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***Cryptosporidium parvum* scavenges LDL-derived cholesterol and micellar cholesterol internalized into enterocytes**

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Abstract

Cryptosporidium spp. are responsible for devastating diarrhea in immunodeficient individuals. In the intestinal tract, the developmental stages of the parasite are confined to the apical surfaces of epithelial cells. Upon invasion, *Cryptosporidium* incorporates the microvillous membrane of the enterocyte to form the parasitophorous vacuole (PV) and sequesters itself from the host cytoplasm by rearranging the host cytoskeleton. *Cryptosporidium parvum* has minimal anabolic capabilities and relies on transporters and salvage pathways to meet its basic metabolic requirements. The cholesterol salvage pathway is crucial for the development of protozoan parasites. In this study, we have examined the sources of cholesterol from *C. parvum* infecting enterocytes. We illustrated that the intracellular stages of *Cryptosporidium* as well as the oocysts shed by the host, contain cholesterol. Incubation of infected enterocytes in lipoprotein-free medium impairs parasite development and results in substantial decrease in cholesterol content associated with the PV. Among lipoproteins, LDL constitutes an important source of cholesterol for *Cryptosporidium*. Dietary cholesterol incorporated into micelles is internalized into enterocytes by the NPC1L1 transporter. We showed that *C. parvum* also obtains cholesterol from micelles in enterocytes. Pharmacological blockade of NPC1L1 function by ezetimibe or moderate down-regulation of NPC1L1 expression decreases parasite infectivity. These observations indicate that, despite its dual sequestration from the intestinal lumen and the host cytoplasm, *C. parvum* can, in fact, obtain cholesterol both from the gut's lumen and the host cell. This study highlights the evolutionary advantages for epicellular pathogens to access to nutrients from the outside and inside of the host cell.

Introduction

Cryptosporidium spp. are the etiological agents of cryptosporidiosis, a life-threatening diarrheal disease in immunocompromised individuals such as HIV/AIDS patients (Tzipori and Ward, 2002). So far the therapeutic options for cryptosporidiosis are limited, and the *Cryptosporidium* parasites exhibit an intrinsic refractoriness to drugs that are parasitocidal for related apicomplexan organisms, e.g., *Toxoplasma gondii* or *Plasmodium* spp. (Blagburn and Soave, 1997). The basis for this drug resistance may be due to *Cryptosporidium*'s peculiar niche at the brush border of epithelial cells and the uniqueness of the parasite's lifestyle (Tzipori and Ward, 2002; Valigurova *et al.*, 2008). In humans, cryptosporidiosis is caused by *Cryptosporidium hominis* and *C. parvum*. The infection usually begins in the upper small intestine where epithelial cells are invaded by sporozoites, the motile

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extracellular stage of the parasite that is released from infectious oocysts in the gut (Current and Garcia, 1991). Intracellular sporozoites form a parasitophorous vacuole (PV) that remains at the apex of the host enterocyte, occupying an intraepithelial, yet extracytoplasmic location that sequesters the parasite from the intestinal lumen and the host cell's cytoplasm. A sporozoite converts first into a trophozoite form 24 h post-infection (p.i), then each trophozoite undergoes one round of asexual multiplication (merogony) to give rise to 6 to 8 merozoites per PV within 24 h (type I meront). Type I merozoites escape from the PV and invade adjacent epithelial cells, which initiates the auto-infective cycle in the infected host. Type I merozoites form type II meronts containing four type II merozoites per PV 48 h p.i. Once liberated, some type II merozoites can undergo sexual multiplication (gametogony) producing male microgamonts and female macrogamonts. Upon fusion of the gamonts, the formed zygote is protected by a resistant oocyst wall. In the infected host, oocysts sporulate to produce *in fine* four infective sporozoites and they are passed in the feces and into the environment (Thompson *et al.*, 2005).

Many aspects of the nature of *Cryptosporidium* interactions with epithelial cells remain unclear. The main site of contact between the maturing parasite and the host epithelial cell seems restricted to an extensively folded membrane structure from parasite and host origin, called the feeder organelle (Valigurova *et al.*, 2007; Fig. S1). *Cryptosporidium* crucially depends on host resources since it has lost the ability to synthesize most basic metabolites, e.g., amino acids, nucleotides, and lipids. Several studies have demonstrated that this parasite relies on an extensive collection of transporters and redundant salvage pathways to meet its anabolic needs (Thompson *et al.*, 2005; Mauzy *et al.*, 2012). Indeed, the genome of *C. parvum* contains not less than 80 genes with strong similarity to known transporters and one hundred genes with transporter-like properties. These transporters constitute ideal therapeutic targets to interfere with the intracellular development of *Cryptosporidium* through nutrient starvation.

The epithelial cells of the small intestinal or enterocytes are highly specialized in the intake, transport and secretion of molecules coming from the intestinal lumen. Obviously, the high nutrient environment offered by enterocytes may represent a privileged habitat for the extreme parasite *Cryptosporidium*. Among the metabolic activities of enterocytes, the maintenance of cholesterol homeostasis is a key function. These epithelial cells are actively involved in the: i) uptake of dietary cholesterol present in micelles via the Niemann-Pick C-1 like-1 (NPC1L1) protein located at the apical surface; ii) cholesterol synthesis from acetyl-CoA via the mevalonate pathway (key-enzyme: hydroxymethylglutaryl-CoA (HMG-CoA) reductase); iii) LDL endocytosis from the plasma mediated by a process of receptor-mediated endocytosis; iv) high-density lipoprotein (HDL) internalization via the scavenger receptor class-B type I (SR-BI); v) cholesterol esterification via acyl-CoA:cholesterol acyltransferase (ACAT) and export through secretion of chylomicrons; and vi) efflux of cholesterol through ATP-binding cassette (ABC) ABCG5/8 transporters (Stange and Dietschy, 1983; Sviridov *et al.*, 2003; Turley and Dietschy, 2003). Due to its unique properties in lipid turnover and abundance, the small intestine is colonized by many pathogens that are unable to synthesize most of their lipids and need lipids to replicate. Among enteric pathogens, the parasites *Entamoeba* and *Giardia* have evolved to take advantage of the sterol-rich environment of the intestinal mucosa as they are equipped to scavenge cholesterol from both micelles and lipoproteins (Das *et al.*, 2002).

