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HOST CELL P-GLYCOPROTEIN IS ESSENTIAL FOR CHOLESTEROL UPTAKE AND REPLICATION OF TOXOPLASMA GONDII.

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P-glycoprotein (P-gp) is a membrane-bound efflux pump which actively exports a wide range of compounds from the cell and is associated with the phenomenon of multidrug-resistance. However, the role of P-gp in normal physiological processes remains elusive. Using P-gp deficient fibroblasts, we showed that P-gp was critical for the replication of the intracellular parasite Toxoplasma gondii, but was not involved in invasion of host cells by the parasite. Importantly, we found that the protein participated in the transport of host-derived cholesterol to the intracellular parasite. T. gondii replication in P-gp deficient host cells not only resulted in reduced cholesterol content in the parasite but also altered its sphingolipid metabolism. In addition, we found that different levels of P-gp expression modified the cholesterol metabolism in uninfected fibroblasts. Collectively our findings reveal a key and previously undocumented role of P-gp in host-parasite interaction and suggest a physiological role of P-gp in cholesterol trafficking in mammalian cells.

P-glycoprotein (P-gp, ABCB1, MDR1) is one of the most intensively studied members of the ABC transporter superfamily. With remarkably broad substrate recognition, P-gp drives the ATP-dependent efflux of toxic metabolites and xenobiotics from the cell (1) and is thus a central mediator of drug bioavailability. Importantly, P-gp over-expression following drug treatment is responsible for the multidrug-resistance (MDR) phenotype, a major reason for chemotherapy failure not only in cancer cells (2) but also in pathogenic microorganisms (3,4). Aside from its well known role in drug efflux, P-gp is also expressed at basal levels in many different tissues, yet the normal physiological functions of the protein remain poorly understood.

The possibility that physiological levels of host P-gp play a role in host-pathogen interaction, other than mediating drug resistance, has not been investigated so far. We addressed this question using Toxoplasma gondii as a model pathogenic parasite. T. gondii is the causative agent of toxoplasmosis, a potentially fatal disease not only for immunocompromised patients and fetuses but according to recent insights also emerging as a life threatening infection in immunocompetent individuals (5). T. gondii infects virtually all nucleated host cells and resides in a highly specialized vacuole, called the parasitophorous vacuole (PV), which is formed by invaginating the host cell membrane at the time of invasion. The PV is not competent for lysosome fusion, thus avoiding acidification (6), but it is closely associated with host organelles, including lysosomes, mitochondria and ER (rev. in (7)). Even though the PV does not intersect directly with host vesicular traffic, T. gondii remains dependent on host cells for a number of critical nutrients. Significant progress has been made in our understanding of the mechanisms T. gondii uses to scavenge nutrients from its host, especially in the case of lipid molecules. An important recent example was the identification of H.O.S.T., a unique system of tubular structures formed by the parasite to sequester cholesterol-containing endolysosomes from the host cytoplasm into the PV (8). However, the molecular mechanisms of the traffic from the host cell to the PV are not completely elucidated and the existence of transporters has been proposed frequently.

To analyze whether the P-gp transporter plays a role in T. gondii biology we compared parasite replication in wild-type (WT) mouse embryonic
fibroblasts with double knock out (DKO) fibroblasts in which neither of the two murine P-gp isoforms are expressed (9). In parallel, we also analyzed DKO cells complemented with the human P-gp homologue (DKO/P-gp) (10), which restored P-gp functionality to DKO cells and allowed P-gp expression levels higher than those found in WT cells (fig. S1). In this way, our model did not depend on either drug-selected P-gp over-expressing cells, which may acquire adaptation mechanisms different from P-gp over-expression during the development of the MDR phenotype, or P-gp inhibitors, several of which are known to have side effects on host metabolism.

**EXPERIMENTAL PROCEDURES**

**Biochemical reagents.** Unless otherwise stated, all chemicals were purchased from Sigma, cell culture reagents from Gibco-BRL, and radiolabeled lipids from Amersham Pharmacia Biotech. Anti-P-gp monoclonal antibody C219 was purchased from Alexis Biochemicals; anti-Lamp1, anti-giantin and anti-tubulin were a kind gift from J. Pieters, J. Rohrer and M.A. Hakimi, respectively. Conjugated secondary antibodies were from Invitrogen. Reconstituted high density lipoproteins and apolipoprotein A-I were a kind gift from P. Lerch (CSL Behring, Bern, Switzerland). NDB-cholesterol was from Avanti Polar Lipids.

**Mammalian cell and parasite culture.** Mouse embryonic fibroblasts (MEF) double knocked out for P-gp (77.1, Mdr1a-/-/Mdr1b-/-) (9), triple knocked out for P-gp and MRP1 (3.8, Mdr1a-/-/Mdr1b-/-/Mrp1-/-) (11) and parental cells were kindly provided by A. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Nederlands). Mdr1 transfected 77.1 (10) and G185 NIH 3T3 fibroblasts (12) were a generous gift from M.M. Gottesman (National Cancer Institute, National Institutes of Health, Bethesda, MD).

Cells were routinely cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 U of penicillin/mL, and 50 µg of streptomycin/mL at 37°C with 5% CO2. Mdr1 transfected 77.1 and G185 cells were maintained in 20 and 60 ng/mL colchicine, respectively. Colchicine was removed from the culture medium during parasite infection.

