015). Images were collected using a Zeiss Elyra PS.1 microscope in the structured illumination (SR-SIM) mode.

2.6. Cryo-immunomicroscopy

For cryo-immunolabeling, the material was processed as described previously (Lemgruber et al 2011). Briefly, the samples were fixed in 0.1 M sodium cacodylate buffer, pH 7.2, containing 4% freshly prepared formaldehyde and embedded in gelatin. The samples were infiltrated overnight in 2.1 M sucrose and rapidly frozen by immersion in liquid nitrogen. Cryosections were obtained at -100°C using an Ultracut cryo-ultramicrotome (Leica Microsystems). The cryosections were thawed in methyl-cellulose, blocked in PBS containing 3% bovine serum albumin and then incubated in the presence of a mouse monoclonal anti-dynamin antibody (Invitrogen, Carlsbad, CA, USA) for 1 hour. The cryosections were washed in blocking buffer and then incubated with 15 nm gold-labeled anti-mouse IgG (BBInternational) and observed using a FEI Tecnai Biotwin transmission electron microscope.

3. Results and discussion

The importance of dynamin on *T. gondii* invasion was previously demonstrated by the inhibitory effect of dynasore, which is a potent blocker of this GTPase (Caldas et al 2009). In order to investigate the participation of dynamin during the parasite-host cell interactions, we

performed immunolabeling assays during three major steps of the *T. gondii* cellular cycle: invasion, development and egress.

3.1. Invasion

The intricate signaling dynamics that characterize Apicomplexan invasion, from the polarization of the parasites towards the host cell surface and the secretion of adhesion molecules to PV formation, were carefully reviewed by Sharma & Chitnis (Sharma and Chitnis 2013). Molecular sieving performed at the moving junction during PV formation is determinant to this vacuole's non-fusogenic nature (Mordue et al 1999; Straub et al 2011). Nevertheless, this vacuole must detach from the host cell plasma membrane, and dynamin is responsible for the release of endocytic vesicles from the plasma membrane. Thus, this GTPase was localized by immunofluorescence to track its position during *T. gondii* invasion.

In order to achieve this, the best scenario should rely on the synchronized *T. gondii* invasion of host cells in culture. Then parasites interacted with the host cells for 15 min at 4°C and were transferred to 37 °C for 10 min, after which the samples were fixed and processed for the immunofluorescence assay. The inhibition of dynamin by dynasore was previously demonstrated to block PV translocation to the perinuclear region (Caldas et al 2009). As expected, dynamin is present at the site of parasite entry, forming a ring around the nascent vacuole (Fig. 1A-B). However, dynamin appears to act at more than one site during the pinching off of the forming PV, as shown in Fig. 1C. Dynamin labeling was intense in the area corresponding to the moving junction, but a second area of labeling was seen at the front edge of the nascent vacuole. This secondary site of dynamin labeling may be caused by the pinching of vesicles containing ROP proteins, as reported by Bradley and Sibley (2007) and preceded by seminal descriptions of the kinetics of *T. gondii* protein secretion during vertebrate host cell invasion (Dubremetz et al 1993; Carruthers and Sibley 1997).

Immediately after invasion, the parasites were already internalized into a PV. Fig. 1D-E shows an internalized parasite, for which labeling for dynamin was always negative.

Dynamin was also tracked during invasion via cryo-immunomicroscopy, and secondary antibodies conjugated to 15 nm colloidal gold particles were observed at the interface between the parasite and the host cell (Fig. 2A). This event occurred at the site where the secretion of the rhoptry contents is believed to occur during the early stages of the interaction, which will lead to the formation of the moving junction.

Labeling was intense at the sites of contact between the nascent PV and the apical portion of the invading parasite, to which dynamin appears to be recruited (Fig. 2B). Interestingly, strongly electron-dense loci also appear to correspond to the stable intracellular clamp that is observed during the moving junction, where the parasites anchor and the molecular sieving of PV proteins takes place (Straub et al 2011). This labeling of the interaction sites between the tachyzoite and the host cell plasma membrane surface, after the initial contact and/or the parasite's reorientation of its apical end towards the host cell, also suggests the recruitment of dynamin.