Almost nothing is known about the sterol metabolism of *Cryptosporidium* or the potential need of this lipid for the parasite. A previous study reported the role of host membrane cholesterol for *C. parvum* invasion. Upon entry, the parasite induces the clustering of the host microdomains that are rich in cholesterol and sphingolipids to facilitate its attachment to epithelial cells (Nelson *et al.*, 2006). This process is important as it further triggers host

actin remodeling at infection sites, which allows the parasite to rest on a bed of host actin filaments at the apex of the cells. Two oxysterol-binding-protein-related proteins (ORP) have been partially characterized in *C. parvum*, and one of them is localized to the PV membrane (Zeng and Zhu, 2006). In mammalian cells, this class of proteins triggers cholesterol release from cells and catabolism (Costet *et al.*, 2003). However, in contrast to mammalian ORP, the ORP homologues in *C. parvum* show no binding affinity for cholesterol, which indicates that they are likely not involved in cholesterol-sensing in the parasite. Our extensive search in the genome of *C. parvum* (Abrahamsen *et al.*, 2004) could not retrieve any homologues for sterol biosynthetic enzymes, proteins of the reverse cholesterol transport such as SR-BI or lecithin:cholesterol acyltransferase (LCAT; Tall *et al.*, 2002), or the soluble sterol carrier protein-2 (SCP-2) that shuttles sterols within cells (Prinz, 2007). Nevertheless, the parasite genome contains clear homologues for sterol-related transporters such as NPC1 and ABCG transporters, as well as for acyltransferases that esterify neutral lipids (e.g., sterols or triacylglycerols) for storage in cytoplasmic lipid bodies (Chang *et al.*, 1997), suggesting the existence of rudimentary sterol homeostatic pathways operational in *C. parvum*.

As any eukaryotic cell, *Cryptosporidium* must contain cholesterol in its membranes. Cholesterol is an important multifunctional lipid involved in membrane organization and activities. In this study, we examined whether the PV of *C. parvum* is accessible to host cholesterol. We have analyzed the sites of sterol accumulation in *C. parvum*-infected epithelial cells, and explored the exogenous and endogenous sources of host cholesterol, potentially exploited by the parasite. We demonstrated that *Cryptosporidium* developing in enterocytes contains significant amounts of cholesterol. The parasite critically relies on host cholesterol for normal development since removal of cholesterol from the medium, and to a lesser extent from host cell intracellular pools, arrests its reproduction. This investigation illustrates the aptitude of *C. parvum* to intersect more than one cholesterol trafficking pathway in enterocytes to satisfy its needs, which allows the parasite to circumvent the blockade of one of the salvage pathways. Interfering with sterol scavenging pathways by pathogenic microorganisms has proven to be very powerful in combating the infections they cause. With respect to preventing *Cryptosporidium* infections, evaluation of the different steps in host cholesterol uptake by the parasite as effective points of attack would yield novel approaches to halt parasite dissemination in the intestine.

Results

Throughout its lifecycle, *Cryptosporidium parvum* contains sterols

No pathway for sterol synthesis can be identified in the *Cryptosporidium* genome. As a first approach to assess the presence of sterols in *Cryptosporidium* infecting enterocytes, we have exploited the properties of filipin, a fluorescent dye that selectively binds and detects sterols within membranes (Volpon and Lancelin, 2000). Caco-2 cells were infected for 24 h with *C. parvum* sporozoites prior to filipin staining and examination by fluorescence microscopy (Fig. 1A). As a positive control for the filipin labeling, we labeled the related apicomplexan parasite *Toxoplasma gondii* with filipin. *T. gondii* scavenges cholesterol from plasma LDL and inserts this lipid into its membranes (Coppens *et al.*, 2000). An intensive fluorescence signal was observed on the sites of *C. parvum* infections, similar to the staining associated with intracellular *Toxoplasma* (values of filipin intensity in A.U.: 0.76 ± 0.23 and 0.65 ± 0.17 for *C. parvum* and *T. gondii*). At 24 h post-infection (p.i.), *C. parvum* has converted into a circular, uninucleated trophozoite, the transitional stage from sporozoite to merozoite. At the trophozoite stage, it is difficult to discriminate between a filipin staining on the host plasma membrane, the PV membrane and the parasite plasma membrane due to close contact between these membranes. In addition, during invasion, *C. parvum* wraps itself in the host microvillous membranes (that contain cholesterol) that will become the outer

membrane of the PV. In order to verify that filipin stains *C. parvum* membranes but not the PV membrane, we infected Caco-2 cells with the parasites for 48 h, allowing them to multiply and form nucleate meronts containing 6 to 8 individualized merozoites per PV prior to filipin labeling (Fig. 1B–C). Results show a clear staining on the plasma membrane of each merozoite and intraparasitic membranes. In parallel, the parasite membrane of the parasite was immunostained for the two surface glycoproteins GP40 and GP900 (Cevallos *et al.*, 2000) and a comparable pattern demarking the parasite surface could be identifiable with filipin and anti-GP40/900 antibodies, confirming the incorporation of sterols into the parasite plasma membrane. A bright fluorescent spot was also visible at the site of attachment of the trophozoite to the host cell (Fig. 1D).

We also examined the sterol content in oocysts of *C. parvum* (Fig. 1E). Oocysts are impermeable, with a cyst wall made of a filamentous outer layer that is carbohydrate-rich. No filipin signal was detected on the oocyst. However, oocysts that had been treated with Na hypochlorite and Na taurocholate to induce sporozoite excystation and expose their interior, a positive filipin staining was observed on the cytoplasmic mass of sporozoites. Egressed sporozoites were also labeled (value of filipin intensity in A.U.: 0.45 ± 0.15). We confirmed that the UV fluorescence associated with oocytes and sporozoites was due to filipin-sterol complexes and not originating from remnants of excystation enhancers: no fluorescent signal on oocytes and sporozoites was detected in the absence of filipin (Fig. S2).

Altogether, these observations point out that the intestinal forms of *C. parvum* are able to divert exogenous sterols and insert these lipids into their membranes. This indicates that the parasite has developed mechanisms to intercept cholesterol from lipid pathways in enterocytes. The persistence of cholesterol in the extracellular forms of *C. parvum* suggests an important role for this lipid throughout the parasite lifecycle.

***Cryptosporidium parvum* is auxotrophic for plasma LDL and diverts cholesterol from these lipoproteins**

We next wanted to examine the host cell source/s of sterols for intracellular *Cryptosporidium*. Fig. 2A summarizes the different cholesterol trafficking pathways, sources and destinations of cholesterol in enterocytes, as detailed in the Introduction section. Among the transporters that deliver cholesterol to enterocytes, apolipoproteins from the plasma and micelles from the intestinal lumen represent potential sources of cholesterol for *C. parvum* (Fig. 2B). In addition, the parasite may exploit cholesterol that is synthesized *de novo* within its host cell.

