FULL PAPER *Parasitology*

Host Cholesterol Synthesis Contributes to Growth of Intracellular *Toxoplasma gondii* in Macrophages

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ABSTRACT. The intracellular protozoan *Toxoplasma gondii* lacks the ability to synthesize sterol and scavenges cholesterol from the low-density lipoprotein receptor (LDLR) pathway of its host to facilitate replication. Sterol biosynthesis inhibitors, however, have a demonstrated anti-*Toxoplasma* effect. In this study, we examined the host mevalonate pathway as a novel source of cholesterol for *T. gondii* and its effects on parasite growth in macrophages. Parasite growth did not significantly change in the absence of LDLR or when LDL was exogenously supplemented. Lovastatin and compactin, both inhibitors of hydroxymethylglutaryl-CoA (HMG-CoA) reductase in the mevalonate pathway, significantly inhibited *T. gondii* growth in both wild-type and LDLR-knockout macrophages. Parasite growth was also suppressed by squalestatin, an inhibitor of squalene synthase, despite mevalonate producing isoprenoid intermediates in host cells. The present study demonstrates that lovastatin, compactin and squalestatin have anti-*Toxoplasma* activities and that the host cholesterol synthesis may contribute to parasite growth in macrophages.

KEY WORDS: cholesterol, lipid metabolism, macrophage, parasitology, *Toxoplasma gondii*.

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Cholesterol homeostasis is highly regulated in higher eukaryotic cells at transcriptional, translational and post-translational stages [3, 11]. Cholesterol is synthesized in the endoplasmic reticulum (ER) via the key enzyme of the mevalonate pathway, hydroxymethylglutaryl-CoA (HMG-CoA) reductase (EC 1.1.1.88) and further utilized by the cell for membrane biogenesis or synthesis of cholesterol derivatives. Plasma low-density lipoproteins (LDLs) provide another important source of cholesterol. These are internalized by the LDL receptor (LDLR) or scavenger receptors and delivered to late endosomes/lysosomes for hydrolysis. Deposition of excess cellular cholesterol in the form of cholesteryl esters (CE) is catalyzed by the resident ER acyl-CoA: cholesterol acyltransferase (ACAT), leading to the biogenesis of lipid bodies [15, 16].

The obligate intracellular parasite *Toxoplasma gondii* is unable to synthesize sterol and acquires cholesterol from the host LDLR pathway [5]. This ubiquitous eukaryotic parasite is an important opportunistic pathogen of vertebrates and is capable of infecting a wide range of warm-blooded hosts, including humans [14]. *T. gondii* replicates exclusively inside host cells in a specialized, non-fusogenic, parasitophorous vacuole (PV) and requires high levels of selected lipids for membrane biogenesis [18]. While this parasite has the autonomous capacity to synthesize phospholipids, it also readily scavenges precursors of these lipids from the host cell [4, 12]. *T. gondii* is auxotrophic for LDL-derived cholesterol and interference with cholesterol acquisition from the host also impairs parasite growth [5]. Characterization of the CE synthetic enzymes, CE synthesis and ACAT enzymatic activity has been described for *T. gondii* [19, 24] and suggests this parasite incorporates and further metabolizes host cholesterol during infection.

Sterol biosynthesis inhibitors have a demonstrated selective effect on *T. gondii* proliferation. These include the statins (or HMG-CoA reductase inhibitors), a class of drug used to lower plasma cholesterol level, that inhibit the enzyme HMG-CoA reductase. Multiple statins have been reported to have a selective inhibitory effect on *T. gondii*, including simvastatin [7] and the azasterols, which are known inhibitors of Δ24(25)–sterol methyltransferase in protozoa and fungi [8, 9]. Squalene synthase inhibitors and quinuclidine derivatives also produce anti-proliferative effects [17]. However, no enzymes homologous to squalene synthase have been identified in the *T. gondii* database (www.toxodb.org) [17].

Since the pathway of sterol synthesis may represent an important drug target in the development of novel anti-*Toxoplasma* therapies, it is crucial to understand why inhibition of host cholesterol synthesis affects parasite growth. This study therefore aimed to evaluate the effects of host cholesterol synthesis on *T. gondii* growth in the absence of lipoprotein and using statins and squalene synthase inhibitor in LDLR deficient macrophages.

Materials and methods: *Parasite and cell cultures*: *Toxoplasma gondii* strain RH was maintained in human foreskin fibroblast (HFF) cells cultured in Dulbecco's modified Eagle medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS). CHO-K1 cells were cultured in Ham's F-12 medium (Gibco BRL, Grand Island, NY) supplemented with 10%

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heat-inactivated FBS. Tachyzoites were purified using PBS (4°C) to remove host-cell debris. Cell pellets were resuspended in medium (4°C) and passed through a 27-gauge needle and 5.0-μm-pore filter (Millipore, Bedford, MA).