β-galactosidase expressing *T. gondii* and *N. caninum* were a kind gift from J. Boothroyd and D. Sibley, respectively. Parasites were maintained by serial passages in MEF, harvested from infected host cells by passage through a 26-gauge needle and purified by separation on Sephadex-G25 columns (Amersham Biosciences, Otelfingen, Switzerland) as described (13). Purified parasites were counted in a hemocytometer chamber and used for a new cycle of host cell invasion at the multiplicity of infection (MOI) of 1. Parasite burden was quantified after 24 hrs by direct parasite counting or after 48 or 72 hrs by colorimetric detection of parasite β-galactosidase using chlorophenol red-β-D-galactopyranoside as substrate, as described (14). In some experiments, parasite quantification in infected host cells was performed by flow cytometry analysis on a FACSCalibur flow cytometer (Becton & Dickinson, Basel, Switzerland), after staining permeabilized cells with a rabbit polyclonal anti-*T. gondii* tachyzoite antiserum (15) at 1:2000 dilution, followed by fluorescein-conjugated anti-rabbit secondary antibody at 1:300 dilution. Parasite invasion was determined by dual color immunostaining with the described anti-*T. gondii* antiserum to differentiate intracellular and extracellular parasites, as described (16).

**Lipid analyses.** For cholesterol transport to intracellular *T. gondii*, infected host cells were labeled with 0.5 µCi/mL [3H]cholesterol for 5 hrs. After extensive washing with PBS and 0.05% fat free BSA in PBS, parasites were isolated as previously described, counted and the associated radioactivity measured by liquid scintillation. Background radioactivity resulting from host cell debris was determined using similarly processed uninfected host cells and subtracted from parasite samples.

For cholesterol uptake analysis, host cells were incubated with 0.5 µCi/mL [3H]cholesterol for 5 hrs, extensively washed and cell-associated radioactivity measured by liquid scintillation. Steady state cholesterol mass was assessed using the Amplex Red kit (Invitrogen, Basel, Switzerland) or by lipid extraction according to (17) and high performance thin layer chromatography (HPTLC) separation of aliquots corresponding to equal protein content on Silica Gel 60 plates in benzene/2-propanol/water
(100:10:0.25). Bands were visualized with 10% CuSO₄ in 8% aqueous phosphoric acid and quantified by densitometry.

Liquid chromatography-mass spectrometry (LC-MS) analysis of lipids from *T. gondii* grown in WT or DKO host cells was performed as described (18) in a Waters Aquity UPLC system connected to a Waters LCT Premier orthogonal accelerated time of flight mass spectrometer (Waters, Millford, MA), operated in positive electrospray ionization mode.

For analysis of sphingolipid synthesis in *T. gondii*, purified parasites were labeled with 0.5 μCi/mL [³H]palmitic acid for 3 hrs, lipids extracted as before and aliquots corresponding to equal protein content separated by HPTLC using chloroform/methanol/25% NH₄OH (65:25:4.5). Radiolabeled bands were visualized by use of a tritium-sensitive screen (Perkin-Elmer, Boston, Mass.) in a Personal Molecular PhosphoImager FX (Biorad), identified according to co-migrating standards visualized by iodine vapors and quantified using ImageQuant software (Amersham, Otelfingen, Switzerland).

For kinetic of cholesterol esterification, host cells were labeled with 0.5 μCi/mL [³H]cholesterol for 5, 24 and 48 hrs, lipids were extracted as before and separated by HPTLC using benzene/2-propanol/water (100:10:0.25) as solvent system. After iodine vapors visualization, bands co-migrating with cholesterol and cholesteryl esters standards were cut out of the plates and the associated radioactivity measured by liquid scintillation. Data for cholesterol esterification are reported as percent of [³H]cholesterol esters from total cellular [³H]cholesterol.

For cholesterol extraction with methyl-β-cyclodextrin (CD) and efflux to reconstituted high density lipoproteins (rHDL) or apolipoprotein A-I (apoAI), cells were labeled with 0.5 μCi/mL [³H]cholesterol for 24 h, prior fixation in acetone at -20°C for 10 min. Anti-P-gp monoclonal antibody C219 was used at 1:10 dilution according to the manufacturer instruction, followed by fluorescein-conjugated secondary antibody at 1: 200 dilution. *T. gondii* was stained with a rabbit polyclonal anti-*T. gondii* tachyzoite antiserum (15) at 1:2000 dilution, followed by texas red-conjugated anti-rabbit secondary antibody at 1:300 dilution. Nuclei were visualized with 4’, 6-diamidino-2-phenylindole (DAPI).

For endo-lysosome localization, host cells grown on glass slides were infected with *T. gondii* or *N. caninum* for 24 h, incubated with 6 μg/mL fluorescein-conjugated cholera toxin B subunit for 60 min at 37°C and analyzed after fixation. For lysosome and Golgi localization, cells were seeded as before, fixed and stained with anti-Lamp1 (1:50) or anti-giantin (1:750) antibodies followed by fluorescein-conjugated secondary antibody.