We first explored whether *C. parvum* accesses cholesterol associated with plasma apolipoproteins. We examined the contribution of LDL and HDL to support *C. parvum* infection. *C. parvum*-infected Caco-2 cells were incubated in the presence of various amounts of lipoproteins in the medium and the parasite development was monitored for 36 h (Fig. 3A). During that period of time, the parasite is undergoing merogony to generate between 6 to 8 type I merozoites per PV. *C. parvum* infectivity was assessed by enumeration of the PV that contain merozoites (normal development) and PV that contain a single parasite at the trophozoite (delayed development). Parasites were identified after immunolabeling with anti-GP40/GP900 antibodies. Incubation of infected cells with 10% FCS in the medium (control condition containing 0.1 and 0.3 mg/ml of LDL and HDL, respectively) resulted in the formation of ~95% of large PV containing dividing merozoites. Removal of total lipoproteins from the culture medium significantly impaired parasite growth as ~97% of the PV still contained undivided trophozoites and the PV were 3-times smaller than those developing in complete serum.

We next wanted to determine the nature of the lipoproteins, e.g., LDL or HDL internalized into enterocytes that influence the parasite's replication. Infected cells were incubated in medium containing excess LDL (1 mg/ml of LDL added to lipoprotein-deficient serum; LPDS) prior to PV observation. LDL alone restored parasite growth close to normal levels as merozoites were visible in ~90% of the PV. The related parasite *T. gondii* multiplies faster in medium supplemented with excess LDL (Coppens *et al.*, 2000). In contrast, we never observed a stimulation of *Cryptosporidium* growth upon excess LDL (not shown). In parallel experiments, we added HDL in excess (2 mg/ml of HDL in LPDS) to the culture medium prior to monitoring *C. parvum* growth. Results did not show any beneficial effect of HDL on the parasite development since less than 5% of PV contained merozoites.

We then wanted to examine whether there is any correlation between the effect of plasma lipoproteins on *C. parvum* replication and cholesterol content in parasites. Caco-2 cells were infected with *C. parvum* for 36 h in the same culture conditions as described above prior to filipin staining and quantification of fluorescence levels (Fig. 3B). Microscopic observations confirmed the growth defect of *C. parvum* in 10% LPDS alone or with added HDL. A weaker fluorescence signal was detected on these parasites with a ~70–80% decrease in the filipin intensity levels compared to the staining on parasites cultivated in complete serum or medium with excess LDL. No increase in the intensity of the fluorescence signal was observed on parasites exposed to excess LDL.

Finally, to directly demonstrate that *C. parvum* retrieves cholesterol from LDL, we incubated infected cells with NBD-cholesterol incorporated into LDL for 2 h and observed the parasites by live fluorescence microscopy (Fig. 4A). Data show that at 24 h and 48 h p.i., the trophozoites and merozoites were brightly fluorescent, indicating that they have incorporated cholesterol derived from LDL into their membranes. When NBD-cholesterol-LDL was added to the medium at late stage of infection (60 h p.i.), a same fluorescence staining was visible on egressing merozoites, indicating that the parasites are incorporating LDL-cholesterol all over its intracellular development in enterocytes. On the contrary, upon incubation of infected cells with NBD-cholesterol inserted into HDL, neither trophozoites (Fig. 4B) or nor merozoites (not shown) were fluorescently labeled, confirming the absence of utilization of HDL by *C. parvum*.

Altogether, these data establish that *C. parvum* requires host plasma LDL for optimal development in enterocytes, likely to benefit from the lipid content of these specific lipoproteins. The parasite is capable of diverting cholesterol from LDL and incorporating this lipid into its membranes.

Excess LDL stimulate lipid body biogenesis in *Cryptosporidium*

We next explored whether *C. parvum* has the ability to store cholesterol, especially when supplied with excess from LDL. In eukaryotic cells, the storage structures for neutral lipids, e.g. sterols and triglycerides, are cytoplasmic lipid bodies (Murphy, 2012). When *C. parvum*-infected Caco-2 cells were incubated in the presence of 10% FCS for 24 h, then stained with the fluorescent lipid body dye Nile Red, a positive fluorescence staining was observed within the parasites (Fig. 5A). The diameter of the parasite's Nile red-labeled structures was ~0.1 μ m. These observations reveal that *C. parvum* does accumulate neutral lipids. Interestingly, when *C. parvum*-infected Caco-2 cells are exposed to excess LDL in the culture medium during 24 h prior to Nile Red staining, larger, well-defined fluorescent structures were observed in the parasite's cytoplasm (Fig. 5A). These structures resemble typical lipid bodies, except that they have heterogeneous shapes, e.g., spherical, oval, or elongated. About 95% of the parasite population contained 1 to 3 lipid bodies with a mean diameter of 0.2 μ m.

To confirm the nature of Nile Red-stained structures in *C. parvum* upon addition of excess LDL, we performed EM studies on infected cells 24 h p.i. Fig. 5B shows several trophozoites attached to apical membrane of an enterocyte via the feeder organelle. Homogenous electron-dense structures without membranous profiles were evident in these parasites, and were morphologically similar to mammalian lipid bodies. In eukaryotic cells, ER is the site of lipid syntheses and lipid body formation, and ER is often seen associated with lipid bodies. Examination at higher magnification illustrated a physical contact of the lipid bodies of *C. parvum* with ER elements with areas of clear attachment (Fig. 5C). Interestingly, more elaborate membranous structures were visible at the periphery of the lipid bodies, and may correspond to enlarged tubules emanating from the ER (Fig. 5D). Finally, about 20% of parasites exhibited enormous lipid bodies with a mean diameter of $0.38 \pm 0.05 \mu\text{m}$. These enormous lipid bodies occupied a vast portion of the parasite cytosol and were even protruding from the parasite (Fig. 5E–F).

These data suggest that *C. parvum* is equipped to store lipids, e.g. cholesterol derived from LDL within lipid bodies in the cytoplasm.

Blockade of *de novo* cholesterol synthesis in enterocytes slightly reduces *Cryptosporidium* growth

We next explore the potential involvement of the cholesterol biosynthetic pathway on parasite development. Infected monolayers were incubated in the presence of pharmacologic inhibitors of the mevalonate pathway. We first examined the effect of lovastatin that blocks the activity of HMG-CoA reductase, the major regulatory enzyme of the mevalonate pathway (McKenney, 1988), on *C. parvum*-infected Caco-2 cells (Fig. 6A). Cells were pretreated with 10 μM lovastatin for 24 h, then infected for 36 h in the presence of the drug. *C. parvum* showed a reduction in multiplication rate upon lovastatin treatment as only one third of the PV contained merozoites and PV were in the average 3-times smaller than PV in untreated conditions (Fig. 6A). Examinations of parasite morphology and sterol content confirmed growth defects and showed reduced filipin staining by ~50%. However, lovastatin blocks the synthesis of mevalonate, and therefore not only the production of cholesterol but also that of several nonsterol isoprenoids that are critical for the growth and proliferation of eukaryotic cells. Lovastatin may interfere with parasite replication as a result of the depletion of diverse mevalonate-derived molecules essential for *C. parvum* viability. We then probed the effect of zaragozic acid (or squalenstatin), a competitive inhibitor of squalene synthase, the first enzyme of the mevalonate pathway that determines the switch toward sterol biosynthesis (Bergstrom *et al.*, 1995), on parasite development. Fig. 6A shows a slight growth reduction of *C. parvum* upon zaragozic acid exposure as the majority of the PV (~88%) contained merozoites, compared to untreated parasites. Intensity of filipin staining on zaragozic acid-treated parasites showed reduced levels by ~20% compared to control levels (Fig. 6B). The growth inhibitory effect of zaragozic acid on parasite was further confirmed by ELISA (Fig. 6C). Compared to untreated parasites, a growth delay of ~25% was observed for parasites incubated with at 15 μM of drug. However, increasing the drug concentrations up to 60 μM did not result in decrease in *C. parvum* replication.

