Neospora caninum Recruits Host Cell Structures to Its Parasitophorous Vacuole and Salvages Lipids from Organelles

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Toxoplasma gondii and Neospora caninum, which cause the diseases toxoplasmosis and neosporosis, respectively, are two closely related apicomplexan parasites. They have similar heteroxenous life cycles and conserved genomes and share many metabolic features. Despite these similarities, T. gondii and N. caninum differ in their transmission strategies and zoonotic potential. Comparative analyses of the two parasites are important to identify the unique biological features that underlie the basis of host preference and pathogenicity. T. gondii and N. caninum are obligate intravacuolar parasites; in contrast to T. gondii, events that occur during N. caninum infection remain largely uncharacterized. We examined the capability of N. caninum (Liverpool isolate) to interact with host organelles and scavenge nutrients in comparison to that of T. gondii (RH strain). N. caninum organizes the host microtubular cytoskeleton and attracts endoplasmic reticulum (ER), mitochondria, lysosomes, multivesicular bodies, and Golgi vesicles to its vacuole though with some notable differences from T. gondii. For example, the host ER gathers around the N. caninum parasitophorous vacuole (PV) but does not physically associate with the vacuolar membrane; the host Golgi apparatus surrounds the N. caninum PV but does not fragment into ministacks. N. caninum relies on plasma lipoproteins and scavenges cholesterol from NPC1-containing endocytic organelles. This parasite salvages sphingolipids from host Golgi Rab14 vesicles that it sequesters into its vacuole. Our data highlight a remarkable degree of conservation in the intracellular infection program of N. caninum and T. gondii. The minor differences between the two parasites related to the recruitment and rearrangement of host organelles around their vacuoles likely reflect divergent evolutionary paths.

Coccidian parasites are obligate intracellular pathogens of the phylum Apicomplexa and responsible for significant human and veterinary diseases. Among them, Toxoplasma gondii and Neospora caninum are very closely related tissue-dwelling Coccidia that share many biological features (1). The two parasites diverged ~28 million years ago, but their genome size and gene content and expression have been remarkably conserved; among the genes shared by T. gondii and N. caninum, 40% have no orthologues in any other Apicomplexa (e.g., Plasmodium and Cryptosporidium) (2). T. gondii affects up to one-third of the human population and is responsible for severe infections associated with the central nervous system (3). In healthy individuals, toxoplasmosis is usually asymptomatic, with the parasite remaining encysted in brain and muscle cells throughout the host’s lifetime. Reactivation of this latent infection occurs under immune-deficiency conditions, which can lead to fatal encephalitis (4). Congenital infection with T. gondii can cause neurologic defects in the fetus and abortions in both humans and animals, particularly in sheep and goats (5, 6). N. caninum is the agent of the disease neosporosis, which is associated with neuromuscular degeneration and neonatal mortality in animals, particularly in dogs and cattle (6–8). Once in their hosts, N. caninum parasites also transform into cyst forms that persist in the brain and muscles (9). Both T. gondii and N. caninum have a heteroxenous life cycle, characterized by asexual replication in an intermediate host and sexual reproduction in the small intestine of a definitive host: Toxoplasma completes its sexual cycle in Felidae and Neospora in Canidae. While T. gondii can infect virtually all warm-blooded animals, N. caninum has a more limited host range. Notably, N. caninum does not cause any recognized disease in humans despite the detection of antibodies against N. caninum antigens in humans (~6% in healthy individuals and up to 40% in HIV-infected patients) (10, 11).

The differences between T. gondii and N. caninum in zoonotic capabilities and host preferences emphasize the relevance of comparative studies to identify organism-driven mechanisms in the program of infectivity of the two pathogens. Comparisons of genomes and transcriptomes have revealed defining differences between these parasites in gene products with roles in host defense (2). For example, N. caninum has twice as many genes coding for surface glycosylphosphatidylinositol-linked proteins (SAG1-related sequences, or SRS) as T. gondii. As these proteins mediate attachment to host cells and immune activation (12), this difference could represent an evolutionary advantage for evolving a narrower host range. A second example involves rhoptry-derived kinases that are secreted by these parasites into host cells to interfere with host signaling pathways; N. caninum encodes fewer of these virulence-associated rhoptry proteins than T. gondii (2). In particular, the ROP18 kinase, which inactivates host immunity-
related GTPases that would otherwise disrupt the membranes of parasitophorous vacuoles (PV) (13–16), is reduced to a pseudo-gene in Neospora (17).

Investigations of host cell invasion by N. caninum are largely inspired by studies on T. gondii, and small differences in the invasion processes of these parasites have been identified. The proliferative tachyzoite forms of these parasites invade a large variety of cultured cells, wherein they multiply in parasitophorous vacuoles. By virtue of their obligate intracellular lifestyle, these parasites cannot survive under axenic conditions, and N. caninum tachyzoites are particularly vulnerable to the harmful effects of extracellular maintenance and rapidly lose their capacity for invasion. Active invasion of mammalian cells by these parasites involves the coordinated release of proteins from the parasite’s secretory organelles. First, micronemes release adhesins that mediate the attachment of the parasites to the host plasma membrane (18). This process is accompanied by the proteolytic cleavage of micronemal proteins by cytoxine proteases and rhomboid proteases (19–22), and T. gondii and N. caninum differ with regard to their susceptibilities to protease inhibitors (23). Second, proteins from rhoptries are released at the parasite-host cell interface to form a tight junction between the plasma membranes of the invading parasite and the host cell (24). The ring-like moving junction serves as a filter to eliminate host transmembrane proteins from the nascent PV, thereby avoiding subsequent recognition and fusion with host lysosomes. Finally, these parasites modify the environment of their PV by secreting proteins from dense granules (25–27). A striking morphological difference between replicating T. gondii and N. caninum is their organization inside the PV; Toxoplasma parasites form rosettes around a central residual body, with the parasite’s apical end facing the PV membrane, while Neospora parasites have no specific spatial organization within their PV. One hallmark of a T. gondii infection is the ability of the parasite to alter host cell pathways by using the PV membrane as a signaling platform or by secreting bioactive factors into the host cytoplasm and nucleus (28, 29). T. gondii is also notorious for interacting with many host cell structures and organelles that surround the PV (30). Despite the discovery of N. caninum 30 years ago, many aspects of its intracellular behavior, including host cell interaction and nutrient uptake, have not been thoroughly studied.

In this study, we examined the capability of N. caninum to modify the host cell interior and recruit mammalian organelles. Our goal is to identify functional differences in the colonization of host cells between N. caninum and T. gondii to provide unique insights into Neospora host specificity and pathogenesis at the cellular level. N. caninum spends most stages of its life cycle as an intravascular parasite as it survives poorly axenically. Thus, to effectively control Neospora infections, it is important to decipher the steps that lead to its successful intracellular development, which may uncover potential targets for chemotherapeutic intervention as neosporosis is a major root cause of economic loss worldwide (31). Here, we show that N. caninum diverts many host cell structures and organelles to its PV, notably to exploit their nutrient content, thereby demonstrating conserved mechanisms between this parasite and T. gondii. However, small differences in the modus operandi of these parasites are also observed, e.g., in the rearrangement of host secretory organelles around the PV, which could account for divergence in their respective evolutionary paths, as well as reflect differences in the expression of unique genes.

**MATERIALS AND METHODS**

**Reagents and antibodies.** All chemicals were obtained from Sigma (St. Louis, MO) or Fisher (Waltham, MA) unless otherwise stated. Nitrobenzoxadiazole (NBD)-cholsterol and NBD-C6-ceramide were obtained from Molecular Probes (Seattle, WA), and BODIPY TR C5-ceramide was from Invitrogen (Carlsbad, CA). [3H]uracil was purchased from PerkinElmer (Shelton, CT). The following primary antibodies were used: mouse monoclonal and rabbit polyclonal anti-γ-tubulin, rabbit polyclonal anti-giantin (Covance, Emeryville, CA), rabbit polyclonal anti-GRAG7 antibodies (32), mouse anti-KDEL (Enzo Life Sciences, PA), mouse anti-H5C6 Limp (CD63), mouse anti-H4A3 LAMP1 (lysosome-associated membrane protein 1; Developmental Studies Hybridoma Bank, University of Iowa), and mouse monoclonal anti-Tom20 (Santa Cruz, CA).

The N. caninum mouse anti-NcP1-S and 21H7A antibodies were generous gifts from Vern Carruthers (University of Michigan) (33) and Peter Bradley (University of California, Los Angeles) (34), respectively. Secondary antibodies used for immunofluorescence were conjugated to Alexa Fluor 488, Alexa 594, or Alexa 350 (Invitrogen, Carlsbad, CA).

