Published in final edited form as:

Microbes Infect. 2008 ; 10(14-15): 1440–1449. doi:10.1016/j.micinf.2008.08.014.

Toxoplasma gondii actively remodels the microtubule network in host cells

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Abstract

Toxoplasma gondii infection triggers host microtubule rearrangement and organelle recruitment around the parasite vacuole. Factors affecting initial stages of microtubule remodeling are unknown. To illuminate the mechanism, we tested the hypothesis that the parasite actively remodels host microtubules. Utilizing heat-killed parasites and time-lapse analysis, we determined microtubule rearrangement requires living parasites and is time dependent. We discovered a novel aster of microtubules (MTs) associates with the vacuole within 1 h of infection. This aster lacks the concentrated foci of gamma (γ)-tubulin normally associated with MT nucleation sites. Unexpectedly, vacuole enlargement does not correlate with an increase in MT staining around the vacuole. We conclude microtubule remodeling does not result from steric constraints. Using nocodazole washout studies, we demonstrate the vacuole nucleates host microtubule growth in-vivo via γ -tubulin-associated sites. Moreover, superinfected host cells display multiple γ -tubulin foci. Microtubule dynamics are critical for cell cycle control in uninfected cells. Using non-confluent monolayers, we show host cells commonly fail to finish cytokinesis resulting in larger, multinucleated cells. Our data suggest intimate interactions between *T. gondii* and host microtubules result in suppression of cell division and/or cause a mitotic defect, thus providing a larger space for parasite duplication.

Keywords

Toxoplasma; Protozoa; Microtubules; Microtubule organizing center; Host-pathogen interactions; Parasite vacuole

1. Introduction

Invasion of a host cell allows a pathogen to avoid various aspects of the immune system including antibody-mediated responses. This lifestyle however, requires development of strategies that exploit or manipulate the host in order to create an intracellular environment safeguarded from host defenses while maintaining accessibility to host nutrients. One of the most common strategies employed by intracellular pathogens is to modify the compartment in

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Appendix A. Supplementary Material Supplementary material (Figs. S1–S3) associated with this article can be found at <http://www.sciencedirect.com>, at doi: 10.1016/j.micinf.2008.08.014.

which they reside [1–3]. The apicomplexan parasite, *Toxoplasma gondii* alters trafficking within the host cell to deliver host vesicles and organelles [4–6] to the parasite vacuole membrane (PVM) for nutrient acquisition purposes.

Contact with host cells triggers adhesion and active penetration by *T. gondii*. Three specialized secretory organelles, micronemes, rhoptries and dense granules release their contents to aid in invasion and establishment of infection [7]. Micronemal proteins aid in adhesion, rhoptries contribute lipids and protein to vacuole formation, and dense granule proteins are important in modification and maintenance of the PV [1,6–9]. Host endoplasmic reticulum (ER) and mitochondria decorate the PVM [10,11]. The secreted parasite protein, ROP2, mediates adhesion of host mitochondria to the PVM [6,11,12] and functional microtubules are necessary for mitochondrial delivery to the *T. gondii* vacuole [11]. While functional MTs have been implicated in mitochondrial delivery and movement of PVs, MTs themselves were never directly visualized in these studies [5,11,12].

Few studies have directly assessed microtubule interactions with the PVM, the timing of MT remodeling, and the extent to which these interactions are the result of steric constraints. The studies outlined here address these issues. Interestingly, cytoskeletal rearrangement is not observed during parasite invasion [13] and this includes recruitment of host MTs around the *Toxoplasma* vacuole [14]. In contrast, other studies report host MTs decorate the PVM 4–6 h post-invasion [4,15,16]. Here we resolve the apparent discrepancy between these disparate observations by demonstrating recruitment does not become readily discernable until 1-h post-invasion.

A clearer picture of the role of MTs in nutrient acquisition has recently begun to emerge. Cholesterol scavenging from the host occurs via vesicular transport, and like mitochondrial delivery, requires functional host MTs [4,16]. Further, MTs and the host cytoskeleton filament vimentin, coat the PVM [4,17], and host microtubule-based invaginations of the PVM serve as conduits for host lysosome sequestration [4]. *T. gondii*, but not the related apicomplexan parasite *Neospora caninum*, triggers host MT rearrangement, indicating a parasite-specific phenomenon [4]. Building on this observation, we asked if MT recruitment requires active parasite intervention and identify several important factors that influence MT remodeling.