Freeze-fracture electron microscopy previously showed a pore-like structure in the PVM, which is located in the same region as the parasite's apical end (Dubremetz 2007). We hypothesize that this structure results from the effect of a complex of solutes and vesicular content that is secreted by the parasite (Bradley et al 2007; Ravindran and Boothroyd 2008), which generates conditions for its own internalization into the cytosol. The dynamin detected at the parasite-PVM interface could have been trapped there during the process of PV formation and may contribute to the crossing of the PVM by secreted vesicles. Recent studies using helium ion microscopy revealed pore-like openings in the intravacuolar face of the PV, varying from 10 to 200 nm in diameter. These openings appear to increase in diameter relative to the size of the PV (De Souza and Attias 2015). In addition to bacterial secretion systems (types i-iv), secreted vesicles have been studied more recently as a keystone of pathogenic infection, in spite of the difficulty caused by the fact that both the pathogen and the host are able to release such vesicles to the extracellular medium, with consequences for disease pathogenesis (Schorey et al 2015). Nevertheless, we cannot forget that in both the host cell and the parasite, a large number of cellular processes depend on dynamin, such as membrane remodeling, endocytic

processes and cytoskeleton interactions (Williams and Kim 2014). For this reason, labeling was distributed sparsely throughout the cytoplasm.

3.2. Development

Dynamin has long been known for its role during endocytosis (Van der Bliek and Meyerowitz 1991; Chappie et al 2011), but its GTPase activity was later found to depend on an oligomerization cycle. As this cycle is determined by the interacting molecule (i.e., lipids or actin filaments) (Gu et al 2014), this discovery was important for improving our understanding of additional roles of dynamin (e.g., direct and indirect actin-driven processes) (Orth and McNiven 2003; Schlunck et al 2004; Gu et al 2010).

Because dynamin is known to be capable of nucleating actin tails via its proline-rich domain (Lee and De Camilli 2002; Orth et al 2002), dynamin could participate in the process of PV translocation to the perinuclear region. This translocation, which presupposes a rearrangement of the host cell cytoskeleton, is present in pathogens, such as *Chlamydia trachomatis* (Romano et al 2013).

Cells were processed for SR-SIM at 24 hpi, and dynamin labeling revealed an unexpected pattern. Punctual dynamin foci were observed inside the PVs (Fig. 3). SR-SIM allowed an accurate localization (Fig. 3; renderization in the supplemental material), and higher magnifications revealed that dynamin is located primarily at the inner border of the PVs, not only in LLC-MK2 cells (Fig. 4A-B) but also in peritoneal macrophages (Fig. 4C-D and supplemental material). However, cryo-immunomicroscopy indicated that this localization might occur in both the tubulovesicular network (Fig. 4E) and the inner side of the PV membrane (Fig. 4F).

It is worth noting that dynamin-like proteins appear to play a role in *T. gondii* apicoplast fission (van Dooren et al 2009) and the generation of secretory pathway vesicles involved in the formation of secretory organelles (Breinich et al 2009). However, in the present study, which is focused on host cell dynamin, the data may indicate a distinct phenomenon. Although hard to

envision, the role of dynamin in parasite maturation must not be underestimated, considering that dynamin may act to stabilize high-curvature membranes and/or mediate changes in the physical properties of the PV membrane, similar to the changes that occur during endosomal processes (Anitei and Hoflack 2012).

3.3. Egress

Once considered to be the reverse of invasion (Hoff and Carruthers 2012), *T. gondii* egress is now understood as a distinct step of the cellular cycle. *Toxoplasma* egress is an active process, which appears to result from mechanical stress caused by PV growth and subsequent host cell ionic imbalances, leading to calcium signaling and parasite actin activation (Moudy 2001; Lourido et al 2012). During egress, no translocation of perinuclear PVs to the periphery or plasma membrane fusion occurs, and the parasites escape by entering the host cell cytosol and crossing the plasma membrane. When this process occurs collectively, cell lysis is observed (Caldas et al 2010).