These data suggest a modest contribution of cholesterol produced by the mevalonate pathway to *C. parvum* growth and sterol supply for the parasite, compared to the source of cholesterol from LDL.

***Cryptosporidium parvum* acquires cholesterol from dietary micelles**

The apical location of the PV of *C. parvum* in enterocytes may be advantageous for the parasite to take up nutrients from the gut. Cholesterol originating from diet and incorporated into micelles could be an alternative source of cholesterol for intraintestinal *C. parvum*. We

probed the capacity of the parasite to divert dietary cholesterol by incubating *C. parvum*-infected Caco-2 cells with a micellar solution containing NBD-cholesterol for 2 h prior to observations by live microscopy 24 h p.i. (Fig. 7A, panels a and b). Data illustrate a positive fluorescent staining associated with intracellular parasites, indicating that *C. parvum* has access to cholesterol present in micelles. We next examined the influence of micellar cholesterol on parasite growth. Intracellular parasites were cultivated for 36 h in medium devoid of lipoproteins and containing micellar preparations of cholesterol (Fig. 7B). Under these conditions, ~95% of the PV underwent merogony, in sharp contrast to PV maintained in LPDS alone. No significant difference in PV size and content was observed with PV incubated in complete serum.

Dietary cholesterol is internalized into enterocytes after binding to the NPC1L1 transporter situated at the apical membrane. In order to clarify whether *Cryptosporidium* exploits the function of host NPC1L1 to acquire micellar cholesterol, we used the pharmacological compound ezetimibe. Ezetimibe binds to NPC1L1, which results in the blockade of NPC1L1 endocytosis into clathrin-coated vesicles, thereby the internalization of cholesterol into enterocytes (Ge *et al.*, 2008; Wang *et al.*, 2009). Caco-2 cells were infected with *C. parvum* for 24 h in the presence of ezetimibe at the concentrations from 25 to 100 μ M, known to block NPC1L1 endocytosis, prior to monitoring parasite replication by ELISA (Fig. 8A). Results indicate a significant decrease in parasite growth upon drug treatment that was proportional to the ezetimibe concentrations with a maximal growth reduction of ~65% with 100 μ M ezetimibe. We confirmed these data by microscopic observations showing dramatic alterations in PV shape and size with increasing ezetimibe concentrations (Fig. 8B). At 100 μ M ezetimibe, all the PV were arrested at the trophozoite stage.

To directly assess whether NPC1L1 facilitates the delivery of dietary cholesterol to the PV, we evaluated the effects of RNA interference (RNAi)-mediated NPC1L1 knockdown on Caco-2 cells. The RNAi experimental approach resulted in a reduction by ~30% in NPC1L1 protein expression in the cells after 2 days, as evaluated by Western blot using anti-NPC1L1 antibodies (Fig. 8C, panels a and b). Two days after transfection, cells were infected and maintained in a medium lacking plasma lipoproteins and supplemented with micellar cholesterol. Thirty-six hours p.i., cells were fixed to visualize the PV morphology and content. Results show a ~2-fold decrease in meront size and number in NPC1L1 knockdown cells compared to PV from control cells that have been transfected with non-targeting control siRNA or and that have been not silenced, which confirms our observations with ezetimibe.

These data indicate that *C. parvum* has the ability to divert micellar cholesterol internalized into the enterocytes via NPC1L1 and that this cholesterol transporter facilitates this process. Thus, dietary cholesterol contributes to some extent to parasite development.

Discussion

Many pathogens are auxotrophic for sterols and need to obtain these lipids from their hosts to maintain the structural and functional integrity of their organelles and membranes, and *in fine* to produce viable progeny (summarized in Wenk, 2006). Among them, apicomplexan parasites cannot synthesize any sterols and scavenges host cholesterol in order to establish their infectious cycle. For example, *Toxoplasma* retrieves cholesterol most exclusively from plasma LDL, and stops dividing in the absence of these lipoproteins in the medium (Coppens *et al.*, 2000). *Plasmodium* spp. are more versatile as they are apt to salvage cholesterol from plasma LDL and HDL, as well from the *de novo* synthetic pathway, according to the parasite stage (Grellier *et al.*, 1991; Labaied *et al.*, 2011). *Eimeria bovis* also seems to scavenge cholesterol, based on observations that several proteins involved in

cholesterol metabolism are up-regulated in its host endothelial cell, e.g., squalene oxidase, INSIG1 and SCAP (Taubert *et al.*, 2010). In this study, we have investigated the source/s of host cholesterol for *Cryptosporidium parvum* that multiplies in intestinal epithelial cells. Enterocytes are characterized by a high rate of *de novo* sterol synthesis and a massive exogenous delivery of cholesterol from the blood and from the intestine lumen, making these cells central stations for cholesterol turnover. We demonstrated that *C. parvum* has access to three sources of cholesterol: the parasite scavenges the needed cholesterol from plasma LDL and micelles, and to a lesser extent from the enterocytic cholesterol biosynthetic pathway. Once internalized, cholesterol remains associated to the parasite until the oocyst stage.

During enterocyte invasion, *Cryptosporidium* spp. encase themselves into a membranous fold of the host plasma membrane and remain at the apical region of the cell. The vacuole is separated from the host cytoplasm by a highly folded membraneous structure referred to as the feeder organelle, an electron-dense desmosome-like boundary of unknown composition and a specialized concentration of host actin filaments (Fig. S1). The unique epicellular location of the PV may confer an advantage to the parasite of having access to nutrients situated both outside and inside of the host cell. The genomes of *C. parvum* and *C. hominis* exhibit compaction, and both species appear to have limited biosynthetic capabilities (Abrahamsen *et al.*, 2004; Xu *et al.*, 2004). This implies that the survival of these pathogens must depend on their abilities to exploit as many resources as possible of the host, e.g., nutrients and energy to meet their basic metabolic requirements. Hence, many redundant salvage pathways have been identified in *Cryptosporidium* (summarized in Thompson *et al.*, 2005). To this point, the intestinal epithelium that abounds in nutrients represents a suitable environment for a parasite that is auxotrophic for so many essential metabolites. Moreover, a previous study reported the predilection of *C. parvum* for infecting dividing enterocytes that contain more metabolites than stationary cells (Widmer *et al.*, 2006).