Microscopy analyses were performed on a Leica DM IRBE fluorescence microscope or on a Leica SP2 A OBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany), using
the appropriate settings. Image stacks of optical sections were further processed using the Huygens deconvolution software package version 2.7 (Scientific Volume Imaging, Hilversum, NL). *Transmission electron microscopy analysis.* Host cells grown on sapphire disks were infected with *T. gondii* and incubated at 37°C for 24 h, prior to fixation with 0.25% glutaraldehyde and freezing in a high-pressure freezing machine (HPM 010, BAL-TEC) as described by (19). Frozen cells were transferred into a freeze-substitution unit (FS 7500, Boeckeler Instruments, Tucson, Arizona, USA) precooled to -88°C for substitution with acetone and subsequent fixation with 0.25% glutaraldehyde and 0.5% osmium tetraoxide at temperatures between -30°C and +2°C as described in detail (20) and embedded in Epon. 50 to 60 nm thick sections were stained with uranyl-acetate and lead-citrate and analyzed in a transmission electron microscope (CM12, Philips, Eindhoven, The Netherlands) equipped with a CCD camera (Ultrascan 1000; Gatan, Pleasanton, CA) at an acceleration voltage of 100 kV. *Cell viability following cholesterol loading.* Host cells were loaded with cyclohextrin-cholesterol complexes (Sigma) for 24 hrs at the concentrations indicated in the figure legend, washed in medium and incubated at 37°C for additional 24 hrs. Cell viability was tested by using the AlamarBlue® assay (Biosource, Camarillo, CA), according to the manufacturer instructions. *P-glycoprotein functional assay.* P-gp activity was assessed by cellular retention of the P-gp substrate rhodamine 123 (Rho). Briefly, cells were incubated with 0.5 µg/mL Rho in PBS for 30 min at 37°C, washed in PBS and incubated in medium at 37°C for the time points indicated in the figure legend. The kinetic of rhodamine retention was quantified by flow cytometry. *Western blot analysis.* Cell lysates for immunoblots were prepared by sonicating cells at 10^7/ml in 50 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5 mM DTT, 0.5 mM phenylmethylsulfonylfluoride and complete protease inhibitor mixture (Calbiochem). Samples corresponding to 40 µg proteins were mixed with SDS-PAGE loading buffer and incubated 10 min at room temperature to prevent P-gp aggregation. Samples were separated on 7.5% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed using anti-P-gp C219 (1:50), and anti-tubulin (1:2000) monoclonal antibodies. Immunoreactive bands were visualized with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL). *Determination of protein concentration.* Protein content was determined using the Bio-Rad Protein Assay according to the instructions provided by the manufacturer. Bovine serum albumin was used for the standard curve. *Statistical analyses.* Data are expressed as means ± SE. 1-way analysis of variance (ANOVA) was performed (GraphPad Prism 4.0c, GraphPad Software, Inc.) and a probability value < 0.05 was considered statistically significant. When the overall probability value was < 0.05, the Dunnett multiple-comparisons test was used as a posttest to determine if there was a significant difference between values of control (reference sample) and samples of interest. **RESULTS**

**Host cell P-gp is essential for normal parasite replication.** To determine whether the activity of host P-gp plays a role in parasite replication, host cells expressing different levels of the protein (fig. S1) were infected with *T. gondii* expressing β-galactosidase, which allows parasite quantification by colorimetric reaction (21). Direct parasite count at 24 hrs post-infection (p.i.) revealed the presence of smaller vacuoles containing fewer parasites in P-gp deficient host cells (fig. 1A). Importantly, complementation of P-gp deficient cells with the human P-gp homologue fully restored parasite replication. Analysis of parasite burden by visualization of parasite vacuoles at 48 hrs p.i. (fig. 1B) and colorimetric reaction at 48 and 72 hrs p.i. (fig. 1C) confirmed that *T. gondii* replication was strongly inhibited in the absence of host P-gp. In addition, P-gp complemented host cells generated a higher *T. gondii* burden than that found in WT cells. To further confirm that P-gp activity confers a replication advantage for the parasite, we used WT 3T3 fibroblasts transfected to overexpress P-gp as host cells (12). Parasite quantification was performed either at the level of single host cells using flow cytometry (fig. S2A) or as parasite burden of the whole host cell monolayer by
colorimetric reaction (fig. S2B). Both analyses indicated a positive correlation between P-gp expression levels and parasite replication. Absence of the multidrug transporter MRP1, in addition to the two P-gp isoforms (triple KO cells) (11), did not further decrease parasite replication (data not shown). Collectively, these data unambiguously show that the presence of an active P-gp in the host is directly involved in a process controlling *T. gondii* replication.

**Host cell P-gp is not involved in parasite invasion.** The reduction in parasite number seen in P-gp deficient cells could have resulted from either decreased parasite replication or reduced invasion efficiency. In order to assess whether the absence of P-gp on the host plasma membrane compromises the ability of *T. gondii* to enter host cells, an invasion assay was performed using parasites harvested from WT host cells and allowed to infect cells expressing different levels of P-gp. The number of intracellular parasites was comparable in host cells expressing basal P-gp levels, P-gp deficient and P-gp complemented at 2 hrs post infection (fig. 1D), indicating that host P-gp is not required for parasite invasion and that the reduced parasite burden observed in DKO cells is directly involved in a process controlling *T. gondii* replication.

**Host cell P-gp deficiency inhibits cholesterol transport to *T. gondii*.** The reduced parasite replication observed in P-gp deficient host cells led us to hypothesize that the absence of P-gp may disturb the supply of lipid components necessary for assembly of new parasite membranes. To test this hypothesis, we evaluated cholesterol trafficking and metabolism in the parasite, as *T. gondii* is auxotrophic for cholesterol and depends solely on the host for its supply (22). When we tested the transport of exogenous radiolabeled cholesterol to the parasite, we found that in the absence of host P-gp cholesterol delivery was strongly reduced (fig. 2A). Importantly, P-gp DKO cells showed normal uptake of exogenous cholesterol (fig. S3), indicating that the reduced cholesterol transport to the parasite is not caused by a compromised uptake of this lipid by the host cells. In addition, the increased *T. gondii* replication in P-gp complemented host cells coincided with enhanced cholesterol delivery to the parasites, suggesting that the level of cholesterol transport was regulating parasite replication rate.