2. Materials and methods

2.1. Chemicals, antibodies and other reagents

All chemical reagents were purchased from Fisher Scientific, VWR or Sigma-Aldrich unless otherwise indicated. Fisher-Sigma-Genosys or Integrated DNA Technologies, Inc (Coralville, IA) provided primers. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). All cell culture reagents were obtained from Gibco (Invitrogen, Carlsbad, CA). Antibodies were obtained from Sigma (monoclonal α -tubulin and polyclonal γ -tubulin antibody) and Molecular Probes (Alexa Fluor 488 goat anti-mouse secondary antibody IgG and Alexa Fluor 546 goat anti-rabbit secondary antibody IgG).

2.2. Host cells and parasites

RH tachyzoites and *N. caninum* were grown in the host cells (HFF) as previously described [18–20]. David Roos (University of Pennsylvania) generously provided the FNR-RFP cell line. *N. caninum* was a generous gift from Daniel Howe (MH Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky). Live cell imaging was performed in a stable BCS-1 line expressing GFP-tubulin at native levels and were maintained as previously described [21]. Macrophage studies were done with the RAW 264.7 macrophage-

like cell line and cultured as previously described [22]. MaryAnn McDowell (University of Notre Dame) generously donated this cell line.

2.3. Microcopy

2.3.1. Fluorescence microscopy—Immunofluorescent assays (IFAs) were carried out as previously described [19] with the following modifications.

2.3.1.1. Wide field: Infected host cells (HFF) were fixed in methanol (at -20°C) for 5 min and blocked in 5% BSA. Primary and secondary antibodies were both used at 1:1000. For heat-killed studies, parasites were subjected to a heat treatment of 56°C for 3 h. Parasite viability was assessed with Trypan Blue (Cellgro, Mediatech, Inc.). All samples were viewed using a Leica DM IRE2, images were captured and processed using Openlab software as previously described [18,19].

2.3.1.2. Confocal microscopy: Samples were viewed using a Leica TCS SP2 (True Confocal Scanner) by Leica Microsystems. Instead of using filter sets, it uses an acousto-optical beam splitter (AOBS) crystal to separate the emissions wavelengths coming from the samples. The system is attached to a Leica DM IRE2 automated inverted microscope using a 100 W mercury bulb for fluorescence imaging, and the following objective lenses were used: 63 $\times$ oil, and 100 $\times$ oil. Confocal images were analyzed using ImageJ software (NIH) and the following plug-ins: Z-coded stack, Volume View and Maximum intensity projection [23,24].

2.3.2. Thin section transmission electron microscopy—Host cells were plated onto aclar discs rather than glass coverslips and infected with RH. The infection was allowed to proceed for 2 h at 37°C , cells were mock-treated or treated with taxol (final concentration of 1 μM) and incubated an additional 2 h at 37°C . The samples were then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature, washed three times in 0.1 M cacodylate buffer and post-fixed for 1 h in 1.0% osmium tetroxide in the same buffer at room temperature. Samples were washed three times in water, stained for 1 h at room temperature in 2.0% uranyl acetate, washed in water and dehydrated in a graded series of ethanol. Subsequently, samples were embedded in Spurs, sections cut ultra thin at 80–90 nm, and viewed using a Hitachi H-600 transmission electron microscope.

2.3.3. Live image analysis—BCS-1 cell lines were plated on glass coverslips and grown for 48 h. They were subsequently infected with RH parasites and incubated for 16 h, washed twice with PBS and imaged live on the Leica DM IRE2 microscope as described above.

2.4. Morphometric analysis

Cells were infected with RH parasites using ENDO buffer and the Kafsack et al. protocol [25], a protocol designed to improve invasion synchrony four-fold. After synchrony and invasion, the infected cells were processed for IFA at 5, 30, and 60 min, 4 h and 24 h post-infection.

2.4.1. MT recruitment—PVs were assessed for location and MT accumulation was scored as absent (–) or present (+) around each PV. Three independent biological trials were carried out and 100 parasite vacuoles (PVs) were quantified per trial. Contingency table analysis (Systat V.10; Systat, SPSS Inc. 2000) demonstrated the proportion of PVs associated with the host nucleus increases significantly over time ($P = 0.00005$) (Fig. 2, Table not shown).