**Cell lines and culture conditions.** Human foreskin fibroblasts (HFF), HeLa cells, and CHO-K1 cells were obtained from the American Type Culture Collection (Manassas, VA). Bovine turbinate cells (BT24 cells) were generously provided by Dan Howe (Gluck Equine Research Center, Lexington, KY). The somatic 2-2 mutant CHO cells lacking functional Niemann-Pick type C1 (NPC1) was kindly provided by L. Liscum (Tufts University) (35). All cells were grown as monolayers and cultivated in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and penicillin-streptomycin (100 units/ml per 100 μg/ml) and maintained at 37°C in 5% CO2. Preparation of low-density lipoprotein (LDL) and lipoprotein-deficient serum (LPDS) was described previously (36).

**Parasite cultivation.** The tachyzoites from the Nc-Liv isolate (Liverpool, United Kingdom) (37) of N. caninum and from the RH strain (type I lineage) of T. gondii were used throughout this study. In one experiment, the Prugniaud strain (type II lineage) of T. gondii was used. The parasites were propagated in vitro by serial passage in monolayers of HFF (38).

**Plaque assay.** BT24 cells or HFF were grown until confluence in a six-well plate as both cell lines have growth contact inhibition. Two hundred parasites were added to each well, and the plates were incubated at 37°C for 6 days. The cells were fixed and stained as described previously (39). The plates were scanned (ScanWizard 5; Microtek), and the area of each plaque was measured using Volocity software (PerkinElmer, Waltham, MA) by tracing each plaque using the region of interest (ROI) tool. The mean areas and standard deviations (SD) of the plaques were calculated from three independent experiments, and the P values were calculated using a Student t test with Excel software (Microsoft, Redmond, WA). The total number of plaques in each well was also counted and analyzed in the same manner. The mean area and number of plaques were calculated from three independent experiments using three wells of a six-well plate per experiment and condition.

**Uracil incorporation assay.** CHO cells were grown until confluent in 24-well plates before infection with 5 × 104 parasites for 4 h at 37°C, washed with phosphate-buffered saline (PBS), and incubated for 24 h in α-MEM. Cells were then incubated with 1 μCi of [3H]uracil for 2 h at 37°C, and the samples were processed as described previously (38).

**Lipid uptake.** To monitor the uptake of ceramides by intracellular N. caninum, HFF were infected for 24 h, washed with PBS, and incubated in serum-free-medium containing 5 μM NBD-C6-ceramide complexed to bovine serum albumin (BSA) for different time periods prior to observations by live microscopy. To examine cholesterol uptake, HFF were infected for 24 h, washed with PBS, and incubated in the presence of the serum containing 1 μM NBD-Cholesterol incorporated into lipoproteins for the various time periods (36).
Mammalian cell transfection with GFP-Rab constructs. Green fluorescent protein (GFP)-Rab43, GFP-Rab30, and GFP-Rab14 constructs were generously provided by John Presley (McGill University, Canada). HFF were used for transfection using an Amaxa Nucleofector Kit V according to the manufacturer’s conditions (Lonza, Basel, Switzerland). Cells were transfected with 2 μg of plasmid DNA and left to recover overnight prior to parasite infections. The cells were infected with *N. caninum* for 45 min at 37°C, washed with PBS to remove extracellular parasites, and incubated for 30 h at 37°C. The cells expressing GFP-Rab14 were incubated with serum-free medium containing 5 μM BODIPY TR C5-ceramide conjugated to BSA for 20, 30, or 40 min.

**Fluorescence microscopy and image analysis.** For immunofluorescence assays (IFA), cells were fixed either with 4% formaldehyde (Polysciences, Warrington, PA) plus 0.02% glutaraldehyde in PBS for 15 min or with cold 100% methanol for 5 min (for immunostaining with γ-tubulin and α-tubulin). IFA were performed as described previously (41) except that the permeabilization step with 0.3% Triton X-100 in PBS was omitted for samples fixed with methanol. Coverslips were mounted using ProLong antifade mounting solution (Invitrogen). The fluorescence dye filipin was used for cytotochemical detection of 3β-hydroxy sterols within membranes of *N. caninum*-infected cells as described previously (36). LysoTracker Red was used to track the distribution of acidic (endocytic) organelles in live cells according to the manufacturer’s instructions (Molecular Probes). Infected HFF expressing a GFP-Rab construct and/or incubated with BODIPY TR C5-ceramide were washed with PBS and fixed with 4% paraformaldehyde (electron microscopy [EM] grade) (Electron Microscopy Sciences, Hatfield, PA) and 0.02% glutaraldehyde (EMS) in PBS for 20 min. Samples were then washed with PBS and mounted using ProLong antifade mounting solution (Invitrogen). Cells were viewed with one of the following: (i) a Nikon Eclipse B800 microscope (Nikon, Melville, NY) equipped with an oil immersion Plan Apo 100× objective (numerical aperture [NA], 1.4), a Spot RT charge-coupled-device (CCD) camera (Diagnostic Instruments, Sterling Heights, MI), and Image-Pro Plus software (Media Cybernetics, Silver Spring, MD), or (ii) a Nikon Eclipse 90i equipped with an oil immersion Plan Apo 100× objective (NA, 1.4) and a Hamamatsu GRCA-ER camera (Hamamatsu Photonics, Hamamatsu, Japan). Optical z-sections with 0.2-μm spacing were acquired using Volocity software (PerkinElmer, Waltham, MA). The images were deconvolved using an iterative restoration algorithm, and the registry was corrected using Volocity software. Photoshop (Adobe) was used to adjust levels and to crop and resize images obtained from both microscopes. The Pearson correlation coefficient (PCC) and the positive product of the differences of the mean (PDM) images were calculated using Volocity software. Thresholds were set automatically using the method of Costes et al. (42).

**Electron microscopy and morphometric analysis.** For transmission EM, BT24 cells infected with *N. caninum* or HFF infected with *T. gondii* for 20 h were fixed in 2.5% glutaraldehyde (EM) in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature and processed as described previously (43) before examination with a Philips CM120 electron microscope (Eindhoven, the Netherlands) under 80 kV. To track the distribution of host endolysosomes, Vero cells infected with *N. caninum* for 30 h were incubated with 0.5 mg/ml of LDL particles adsorbed to 15-nm gold particles as described previously (44) before fixation. To determine the extent of association of host mitochondria with *N. caninum* PV at the ultrastructural level, about 50 images of PV of *N. caninum* in BT24 cells at 20 h postinfection (p.i.) were randomly taken at a magnification of ×15,000 using a grid containing points spaced at 0.1 cm. Morphometry was performed as follows: the surface densities (membrane surface area in μm²/volume in μm³) of each PV (S, PV) and host mitochondrion associated with the PV membrane and mitochondrial membrane images. Combining the S, for each PV and each mitochondrion, the percentage of host mitochondria associated with the PV membrane was obtained by calculating the mean ratios of S,M/S,PV. Data were compiled and analyzed using Microsoft Excel 2007.

**Quantitative analysis with MetaScopics.** We created and used MetaScopics, an image analysis web application developed to provide simple and effective tools for leveraging user input to quantify protein or organelle recruitment, perform morphological analysis, and determine object distances (see illustrations in Fig. S1 in the supplemental material). MetaScopics algorithms operate on a per image basis. After the upload of fluorescent images of infected cells and partitioning into groups (e.g., parasites, time points of infection, cell types, etc.), each algorithm quantifies a specific metric over individual images. This metric is then aggregated over each group of images, and the group aggregations are compared. To quantify host mitochondria, ER, microtubule, lysosome, and multivesicular body (MVB) recruitment around the PV, we used the MetaScopics algorithm “intensity by distance.” This operates via user input (e.g., drawing a boundary around an object) and a shell-pixel counting method (e.g., generating concentric 1 pixel-wide layers surrounding the object). First, the user draws a boundary around an object (see Fig. S1A, PV in red), selects a color of interest (see Fig. S1A, mitochondria in green), and selects a maximum distance (see Fig. S1B, 200 pixels, indicated in white). Then, the MetaScopics algorithm sums the color intensity of each pixel within a layer (i.e., distance of 1 pixel from the PV or previous layer) and determines the percent intensity by calculating the ratio of pixel intensity per layer to the total pixel intensity of all layers (see Fig. S1B, 200 pixels, indicated in white). Each layer corresponds to a given concentric distance from the object boundary in pixels. Figure S1C in the supplemental material shows the percentage of the total intensity of each layer (1-pixel width) surrounding the boundary of the PV in Fig. S1A within a 200-pixel maximum distance (see Fig. S1B). Figure S1D displays the percent intensity of Tom20 staining (mitochondria) versus distance in pixels averaged over many images of *T. gondii* and *N. caninum* PV. A maximum distance of 100 pixels was used, representing approximately 7 μm, a physiologically relevant distance.