Mice: C57BL/6J female mice (6–7 weeks of age) were obtained from Clea Japan (Tokyo, Japan). LDL receptor knockout (LDLR KO) mice, bred on a C57BL/6J genetic background, were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed under specific pathogen-free conditions in the animal facility of the National Research Center for Protozoan Diseases at the Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan, before experimental use at 7–8 weeks of age. All mice were cared for and used under the Guiding Principles for the Care and Use of Research Animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine.

Reagents: Lovastatin and compactin were purchased from Wako Pure Chemical Industries (Osaka, Japan) and squalestatin was obtained from Sigma (St. Louis, MO). Human LDL (density 1.019–1.063 g/mL) was purchased from Biomedical Technologies Inc. (Stoughton, MA). Lipoprotein-deficient serum (LPDS) was prepared by ultracentrifugation of FBS after the density was increased to 1.215 g/mL with KBr [5]. The cholesterol concentration in LPDS was lower than the minimum detectable level by a commercial detection kit (Cholesterol E-test Wako, Wako Pure Chemical Industries).

Monolayer cultures of peritoneal macrophages: Mouse peritoneal macrophages were collected by peritoneal washing with 5 m*l* of PBS (4°C) four days after intraperitoneal injection of 1 m*l* of brewer modified 4.05 % BBL™ thioglycolate medium (Becton and Dickinson, Sparks, MD). Cells were centrifuged at 800 × *g* for 10 min and suspended in RPMI-1640 medium containing 10% FBS. The macrophages suspension was applied to 24-well tissue culture microplates at 1×10^6 cells/well. Suspensions were incubated at 37°C for 3 hr, washed thoroughly to remove nonadherent cells, and further incubated at 37°C.

Growth analyses of parasite and macrophage: Mouse peritoneal cells (1×10^6) were applied to 24-well tissue culture microplates and after removing nonadherent cells, the adherent cells were infected with *T. gondii* tachyzoites of the (5×10^4). After incubation at 37°C, [5,6-^{3}H] uracil (Moravek Biochemicals, Brea, CA) was added to the plates at 1 μCi/well and the cell mixtures were further incubated for 2 hr at 37°C. Cell mixtures were fixed using 10% trichloroacetic acid for 30 min and further incubated with 0.2 N NaOH for 30 min at 37°C. Parasite radioactivity was measured using a beta counter (Perkin-Elmer, Boston, MA). Macrophage viability was measured by plating cells on 96-well microplates at a density of 2.5×10^5/well and incubating for 20 hr at 37°C. Ten μ*l* of Cell Counting Kit-8 (Dojindo Laboratories, Japan) reagent was added to each well and after a 3 hr incubation at 37°C in 5% CO_2, the optical density was determined at 450 nm (Corona Electric, Tokyo, Japan).

Real-time PCR analysis: Total RNA was extracted from cells using Trizol reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Reverse transcription of 2 μg of RNA was performed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) in a final volume of 25 μL. The PCR mix contained 100 *n*g of reverse-transcribed total RNA, 0.8 μM of both forward and reverse primers, SYBR Green PCR buffer, 2 mM $MgCl_2$, 1 mM dNTP + dUTP, 0.625 U AmpliTaq Gold, 0.25 U AmpErase UNG. PCR was performed using the Applied Biosystems Prism 7700 Sequence Detection System. Relative amounts of all mRNAs were calculated using the comparative C_T method (User Bulletin no. 2, Perkin-Elmer, Boston, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as the invariant control. Specific primers for each gene were designed using PRIMER EXPRESS software (Perkin-Elmer, Boston, MA). The primer sequences (sense and antisense) used were as follows: mouse HMG-CoA reductase, 5'-CTT GTG GAA TGC CTT GTG ATT G-3'and 5'-AGC CGA AGC AGC ACA TGA T-3'; mouse squalene synthase, 5'-CCA ACT CAA TGG GTC TGT TCC T-3' and 5'-TGG CTT AGC AAA GTC TTC CAA CT-3'; mouse LDLR, 5'-AGG CTG TGG GCT CCA TAG G-3' and 5'-TGC GGT CCA GGG TCA TCT-3'; mouse GAPDH, 5'-TGT GTC CGT CGT GGA TCT GA-3' and 5'-CCT GCT TCA CCA CCT TCT TGA T-3'.