2.4.2. Mean fluorescent intensity/area—The ratio of MT mean fluorescent intensity (MFI) to area of vacuole was calculated using OpenLab 3.2 software (Improvision, Coventry, UK). All pictures were taken with the same exposure time and binning to ensure a balanced

comparison of staining. We manually created a region of interest (ROI) specific to each vacuole (in size and area). Pixel area and the threshold intensity values (max, min, mean) were measured within this ROI in Openlab using the Advanced Measurements Module. Three biological replicates of each time point were carried out and 10 vacuoles per replicate were calculated. Error bars and graphical analyses were calculated/performed in Microsoft Excel (Fig. 2B, inset).

2.4.3. Foci of γ -tubulin—Location and number of γ -tubulin foci within infected and uninfected host cells were determined at 24 h post-infection. Samples were fixed and processed for double immunofluorescent labeling using α - and γ -tubulin antibodies. Three independent biological trials were carried out and 15 fields were quantified per trial. The numerical data were collated and assessed by analysis of variance (using one-way ANOVA) [26]. Host MTOC (as judged by γ -tubulin staining) association with the nucleus decreases in infected host cells (ANOVA, $P = 0.01$).

2.5. Nocodazole treatment

RH tachyzoites were cultured as previously described [18–20]. Sixteen hours post-infection host MT network was perturbed using nocodazole at 10 μ M for 3 h at 37 °C. The drug was then washed out and cells allowed to recover for 5 min. Samples were fixed and processed for IFA using α - and γ -tubulin antibodies.

3. Results

3.1. Microtubule recruitment is time dependent and requires living parasites

MTs in uninfected host cells radiate from the host nucleus and form an ordered network throughout the cytoplasm (Fig. 1A). Whereas in infected cells, MTs form circular, basket-like structures that surround the PV (Fig. 1A, [4]). Recruitment of MTs to the PV does not appear to be cell-type-specific as the phenomenon occurs in several cell types such as HFF cells (Fig. 1A [4]), BCS-1 cells (Fig. 1D) and Raw 264.7 macrophages-like cell line (Fig. 1B, white arrowhead). Uninfected monolayers do not display similar structures (Fig. 1A). There is a possibility that the cell could simply be “walling off” an object that is as large as a parasite-filled vacuole. To test the hypothesis that microtubule rearrangement observed in infected host cells requires active parasite intervention, we incubated live and heat-killed parasite with Raw 264.7 macrophages (HFF host cells are skin cells and not phagocytic). After 4 h incubation, the host cells were fixed and processed for IFA. Host MT rearrangement into ordered circular arrays occurs in the presence of living parasites (Fig. 1B, white arrowhead). In contrast, macrophages containing heat-killed parasites demonstrate no such ordered structures surrounding the vacuole containing the heat-killed parasites (Fig. 1B, white arrow), even in cases where two or more parasites are taken up into a single vacuole (data not shown). Furthermore, large vacuoles of *N. caninum* do not display ordered arrays of MTs as observed for *T. gondii* (Fig. 1C [4]); demonstrating that MT hijacking is specifically triggered by *T. gondii* vacuoles and is not the result of non-specific stress response to infectious insult. While these complex structures appeared to be host MTs, it remained theoretically possible that we were observing an accumulation of *Toxoplasma*-derived MTs at the host-PV interface. Both poly- and monoclonal α -tubulin antibodies recognize the parasite's tubulin (data not shown). To test the origin of the MTs surrounding the PV, live cell imaging of infected BCS-1 cells expressing α -tubulin-GFP was carried out. MTs surrounding the PV are host-derived (Fig. 1D) and display a characteristic pattern mirroring the basket of MTs observed using α -tubulin antibody (Fig. 1A).