The small intestine is a major site of cholesterol biosynthesis and lipoprotein degradation. Within this organ, *Cryptosporidium* primarily colonizes the jejunum and the ileum that are rich in bile salts and dietary cholesterol (Field *et al.*, 1990; Thomson *et al.*, 1993). Micelles are made of monoglycerides and fatty acids that associate with bile salts and phospholipids. If provided by the diet, cholesterol is incorporated into micelles. Micelles are absorbed at the anterior pole of enterocytes, and these cells internalize dietary cholesterol via the NPC1L1 transporter. The knockdown of the *NPC1L1* gene in Caco-2 cells causes a markedly decreased ability of the enterocytes to capture micellar cholesterol (Sane *et al.*, 2006). The pharmacological drug ezetimibe selectively binds to NPC1L1, leading to the reduction in cholesterol absorption by enterocytes (Ge *et al.*, 2008). Located at the apex of enterocytes, *Cryptosporidium* is well positioned to intercept the traffic of lipids transferred to enterocytes by micelles. Obviously, the growth of *Cryptosporidium* is affected in enterocytes with reduced level of NPC1L1 expression or in ezetimibe-treated enterocytes, suggesting that NPC1L1 activity, thus micellar cholesterol uptake, contributes to the parasite growth.

The PV membrane of *Cryptosporidium* originates from the host plasma membrane, and is later on extensively modified by the parasite. It is conceivable that the PV membrane may have retained some absorptive properties of the plasma membrane of enterocytes. NPC1L1 internalization is mediated by vesicular endocytosis in enterocytes. NPC1L1 has been detected in intracellular compartments including endosomes, lysosomes and mitochondria, suggesting that NPC1L1 may also act intracellularly, mediating the movement of cholesterol from plasma membrane to organelles (Hui and Howles, 2005; Skov *et al.*, 2011). How can *C. parvum* acquire micellar cholesterol? The parasite may either engulf host NPC1L1-containing organelles inside the PV, as similarly described for *Toxoplasma* that sequesters host endocytic vesicles in the PV lumen (Coppens *et al.*, 2006). Alternatively, the parasite

can express transporters/translocators for cholesterol on the PV membrane which can desorb cholesterol from NPC1L1. In support to this idea, it is known that *C. parvum* expresses at the PV membrane an oxysterol-binding-protein-related protein, a fatty acyl-CoA binding protein and a long-chain fatty acid elongase, all possibly involved in lipid translocation across the vacuolar membrane (Zeng and Zhu, 2006; Zeng *et al.*, 2006; Fritzler *et al.*, 2007).

After invasion, the portion of the PV membrane that is in contact with the enterocyte cytoplasm loses its structural integrity and fuses with the parasite plasma membrane to form the feeder organelle (Fig. S1). The function of this structure is still unknown. The feeder organelle has been thought to form a tunnel connecting the cytoplasm of the parasite and of the host cell (Lumb *et al.*, 1988). So far no experimental evidence has demonstrated a trafficking of molecules between the PV and the host cell. However, if true, it is tempting to propose that *Cryptosporidium* may use this feeding tube to suck out the host cell content to acquire nutrients. In support to this hypothesis, our morphological observations illustrate an intense filipin staining on the feeder organelle, suggesting that this structure may be a passageway of cholesterol from the host cell to the parasite. If true, this form of ‘cellular vampirism’ can be viewed as a remarkable adaptation for a pathogen whose the PV stands away from the host cytoplasm and organelles.

In addition to the diversion of cholesterol packaged into micelles, *C. parvum* can utilize cholesterol internalized by plasma LDL, and more modestly cholesterol synthesized in the host cell. The process of LDL-cholesterol delivery to the parasite, either vesicular from endocytic compartments or molecular from cholesterol carrier, is unknown but it may also involve the feeder organelle as a portal to reach the parasite’s niche. The enteric pathogen *Giardia lamblia* is unable to synthesize cholesterol (Jarroll *et al.*, 1981). This extracellular parasite obtains its cholesterol from the small intestine. Cholesterol deprivation induces the growth arrest and encystation of *Giardia* (Lujan *et al.*, 1996). Like *Giardia*, *C. parvum* incubated in medium devoid of LDL slows down its growth but does not die. A possible explanation may be due to the ability of *C. parvum* to retrieve cholesterol from many different pathways that are operational in enterocytes. Interestingly, *Plasmodium* developing in hepatocytes, other professional cholesterol synthesizer and recycler cells, has also the capacity to scavenge cholesterol from several sources (Labaied *et al.*, 2011). This suggests that both *Cryptosporidium* and *Plasmodium* are superbly adapted to exploit the opulent environment of their respective host cells in order to support their fast growth. In *C. parvum*, the transporters implicated in the cholesterol salvage pathways may differ depending on the sources of the lipid. This possibility would be not surprising since redundant salvage pathways has already been described for amino acids and sugars, for which eleven and nine putative transporters respectively, have been identified in the genome of *C. parvum* (Thompson *et al.*, 2005).

Profusion of LDL in the culture medium leads to the formation of very large lipid bodies in the cytoplasm of *C. parvum*, similarly described for *Toxoplasma* (Nishikawa *et al.*, 2005). The reliance of *C. parvum* on LDL suggests that LDL-cholesterol is probably one of the lipids accumulated into the parasite lipidic inclusions. *T. gondii* can esterify cholesterol for subsequent storage in lipid bodies using two acyl-CoA:cholesterol acyltransferases (ACAT; Nishikawa *et al.*, 2005 and our unpublished data). In both *C. parvum* and *T. gondii*, the number and size of lipid bodies correlate with the amounts of LDL in the medium. The storage of cholesterol within enlarged cytoplasmic inclusions may represent a protective mechanism against cholesterol overloading and membrane damages, developed by these parasites. This function seems crucial for *T. gondii* since genetic ablation of each individual ACAT results in growth impairment whereas dual ablation of the two ACAT is not tolerated by the parasite. Although it remains to be clarified whether *C. parvum* is capable to synthesize cholesteryl esters, our blast search using the sequence of *T. gondii* ACAT

retrieves two membrane-bound *O*-acyltransferases from the *Cryptosporidium* genome (NCBI #: XP_626337.1 and XP_668402.1). It would then be interesting to test the potential vulnerability of *C. parvum* towards ACAT inhibitors to evaluate at first the importance of cholesterol storage pathways for this parasite.