The dynamin inhibitor dynasore (Macia et al 2006) did not block *T. gondii* egress (Caldas et al 2013), and this finding was expected, taking into account previous knowledge of this large GTPase (Williams and Kim 2014). To corroborate these data, we performed an immunofluorescence assay of *T. gondii* calcium ionophore-induced egress (Endo et al 1982), and no dynamin was detected at this step of the parasite cell cycle (Fig. 5A-B). The same outcome was shown for natural egress from the host cell, as observed by SR-SIM (Fig. 5C).

Because dynamin and dynamin-like proteins are involved in endocytosis, cellular trafficking, and vacuole fission and fusion (Williams and Kim 2014), it is also necessary to take into account the participation of this GTPase in the possible communication between different PVs. It is also worth noting that the immediate association of dynamin with these endocytic processes must be considered, as host cell endocytic structures and Golgi vesicles can be found inside PVs (Romano et al 2013; Coppens 2014). In addition, the parasite secretes proteins involved in the recruitment of host mitochondria, from which the tachyzoites are believed to hijack nutrients (Sinai et al 1997; Pernas et al 2014; Mercier and Cesbron-Delauw 2015).

Additionally, parasite replication within PVs leads to an increase in PV size, resulting in a mechanical stress to host cell (Moudy 2001; Magno et al 2005). However, this increase in PV size causes a large amount of tension in the PV membrane, and host cell dynamin can oligomerize during this high-curvature process, contributing to these changes in physical membrane properties. Altogether, these data provide insights into the role of dynamin during pathogen-host cell interactions.

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Conflict of interest

We the authors declare no conflict of interest in writing this article.

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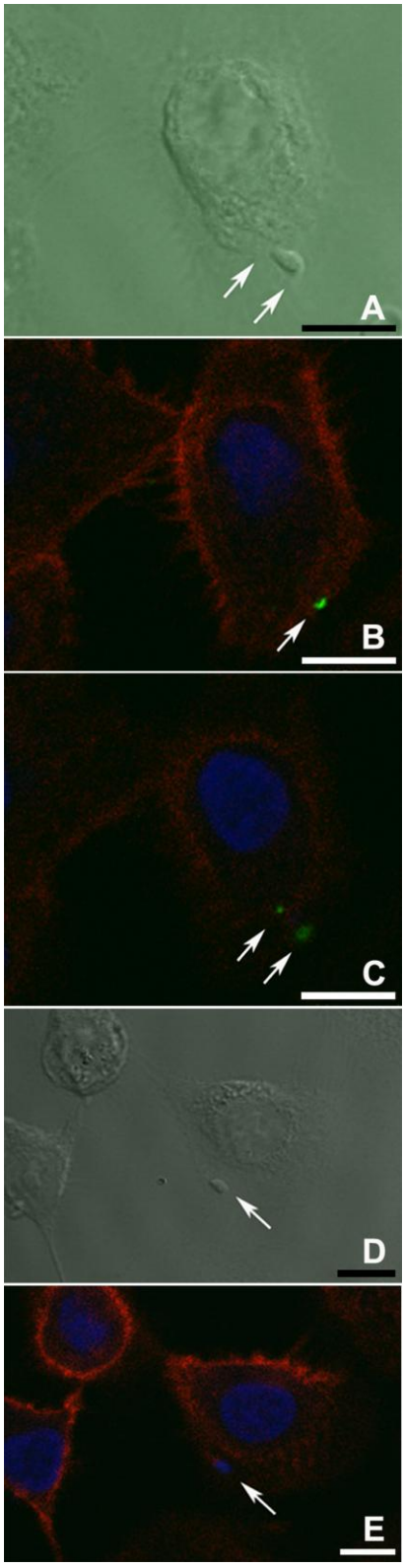

Figure 1: Immunofluorescence assay of the distribution of dynamin during *T. gondii* invasion. **(A)** During the invasion of a single tachyzoite, the arrows point to loci near the apical ends and backsides of the parasite. In distinct slices of the same sample,

dynamin labeling (green) occurs at the expected site of PV pinching off (arrow) **(B)**, and panel **(C)** shows the detection of dynamin at sites (arrows) that represent the beginning and the end of *T. gondii* invasion and PV formation. **(D-E)** Parasites that were completely internalized do not show dynamin labeling. The red staining represents the actin distribution, and DAPI staining (blue) represents the nuclear DNA. Bars: 10 µM.