T. gondii infection does not trigger MT recruitment at 5 min post-infection (Fig. 2A [14]). It has been postulated that hMTs begin accumulating around the PV 4–6 h post-invasion [4]. To

determine timing of host MT recruitment, host cells were infected using the Kafsack et al. protocol [25] (a protocol designed to improve invasion synchrony four-fold). The infected host cells were then processed for immunofluorescence at several different times post-infection (PI) including time points prior to the first parasite division (Fig. 2A). The basket of host MTs begins formation around the PV at 30 min PI (Fig. 2A) and the characteristic circular structure surrounding the PV is observed at 1 h PI. Unexpectedly, the formation of the MT net containing a large foci of MT at one end of the vacuole (Fig. 2A,B) occurs prior to parasite division. This structure does not appreciably change even as the vacuole enlarges (Fig. 2A, compare 4 with 24 h). Moreover, one might expect that as the vacuole enlarges, the amount of MTs would increase accordingly. To test this possibility, we determined the ratio of MT mean fluorescence intensity (MFI) to vacuole area by comparing vacuoles containing one parasite (4 h) and larger vacuoles containing multiple parasites (24 h). The MFI/area does not increase as the area of the vacuole increases; rather the ratio of MFI/area significantly decreases when comparing 4–24 h infection (Fig. 2B, inset). Lastly, we determined that PVs association with the host nucleus increase over time (Fig. 2C) and this positively correlates to microtubule recruitment.

3.2. Distinctive features of the MT structures associated with the PVM

Our preliminary results reveal that basic building blocks of hMTs concentrate in ordered arrays around the PV (Figs. 1 and 2). A structure called the microtubule-organizing center (MTOC) initiates the growth of MTs in a process called nucleation [27–30]. All MTOCs share the ability to nucleate MTs [27,28,30]. In addition to nucleating MTs, MTOCs also dictate the number and arrangement of MTs. All MTOCs examined, in all species, stain with antibodies directed against γ -tubulin [27,31]. Given that the host MTOC has been reported to associate with the PV [4], it seemed likely that MTs surrounding the PV would emerge primarily from a major foci of γ -tubulin. To probe the relationship between γ -tubulin foci and the microtubules surrounding the PV, infected host cells were stained with antibodies directed against both α and γ -tubulin and then analyzed both by wide-field and confocal microscopy. Normally, radial arrays of MTs are associated with γ -tubulin foci in eukaryotic cells (Fig. 1A). Close examination of the PV by wide-field microscopy reveals a novel MT arrangement (green, Fig. 3A) restricted to one side of the vacuole (yellow arrow) that does not correspond to a “traditional” MTOC as defined γ -tubulin staining (Fig. 3A white arrowhead, bottom panel). This structure is observed on every PV and appears as early as 1 h post-infection (Fig. 2A). Confocal analysis of the PV demonstrates that the MTs around the PV form an ordered array that appears to wrap over and around the PV (Figs. 3B and S1). Top sections of the vacuole reveal a “sea-shell” pattern of MTs (Fig. 3B, model inset) with a common area of MT emergence arising from one side of the PVM (Fig. 3B, arrow). This concentration of microtubules is observed in a series of wishbone shaped structures (Fig. 3A,B inset). Confocal reveals that the PVM is often enriched in γ -tubulin and that the concentration of MTs (Fig. S1A, yellow arrow) restricted to one side of the vacuole does not correspond to a large foci of γ -tubulin (of a size and staining intensity normally associated with a host MTOC) in any optical section (Fig. S1A). At the ultrastructural level, taxol treated cells displayed similar wishbone shaped structures associated with the PV 4 h after infection (Fig. 3C, star).

3.3. The PVM nucleates the growth of host MT in-vivo via foci of γ -tubulin

Growth of hMTs occurs in a characteristic fashion with hMTs growing in a distinctive radial/star-like array called an aster. Aster formation generally takes place near or around the host nucleus (Fig. 4A). This is followed by elongation and formation of the mature host MT network. Given that recruitment is dependent upon living parasites, are often enriched in γ -tubulin and unique MT structures are in close association with the PV, we hypothesized that the PV may possess the ability to nucleate the growth of and/or bind microtubules. To test this, infected host cells were treated with nocodazole to depolymerize the microtubule network (Fig. 4B). The drug was then washed out and the cells allowed to recover. Re-growth of host MTs was

monitored with respect to the PV. MTs were observed growing directly from a PV-associated focus of γ -tubulin on one side of the vacuole (Fig. 4, yellow arrow). Less frequently, we observed MTs arising along PV perimeter at sites containing reduced (but not absent) amounts of γ -tubulin (Fig. 4, white arrow). Gamma-tubulin pools exist in the host cytosol [32], and therefore it is likely that small amounts of γ -tubulin are present, but not distinguishable above background, at these sites on the PVM and nucleating growth.