In addition, we monitored the cholesterol transport in vivo by using the fluorescently labeled analogue NBD-cholesterol (fig. 2B). Similar to the observations during the radiolabeled cholesterol incubation, transport of NBD-cholesterol to parasites grown in DKO cells was reduced compared with WT cells and a pattern of punctuated structures, reminiscent of endocytic vesicles, accumulated around the parasite vacuoles. Conversely, parasites infecting P-gp complemented cells showed high level of fluorescence, indicating a robust trafficking of cholesterol to the vacuole.

To test whether decreased cholesterol availability in absence of host P-gp was directly responsible for the reduced *T. gondii* replication, we evaluated the parasite burden in DKO cells loaded with exogenous cholesterol following infection. This treatment improved parasite replication in a dose dependent manner (fig. 2C), confirming that insufficient cholesterol availability was indeed the limiting factor for parasite replication.

To investigate the mechanism of P-gp involvement in the cholesterol trafficking to *T. gondii*, we inhibited the vesicular transport of host cholesterol from lysosomes to both plasma membrane and endoplasmic reticulum with the class-2 amphiphile U-18666A (23). Treatment with U-18666A has been shown to decrease the cholesterol delivery to intracellular parasites (22). This broad inhibition of cholesterol trafficking severely affected *T. gondii* replication in WT host cells, with higher levels of inhibition than the ones observed in P-gp defective cells (fig. 2D). On the other hand, parasite replication in DKO cells was further inhibited by only 10% upon U-18666A treatment. The absence of a significant cumulative effect between lack of P-gp and inhibitor treatment indicates that P-gp may operate, directly or indirectly, on a similar pathway of cholesterol trafficking that is essential for parasite replication.

**P-gp associates with the parasite vacuole.** Our previous results suggest that P-gp may be involved in the cholesterol trafficking to the parasite via the endocytic pathway. To investigate this possibility we localized host P-gp in infected cells. P-gp is mainly present in the plasma membrane of the cells, but a minority also localizes in intracellular compartments, including endo-lysosomes (24,25). As endo-lysosomes have been shown to be recruited to the PV in *T. gondii* infected cells (8),
we reasoned that P-gp also associates with the parasite vacuole. To test this hypothesis, we infected P-gp complemented host cells, whose level of P-gp expression allows analysis by immunofluorescence using the P-gp specific monoclonal antibody C219 (fig. S1A). This analysis showed that P-gp was predominantly present on the host plasma membrane as expected. In addition, P-gp was also found closely associated with the PV (fig. 3A, B).

**Host organelles are recruited to the PV in absence of host P-gp.** *T. gondii* scavenges cholesterol from host endo-lysosomal compartments (22), and these organelles are actively recruited by the parasite to the PV in a time dependent manner after infection (8). To test whether the decreased cholesterol delivery to the parasite in P-gp deficient cells was due to a failure in recruiting the cholesterol rich endo-lysosomes, we probed the localization of these organelles in infected cells (fig. 3C). First we analyzed the labeling of endocytic vesicles using cholera toxin B-subunit (CTX), which binds to the raft-associated sphingolipid GM1 and enters the cell by retrograde transport in the secretory pathway (26). CTX positive structures were equally found around the PV of parasites infecting WT, DKO and P-gp complemented host cells. Moreover, Lamp1 (Lysosome-associated membrane protein 1) staining further confirmed that lysosomes are recruited to the PV in DKO cells. Finally, host Golgi, which was previously shown to redistribute at the PV (8), was also found close to the PV surface in all tested cell lines. Collectively, our results show that the absence of host P-gp does not inhibit the parasite-mediated recruitment of host organelles to the PV.

**Cholesterol accumulates outside the PV in absence of host P-gp.** Despite the normal recruitment of lysosomes around the PV in DKO host cells, the accumulation of NBD-cholesterol observed in this cell type (fig.2B) prompted us to analyze whether the intracellular distribution of endogenous cholesterol is altered in absence of host P-gp. Staining of unesterified cholesterol with the poliene antibiotic filipin (27) in infected WT cells showed labeling of parasite membranes and perinuclear vesicular structures, previously reported to be endo-lysosomal compartments (fig. 4A). On the contrary, in DKO cells the filipin-positive vesicular structures were bigger and more intensely labeled and accumulated outside the PV. Importantly, P-gp complementation prevented the cholesterol accumulation, indicating that the cholesterol accumulation is P-gp dependent. **Inhibition of cholesterol transport to the parasite vacuole does not depend on H.O.S.T. formation.**

Next we assessed whether the defective transport of cholesterol to the parasite resulted from a faulty formation of the H.O.S.T. system, a recently described structure that can supply the parasite with cholesterol contained in host endo-lysosomes (8). Analysis by electron microscopy found normal recruitment of host organelles to the PV, including mitochondria, both in WT and DKO host cells (fig. 4B, arrowheads). Importantly, parasites grown in DKO host cells were able to form H.O.S.T. structures with clearly visible tubules and vesicles (fig. 4B, arrows and magnified images). Thus, host P-gp is not required for the formation of structurally normal parasite vacuoles. In this respect, the observed inhibition of cholesterol transport despite the formation of H.O.S.T. suggests that either P-gp acts at a later stage than the development of these structures, or it affects a H.O.S.T. independent mechanism of cholesterol delivery. To test the latter hypothesis, we analyzed the replication of *Neospora caninum*, an apicomplexan parasite related to *T. gondii* whose intracellular vacuoles recruits host endo-lysosomes (fig. S4), but are devoid of H.O.S.T. (8). As with *T. gondii, N. caninum* replication was inhibited in absence of host P-gp, and could be rescued by P-gp complementation (fig. 4C). In addition, *N. caninum* growth in DKO host cells resulted in decreased free cholesterol content and cholesteryl ester storage in lipid droplets (fig. 4D), suggesting that P-gp mediated cholesterol supply to the parasite operates via a mechanism independent of the H.O.S.T. system found in the PV.