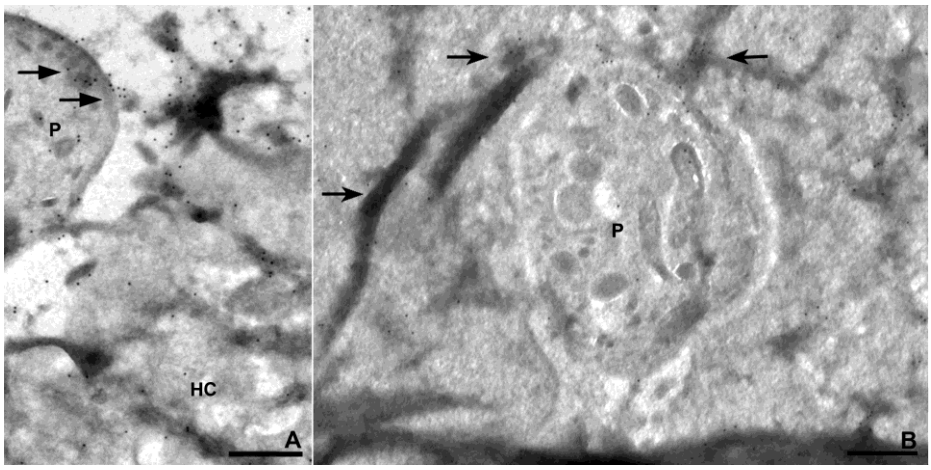

Figure 2: Cryo-immunomicroscopy of dynamin during *T. gondii* invasion. **(A)** 15 nm immunogold particles accumulated at sites of rhoptry secretion (arrows) during interactions of the tachyzoite with the host cell surface. **(B)** Labeling was observed at loci where dynamin is recruited (arrows) during parasite (P) invasion. Bars: 500 nm.