A summary statistic, called average distance, for recruitment is created for each image by finding the average distance for the color of interest weighted by pixel intensity from the user input boundary. This statistic is calculated by summing the product of the total number of pixels of a given color at a given distance (e.g., 100 pixels away; 7 μm away) with that distance and dividing by the total color intensity, thus normalizing and ensuring that the quantification is weighted by intensity. In the equation below describing this process, \(P_{\text{intensity}}\) describes the intensity of a pixel and \(P_{\text{distance to boundary}}\) is the distance of that pixel from the user-defined boundary. The summary statistic (intensity-weighted distance) is used to compare images from different conditions using box plots:

\[
\text{Average distance} = \frac{\sum P_{\text{intensity}} \times P_{\text{distance to boundary}}}{\sum P_{\text{intensity}}}
\]

To assess host Golgi compartment proximity to the PV, we used the algorithm “centroid to surface distance” from MetaScopics, which provides another useful metric to determine recruitment. This method is more appropriate than the intensity by distance algorithm for objects that are compact or separated by large distances from the PV as the intensity by distance task is more advantageous for focusing on close interactions with spread organelles or structures. First, the user selects the color of the centroid object (e.g., Golgi apparatus) and the color of the other object (e.g., PV). The algorithm finds the centroid of the object by first finding all pixels with the user selected color (Golgi apparatus). Next, the x and y locations of these pixels are averaged, arriving at the x and y centroid coordinates. Once the centroid is found, all pixels corresponding to the PV are scanned, and the distance from the pixel closest to the centroid is used to define the centroid to surface distance metric. This process is visualized in Fig. S1E and F in the supplemental material. The metric centroid to surface distance provides a good relative metric for recruitment. Because a strongly recruited object may become fragmented or distorted around its recruiter, the centroid provides a heuristic for the
RESULTS

Neospora caninum infects and replicates in bovine and human cells with the same efficiency. Our analyses of the host cell-parasite interaction were conducted in parallel on N. caninum and T. gondii in cultured mammalian cells. These studies were performed on the Nc-Liv isolate of N. caninum collected from tissues of infected dogs (37). Among all the N. caninum isolates, Nc-Liv shows the highest proliferative capacity and parasite burden in both the brain and placenta and therefore has the highest virulence in hosts (45). The behavior of Nc-Liv in vitro was compared with that of the RH strain of T. gondii [referred to here as T. gondii (RH)], which is also the most highly virulent strain for this parasite. Since cattle, and never humans, are important intermediate hosts of N. caninum, we first wanted to examine whether bovine cells may confer an advantage over human cells in supporting the intracellular development of Nc-Liv. The growth rate of Nc-Liv was monitored in bovine cells (bovine nasal turbinate epithelial cells, or BT24 cells) in comparison with human cells (human foreskin fibroblasts, or HFF) by parasite enumeration at 24 h and 48 h postinfection (p.i.). Figure 1A shows no significant difference in parasite numbers between the two cell lines (doubling time of 7.9 ± 1.4 h and 10.2 ± 3.9 h in BT24 cells and HFF, respectively). In addition, we performed plaque-based assays and measured the size of the lysed area in Nc-Liv-infected monolayers of BT24 cells or HFF. Cells were infected and incubated for 6 days to allow for multiple rounds of replication and egress. No statistical difference was observed in either plaque number or size between Nc-Liv grown in BT24 cells and those grown in HFF (Fig. 1B). This suggests that the host cell specificity does not influence the proliferation of Nc-Liv in vitro.

Nc-Liv grows more slowly than T. gondii (RH) in vitro regardless of cell type. We compared the efficiency of Nc-Liv and T. gondii (RH) to infect and lyse cell monolayers (Fig. 1C). Data from plaque assays were normalized with values of plaque number and size during an Nc-Liv infection in BT24 cells or HFF. BT24 cells have previously been used to cultivate T. gondii, and no difference was observed for productive infection of the parasite in BT24 cells and HFF (46). T. gondii (RH) formed significantly more abundant and larger lytic areas than those observed for Nc-Liv, whether inoculated in BT24 cells or HFF. This indicates that Nc-Liv parasites have a lower developmental rate than T. gondii (RH) parasites in cultured cells.

Development of MetaScopics, a new image analysis software for the quantification of host organelle-PV interactions. Using image analysis, we compared the relative ability of N. caninum and T. gondii to attract host cell structures and organelles to their PV. Mammalian cytoskeletal elements and organelles were fluorescently labeled to track their distribution in infected cells. To compare the intensity of the fluorescent signal around the PV of N. caninum and T. gondii and therefore to evaluate the respective competence of these parasites for recruiting host cell structures, we developed a new image analysis program, named MetaScopics. MetaScopics consists of several algorithms for quantitative analysis of microscopy images. Information on computational principles underlying the algorithms of MetaScopics and applications is available in the supplemental material and in Materials and Methods.

One algorithm used in this study, named intensity by distance, measures the percent intensity of a fluorescent marker (associated with an organelle) as a function of the distance of this marker from the perimeter of a PV, which is labeled with a different fluorescent marker. The output of this algorithm allows the comparison of distances between groups of images. Robust PV association of a given marker correlates with smaller distances between the marker and the PV perimeter. This algorithm is best suited for structures that pervade the cell, e.g., the ER, cytoskeleton, mitochondria, lysosomes, or MVB, or that have poorly defined boundaries, e.g., microtubules. In a second algorithm, titled centroid to surface distance, MetaScopics calculates the centroid of a given object (fluorescently labeled organelle) and measures the distance from the centroid to the nearest surface of the PV stained with a different fluorochrome. By tracking distances between objects designated by fluorescence markers, MetaScopics allows the user to derive changes in distance attributable to different experimental conditions. This algorithm is suitable for organelles with well-defined structures or localizations in the cell, e.g., the Golgi apparatus. To validate the centroid to surface distance task, we applied the algorithm to a well-known biological event, the change in morphology of the Golgi apparatus upon treatment with nocodazole. This drug interferes with microtubule polymerization, leading to the destabilization of the microtubular network and changes in organelle positioning in cells. In HFF exposed to 300 nM nocodazole for 90 min, the Golgi apparatus is disrupted, and Golgi fragments are spread in a broad area around the nucleus (47) (see Fig. S2A in the supplemental material). The centroid to surface distance task was used to measure the distance between the centroid of the Golgi apparatus and the nucleus in drug-treated and control cells. MetaScopics analysis measured the average distance of the Golgi apparatus centroid to the nucleus as 3.18 μm in untreated cells and as 0.11 μm in nocodazole-treated cells, in accordance with a perinuclear localization of Golgi fragments induced by the drug (see Fig. S2B).

Like T. gondii (RH), Nc-Liv attracts host mitochondria to its PV and retains these organelles close to the vacuolar membrane. Mitochondria are dynamic organelles involved in a wide range of processes, such as ATP generation, cell death, immune signaling, chemotaxis, and calcium homeostasis (48). These organelles also play a central role in phospholipid metabolism and lipid exchange with the ER (49, 50). Many intracellular pathogens, including T. gondii, have evolved to manipulate the surveillance or bioenergetic pathways of host mitochondria for their benefit (51–54). Rapidly after penetration into the cell, T. gondii (RH) induces a dramatic change in the spatial distribution of host mitochondria. These organelles become concentrated around the PV and closely associate with the vacuolar membrane, with a distance of ~12 nm (55). By 4 h and 24 h p.i., 18 and 23% of the PV membrane, respectively, is covered by host mitochondria.

As T. gondii and N. caninum share several metabolic features,
we determined whether Nc-Liv, similarly to *T. gondii* (RH), co-opts host mitochondria. In infected cells, mitochondria were immunolabeled for Tom20, and their distribution was assessed by fluorescence microscopy at 24 h p.i. A concentric fluorescent signal was observed around the PV of Nc-Liv in both BT24 cells and HFF, resembling the perivacuolar gathering of host mitochondria in *T. gondii* (RH)-infected cells (Fig. 2A). We used MetaScopics to calculate the intensity-weighed distances of host mitochondria to the PV of *T. gondii* (RH). Host mitochondria were statistically closer to the PV of *T. gondii* (RH) than to those of Nc-Liv (Fig. 2B). This indicates that these host organelles are more concentrated around the PV of *T. gondii* (RH) than around those of Nc-Liv, as calculated within an arbitrarily delineated area of 7 μm radiating from the PV. Identical statistical results were obtained by selecting perimeters of 3.5 and 10 μm around the PV boundary (data not shown).