3.4. *T. gondii* infected cells display supernumerary foci of γ -tubulin

While it has been reported that host MTOC associates with the parasite vacuole [4], the frequency of association and the extent to which position and number of parasite vacuoles affect MTOC location and/or number are unknown. Note: it has not been shown that the focus of γ -tubulin associated with the PV contains centrioles [4], consequently this focus cannot formally be called an MTOC. Thus, the hub of MT radial arrays that stain at their focus with γ -tubulin will be referred to as γ -tubulin foci (of a size consistent with the host MTOC). To test the extent to which *T. gondii* affects the location and number of γ -tubulin foci present within the host cell, host cells were infected and processed for IFA 24 h post-infection (Fig. 5). Normally, the host MTOC is found anchored in close proximity to the host nucleus (Fig. 5A,B) in uninfected host cells. However, we demonstrated that the anchorage of the γ -tubulin foci consistent in size with the host MTOC, significantly decreases (ANOVA, $P = 0.01$) in infected cells (Fig. 5B). Three major phenotypes are observed for γ -tubulin foci-PV interactions (Fig. 5A,B). These phenotypes are: (1) no γ -tubulin focus association; (2) γ -tubulin focus links the PV to the nucleus; and/or (3) γ -tubulin focus consistent in size with the host MTOC completely detaches from the host nucleus and associates solely with the PV. These data were quantitated and the results and model phenotypes are shown in (Fig. 5B). Superinfected cells display multiple foci of γ -tubulin (Fig. 5C). In uninfected cells, the ratio of host γ -tubulin foci/nucleus is approximately 1:1. The ratio in infected host cells increases to approximately 1.7:1 γ -tubulin foci/nucleus. The latter ratio is deemed a conservative estimate as only those concentrations of γ -tubulin mimicking the size and shape of a normal MTOC were counted. Multiple apparent MTOC fragments are often observed in superinfected host cells (data not shown).

3.5. *T. gondii* infected host cells commonly fail to finish cytokinesis

It seemed likely parasite co-option of host MTs and the presence of multiple foci of γ -tubulin would affect host progression through the cell cycle. To test this hypothesis, we modified our invasion protocol. Usually, IFAs are carried out utilizing confluent host cells (contact-inhibited cells do not divide). To test the extent to which *T. gondii* infection affects cytokinesis, a non-confluent monolayer was infected and processed for immunofluorescence 48 h after infection using either α -tubulin to follow spindle formation/cytokinesis or γ -tubulin to follow centrosome duplication and separation. Infected cells were also stained with the DNA dye DAPI to assess the number of host nuclei per host cell. Infected host cells display multiple nuclei and fragmented Golgi (Figs. 6 and S3A,B,D). Comparison of infected and uninfected host cells in three biological trials reveals that infected host cells display not only more nuclei, but also nuclei that are larger and misshapen (see infected host cell, small white arrow in Fig. 6C, and Fig. S3A,B). Uninfected cells are observed finishing cytokinesis (Fig. 6C, see large white arrow pointing to mid-body containing the remains of the central MT spindle), while infected cells are multinucleate (Fig. 6A,C, small white arrow). Additionally, infected host cells often possess bi-lobed nuclei (Figs. 6A and S3A,B). This is observed at the light and ultrastructural level in infected host cells (Figs. 6 and S3A,B). As judged by IFA, infected host cells appear capable of entering S phase and perhaps initiating mitosis (Fig. S3), however cytokinesis is frequently blocked resulting in the creation of multiple nuclei and/or large, misshapen nuclei.