**Host cell P-gp deficiency affects *T. gondii* sphingolipid metabolism.** Our previous results showed that cholesterol transport to the parasite is defective in P-gp deficient host cells. We then investigated whether this impaired transport affected the parasite’s lipid content. Lipid analyses of *T. gondii* maintained in DKO host cells revealed that the steady state of cholesterol content was comparable to WT after one lysis passage (23 ± 1.6 µg/mg protein) but decreased after prolonged growth in these cells (four lysis passages) (fig. 5A). Similar to the observations in *N. caninum*, a
marked decrease in cholesteryl esters stored in lipid droplets was observed in parasites isolated from DKO cells (fig. S5). Since cholesterol is an important regulator of membrane fluidity, membrane domains and signaling processes (rev. in (28)), we investigated whether the reduced cholesterol content observed in *T. gondii* grown in DKO host cells affected the lipid profile of the parasite’s membranes. Unexpectedly, liquid chromatography-mass spectrometry (LC-MS) analysis of parasite sphingolipids revealed a considerably higher amount of ceramide, ceramide phosphatidylethanolamine, sphingomyelin and lactosylceramide, while the levels of glucosylceramide did not change (fig. 5B). Given the crucial role of ceramide in cell physiology both as a precursor of complex sphingolipids and as a second messenger regulating a variety of cellular processes (rev. in (29)), we analyzed whether neosynthesis of this lipid was up-regulated in *T. gondii* grown in DKO host cells. Metabolic labeling of extracellular parasites revealed that ceramide synthesis was higher in *T. gondii* isolated from DKO host cells after four lysis passages than in WT cells (fig. 5C), supporting the hypothesis that the higher ceramide levels observed are indeed an active response of the parasite. These results suggest that reduced cholesterol availability triggers compensatory mechanisms in *T. gondii*, presumably to adapt to the intracellular environment of DKO cells and/or to adjust the lipid composition of its membranes.

**Absence of P-gp alters normal cholesterol metabolism in uninfected fibroblasts.** The observed inhibition of cholesterol transport to the parasite and the accumulation of cholesterol-loaded organelles around the PV in P-gp deficient host cells prompted us to analyze whether P-gp is also involved in cholesterol trafficking in uninfected host cells. To test this hypothesis, we investigated whether P-gp expression levels correlated with alterations in cholesterol metabolism in fibroblasts. Analysis of intracellular cholesterol distribution visualized with filipin revealed that P-gp DKO cells accumulated cholesterol in perinuclear vesicles compared with WT and P-gp complemented cells (fig. 6A). In addition, despite comparable uptake of radiolabeled cholesterol (fig. S3), DKO cells showed a time dependent increase in cholesterol esterification compared with WT cells (fig. 6B). On the other hand, P-gp complementation of DKO cells lowered the levels of cholesterol esterification. This correlation between the absence of P-gp and increased cholesterol esterification was evident not only in the kinetics of the process, but also in the steady state cholesteryl ester content (fig. S6). Next we evaluated cholesterol levels in the plasma membrane by extraction with methyl-β-cyclodextrin (CD) or efflux to the extracellular acceptors reconstituted HDL (30) and apolipoprotein A-I (apoA-1). We found that less cholesterol could be extracted with CD from the plasma membrane of DKO cells and that P-gp complementation increased the level of cholesterol extraction (fig. 6C). Furthermore, P-gp complementation also enhanced the ABCA1-mediated efflux of cholesterol to its natural acceptor HDL (fig. 6D), and apoA-I (fig. 6E). These data reveal that cells deficient in P-gp accumulated free and esterified cholesterol in perinuclear vesicular structures and cytosolic lipid droplets respectively. On the other hand, cells expressing high levels of P-gp showed increased amounts of cholesterol in the plasma membrane which then resulted in increased efflux to extracellular acceptors. To further test whether WT cells were more prone to accumulate free cholesterol in the plasma membrane than DKO cells, we exploited the toxicity induced by destabilizing the plasma membrane via exogenous cholesterol loading. Analysis of cell viability showed that DKO cells were minimally affected by exogenous cholesterol loading, whereas viability decreased with increasing levels of P-gp expression in WT and P-gp complemented cells (fig. 6F). As previously reported in cholesterol-loaded macrophages (31), blocking cholesterol transport from lysosomes to the plasma membrane using the hydrophobic amine U-18666A (23) prevented the cholesterol toxicity in P-gp expressing cells, but did not alter the viability of DKO cells (data not shown). Thus, these data suggest that inhibition of intracellular cholesterol transport mimics the increased survival of DKO cells during cholesterol loading.