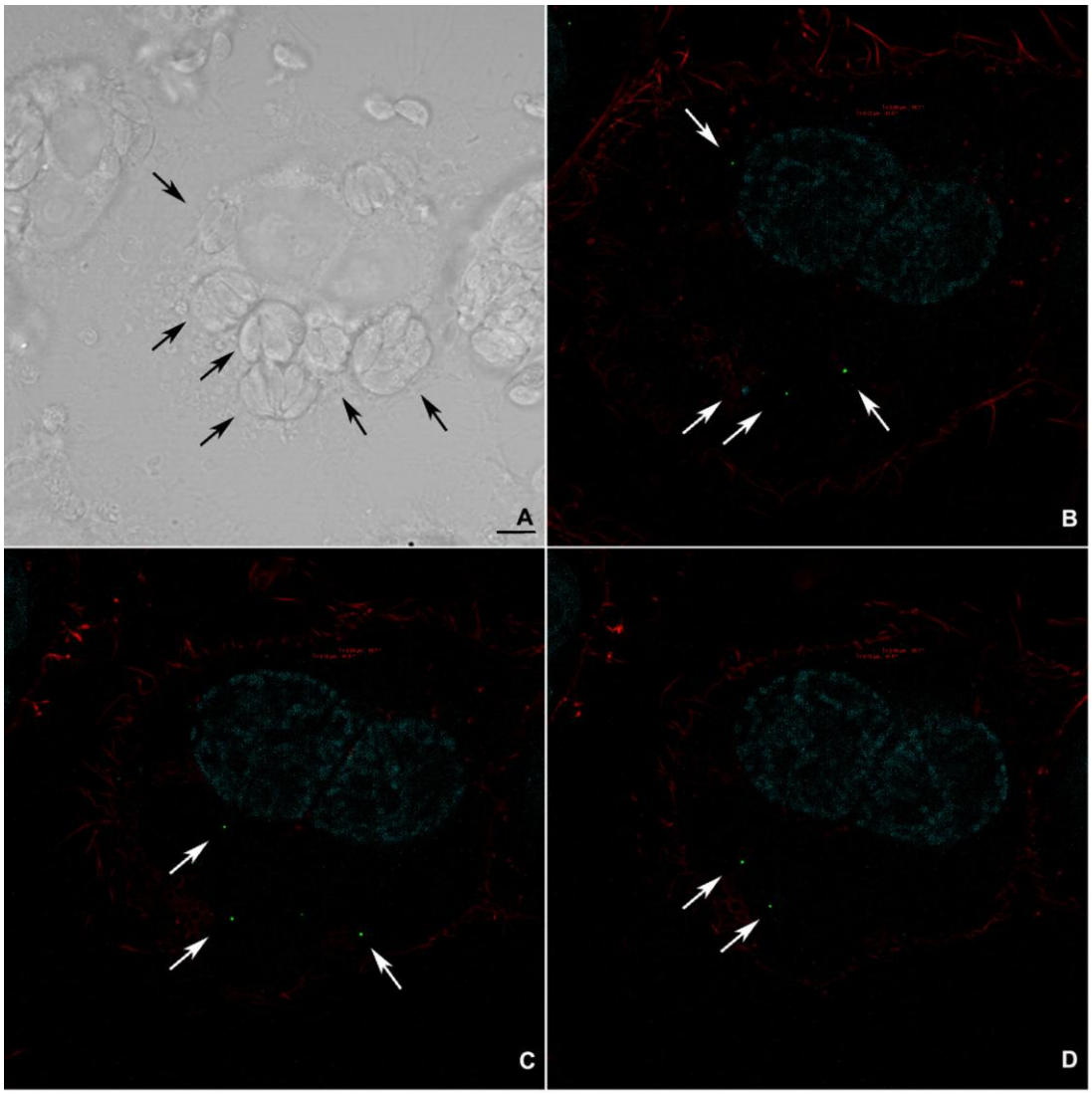

Figure 3: SR-SIM of cells labeled for dynamin and actin at 24 hpi. **(A)** The PVs (black arrows) within host cells at 24 hpi contain dynamin (white arrows) in different slices of the sample **(B-D)** (green). The red staining represents the actin distribution, and DAPI staining (blue) represents the nuclear DNA.