4. Discussion

Microtubules direct intracellular transport of vesicles and position organelles [33] and host organelles modify the parasite vacuole [10,11]. We show that the parasite actively recruits host MTs. Steric constraints within the host cell could contribute to the MT remodeling observed around the host cell, however we consider this unlikely for several reasons. First, host MT rearrangement occurs specifically in response to infection by the apicomplexan parasite *T. gondii*, whereas infection by the apicomplexan parasite *Neospora caninum* does not elicit this response (Fig. 1C [4]). Second, we tested the hypothesis that *T. gondii* actively stimulated this response. MT rearrangement occurs in the presence of living parasites while vacuoles containing heat-killed parasites do not exhibit the same ordered basket/circular structure (Fig. 1). Third, a high percentage of PVs display host MT accumulation at 4 h post-infection (PI) prior to parasite division and the circular structure surrounding the vacuole does not appreciably change in larger vacuoles containing multiple parasites. Moreover, the MT MFI/area ratio decreases after 4 h (Fig. 2B, inset) suggesting that the number of MTs associated with the PVM at 4 h, remains constant despite an increase in vacuole size (Fig. 2B, inset). Lastly, parasite-specific intervention seems likely given that the PVM nucleates growth of host MTs via a focus of γ -tubulin that appears tethered to the vacuole (Fig. 3).

We observe PV association with the host nucleus is often mediated by a focus of γ -tubulin consistent in size to the host MTOC. Microtubule organizing centers stain with γ -tubulin and may serve as the mechanism through which *T. gondii* remodels host MTs. *T. gondii* infection results in an increase of the number of γ -tubulin staining foci within the host cell. The parasite may be disrupting mitotic spindle separation or decoupling MTOC duplication from mitosis as indicated by the multiple nuclei (Figs. 6 and S3). One possible benefit of allowing the cell to duplicate DNA is that cells become larger and provide more space for parasite duplication. Furthermore, this type of cycling allows for the production of more nutrient materials; infected host cells display more ER and mitochondria than uninfected host cells on the ultrastructural level (data not shown).

Host organelle decoration of the PVM is a well-documented phenomenon [6,11,34,35], however, the identities of secreted parasite proteins capable of recruiting and/or interacting directly with host organelles or MTs have largely been elusive. One protein, ROP2, has been identified as a molecular rivet 'tethering' the host mitochondria to the PVM [6]. A second protein, GRA7, sequesters host endocytic organelles by acting as a garrote around MT-based invaginations of the PV membrane [4,17]. Although many pieces seemed to be falling into place regarding the mechanism of MT recruitment, several unknowns remain. What molecular mechanism does *T. gondii* use to hijack the host cell cytoskeleton? In other words, do secreted parasite proteins drive MT rearrangements? Are molecular motors involved in the movement of the host organelles? Given that parasites must secrete protein to actively invade the host cell and active invasion is required for MT rearrangement (Fig. 1B), we hypothesize that secreted parasite proteins are likely involved in initiating host MT structural changes and host organelle recruitment.

Parasite control of host MTs may be occurring primarily via capture and subsequent tethering of the host MTOC to the PV. Secondary control may be exerted at specialized, parasite protein-containing sites that bind and/or nucleate host MT, albeit with less affinity than the host MTOC. This hypothesis arises from our observations that the PV nucleates growth of MTs primarily via foci of γ -tubulin of a size consistent with the host MTOC (Fig. 3) although re-growth is occasionally observed at sites containing reduced amounts of γ -tubulin. These specialized sites are likely concentrated on one side of the vacuole and correspond to the unique structures observed in Fig. 3. Thus the PVM may function as a specialized docking site capturing growing MTs and MTOCs. MTs grow and shrink through the addition and subtraction of tubulin

heterodimers at their ends. Upon binding their target, MTs are stabilized [36]. Similarly, the PVM may capture growing host MTs as they undergo normal rounds of growth and catastrophe. Our preliminary data suggest host MTs associated with the PVM are more stable than cytosolic MTs (data not shown). The unique structures observed in Fig. 3 may function as docking sites. Alternatively they could serve as low-affinity nucleation sites because they likely contain low amounts of γ -tubulin (i.e. difficult to distinguish from background because of the presence of cytosolic pools of γ -tubulin). These structures are wishbone shaped at the light level and also at the ultrastructural level. The taxol treatment used to stabilize the MTs with respect to the PV during the EM fixation procedure can cause MT bundling. Consequently, the structures observed at the ultrastructural level may not be as representative as the structures observed at the light level. Future studies will address the composition and function of these novel structures.