In *T. gondii*, the recruitment of host mitochondria is strain dependent. RH parasites express *T. gondii* mitochondrion association factor 1 (TgMAF1) at the PV membrane, a mediator of PV association with host mitochondria (56). The cystogenic Prugniaud strain of *T. gondii*, however, does not express TgMAF1, and at 12 h p.i. less than 2% of the PV are associated with

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**Fig 1** Growth specificity of Nc-Liv in vitro. (A) Comparison of the replication rate of Nc-Liv in BT24 cells and HFF. Cell monolayers of BT24 cells and HFF seeded in six-well plates were infected with the same-sized inocula of Nc-Liv and then washed after 2 h. Cells were scraped 24 h or 48 h p.i., and the total number of parasites in each well was assessed using a hemocytometer. Data are means ± SD (n = 3 separate assays). (B) Comparison of the growth rate of Nc-Liv in BT24 cells and HFF using plaque assays. Panel a illustrates the representative area of monolayers of BT24 cells or HFF destroyed by the parasite. The mean numbers (b) and areas (c) of the plaques ± SD were calculated from three independent experiments. (C) Comparison of the growth rate of Nc-Liv and *T. gondii* (RH) in BT24 cells and HFF using plaque assays. The mean numbers and areas of the plaques ± SD are expressed as a percentage relative to the control value (infection with Nc-Liv), which was set as 100%, for three independent experiments. Results were statistically significant (*, P < 0.05; **, P < 0.01).

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host mitochondria. We compared the distribution of host mitochondria in HFF infected with Nc-Liv and T. gondii (Prugniaud). Early in infection, no interaction of host mitochondria was observed for either parasite (see Fig. S3 in the supplemental material). In a coinfection assay, the antibody 2H7A was used to identify Nc-Liv (red, PV). An asterisk identifies a coinfect cell. Representative extended-focus images are shown. Arrowheads and arrows pinpoint the PV of T. gondii (RH) and Nc-Liv, respectively. (B) Quantitative comparison of mitochondrion recruitment by Nc-Liv and T. gondii (RH) by MetaScopics analysis. Box plots show the average distances, weighted by intensity, of the host mitochondria to the PV boundary, as calculated for host mitochondrial profiles included in a 7-μm radius of the PV (data from 94 infected HFF at 24 h p.i.). The lines inside the box are the median values; the numbers written under the plot are the mean fluorescence intensities. A comparison between the values for the two parasites is statistically significant (*, P < 0.004). (C) Ultrastructural analysis of the host mitochondrion-PV interaction. EM of Nc-Liv-infected BT24 cells (a to d) and T. gondii (RH)-infected HFF (e and f) showing the distribution of host mitochondria (hm) relative to the PV. a, amylopectin granule. Scale bar, 0.5 μm.

As in T. gondii (RH)-infected cells, many host mitochondria gathered around the N. caninum PV, often in multiple layers (Fig. 2C, panels a and b compared to panel e). Observations at high magnification show a close apposition of the outer membrane of the host mitochondria with the PV membrane of Nc-Liv, with a mean distance of 16 ± 4 nm, as similarly documented for T. gondii (RH) (Fig. 2C, compare panels c and d with panel f). Morphometric analyses were undertaken to quantify the extent of the host mitochondrion-PV membrane association in Nc-Liv-infected BT24 cells and ascertain the representativeness of our microscopic observations. The extent of association is presented as the percentage

FIG 2 Host mitochondria interaction with the PV of Nc-Liv. (A) Immunofluorescence assays (IFA) of host mitochondria in T. gondii (RH)- or Nc-Liv-infected cells. HFF or BT24 cells were infected with T. gondii (RH) and/or Nc-Liv, fixed, and stained with 4',6'-diamidino-2-phenylindole (DAPI; blue, nucleus) and antibodies against Tom20 (green, mitochondria). In a coinfection assay, the antibody 2H7A was used to identify Nc-Liv (red, PV). An asterisk identifies a coinfect cell. Representative extended-focus images are shown. Arrowheads and arrows pinpoint the PV of T. gondii (RH) and Nc-Liv, respectively. (B) Quantitative comparison of mitochondrion recruitment by Nc-Liv and T. gondii (RH) by MetaScopics analysis. Box plots show the average distances, weighted by intensity, of the host mitochondria to the PV boundary, as calculated for host mitochondrial profiles included in a 7-μm radius of the PV (data from 94 infected HFF at 24 h p.i.). The lines inside the box are the median values; the numbers written under the plot are the mean fluorescence intensities. A comparison between the values for the two parasites is statistically significant (*, P < 0.004). (C) Ultrastructural analysis of the host mitochondrion-PV interaction. EM of Nc-Liv-infected BT24 cells (a to d) and T. gondii (RH)-infected HFF (e and f) showing the distribution of host mitochondria (hm) relative to the PV. a, amylopectin granule. Scale bar, 0.5 μm.
of the PV surface in direct physical contact with host mitochondria, as shown in panel d of Fig. 2C. For a 20 h infection, ~8% of the PV membrane was associated with host mitochondria, which is less than that calculated for T. gondii (RH) (23% at 24 h) (56).

While host ER elements are closely apposed to the PV membrane of T. gondii (RH), ER elements gather around the Nc-Liv PV but do not tightly associate with the vacuolar membrane. The distribution of the ER is also modified in T. gondii (RH)-infected cells as ER elements surround the PV and are in intimate association with the PV membrane (55). From 4 h p.i. and onwards, half of the PV membrane was covered by host ER tubules. The perivacuolar ER elements were located within ~20 nm of the vacuolar membrane, and ribosomes were restricted to the opposite face of the ER from the PV.

Inspection of the organization of the host ER in Nc-Liv-infected cells by IFA using anti-KDEL antibodies revealed intense staining around the PV. This perivacuolar fluorescent signal was more pronounced in BT24 cells than in HFF but was similar to that of the T. gondii (RH) PV in fibroblasts (Fig. 3A), suggestive of an interaction between the host ER and the Nc-Liv PV. The spatial

FIG 3 Host ER interaction with the PV of Nc-Liv. (A) IFA of host ER in T. gondii (RH)- or Nc-Liv-infected cells. HFF or BT24 cells were infected with T. gondii (RH) or Nc-Liv, fixed, and stained with 4',6'-diamidino-2-phenylindole (DAPI; blue, nucleus) and antibodies against KDEL (green, ER). Representative extended-focus images are shown. Arrowheads and arrows pinpoint the PV of T. gondii (RH) and Nc-Liv, respectively. (B) MetaScopics analysis of the kinetics of host ER recruitment by Nc-Liv. Box plots show the average distance, weighted by intensity, of host ER to the PV boundary, as calculated for host ER profiles detected within a 7-μm radius of the PV (data from 158 infected HFF at 24 h p.i.). The lines inside the box are the median values; the numbers written under the plot are the mean fluorescence intensities. A comparison between the values for the ER distributions at 8 h and 24 h is statistically significant (*, P < 0.0001). (C) Ultrastructural analysis of host ER-PV interaction. EM of Nc-Liv-infected BT24 cells (a to e) and T. gondii (RH)-infected HFF (f to h) showing the distribution of host ER (hER) relative to the PV. Arrowheads show the distribution of ribosomes on the ER side facing the host cytoplasm and not the T. gondii (RH) PV membrane. Scale bar, 0.5 μm.
distribution of host ER elements relative to the Nc-Liv PV was measured using MetaScopics at different time points of infection. The distance between the host ER and the PV decreased significantly between 8 and 24 h p.i., with no significant decrease observed between 24 h and 48 h (Fig. 3B). This suggests that the host ER recruitment occurs prior to 24 h p.i.

EM observations of Nc-Liv-infected BT24 cells confirm the bundling of the host ER around the PV at 20 h p.i. (Fig. 3C, panel a). Perivacuolar ER elements were particularly enlarged (Fig. 3C, panels b and c), compared to those of the ER in uninfected BT24 cells or in T. gondii (RH)-infected cells (Fig. 3C, panel f). Although the massing of the host ER around the PV was impressive, no physical association of ER elements with the PV membrane could be observed, and ribosomes were also distributed on ER tubules facing the PV membrane (Fig. 3C, panels d and e compared to panels g and h). This suggests either that there is no molecular contact between proteins of the PV of Nc-Liv and host ER or that contacts, if any, are transient.