Proliferation at the apex of a host cell holds many evolutionary advantages for microbes (Dumenil, 2011). First, lurked in its host, the pathogen can escape the immune assaults. Second, the pathogen extensively remodels the host plasma membrane around its site of replication. In case of *Cryptosporidium*, the PV is embedded in many membranous folds. In this scenario, the developmental site of the pathogen is consolidated and more resistant to mechanical stresses. Third, an epicellular location facilitates the dissemination of the pathogen, e.g. transmission from one host cell to another one and expulsion from the host, by just shearing off the cell surface. Four, as highlighted by this study, an epicellular localization of the PV offers the advantage for the parasite to receive nutrients dispatched to its host cell from different sources, as it is the case for enterocytes. It is also tempting to conceive that the parasite may express transporters at the PV membrane facing the gut to short-circuit its host cell by catching the first nutrients arriving with the diet. If this situation can be demonstrated, it would be interesting to examine whether the severity of *Cryptosporidium* infections is associated with the diet of the host.

Experimental procedures

Reagents and antibodies

All chemicals were obtained from Sigma Chem. Co. (St. Louis, MO) or Fisher (Waltham, MA) unless indicated otherwise. The nitrobenzoxadiazole (NBD)-cholesterol was purchased from Molecular Probes (Eugene, OR). Lovastatin was a gift from Merck, Sharp and Dohme. Ezetimibe was from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies used were rabbit anti-NPC1L1 from Santa Cruz Biotechnology, mouse α -tubulin and mouse anti-GP40/900 of *C. parvum* (monoclonal 4E9; Cevallos *et al.*, 2000).

Cell lines and culture conditions

The human cell lines used in this study are the intestinal epithelial Caco-2 cells (ATCC HTB-37) for *C. parvum* infections and foreskin fibroblasts (HFF; ATCC CRL-1635) for *T. gondii* infections. Caco-2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), 25 mM HEPES, and 2% penicillin-streptomycin-amphotericin B (Gibco, Langley, OK). HFF were cultured in alpha modified Eagle's medium (α -MEM) with 10% FCS, 2mM L-glutamine and 2% penicillin-streptomycin. All cells were maintained at 37°C in 5% CO₂.

Parasite cultivation

C. parvum oocysts from the Iowa strain were obtained from the University of Arizona Sterling Parasitology Lab (Tucson, AZ) or from Bunch Grass Farm (Deary, ID). Oocysts were stored at 4°C for 2–8 weeks. Prior to use, oocysts were treated with 1.75% sodium hypochlorite for 10 min on ice and 0.75% sodium taurocholate (pH 7.5) for 30 min at 37°C to induce sporozoite excystation, washed three times with PBS by centrifugation at 5000 $\times$ g for 3 min at 4°C, and a mixture of excysted oocysts and free sporozoites resuspended in the culture medium were inoculated to Caco-2 cells.

Preparation of LDL, HDL, lipoprotein-deficient serum (LPDS), NBD-cholesterol associated with lipoproteins or with micelles

Human LDL (density: 1.019 to 1.063 g/ml) and human HDL (density: 1.063 to 1.2 g/ml) were isolated from fresh serum by zonal density gradient ultracentrifugation as described

(Poumay and Ronveaux-Dupal, 1985). LPDS was prepared by ultracentrifugation of fetal calf serum after adjustment of serum density to 1.215 g/ml with KBr (Havel *et al.*, 1955). To incorporate fluorescent cholesterol into LDL or HDL, NBD-cholesterol was dissolved in 25 ml dimethylformamide and added to 210 ml of filtered fresh human plasma for 16 h at 37°C before lipoprotein isolation. To study cholesterol uptake from micelles, micellar solutions were prepared containing 0.2 mM cholesterol and NBD-cholesterol, 0.3 mM monoolein, 10 mM sodium taurocholate, filtered through a 0.2- μ m filter and kept at 37°C. The micellar solution was stirred and dissolved in DMEM (dilution 1/100, v/v) prior to incubation with *C. parvum*-infected Caco-2 cells.

Fluorescence microscopy

Filipin staining: The fluorescence dye filipin (10 mg/ml in dimethyl sulfoxide) has been used for cytochemical detection of 3β -hydroxysterols within membranes of *C. parvum*-infected cells, or extracellular oocysts and (excysted) sporozoites as described (Coppens *et al.*, 2000). To quantify the level of filipin accumulated in the PV, images were collected using sequential scanning, processed and merged using Volocity software (PerkinElmer, Waltham, MA). For quantification of fluorescence intensity, the total intensity in the UV channel was determined for each vacuole and compared with the total fluorescence intensity of the whole same cell using the following equation: [sum intensity (pathogenic vacuole center)/sum intensity (entire host cell)] $\times$ 100. **Nile Red staining:** For detection of lipid bodies using Nile Red by fluorescence microscopy, intravacuolar parasites were fixed in paraformaldehyde and treated as described (Nishikawa *et al.*, 2004). **NBD-cholesterol staining:** To visualize fluorescent cholesterol associated with *C. parvum*, Caco-2 cells were infected with the parasites before addition of LDL, HDL or micelles containing NBD-cholesterol for 2 h and observed as live by fluorescence microscopy. **IFA:** For GP40/900 staining, infected cells were fixed with 4% formaldehyde (Polysciences, Warrington, PA) plus 0.02% glutaraldehyde in PBS for 15 min. IFA were performed as described previously (Karsten *et al.*, 2004) with primary antibodies used at 1/250 dilution. Coverslips were mounted using ProLong anti-fade mounting solution (Invitrogen). Images were acquired on a Nikon Eclipse E800 microscope equipped with a Spot RT CCD Camera and processed using Image-Pro-Plus software (Media Cybernetics, Silver Spring, MD) before assembly using Adobe Photoshop (Adobe Systems, Mountain View, CA).