Despite the apparent physical barrier of the PVM, *T. gondii* actively 'communicates' with its host, by recruiting host cell organelles [7,11] scavenging host cholesterol [16], inhibiting host cell apoptosis [37] and co-option of host gene expression [38]. Secreted parasite proteins have been directly identified or implicated as playing important roles in all of these host—parasite interactions. Thus we expect that secreted parasite proteins will also be shown to play a role in recruitment and remodeling of host microtubules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We gratefully acknowledge Tracy Eggleston for her help with tissue culture. KMH was supported by a grant from the Ellison Medical Foundation (ID-NS-0058-02) and from a University of Notre Dame Faculty Research Program award (FRP). EHH is supported by a grant from the American Cancer Society (ACS RSG CCG-104915) and an NIH ROI (GM107275). A Pollard Graduate Fellowship supported JEH. We thank Holly Goodson and Kevin Vaughan for helpful conversations regarding this work.

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Fig. 1.

An active *T. gondii* infection is necessary for host microtubule recruitment around the parasite vacuole. (A) Host cells were mock infected (uninfected, UI) or infected (I) with RH, fixed and processed for immunofluorescence assay (IFA) by staining with anti- α -tubulin and DAPI (blue DNA dye). An accumulation of microtubules is observed surrounding the parasite vacuole (PV), white arrows. (B) Raw macrophage-like cells were either mock infected with heat-killed parasites (white arrow) or infected with live RH parasites (arrowhead). The infection was allowed to proceed for 4 h and then the samples were fixed and processed for IFA using anti- α -tubulin (white) and DAPI (blue). Microtubule surrounds living parasite in ordered arrays (white arrowhead), whereas this ordered rearrangement is not observed in vacuoles containing heat-killed parasites (white arrow). (C) Alpha-tubulin (white) forms ordered arrays around *T. gondii* (Tg), not *N. caninum* (Nc) vacuoles. Parasites stained with DAPI are blue. (D) A stable BSC-1 line expressing GFP-tubulin (GFP-tub) at native levels was infected with RH parasites stably expressing the apicoplast marker FNR-RFP. Host MTs (green) are observed surrounding the parasites (red) in the merge. PV, parasite vacuole; hN, host nucleus. Scale bars = 10 μ m.

Fig. 2.

Time-course of host microtubule recruitment and PV location during the course of infection. Host cells were infected and processed for IFA at 5.0 min and 0.5, 1, 4 and 24 h post-infection. Three independent biological trials were carried out and representative fields are shown. These data were quantitated and results are graphically depicted in (B) and (C). (A)

Immunofluorescence microscopy of host MTs (stained with anti- α -tubulin) showing that MT recruitment to the PV is time dependent. Parasites and host nucleus are visualized by DAPI (blue). Boxed region is enlarged in the panel below. Representative fields are shown for each time point. Scale bar, 10 μ m. (B) Quantification of microtubule accumulation around parasite vacuoles during the course of infection demonstrates that MT accumulation is time dependent and maximizes at 4 h post-infection. Three independent biological trials were carried out and error bars are shown. Time (h) represents amount of time post-infection of the host monolayer. Inset: MT mean fluorescent intensity per area of vacuole (MFI/area vacuole) was calculated as described in Section 2. (C) Localization of MT-surrounded PVs during the course of infection. Three biological trials were carried out and error bars are shown. Note error bars are so small for the nucleus that they do not appear in the figure. PVs, parasite vacuoles; circles, PVs in the cytoplasm; squares, PVs associated with the nucleus.

Fig. 3.

Peculiarities of MT structures surrounding the parasite vacuole. (A) Immunofluorescent microcopy showing several foci of MTs. In uninfected cells, foci of MTs (yellow arrowhead), correspond to a foci of γ -tubulin (white arrowhead) similar in size to the host MTOC. In infected cells, α -tubulin staining reveals a novel MT arrangement restricted to one side of the vacuole (yellow arrow) that does not correspond to the host MTOC (as judged by γ -tubulin staining, white arrowhead). Cells were stained with DAPI (blue) to visualize nuclei. (B) Confocal microscopy confirms that host MTs surround the PV and also emerge from, and/or are concentrated on, one side of the vacuole (left panel, large yellow arrow). Right panel: reconstruction of confocal stacks and use of Image J software (NIH), Volume View [23,24], were used to “tilt” the stacks to get an “end” on view of the emerging MTs. Top sections of the vacuole reveal a sea-shell pattern of MTs (model inset) with a common area of emergence from the PVM. This concentration of MTs appears in both (A) and (B) and does not correspond to a “traditional” host γ -tubulin foci consistent in size to the host MTOC. Gamma-tubulin staining is not shown in (B) for purposes of clarity (data not shown). See also Fig. S1. (C) Transmission electron micrograph of taxol treated host cells reveals that host microtubules associate with the PV membrane (PVM) in a pattern similar (black star) to what is observed at the confocal and wide-field levels in non-taxol treated cells (A and B). Host cells were infected 2 h, then treated with 1 μ M taxol for an additional 2 h and then fixed and process for TEM. *MTs associated with the PVM; PV, parasite vacuole; *T. gondii*, *Toxoplasma gondii*. Note, cells shown in the IFA in (A,B) were not treated with taxol. Black scale bar, 1.0 μ m; white scale bar, 0.25 μ m; red scale bar, 0.2 μ m.