Host microtubules concentrate around the Nc-Liv PV but not as extensively as with the T. gondii (RH) PV. T. gondii (RH) dramatically modifies the architecture of the host microtubular network as its PV is entirely wrapped by microtubules (32, 57). To determine whether the PV of Nc-Liv is comparably surrounded by host microtubules, we examined the distribution of the host microtubular cytoskeleton in Nc-Liv-infected cells by IFA, using anti-α-tubulin antibodies, and EM. Figure 4A illustrates a concentration of host microtubules around the PV of Nc-Liv in mono-infected cells and in cells coinfected with T. gondii (RH). At the ultrastructural level, bundles of host microtubules closely apposed to the PV membrane could be observed (Fig. 4B). Using MetaScopics, we quantified the distribution of host microtubules around the PV of both parasites and found that host microtubules were more concentrated around the PV of T. gondii (RH) than around that of Nc-Liv in a statistically significant manner (Fig. 4B). To validate the intensity by distance task of MetaScopics, we quantified the concentration of host microtubules around the PV of T. gondii (RH) and Nc-Liv in the presence or absence of the microtubule-depolymerizing agent nocodazole (see Fig. S5 in the supplemental material). Data show a significant decrease in the fluorescent staining for α-tubulin around the T. gondii PV in nocodazole-treated cells in comparison with the fluorescent signal in untreated cells; no significant decrease in the fluorescent staining was observed for the Nc-Liv PV in treated versus untreated cells. This result is in accordance with our data in Fig. 4A, showing a higher concentration of host microtubules around the PV of T. gondii (RH) than around that of Nc-Liv.

Like T. gondii (RH), Nc-Liv displaces the host microtubule-organizing center to its PV. Most animal cells have one microtubule-organizing center (MTOC) during interphase. According to our observations, the MTOC in both HFF and BT24 cells was located on or near the nucleus in 92% and 6% of cells, respectively. T. gondii (RH) hijacks the host MTOC, or centrosome, by detaching this structure from the host nuclear envelope and relocating it to its PV (40). Evidence for a MTOC-PV association in T. gondii (RH)-infected cells is supported by the detection of centrosomal material at the PV surface, including pericentriolar matrix proteins and components of the γ-tubulin ring complex, which are all critical for microtubule nucleation (32, 40, 57, 58).

The recruitment of the host MTOC by Nc-Liv and the MTOC position relative to the PV were investigated by IFA using anti-γ-tubulin antibodies. Occasionally, the host MTOC was present at the PV surface of Nc-Liv (Fig. 4D). Quantitative analysis shows that the host MTOC was (i) associated with ~20% of the PV of Nc-Liv, (ii) equidistant to the host nucleus and the PV in ~25% of infected cells, or (iii) closer to the host nucleus than the PV in half of the infected cells (Fig. 4E). These distributions are similar to those observed during T. gondii (RH) infection. Though Nc-Liv does not recruit microtubules as extensively as T. gondii (RH) (Fig. 4B), this parasite is equally proficient in hijacking the host MTOC, perhaps suggesting that the association of the host MTOC and the wrapping of host microtubules around the PV are not necessarily correlated.

Host endocytic organelles and multivesicular bodies concentrate around the PV of both parasites. Host endocytic organelles represent a rich source of nutrients derived from the extracellular medium for intracellular pathogens. Toxoplasma has developed strategies to attract these organelles while avoiding destruction. The parasite manipulates the host microtubular network to create invaginations of the PV membrane, and through these microtubule-based invaginations, host endolysosomes are retained within the PV (32). The host mammalian target of rapamycin complex 2 (mTORC2)-Akt signaling is then usurped by Toxoplasma to maintain endolysosomes around the PV (58).

We assessed the distribution of host endocytic compartments in Nc-Liv-infected cells using three independent approaches: (i) LysoTracker-containing organelles (Fig. 5A), (ii) endocytic structures loaded with lipoprotein-gold particles (Fig. 5B), and (iii) LAMP1 (lysosome-associated membrane protein 1)-labeled organelles (Fig. 5C, panel a). Findings were concordant from all of these approaches, illustrating an accumulation of endolysosomes around the PV of Nc-Liv. As similarly observed for the T. gondii (RH) PV, the gathering of these organelles at the Nc-Liv PV was maintained throughout parasite replication as 58 to 70% of the LysoTracker-containing structures surrounded the PV at 24 h p.i. EM observations illustrate lipoprotein-containing organelles concentrated at the PV of Nc-Liv. MetaScopics analysis comparing the intensity-weighted distances of host endolysosomes to the PV of Nc-Liv and T. gondii (RH) did not reveal any statistical difference between the two parasites (Fig. 5C, panel b).

Multivesicular bodies (MVB) are temporary storage compartments enriched in sphingolipids and cholesterol at the intersection of the endocytic and exocytic pathways. Nothing is known about the interaction of host MVB with the PV of T. gondii or N. caninum. We used antibodies against the MVB protein marker CD63 to analyze the distribution of MVB in T. gondii (RH)- or Nc-Liv-infected cells (Fig. 5D, panel a). We observed a distribution of CD63-labeled organelles around the PV of each pathogen, and quantification by MetaScopics indicated a statistically higher concentration of MVB around the PV of T. gondii (RH) than around that of Nc-Liv (Fig. 5D, panel b).

Like T. gondii (RH), Nc-Liv contains cholesterol that it scavenges from host endocytic compartments. Cholesterol is the major sterol molecule ubiquitously present in mammalian cells, in which it plays key roles in organizing signaling lipids and proteins within membranes. Mammalian cells obtain cholesterol by internalization of plasma low-density lipoproteins (LDL) or from de novo synthesis in the ER via the mevalonate pathway (59). T. gondii contains cholesterol but cannot synthesize this lipid, which it salvages from host endocytic organelles (36). Toxoplasma recruits...
FIG 4 Host microtubules and MTOC association with the PV of Nc-Liv. (A) IFA of host microtubules in *T. gondii* (RH)- and/or Nc-Liv-infected cells. HFF were infected with *T. gondii* (RH) or Nc-Liv, fixed, and stained with 4',6'-diamidino-2-phenylindole (DAPI; blue, nucleus), and antibodies against α-tubulin (green or red, microtubules) during mono-infection and during a coinfection with anti-GRA7 antibodies to identify *T. gondii* (RH) (red, PV). Representative extended-focus images are shown. Arrowheads and arrows pinpoint the PV of *T. gondii* (RH) and Nc-Liv, respectively. (B) EM of a view of a PV of Nc-Liv in BT24 cells showing host microtubules (hMT) aligned along the PV membrane. Scale bar, 0.5 μm. (C) Quantitative comparison of microtubule recruitment by Nc-Liv and *T. gondii* (RH) by MetaScopics analysis. Box plots show the average distances, weighted by intensity, of the host microtubules to the PV boundary, as calculated for host microtubules in a 7-μm radius of the PV (data from 26 infected HFF at 24 h p.i.). The lines inside the box are the median values; the numbers written under the plot are the mean fluorescence intensities. A comparison between the results for the two parasites is statistically significant (*, P < 0.01). (D) IFA of host MTOC in *T. gondii* (RH)- and/or Nc-Liv-infected cells. BT24 cells or HFF were infected with *T. gondii* (RH) or Nc-Liv, fixed, and stained with DAPI (blue, nucleus), antibodies against γ-tubulin as a marker of the MTOC (green), indicated by yellow arrows during mono-infection, and GRA7 to identify *T. gondii* (RH) (red, PV) in a coinfected cell. Note that the anti-γ-tubulin antibodies also label the MTOC/centrosome of Nc-Liv. Representative extended-focus images are shown. Arrowheads and arrows pinpoint the PV of *T. gondii* (RH) and Nc-Liv, respectively. (E) Quantification of the distribution of the PV of Nc-Liv or *T. gondii* (RH) relative to the host nucleus (hNuc). The distribution of the host MTOC has been classified as follows: on the PV, equidistant to the PV and the host nucleus, and close to the host nucleus. Data, expressed as a percentage of the PV population, are means ± SD of three independent assays for Nc-Liv and means for a representative experiment for *T. gondii* (RH), with a minimum of 150 vacuoles counted in each experiment.
FIG 5 Host endosomal organelle interaction with the PV of Nc-Liv. (A) Live fluorescence microscopy of cells incubated with LysoTracker. HFF or BT24 cells, uninfected or infected with T. gondii (RH) or Nc-Liv, were incubated for 1 h with LysoTracker before observation by live fluorescence microscopy. Arrowheads and arrows pinpoint the PV of T. gondii (RH) and Nc-Liv, respectively. (B) Ultrastructural analysis of Nc-Liv incubated with LDL-labeled organelles. Nc-Liv-infected CHO cells incubated with LDL-gold particles for 24 h show the concentration of host LDL-containing endolysosomes (hE-L) at the PV membrane. Scale bar, 0.5 μm. (C) IFA of host endocytic structures in Nc-Liv-infected HFF is shown in panel a. HFF were infected with Nc-Liv, fixed, and stained with 4',6''-diamidino-2-phenylindole (DAPI, blue, nucleus) and antibodies against LAMP1 (green, late endosomes/lysosomes). Panel b shows a quantitative comparison of host late endosome (LE)-lysosome recruitment by Nc-Liv and T. gondii (RH) by MetaScopics analysis. Box plots show the average distance, weighted by intensity, of LAMP1-positive structures to the PV boundary, as calculated for all of these host organelles within a 7-μm radius of the PV (data from 26 infected cells at 24 h p.i.). (D) Panel a shows an IFA of host multivesicular bodies (MVBs) in T. gondii (RH)- and/or Nc-Liv-infected HFF. HFF were infected with T. gondii (RH) or Nc-Liv, fixed, and stained with DAPI (blue, nucleus) and antibodies against CD63 (green, MVB) during mono-infection and during a coinfection with anti-GRA7 antibodies to identify T. gondii (RH) (red, PV). Arrowheads and arrows pinpoint the PV of T. gondii (RH) and Nc-Liv, respectively. Panel b shows a quantitative comparison of host MVB recruitment by Nc-Liv and T. gondii (RH) by MetaScopics analysis. Box plots show the average distance, weighted by intensity, of MVB to the PV boundary, as calculated for all of the MVB within in a 7-μm radius of the PV (data from 68 infected HFF at 24 h p.i.). The lines inside the box are the median values; the numbers written under the plot are the mean fluorescence intensities. A comparison between the results for the two parasites is statistically significant (*, P < 0.002).
the host ER, but its growth does not rely on ER-synthesized cholesterol.