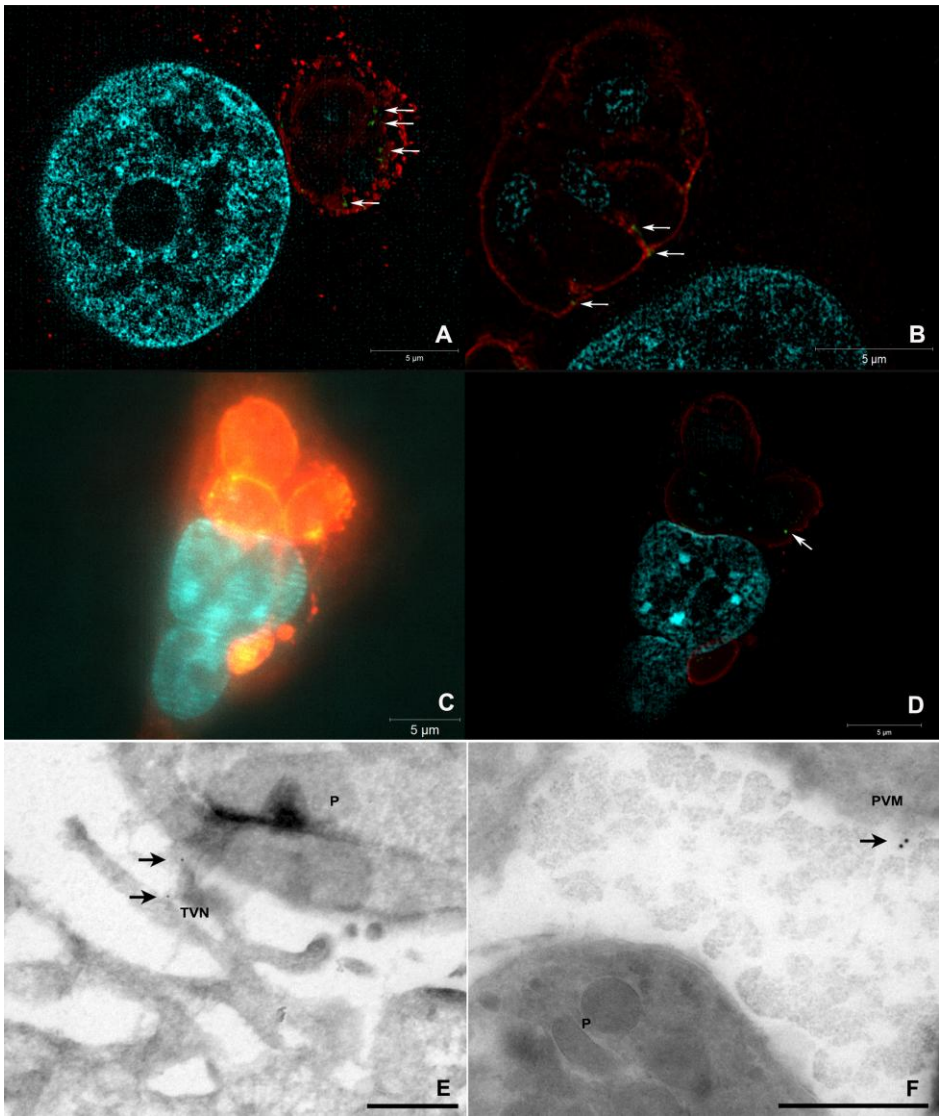

Figure 4: SR-SIM of cells labeled for dynamin and actin at 48 hpi **(A-D)**: The arrows point to dynamin foci (green) that are located primarily in the inner PV membrane (labeled in red) in two different infected LLCMK2 cells **(A-B)**. Wide-field SR-SIM mode shows an infected peritoneal macrophage exhibiting its *T. gondii* PVs (red) **(C)**, and the arrow points to dynamin expression (green) inside the vacuole **(D)**. DAPI staining (blue) represents the nuclear DNA. **(E-F)** show cryo-immunomicroscopy of dynamin during *T. gondii* development**.** The arrows point to the tubulovesicular network (TVN) that develops around the parasites (P) inside the PV **(E)**. **(F)** Dynamin is detected (arrow) on the inner side of the PV membrane (PVM). Bars: 500 nm.

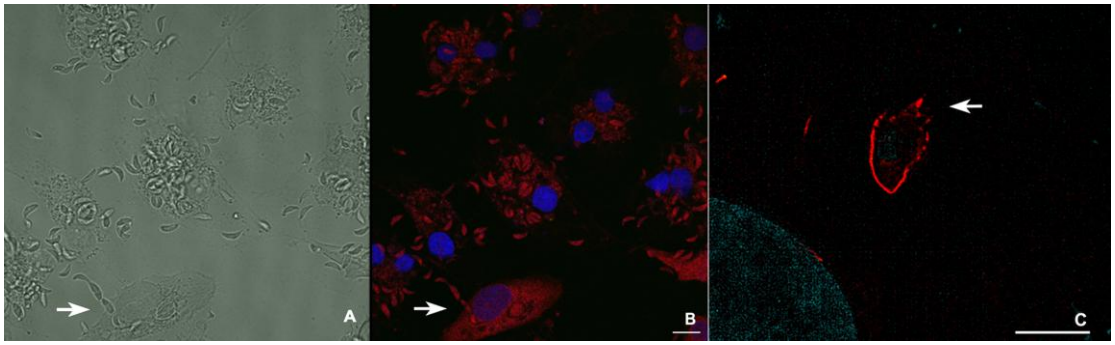

Figure 5: Immunofluorescence assay of the distribution of dynamin during *T. gondii* egress. **(A)** Interferential microscopy of induced tachyzoite egress shows no labelling of dynamin **(B)**. The arrows point to an egressing parasite crossing the host cell plasma membrane. The red staining represents the actin distribution. **(C)** Dynamin was not detected by SR-SIM in a non-induced egressing parasite (red) (arrow). DAPI staining (blue) represents the nuclear DNA. Bar: 10 µm **(A-B)**, 5 µm **(C)**.