Fig. 4. Microtubules reform in the close vicinity of parasite vacuoles. Infected HFF cells were pre-treated without (-NZ) and with 10 μ M nocodazole (+NZ) for 5 h at 37 °C. Drug was washed out and the cells allowed to recover. All samples were fixed and processed for double immunofluorescence labeling using anti- α and anti- γ -tubulin. Cells were stained with DAPI to visualize nuclei. All pictures were taken at the same exposure time. (A) Nocodazole disruption and recovery in uninfected host cells (see also Fig S2). (B) Nocodazole disruption and recovery in infected cells. Microtubules (yellow arrow) reform in close proximity to the parasite vacuole in a characteristic aster pattern. Foci of γ -tubulin staining are also observed associated with the PV. Bottom panel: MTs are observed re-growing from the PV from sites

containing reduced amounts of γ -tubulin (white arrow). Cells were stained with DAPI to visualize nuclei of host and parasites. Anti- α -tubulin (green), γ -tubulin (red), DAPI (blue) in merge. Inset: higher contrast showing γ -tubulin with respect to the parasite. Far right panel is enlargement of area and field of view in order to show the entire cell showing host γ -tubulin staining (small white arrow) with respect to re-growing MTs. Yellow arrow and white arrow, nucleating microtubules; small white arrows (far right panel), foci of γ -tubulin approximately similar in size to the host MTOC; scale bars, 10 μ m.

Fig. 5.

Immunofluorescence microscopy of foci of γ -tubulin location with respect to the parasite vacuole. (A,B) Double immunofluorescence microscopy demonstrates that host MTs (anti- α -tubulin, green) surround, and foci of γ -tubulin (anti- γ -tubulin, red) associated with the parasite vacuole. Three phenotypes for γ -tubulin foci location with respect to the parasite vacuole are observed: (1) no γ -tubulin foci association, (2) γ -tubulin focus links PV with the host nucleus, and (3) γ -tubulin foci associated with the PV membrane only. These phenotypes were quantified and are shown in Fig. 3B. (B) Quantification of γ -tubulin foci location with respect to the host nucleus and parasite vacuole within uninfected and infected host cells were determined and standard errors calculated: (1) no γ -tubulin focus association (38.1 ± 1.1), (2) γ -tubulin focus links PV with the host nucleus (26.5 ± 4.4), and (3) γ -tubulin foci associated with the PV membrane only (35.5 ± 5.5). There is a statistically significant decrease in association of γ -tubulin focus consistent in size to the host MTOC with the host nucleus. It decreases from 91.7 ± 2.2 down to 64.4 ± 5.5 ($P = 0.001$). (C) Immunofluorescence microscopy of superinfected host cells. Superinfected host cells display multiple γ -tubulin foci (white lines). PV, parasite vacuole (black lines). Anti- α -tubulin (green), γ -tubulin (red), DAPI (blue) in merge.

Fig. 6.

Infected host cells display large multi-nucleate structures. Immunofluorescent microscopy of non-taxol treated infected host cells shows multinucleate and bi-lobed nuclei (DAPI, blue) are present within host cells that are infected with *T. gondii*. (A,B,C) The last panel is a merge of stained host cells. Merge, anti- α -tubulin (MT are shown are green), anti- γ -tubulin (MTOCs are shown as red) and DAPI (nuclei are shown as blue) are shown as an overlay. Small white arrow, infected host cell; large white arrow, a remnant spindle is present in the mid-body of an uninfected host cell undergoing cytokinesis.