In vitro *C. parvum* infection assays

To monitor *C. parvum* infectivity *in vitro*, we performed immunofluorescence assays (IFA) and ELISA using antibodies against parasite antigens. **IFA:** experiments were conducted in 24-well plates with Caco-2 cells seeded on coverslips. To examine the effect of lipoproteins on *C. parvum* development, 2.5×10^5 Caco-2 cells per well were cultured in DMEM + 10% FBS for 24 h before infection with 10^5 oocysts. Two hours p.i., cells were washed to replace the medium by fresh DMEM + 10% FBS (as positive control); DMEM + 10 % LPDS; DMEM + 10% LPDS + LDL (1 mg/ml) or DMEM + 10 % LPDS + HDL (2 mg/ml) and incubated for 24 h or 36 h. The size of the *C. parvum* PV and number of meronts were determined after labeling of the parasites with antibodies against GP40/900 prior to microscopic observations. To investigate the effects of sterol-related drugs on *C. parvum* replication, uninfected cells were preincubated with 10 μ M lovastatin for 24 h; 15 μ M zaragozic acid for 24 h; or 0, 50 or 100 μ M ezetimibe for 2 h, then infected with *C. parvum* for 36 h in the presence of the drug, prior to IFA. To assess potential drug cytotoxicity on mammalian cells, Caco-2 cells were treated with these drugs at the same concentrations, collected, and their cell number and viability were determined using a trypan blue dye exclusion assay, compared to untreated cells. **ELISA:** 96-well plates were seeded with Caco-2 cells, and the cells were grown to confluence before treatment with drugs either zaragozic acid (0, 15, 30 or 60 μ M) or ezetimibe (0, 50 or 100 μ M). After washing, 5×10^5

C. parvum oocysts were added into each well and allowed to infect cells in the presence of the drugs at the same concentrations for 36 h. Micelles were added to cells treated with ezetimibe. After washing, infected cells were fixed, permeabilized with methanol for 10 min at room temperature, and washed with PBS. Nonspecific binding was blocked using 1% normal goat serum (NGS) in PBS overnight at 4°C before adding antibodies against GP40/900 in 1% NGS overnight at 4°C. After washing, the plates were incubated with biotinylated goat anti-mouse IgM (SouthernBiotech, Birmingham, AL) for 1 h, then with the avidin-biotin alkaline phosphatase complex (Vector Labs, Burlingame, CA) and its substrate *p*-nitrophenol phosphate for 1 h. The reaction was stopped with 0.1M EDTA, and the plates were read at the absorbance value of 405 nm. Assays were carried out in triplicates and data analyzed by Graphpad Prism (version 7).

RNA interference

BLOCK-iT Pol 11 miR RNAi Expression Vector Kit (Invitrogen) was used to create clones for RNAi in Caco-2 cells. Annealed NPC1L1 miRNAi Select oligos were used to create miRNA clones in the vector pcDNA 6.2-GW/EmGFP, following the protocols supplied in the kit. Primer sequences for *NPC1L1* were sense: 5'-TGCTGTAAGAAGGCCTCCTCCCACAGGTTTTGGCCACTGACTGACCTGTGGGAA GGCCTTCTTA-3' and antisense: 5'-CCTGTAAGAAGGCCTTCCCACAGGTCAGTCAGTGGCCAAAACCTGTGGGAGGA GGCCTTCTTAC-3'. For 24-well plates, 8 µg of plasmid DNA mixed to 3 µg of lipofectamine 2000 Transfection Reagent (Invitrogen) were used to transiently transfect Caco-2 cells. Mixture of non-targeting miRNA plasmid (pcDNA 6.2-GW/EmGFP-miR) supplied in the kit was used as negative control. In each replicate, 0.3×10^6 cells were transfected and immediately added to 1 ml culture medium in the culture plate. Efficiency of siRNA for NPC1L1 was assessed at the protein level by western blotting as described (Labaied *et al.*, 2011) and normalized by expression levels of α -tubulin in Caco-2 cells. Immunoblotting was performed using the primary antibodies against α -tubulin at 1/2,500 dilution and NPC1L1 at 1/1,000 dilution, revealed by appropriate secondary antibodies coupled to horseradish peroxidase (HRP) at 1/15,000 to stain proteins of interest. Detection of immunoreactive proteins by chemiluminescence was performed using the ECL Plus Western Blotting Kit (Amersham). Forty-eight hours post-transfection, cells were infected with 5×10^5 oocysts and incubated in medium plus LPDS and 0.2 mM micellar cholesterol for 36 h. The morphology and number of PV containing merozoites or trophozoites in the silenced cells was then assessed by IFA using antibodies against GP40/900.

Electron microscopy

For thin-section transmission electron microscopy (EM), Caco-2 cells infected with *C. parvum* were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences; EMS, Hatfield, PA) in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature, and processed as described (Fölsch *et al.*, 2001) before examination with a Philips CM120 Electron Microscope (Eindhoven, the Netherlands) under 80 kV.

Protein concentration

Protein content was determined by the bicinchoninic acid protein assay (Pierce Chemical Co, Rockford, IL) using the wavelength scan program of the Beckman DU-640 spectrophotometer (Miami, FL).

Statistical analysis

For comparison of means, *P* was determined by analysis of variance against control (ANOVA 2) with a threshold of *P*-value < 0.05 considered as statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Association of sterols with intracellular and extracellular stages of *Cryptosporidium parvum*

A–B. Filipin staining of Caco-2 cells infected with *Cryptosporidium parvum* or human fibroblasts infected with *Toxoplasma gondii* at the indicated times. Data show an intense fluorescent signal associated with the PV of *C. parvum* (arrow; 24 h and 48 h for the trophozoite and the merozoite stages respectively) as observed for the *Toxoplasma* PV (arrow). In B, intravacuolar merozoites are indicated by arrowheads. Scale bars are 10 (in A) and 5 (in B) μm. **C.** Filipin staining (left panels) or immunofluorescence assays (IFA) using antibodies against the plasma membrane marker GP40/900 (right panels) of *C. parvum*-infected cells for 24 h and 48 h, showing comparable staining of the parasite plasma membrane (arrows). Scale bars are 2 μm. **D.** Filipin staining of trophozoites illustrating a fluorescent signal at the site of attachment of the parasite to the host cell (arrows). The right figure shows a positive filipin staining on intraparasitic structures. Scale bars are 2 μm. **E.** Filipin staining of extracellular oocysts either untreated (cyst wall intact) or treated with taurochoate and bleach to induce excystation (cyst wall open) showing fluorescence inside excysted oocysts and on released sporozoites. Arrow shows the progression of sporulation until sporozoite egress. Scale bars are 3 μm.

Figure 2. Cholesterol homeostasis in enterocytes in the small intestine and potential sources of cholesterol for *C. parvum*

A. In enterocytes, the trafficking pathways of cholesterol are multiple: cholesterol intake from micelles via NPC1L1 (pathway I); cholesterol synthesis via HMG-CoA reductase (pathway II); plasma LDL uptake with LDL receptor (LDL R; pathway III); plasma HDL uptake with SR-BI (pathway IV); Cholesteryl ester (CE) formation via ACAT and efflux via chylomicrons (pathway V); and free cholesterol (FC) efflux via ABCG5/8 (pathway VI). **B.** Schematic representation of *C. parvum*-infected cells showing hypothetical pathways of cholesterol that could be diverted by the parasite. Source I is micellar cholesterol (MC); source II is cholesterol synthesized *de novo* on host ER (ER-C); and sources III and IV are exogenous cholesterol derived from plasma LDL or HDL, respectively. hPM, host plasma membrane; FO, feeder organelle.