**DISCUSSION**

P-gp is a unique transporter due to its broad substrate specificity and its expression constitutes
a major obstacle in the fight against multidrug-resistant tumor cells and pathogens. Yet P-gp also plays a key role in normal physiological processes, including preventing cellular toxicity at the blood brain barrier, as shown in vivo by the extremely high sensitivity of P-gp KO mice to toxic compounds (32).

Our aim was to investigate whether host P-gp plays a role in host-pathogen interaction that is distinct from multidrug resistance. Previous studies using P-gp inhibitors suggested that T. gondii may require a functional P-gp to survive in the host cell (33). In the present work more detailed investigation found that host P-gp is in fact a crucial modulator of T. gondii and N. caninum replication. In addition, we found that host P-gp is not a receptor for the parasite and its absence does not affect parasite invasion or the formation of structurally normal parasite vacuoles. Importantly, we found that transport of cholesterol from the host cell to the parasites, a process on which T. gondii is completely dependent, was impaired in absence of host P-gp. Biochemical analyses revealed that the parasite’s lipid composition was altered when grown in P-gp deficient host cells. Specifically, both free and esterified cholesterol levels were reduced, as expected from a decreased uptake of cholesterol from the host; interestingly, the content of selected sphingolipids also changed compared with T. gondii grown in WT cells. In particular, the level of sphingomyelin, a major structural sphingolipid mainly found on the plasma membrane, was increased two fold. Sphingomyelin has a high affinity for and closely associates with cholesterol and both lipids contribute to the ordered state of plasma membrane domains. While it is well accepted that cholesterol homeostasis is modulated by sphingomyelin content, studies on the regulation of sphingomyelin metabolism by varying the cellular cholesterol levels produced contradictory results in different cell types analyzed (rev. in (34)). However, cholesterol depletion was shown to stimulate sphingomyelin synthesis in fibroblasts (35), and high levels of sphingolipids can retain the ordered state of model membranes (36), suggesting that in conditions of cholesterol depletion, increased sphingomyelin content could still contribute to the maintenance of an ordered plasma membrane structure. Thus it is possible that the increased sphingomyelin content detected in parasites with limited cholesterol levels plays a role in conserving the structural properties of parasite membranes.

Also noteworthy was the increased ceramide content in parasites from P-gp deficient host cells. It is tempting to speculate that this elevation is a compensatory mechanism for the decreased cholesterol in the membranes, as ceramide-cholesterol replacement has been shown to occur in vitro (37). However, the ceramide increase observed does not justify this hypothesis, as similarly for mammalian cells, ceramide in the parasite is about 10 times less abundant than cholesterol. On the other hand, ceramide plays a well known role of second messenger in a variety of cellular processes, including stress response (rev. in (38)), thus it seems more likely that the enlarged ceramide pool helps to fulfill the signaling needs of T. gondii in a host cell environment not optimal for parasite survival. The increased ceramide synthesis observed in labeled parasites isolated from P-gp deficient host cells supports the idea that the content of this lipid is modulated at least in part by an active synthetic process in the parasite. Scavenging of sphingolipids from the host cell can not be excluded at this stage, although it has not been demonstrated to date in this parasite.

While our results clearly indicate that cholesterol transport from the host to the parasite is defective in absence of host P-gp, the data regarding the modality of this transport were quite surprising. So far, T. gondii has been shown to depend on two forms of host-derived cholesterol: free cholesterol contained in endo-lysosomes (22) and esterified cholesterol, present in cytosolic lipid bodies (14). The former requires functional host vesicular trafficking (22,39) and relies on the recruitment of host endo-lysosomes around the PV and their diversion to the PV interior via the formation of H.O.S.T. structures (8). Our data show that P-gp deficient cells were competent for both cholesterol esterification, endo-lysosome recruitment to the PV and H.O.S.T. formation, thus the inhibition of cholesterol trafficking to the parasite cannot be attributed to lack of these processes. However, the observed accumulation of cholesterol in endo-lysosomes in P-gp deficient cells raised the possibility that P-gp contributes to the cholesterol mobilization from lysosomes to the PV, a crucial process for cholesterol trafficking to the PV (8,22). Thus, P-gp in endocytic compartments (25) could
help to mobilize cholesterol from endo-lysosomes either juxtaposed to or internalized in the PV via the H.O.S.T. system. This scenario is also in agreement with the reduced cholesterol content in *N. caninum* when grown in P-gp deficient cells, as the PV of this parasite does not contain H.O.S.T., but is in close proximity with host endo-lysosomes.

Collectively, these results show that host P-gp plays an important role in cholesterol transport to the parasite vacuole. However, there are several observations which imply that P-gp does not control all cholesterol transport to the parasite and that P-gp independent transport pathways must also exist, namely (i) the slight (10%) additional inhibition of parasite replication in DKO cells following U-18666A drug treatment, (ii) the increased parasite replication upon cholesterol loading of DKO cells, and (iii) the parasite’s ability to replicate in DKO cells for several lysis passages.