To assess the presence and distribution of cholesterol in *N. caninum*, we labeled Nc-Liv parasites with filipin, a fluorescent compound that interacts with the 3β-OH group of sterols within membranes (60), and viewed the parasite by microscopy (Fig. 6A). Intracellular Nc-Liv parasites exhibited strong filipin-positive staining (Fig. 6A), as previously observed for *T. gondii* (RH) (36). The parasite’s plasma membrane and apical elongated organelles displayed the most intense labeling. The morphology of the apical organelles is reminiscent of that of rhoptries, organelles that contain cholesterol in *T. gondii* (61, 62).

*Toxoplasma* has the ability to store cholesterol as cholesteryl esters in lipid bodies. To examine whether Nc-Liv has cholesterol stores, we labeled the parasite with Nile Red, a dye that strongly fluoresces in cytoplasmic lipid bodies. Under normal culture conditions (i.e., 10% FBS), *N. caninum* contained an average of 4 lipid bodies detected in *T. gondii* (63). The availability of host LDL has a direct impact on the development of

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**FIG 6** Cholesterol uptake and storage by Nc-Liv. (A) Fluorescence microscopy of Nc-Liv-infected HFF labeled with filipin. HFF were infected with Nc-Liv for 24 h, fixed, and stained with the fluorescent dye filipin for sterols, which shows strong fluorescence associated with the PV (arrow) and the parasite’s plasma membrane and rhoptries (arrowhead). (B) Fluorescence microscopy of Nc-Liv-infected HFF labeled with Nile Red. HFF were infected with Nc-Liv for 24 h, fixed, and stained with Nile Red for lipid bodies (arrowhead). (C) Live fluorescence microscopy of Nc-Liv-infected HFF incubated with exogenous fluorescent cholesterol. HFF infected with Nc-Liv for 24 h (arrows) were incubated with NBD-cholesterol incorporated into lipoproteins ([NBD-C]LP) for 5 to 20 min prior to observation by live fluorescence microscopy, which shows cholesterol on the plasma membrane and then in lipid bodies (arrowheads). (D) Influence of exogenous lipoproteins on Nc-Liv proliferation. Uracl incorporation by Nc-Liv at 18 h or 36 h p.i. in either CHO cells grown in medium containing 10% FBS (CHO ctl), 10% delipidated FBS (LPDS), or 10% LPDS supplemented with 1 mg/ml of LDL (LDL) or in CHO cells with defective NPC1 (NPC1mut). Data are percentages ± SD relative to the control (set as 100%) from four separate experiments done in triplicate. Differences between the results for the control and experimental groups were statistically significant (*, *P* < 0.05). (E) Ultrastructural analysis of Nc-Liv-infected NPC1 mutant cells. EM of Nc-Liv (P) incubated for 36 h in CHO cells lacking functional NPC1. Small PV size with parasite membrane defects and abnormal lipid accumulation in the PV lumen (arrows) were observed. Scale bar, 0.5 μm.
T. gondii (RH). LDL deprivation (e.g., via interference with LDL endocytosis, LDL lysosomal degradation, or cholesterol egress from lysosomes) impairs Toxoplasma growth, whereas the over-abundance of these lipoproteins stimulates parasite replication (36, 64). To determine if Nc-Liv also scavenges cholesterol from host endolysosomes, we incubated infected fibroblasts with fluorescent NBD-cholesterol incorporated into lipoproteins for 5, 10, and 20 min at 37°C and viewed the parasites by live microscopy. We observed an intense labeling of intravacuolar parasites after 5 and 20 min at 37°C and viewed the parasites by live microscopy. From 10 min and onwards, we noticed the appearance of stained cytoplasmic lipid bodies in the parasites, indicative of storage of NBD-cholesterol. Next, we evaluated whether the growth of Nc-Liv was influenced by the availability of exogenous cholesterol. Infected cells were incubated in medium containing FBS (control), lipoprotein-deficient FBS (LPDS), or excess LDL, and parasite replication was measured by radioactive uracil incorporation at 18 h and 36 h p.i. (Fig. 6D). Removal of lipoproteins from the incubation medium resulted in a significant reduction in uracil incorporation into the parasites compared with that in control parasites. The lower levels of uracil incorporation coincided with the presence of PV containing fewer parasites (data not shown). Addition of excess LDL to the culture medium stimulated Nc-Liv replication at 36 h p.i., as previously demonstrated for T. gondii (RH) (36). To determine if Nc-Liv relies on host endocytic organelles for cholesterol acquisition, we monitored parasite growth in NPC1-deficient CHO cells. Mammalian Niemann-Pick type C1 (NPC1) cells display defect in cholesterol mobilization from lysosomes (65). A significant impairment of Nc-Liv growth was observed in NPC1 mutant cells compared to that in CHO wild-type cells, a phenomenon similarly reported for T. gondii (RH) (62). EM studies of parasites infecting NPC1 mutant cells showed abnormal parasite division and accumulation of electron-dense multilamellar structures in the vacuolar space, which is suggestive of lipid disorders (Fig. 6E).

Jointly, these results highlight the dependence of N. caninum on host endocytic structures to access cholesterol and the role of LDL to supply this lipid for the parasite.

N. caninum attracts the host Golgi apparatus to its PV but induces less Golgi fragmentation than T. gondii. The morphology of the host Golgi apparatus is altered in cells infected with T. gondii (RH and Prugniaud strains), as this organelle is fragmented into ministacks that encircle the PV (40). To assess the effect of an Nc-Liv infection on the morphology of the host Golgi apparatus, we inspected the morphology of this organelle by fluorescence microscopy and EM throughout parasite infection in various mammalian cells. In each of the cell types assayed (HeLa cells, HFF, and BT24 cells), the host Golgi apparatus associated with and wrapped around the Nc-Liv PV (Fig. 7A). However, the morphology of this organelle remained more compact in Nc-Liv-infected cells than in T. gondii (RH)-infected cells wherein the host Golgi apparatus was severely dismantled and spread all around the PV (Fig. 7A and B) (40). EM observations illustrate the ultrastructure of Golgi stacks in the vicinity of the PV of Nc-Liv. These stacks were longer and composed of more cisternae than the stacks observed around the T. gondii PV (Fig. 7B). We used MetaScopics to assess the association of the host Golgi apparatus with the Nc-Liv PV. In BT24 cells, the centroid to surface distance of the host Golgi apparatus to the PV was larger when the PV was small and contained few parasites. A similar result was observed in infected HFF cells where the distance from the host Golgi apparatus centroid to the PV decreased progressively from 24 h to 48 h p.i. in a statistically significant manner.