Figure 3. Role of plasma lipoproteins for cholesterol supply to *C. parvum*

A. PV properties determined 36 h p.i. in infected enterocytes after incubation in the presence of 10% FCS (fetal calf serum), 10% lipoprotein-deficient serum (LPDS), excess LDL in LPDS or excess HDL in LPDS. The number of PV containing one parasite (trophozoite) and of PV containing 6 to 8 parasites (type 1 merozoites) was counted. The diameter of these PV calculated in micron are mean values $\pm$ S.D., $n=3$ independent assays (*, $P < 0.01$ compared to 10% FCS). **B.** Fluorescence filipin staining of representative PV grown in the indicated media and quantitative measurement of filipin fluorescence levels associated with the PV 36 h p.i. Fluorescence intensity was expressed as a ratio of total filipin intensity within the PV to total filipin intensity within the host cell. Error bars indicate SEM, $n=10$ per condition (*, $P < 0.01$). Scale bars are $2 \mu\text{m}$.

Figure 4. Salvage of cholesterol from plasma lipoproteins by *C. parvum*

A–B. Fluorescence microscopy of *C. parvum*-infected cells at the indicated infection times incubated with NBD-cholesterol incorporated into LDL ([NBD-C]-LDL) in A or with NBD-cholesterol incorporated HDL ([NBD-C]-HDL) in B. The concentration of fluorescent LDL or HDL was ~80 μg/ml. After 2 h pulse, parasites were chased for 1 h and observed by microscopy to examine the fluorescence associated with intracellular parasites or released merozoites (inset in A). Scale bars are 5 μm.

Figure 5. Stimulation of lipid storage in *C. parvum*-infected cells upon addition of LDL
A. Fluorescence Nile Red staining of *C. parvum* 24 h p.i. associated with internal structures (arrows) in control conditions (10% FCS) and in excess LDL (1 mg/ml). Scale bars are 1 μm. **B–F.** Transmission EM of *C. parvum*-infected cells incubated with 1 mg/ml of LDL for 24 h showing large lipidic inclusions in the cytoplasm of the parasite, which resemble lipid bodies (LB) surrounded by ER elements (insets in C and D). Some inclusions were very large (E–F) and protruding from the parasite (F). FO, feeder organelle; n, nucleus. Scale bars are 0.2 μm.

A

PV properties	Medium conditions		
	untreated	Lovastatin	Zaragozic acid
# trophozoite PV / merozoite PV	3 / 35	45 / 15	5 / 41
PV size (μm)	8.0 ± 1.8	$3.2 \pm 0.4^*$	6.6 ± 1.0

B **C** **Figure 6. Effect of blockade of host cholesterol synthesis on *C. parvum* growth**

A. Analysis of PV properties determined 36 h p.i. in infected enterocytes incubated in the presence of 10 μM lovastatin, 15 μM zaragozic acid, or untreated, as described in the legend of Figure 3. (*, $P < 0.05$ compared to untreated parasites). **B.** Fluorescence filipin staining of representative PV 36 h p.i. grown in the absence or the presence of 10 μM lovastatin or 15 μM zaragozic acid, and quantitative measurement of filipin fluorescence levels associated with the PV. Fluorescence intensity was expressed as a ratio of total filipin intensity within the PV to total filipin intensity within the host cell. Error bars indicate SEM, $n=9$ per condition (*, $P < 0.01$). The trypan blue exclusion assay used to determine the number of viable cells present in suspension did not reveal any difference between treated and untreated cells. Scale bars are 2 μm . **C.** Quantification of *C. parvum* growth by ELISA. Infected caco-2 cells were incubated with zaragozic acid at the indicated concentrations for 36 h before monitoring infection by ELISA. Slight statistically significant differences in the infection levels were found in zaragozic acid-treated parasites compared to untreated parasites as shown by the P values. Data are means $\pm$ SEM from 3 independent populations of *C. parvum*.

Figure 7. Uptake of cholesterol from micelles by *C. parvum*

A. Fluorescence microscopy of live *C. parvum*-infected cells 24 h p.i. incubated with micelles preloaded with NBD-cholesterol. After 2 h pulse, cells were chased and observed by microscopy to examine the fluorescence associated with intracellular parasites (arrow). Scale bars are 5 μm . **B.** Analysis of PV properties determined 36 h p.i. in infected enterocytes incubated in 10% FCS, in the absence of lipoproteins (LPDS) or in the presence of 0.2 mM micellar cholesterol (MC) in LPDS, as described in the legend of Figure 3. (*, $P < 0.01$ compared to control parasites).

Figure 8. Role of host NPC1L1 for cholesterol supply for *C. parvum*

A. Quantification of *C. parvum* growth by ELISA. Caco-2 cells were infected for 36 h with *C. parvum* in the presence of ezetimibe at the indicated concentrations before monitoring infection by ELISA. The trypan blue exclusion assay did not reveal any difference between ezetimibe-treated and untreated cells. Statistically significant differences in the infection levels were found in treated parasites compared to untreated parasites as shown by the *P* values. Data are means $\pm$ SEM from 3 independent populations of *C. parvum*. **B.** IFA of *C. parvum*-infected cells incubated 36 h with ezetimibe at the shown concentrations before staining with antibodies against GP40/900 confirming the ELISA results in A. **C.** Effect of silencing of host NPC1L1 on *C. parvum* growth. Panel a: Immunoblots of lysates from Caco-2 cells that have been transfected with NPC1L1 siRNA expression for 48 h (NPC1L1 siRNA) or non-targeting siRNA using antibodies against NPC1L1 (140 kDa) and α -tubulin (α -tub; 55 kDa). Molecular weight standards are shown at the right. Panel b: Quantification of expression levels of NPC1L1 or α -tubulin in Caco-2 cells transfected with NPC1L1 siRNA or non-specific targeting siRNA by densitometry. Results are means from 3 independent assays. Panel c: Quantification of *C. parvum* infectivity after silencing of NPC1L1 expression, using non-targeting siRNA or under control conditions (non-silenced cells) 36 h post-transfection in LPDS medium containing micellar cholesterol. The PV size was measured and expressed as means $\pm$ SEM, in μm (n=3) and the percents of PV

containing merozoites (meronts) proportional to the PV with trophozoites are shown in histograms. Panel d: Morphological illustrations of the parasites (red-stained with antibodies against GP40/900) grown in NPC1L1 siRNA-treated cells (identifiable by GFP staining; arrow) in comparison to parasites developing in non-silenced cells (arrowheads). Top right panel shows a PV from cells with non-relevant siRNA. Scale bars are 5 μ m.