Considering these results, it seems that the mechanism underlying P-gp mediated cholesterol transport to the parasite is most likely to fall within one of two general scenarios, namely (i) P-gp does not transport cholesterol directly, but is critical in another yet unidentified cellular process essential for cholesterol transport and parasite replication, or (ii) P-gp plays a direct, but seemingly not exclusive, role in cholesterol transport and/or metabolism. The role of P-gp as a transporter of cellular cholesterol has been the subject of considerable debate. While the presence of cholesterol in the membrane surrounding P-gp is crucial for the ATPase activity of the protein (40), results of studies addressing P-gp’s role in cholesterol trafficking have been contradictory. P-gp has been proposed to translocate cholesterol between the plasma membrane leaflets *in vitro* (40). In addition, several reports using chemically selected cell lines or inhibitors associated P-gp over-expression with increased cholesterol transport to the endoplasmic reticulum and consequent esterification (rev. in (41)). However, from these studies it is not clear whether the enhanced cholesterol trafficking observed is due to a direct role of the protein or to an elevated turnover of the cholesterol-rich membranes where P-gp is located (42). Our understanding of P-gp mediated cholesterol transport is further compromised by the contradictory results obtained through the use of P-gp inhibitors with potential side-effects and cell type-specific outcome of P-gp expression (41). In our work using P-gp deficient cells we found that free cholesterol accumulated in vesicular structures located in the perinuclear region and that this accumulation was absent once P-gp activity was restored. In addition, contrary to previous reports using P-gp inhibitors (43,44) or selected MDR cell lines (45), cholesteryl esters accumulated in absence of P-gp. Interestingly, a similar increase in cholesterol esterification was observed in vivo in the liver of P-gp deficient mice (46). Furthermore, we found a positive correlation between cholesterol extractability/efflux from the plasma membrane and P-gp expression. Taken together, our results suggest that P-gp does play a significant role in cholesterol transport in both host-parasite interaction and in normal metabolism of fibroblasts.

Thus, although an indirect effect on cholesterol transport in the absence of P-gp cannot be completely excluded, we observed a number of indications consistent with a model in which P-gp resident in the endo-lysosomes is involved in cholesterol mobilization from these compartments to the PV in case of parasite infection. These indications include: the endo-lysosome recruitment around the PV in infected cells, the cholesterol accumulation outside the PV and the reduced cholesterol supply to the parasite in the absence of host P-gp, and the observation that P-gp mediated parasite inhibition depends on vesicular trafficking of cholesterol. In addition, several alterations in cholesterol metabolism observed in DKO host cells also suggest that P-gp plays a role in cholesterol trafficking from endo-lysosomes to the plasma membrane in uninfected cells. Such observations include the accumulation of free cholesterol in endo-lysosome like structures in the absence of P-gp, the decreased recycling of exogenous cholesterol to the plasma membrane and the increase in cholesterol esterification. On the other hand, increased P-gp expression in host cells correlates with increased cholesterol content in the plasma membrane and efflux, and increased sensitivity to cholesterol loading toxicity.

In summary, our studies using the intracellular parasites *T. gondii* and *N. caninum* provide evidence that host cell P-gp plays a previously unidentified role in host-parasite interaction. Our results reveal that host P-gp is required for
cholesterol transport to the parasite vacuole via a mechanism independent of H.O.S.T. formation, and is also involved in normal cholesterol metabolism in uninfected mammalian fibroblasts.

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**FOOTNOTES**

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Abbreviations: P-gp, P-glycoprotein; MDR, multidrug resistance; PV, parasitophorous vacuole.

**FIGURE LEGENDS**

Fig. 1: Host cell P-gp modulates parasite replication. A. WT, P-gp deficient (DKO) and P-gp complemented (DKO/P-gp) host cells were infected with *T. gondii* at multiplicity of infection (MOI) 1. After 24 hrs post infection (p.i.), intracellular parasites were quantified by direct counting. The distribution of the parasite number in single vacuoles is expressed as percentage of total vacuoles examined (n=30). B. Immunofluorescence analysis of intracellular vacuoles containing parasites at 48 hrs p.i. using anti-*T. gondii* serum, followed by fluorescein-conjugated secondary antibodies. C. Quantification of parasite burden at 48 and 72 hrs p.i. by a colorimetric assay measuring the amount of parasite-expressed β-galactosidase. Results are expressed as percentage of parasite number in WT host cells ± standard error (SE) (n=6). *p < 0.05 and **p < 0.01. D. Host cell monolayers were incubated with
T. gondii at MOI 1. After 2 hrs incubation, 20 fields of infected cells were examined and the number of intracellular invaded parasites determined as described in the material and methods section. Data are expressed as percentage of total parasite number ± SE (n=20).

**Fig. 2:** Host cell P-gp deficiency inhibits cholesterol transport to T. gondii. A. T. gondii-infected host cells were labeled for 5 hrs with 0.5 µCi/mL [3H]cholesterol. Parasites were isolated and [3H]cholesterol incorporation measured by liquid scintillation. Data are expressed as percentage of cholesterol in parasites (dpm/10^6 parasites) isolated from WT host cells ± SE (n=6). B. Host cell monolayers were infected with T. gondii for 24 hrs followed by incubation with NBD-cholesterol and observed by fluorescence microscopy. Asterisks indicate parasite vacuoles. C. After T. gondii invasion, DKO host cells were loaded with the indicated concentrations of cyclodextrin-cholesterol complexes and parasite replication monitored at 48 hrs p.i. as described in Fig. 1. Data are average ± SE (n=3). D. After T. gondii infection, WT and DKO host cells were treated with the class-2 amphiphile U-18666A at the indicated concentrations and parasite replication quantified at 48 hrs p.i. Results are expressed as percentage of parasite number in untreated (cntl) WT cells. Data are average ± SE (n=6).