Like T. gondii, N. caninum scavenges exogenously derived ceramides from the host Golgi apparatus. In mammalian cells, ceramides are synthesized in the ER and then transported to the Golgi apparatus for further modifications (e.g., phosphorylation or glycosylation) or conversion to sphingomyelin, diacylglycerol, or sphingosine. Toxoplasma contains several species of sphingolipids (66, 67) and scavenges ceramides and ceramide-derived lipids from the host Golgi apparatus (40, 68). We investigated whether Nc-Liv also diverts ceramides from the host Golgi apparatus by incubating infected cells with NBD-labeled C6-ceramide. Uninfected fibroblasts exposed 15 min to the lipid dye showed fluorescent staining of the Golgi complex (Fig. 8). Correspondingly, Nc-Liv-infected HFF displayed fluorescent labeling of host Golgi apparatus, which surrounds the PV. As the incubation time increased from 30 to 120 min, a gradual staining of the parasites was observed, with a concentration of the fluorescent signal on the parasite’s plasma membrane and Golgi apparatus. In addition, egress Nc-Liv-parasites contained NBD-labeled lipids. These observations indicate that Nc-Liv, like T. gondii, scavenges ceramides or lipids derived from the host Golgi apparatus and incorporates these lipids into its membranes. To ascertain that Nc-Liv is actively involved in the scavenging of host Golgi apparatus-derived sphingolipids, we pretreated infected cells with the antiparasitic drug pyrimethamine, which inhibits dihydrofolate reductase, thus affecting the parasite’s metabolism. Pyrimethamine-treated, infected cells contained small PV with misshapen parasites, and following NBD C6-ceramide incubation, the Nc-Liv PV were barely fluorescent, confirming that an active metabolism of the parasite is required for lipid uptake from the host Golgi apparatus.

Similarly to T. gondii, Nc-Liv intercepts host Rab Golgi complex-derived vesicles to access their sphingolipid content. Ceramides converted into major sphingolipids in the Golgi apparatus are transported to various cell membranes via Golgi apparatus-derived Rab-associated vesicles (69, 70). We previously showed that Toxoplasma (RH and Prugniaud strains) diverts several host Golgi apparatus-derived vesicles to its PV (40). These vesicles contained Golgi apparatus-associated Rab vesicles, including Rab14, which mediates the trafficking between the trans-Golgi network (TGN), endosomes, and the plasma membrane and delivers sphingomyelin to the plasma membrane (71–73), Rab30, which is associated with many compartments of the Golgi complex and is involved in the maintenance of Golgi complex structure and vesicular trafficking (74–77), and Rab43, which is located in the cis-Golgi (78). T. gondii scavenges sphingolipids from Rab30-, and Rab43-associated Golgi vesicles that accumulate within the PV. This process mirrors the uptake of host endocytic structures by T. gondii, which are also delivered intact into the PV lumen (32).

The close association of the Nc-Liv PV with the host Golgi apparatus may also facilitate the scavenging of sphingolipids present in this organelle by the parasite. For this reason, we examined whether Nc-Liv intercepts the host Rab-mediated vesicular trafficking from the Golgi apparatus. We therefore followed the distribution of host Golgi apparatus-derived vesicles by focusing on those marked with Rab14, Rab30, or Rab43. HFF were transfected with each of the three GFP-Rab constructs, infected with Nc-Liv, and observed by fluorescence microscopy (Fig. 9A). In the infected, transfected fibroblasts, numerous GFP-Rab puncta were
concentrated all round the PV. In large PV, the fluorescence signal was particularly intense around the PV membrane. Viewing individual optical z-slices demonstrated that GFP-Rab puncta were localized on top of the PV as well as inside the PV, as illustrated for Rab14 vesicles (Fig. 9B). Quantification indicates that the GFP foci were perivacuolar in 77%, 79%, and 48% of infected cells expressing GFP-Rab14, -Rab30, and -Rab43, respectively. Similarly, GFP foci were observed intraluminally in 29%, 21%, and 2%
of infected cells that express GFP-Rab14, -Rab30, and -Rab43, respectively.

To determine whether GFP-Rab14-associated vesicles located within the Nc-Liv PV contain sphingolipids, we monitored the uptake of BODIPY TR C5-ceramide in infected HFF expressing GFP-Rab14. We compared the respective localization of the GFP to BODIPY signal around and inside the PV by fluorescence microscopy and measured the level of colocalization using the Pearson correlation coefficient (PCC) (Fig. 9B and C). In uninfected cells incubated with BODIPY TR C5-ceramide from 20 to 40 min, the two fluorescent signals partially colocalized, with a PCC of ~0.65 (Fig. 9B). In infected cells, several GFP-Rab14 foci that
Colocalized with red fluorescent puncta were visible at the edge of and within the PV (Fig. 9B). The level of colocalization was greater for GFP-Rab14 foci associated with the PV as the PCC averaged around 0.9 for the GFP and BODIPY TR signals within and on the PV, whereas the PCC was around 0.6 when calculated over the entire cell. These results suggest that the parasite is capable of hijacking selective Golgi apparatus-derived vesicles, and host ceramides are, at least partially, contained in these Rab14-associated vesicles.

**DISCUSSION**

This study demonstrates that *N. caninum* (Nc-Liv) reroutes host cell structures to its PV and salvages the nutrient content of organelles, e.g., lipids. Based on these results, we concluded that this

![Image of Figure 9](https://example.com/figure9.png)
Host Cell Exploitation by Neospora

parasite has evolved strategies similar to those of *T. gondii* (RH strain) for manipulating the host cell and exploiting mammalian resources, which emphasizes the remarkable conservation of these processes between the two pathogens. However, we identified small functional differences between Nc-Liv and *T. gondii* (RH) related to the growth rate and rearrangement of host organelles around the PV, which likely reflect divergent evolutionary paths.

Compared to *T. gondii* (RH), Nc-Liv grows more slowly in cultured cells, regardless of the cell origin. These observations are in accordance with a recent analysis of the transcriptional activities of Nc-Liv which have revealed that after 6 days of adaptation in cultured cells, the parasite shows signs of conversion from the fast-growing tachyzoite to slower-growing prebradyzoite-like forms, as reflected by a downregulation of typical tachyzoite markers (SRS genes including SAG1) and an upregulation of bradyzoite-specific mRNA (BAG1) (2). During bradyzoite differentiation, *T. gondii* shuts down aerobic respiration in favor of glycolysis and gluconeogenesis and accumulates amyllopectin granules (79). Transcriptional profiles of Nc-Liv grown in vitro indicate a redirection of pyruvate from the tricarboxylic acid (TCA) cycle into gluconeogenesis with downregulation of transcription of pyruvate dehydrogenase and upregulation of lactate dehydrogenase, pyruvate carboxylase, and phosphoenolpyruvate (PEP)-carboxylase kinase. Moreover, our EM observations of Nc-Liv illustrate the presence of amyllopectin granules in the parasite’s cytoplasm. Thus, the lower growth rate of Nc-Liv than that of *T. gondii* (RH) could be explained by the bradyzoite-like behavior of Neospora in cultured cells.

Like *T. gondii* (RH), Nc-Liv attracts and retains host mitochondria to its PV. In *T. gondii* (RH), the tropism of host mitochondria to the PV is unknown, but the retention of these organelles at the PV surface seems to be mediated largely by the parasite mitochondrion association factor 1 (TgMAF1) (56). Earlier studies have suggested that the TgROP2 protein may interact with host mitochondria as it is anchored to the PV membrane via hydrophobic and ionic interactions and contains a matrix mitochondrial targeting signal (80, 81). However, a *T. gondii* (RH) mutant strain that lacks TgMAF1 mostly lost its ability to recruit host mitochondria while *T. gondii* (RH) parasites deficient in TgROP2 expression did not, indicating that TgMAF1 is one of the prime factors for this function (82). The molecular mechanism underlying Nc-Liv PV association with host mitochondria remains to be elucidated. An MAF1 gene is present in the genome of *N. caninum* (GenBank accession XP_003886010.1), and *N. caninum* MAF1 (NcMAF1) shares 69% identity with TgMAF1. If NcMAF1 is secreted into the PV, it might be involved in the association of host mitochondria with the PV membrane as TgMAF1 is for *T. gondii* (RH). This would substantiate our observations illustrating that the Nc-Liv PV display a more important coverage with host mitochondria than the PV of *T. gondii* (Prugniaud), a parasite strain lacking TgMAF1 (56). A ROP2 gene is also present in *N. caninum* and is expressed by the parasite (83). NcROP2 shares 47% identity with TgROP2 and possesses features of ROP2 family proteins, including the R-rich amphipathic helix (RAH) domain at the N terminus that mediates the anchorage of ROP2 to the PV membrane. NcROP2, however, does not have the putative mitochondrial matrix targeting signal (SAFRRT) present in TgROP2, disputing its possible role in associating host mitochondria with the PV of Nc-Liv. The biological relevance of the host mitochondrion-PV association is still unknown, but MAF1 appears to play a role in modulating the host immune response as its deletion results in changes in serum cytokines, thus altering conditions to be conducive to parasite dissemination in animals (56). An additional possibility is that the recruitment of mitochondria may prove beneficial to the parasite by providing metabolites, e.g., lipids and lipoate, synthesized in these organelles (84).