**Fig. 3:** Host P-gp associates with the parasite vacuole. A. Confocal microscopy of P-gp localization in P-gp complemented (DKO/Pgp) cells infected for 24 hrs with T. gondii. Nuclear DNA was stained with DAPI, PV, parasitophorous vacuole. DIC, differential interference contrast image. B. Confocal microscopy of infected cells as described in panel A, showing P-gp (green) and parasite (red) staining. C. Fluorescence microscopy of infected WT, P-gp deficient (DKO) and P-gp complemented (DKO/P-gp) host cells showing that T. gondii PV associated with labeled host endo-lysosomes (cholera toxin (CTX)), lysosomes (Lamp1) and Golgi (giantin). Asterisks indicate parasite vacuoles.

**Fig. 4:** Cholesterol accumulates outside the parasite vacuole in absence of host P-gp. A. WT, P-gp deficient (DKO) and P-gp complemented (DKO/P-gp) host cell monolayers were infected with T. gondii for 24 hrs. After fixation, the intracellular distribution of unesterified cholesterol was visualized with filipin and observed by fluorescence microscopy. Arrows indicate parasite vacuoles. B. Electron microscopy of T. gondii infected WT and P-gp deficient (DKO) host cells at 24 hrs p.i., showing PV-associated mitochondria (arrowheads) and H.O.S.T. structures (arrows). Right, magnification of H.O.S.T. structures. Scale bars, 2 µm. C. Host cells monolayers were infected with N. caninum and parasite replication quantified at 72 hrs p.i., as described. Results are expressed as percentage of parasite number in WT host cells ± SE (n=3). D. Lipids were extracted from N. caninum isolated from WT or DKO host cells after 4 lysis passages. A representative TLC of lipids corresponding to equal protein amount is shown. Free cholesterol (FC) and cholesteryl esters (CE) were visualized with CuSO₄. Inset, Nile red staining of cholesteryl esters-containing lipid droplets in parasites isolated from WT or DKO host cells after 4 lysis passages.

**Fig. 5:** Host cell P-gp deficiency affects T. gondii lipid metabolism. A. Quantification of free cholesterol (FC) and cholesteryl esters (CE) by enzymatic reaction (Amplex red®) in T. gondii isolated from WT or DKO host cells after 4 lysis passages. Data are expressed as percentage of lipid content in T. gondii from WT cells ± SE (n=5). B. LC-MS analysis of sphingolipids of T. gondii isolated from WT or DKO host cells after 4 lysis passages. Data are average ± SE (n=3) of a representative from two experiments done in triplicate. Cer, ceramide; GlcCer, glucosylceramide; LacCer, lactosylceramide; CerPE, ceramide phosphoethanolamine. The predominantly detected C16 species are plotted. SM, sphingomyelin. C. Left, TLC separation of de novo synthesized lipids labeled with [3H]palmitic acid in T. gondii isolated from WT (1) or DKO (2) cells. Right, newly synthesized ceramide is expressed as percentage of ceramide in T. gondii isolated from WT host cells. Data are average ± SE (n=3).

**Fig. 6:** P-gp expression alters cholesterol metabolism in fibroblasts. A. Filipin labeling of WT, P-gp deficient (DKO) and P-gp complemented (DKO/P-gp) host cells monolayers to visualize the intracellular
distribution of unesterified cholesterol. B. Host cells were labeled with [3H]cholesterol and the time course of [3H]cholesterol esterification measured at the indicated time points. Data are percentage of [3H]cholesteryl esters (CE) of total cellular [3H]cholesterol. C. Cells were labeled with [3H]cholesterol for 24 hrs, washed and incubated with 2 mM methyl-β-cyclodextrin. Medium aliquots were taken at the indicated time points and extracted [3H]cholesterol measured by liquid scintillation. Extracted cholesterol is expressed as percentage of total [3H]cholesterol (medium + cell associated radioactivity). Cells were similarly labeled and incubated with 20 µg/mL reconstituted HDL (D) or 10 µg/mL apolipoprotein A-I (E). Cholesterol efflux is expressed as percentage of total [3H]cholesterol as before. F. Cells were loaded with the indicated concentrations of cyclodextrin-cholesterol complexes for 24 hrs and viability measured by AlamarBlue® assay. Results are expressed as percentage of untreated (cntl) cell viability. All data are average ± SE (n=3).
Figure 1

A

B

C

D

Parasites per vacuole

% Vacuoles

WT

DKO

DKO/P-gp

WT

DKO

DKO/P-gp

I. gordii replication (% of WT)

1. gordii invasion (%)

48 hrs

72 hrs

WT

DKO

DKO/P-gp

WT

DKO

DKO/P-gp
Figure 2

A

B

WT DKO DKO/P-gp

C

D

WT DKO

Chol concentration (μg/mL)

Chol Incorporation (% of WT)

T. gondii replication (A630-595)

T. gondii replication (% of WT ctrl)

C0 ctrl 1 μM 5 μM
Figure 3

A.

B.

C.

WT | DKO | DKO/P-gp
---|-----|------
CTX | *   | *    
Lamp1 | *   | *    
Giantin | *   | *    

* indicates specific locations or regions of interest.
Figure 5
Figure 6

A

WT  DKO  DKO/P-gp

B

CE (% of TOT chol)

Time (hrs)

0  10  20  30  40  50

C

Chol extraction (% of TOT chol)

Time (min)

0  20  40  60

D

Chol efflux (% of TOT chol)

Time (hrs)

0  5  10  15  20

E

Chol efflux (% of TOT chol)

Time (hrs)

0  5  10  15  20

F

Cell viability (% of confluent)

Cholesterol loading (µg/mL)

0  20  40  60