The PV of Nc-Liv is enveloped by the host ER but does not physically interact with this organelle. In *T. gondii* (RH), host ER elements are closely apposed to the PV membrane (55). The molecular machinery of this process is still unknown, but two candidates have been proposed. ROP2, which contains an ER-targeting domain exposed to the host cytosol, and GRA3, which interacts with the host ER type II transmembrane protein calcium modulating ligand (CAMLG) (81, 85). A GRA3 gene is present in the genome of *N. caninum* although transcriptional profiles show a strong downregulation of GRA3 in cultured Neospora. Attraction of the host ER to the PV may facilitate the delivery of ER-derived lipids or glucides to *N. caninum* and *T. gondii*. In fact, the growth of *Toxoplasma* is decreased in cells impaired in ceramide production in the ER (40, 86), suggesting an interdependence between parasite infectivity and host sphingolipid metabolism. Also, *T. gondii* synthesizes N-glycans from sugar precursors synthesized in the host ER, implying that it has access to ER molecules (87, 88).

Nc-Liv also has the ability for cross talk with the host microtubular cytoskeleton as its PV is encased by microtubules and is often seen close to the host MTOC to the same extent as observed for *T. gondii* (RH). The role of host microtubules recruited by either parasite needs to be clarified, but it is known that host microtubules are important for *T. gondii* (RH) intracellular development. These cytoskeletal elements are exploited by *Toxoplasma* to facilitate its invasion into a host cell (89); it has been proposed that host microtubules selectively concentrated on one side of the moving junction may help stabilize the site of parasite invasion. After invasion, the PV is positioned at the center of the microtubular network in the host perinuclear region and remains surrounded by host microtubules throughout infection (32, 57). The parasite nucleates host microtubule growth via γ-tubulin-associated sites, which suggests a physical interaction between the PV membrane and host microtubules. EM observations also show invaginations of the PV membrane mediated by host microtubules that serve as conduits to guide host organelles to the PV lumen.

During a *T. gondii* (RH) infection, host centrosome positioning at the PV requires the function of the host mammalian target of rapamycin complex 2 (mTORC2), which activates the Akt signaling pathway (58). In mTORC2-deficient cells infected with the parasite or cells treated with an Akt inhibitor, the host MTOC-PV association is abolished, and the microtubules display an altered distribution. The Akt signaling pathway plays a pivotal role in growth factor regulation of microtubule stability, resulting in the phosphorylation of the glycogen synthase kinase 3β (GSK3β), which is a master regulator of the microtubule cytoskeleton. Moreover, inhibition of GSK3β restores the host centrosome-PV association in infected cells treated with an Akt inhibitor and in mTORC2-deficient cells. Inhibition of GSK3β in untreated cells increases the association of the host MTOC and the PV. It could be interesting to investigate if *N. caninum* also interferes with the host Akt signaling pathway to attract the MTOC or if it operates differently. Controlling MTOC functions may allow these parasites to modulate the host cell cycle by creating centrosomal de-
fects and/or disorganizing mitosis. By stalling host cell division prior to cytokinesis, the parasites may ensure a stable and spacious environment for replication, as offered by a multinucleated cell. To this point, infection of quiescent cells with T. gondii induces an increase in D and E cyclins, thus promoting progression through G1 and transition into S phase, respectively (90). Therefore, in Toxoplasma-infected cells, there is not only an induction of entry into S phase triggered by the parasite but also an arrest in cell cycle progression in the S/G2 transition, based on host cyclin expression levels and accumulation in the infected cells. Additionally, usurping MTOC functions may also permit N. caninum and T. gondii to regulate the movement of host organelles and attract them to their vacuoles. Located at the intersection of the exocytic and endocytic pathways, the MTOC-Golgi complex region of the cell is rich in both endosomes and lysosomes. Therefore, the location of the PV in the peri-Golgi complex/MTOC region of the cell could facilitate the interception of host vesicular trafficking, which may help satisfy the pathogens’ requirements for nutrients.

We previously documented that the attraction and sequestration of host endolysosomal organelles within the PV of T. gondii (RH) may facilitate the delivery of a diverse range of molecules by diverting autophagosomes to its PV (91, 92). Blocking host lysosomal or autolysosomal functions impairs the parasite’s growth under nutrient-limiting conditions. Finally, MVBs may represent a prodigious source of nutrients for T. gondii and N. caninum as reported for Chlamydia trachomatis, which shows growth delay upon inhibition of host MVB biogenesis and disruption of lipid trafficking from MVBs to the bacterium (93, 94).

Like T. gondii (RH), Nc-Liv is auxotrophic for cholesterol and stores this lipid in lipid bodies. To esterify cholesterol, T. gondii expresses two acyl-coenzyme A (CoA):cholesterol acyltransferases (ACAT), TgACAT1 and TgACAT2, that share 56% identity with each other. Under conditions of excess LDL, the parasite takes up cholesterol proportionally to the LDL concentration but controls the massive supply of cholesterol by increasing the activity of TgACAT1 and TgACAT2 to store excess cholesterol. Pharmacological blockade of cholesterol ester synthesis with ACAT inhibitors or genetic ablation of either TgACAT1 or TgACAT2 is highly deleterious for parasite growth (95, 96). The genome of N. caninum contains a single gene homologue to an ACAT enzyme (accession number NCLIV_024240; www.ToxoDB.org). NcACAT shares 71% identity with TgACAT1 and possesses the canonical cholesterol-binding site (HSY), suggesting that Neospora has the capacity to synthesize cholesteryl esters for storage in lipid bodies.

Nc-Liv growth depends on host endocytic structures for a cholesterol supply from LDL. We cannot exclude the possibility that other sources of cholesterol may be exploited by N. caninum as is the case for other Apicomplexa. For example, Plasmodium berghei retrieves cholesterol from the de novo synthetic pathway (97), and Cryptosporidium parvum salvages cholesterol originating from micelles (98). How N. caninum accesses host cholesterol from lysosomes and internalizes this lipid remains to be elucidated. T. gondii expresses a lipid-translocating importer of the ATP-binding cassette (ABC) transporter G subfamily (ABC-G family) at the plasma membrane and in the PV, and this translocator delivers cholesterol to the parasite’s interior (99). A homologue for this Toxoplasma ABCG is present in the N. caninum genome (76% identity; GenBank accession number XP_003881240.1).

Like T. gondii, Nc-Liv scavenges exogenously derived ceramides from the host Golgi apparatus that is reorganized around the PV. The proximity of the PV of T. gondii (RH and Pruginiaud) and Nc-Liv to host Golgi elements may facilitate the retrieval of nutrients, e.g., sphingolipids that are manufactured in this organelle. Toxoplasma is capable of de novo sphingolipid synthesis (100–102), but it relies on the salvage of host sphingolipids from many sources. Indeed, exogenously added ceramides processed in the Golgi apparatus enhance parasite replication, whereas blockade of host ceramide production leads to parasite growth impairment (40). The recruitment of the host Golgi apparatus and its size reduction in Nc-Liv- or T. gondii (RH)-infected cells may facilitate the interception of host Golgi apparatus-derived vesicles by the parasites. Such a scenario has been described for C. trachomatis, which scavenges host Golgi apparatus-derived lipids (103, 104). The bacterium fragments the host Golgi apparatus by co-opting the Golgi apparatus-specific brefeldin A resistance guanine nucleotide exchange factor 1 (GBF1) (105), which is required for the assembly of the Golgi stacks and for vesicle-mediated sphingolipid acquisition by the bacterium. C. trachomatis also cleaves golgins, which are important for the structural organization of the Golgi apparatus, leading to the fragmentation of the Golgi apparatus (106). Interfering with Golgi fragmentation in C. trachomatis-infected cells impairs bacteria growth. The mechanism leading to the dismantling of the host Golgi apparatus during Toxoplasma infection is still unknown, but it does not involve the proteolytic cleavage of the Golgi matrix proteins, golgin-160 and golgin-97 (40). Finally, the interception of host Golgi Rab vesicles by Nc-Liv, followed by the sequestration of these vesicles in the PV to access their sphingolipid content, is also a process shared with T. gondii. Many proteins secreted by these parasites are embedded in the PV membrane and localize thus at the interface between the PV and mammalian cell structures. Identification of specific parasite effectors that interact with host Rab proteins to mediate the docking of Rab vesicles onto the PV membrane would reveal unique molecular players and open new therapeutic approaches to fight against these pathogens.

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