RESULTS

Effects of cholesterol from host LDL on T. gondii growth in macrophages and CHO cells: *T. gondii* growth in wild-type and LDLR KO macrophages in medium containing 10% FBS was compared to examine the role of host LDLR. The ^{3}H-uracil incorporation assay revealed that there was no significant difference on the parasite growth between wild-type and LDLR KO macrophages (data not shown). Parasite growth in macrophages was further measured after treatment of macrophages with exogenous LDL in medium containing 5% LPDS to confirm the effects of cholesterol derived from host LDLR-mediated endocytosis (Fig. 1). CHO cells were used as a positive control because it has shown that *T. gondii* growth was enhanced in CHO cells treated with excess LDL [5]. Although exogenous LDL treatment significantly enhanced parasite growth in CHO cells, it did not increase growth in wild type or LDLR KO macrophages. Moreover, the parasite growth was slightly inhibited at 100 μg/mL LDL in both types of macrophages. High levels of cellular cholesterol may affect macrophage viability [13, 26]. Since LDL uptake can occur via LDLR-independent pathway(s), low affinity adsorptive endocytosis, fluid phase endocytosis (pinocytosis) or other receptors may affect *T. gondii* in macrophages treated with 100 μg/mL LDL [25]. We observed that viabilities of wild type and LDLR KO macrophages treated with 100 μg/mL LDL were slightly inhibited (data not shown). These results indicated that LDL-derived cholesterol is not a major contributor to *T.*

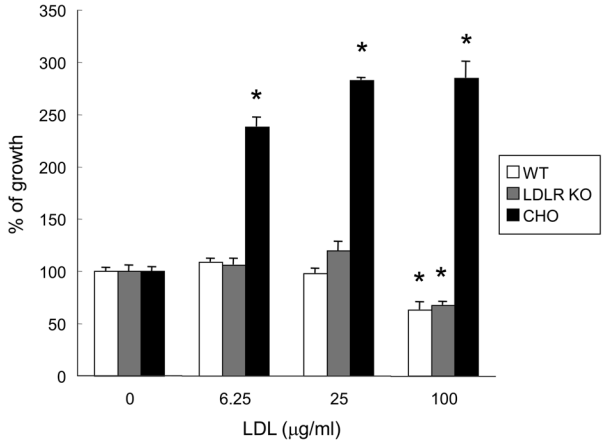

Fig. 1. Uracil incorporation was assayed using CHO cells, wild-type (WT) and LDLR KO macrophages infected with the RH strain of *T. gondii* for 20 hr in medium containing 5% LPDS supplemented with LDL at the indicated concentrations. Data are expressed as a percentage relative to the each control (incubation in the presence of 5% LPDS without LDL), which was taken as 100% ± the standard deviation of triplicate samples. Statistical analysis of the data was carried out using one-way ANOVA followed by Tukey's multiple comparison tests. (*) Values of $P<0.01$ were considered significant. These experiments were repeated three times with similar results.

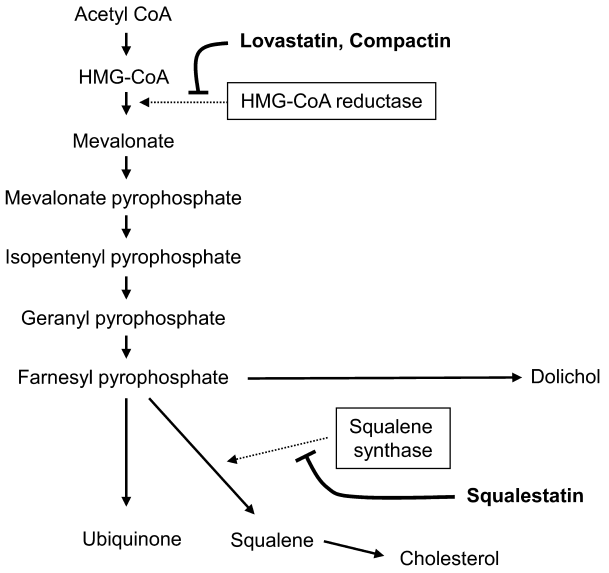

Fig. 2. Cholesterol biosynthesis pathway in mammalian cells. Cholesterol biosynthesis is dependent on the mevalonate pathway that metabolizes HMG-CoA to squalene. HMG-CoA reductase is the rate-controlling enzyme in the mevalonate pathway, and is the target of statins, including lovastatin and compactin. Squalene synthase is the initial committed enzyme in sterol synthesis and is responsible for the reductive condensation of farnesyl pyrophosphate to form squalene. Squalestatin is an inhibitor of squalene synthase. Mevalonate is also a source of isoprenoid intermediates, such as dolichol and ubiquinone.

gondii growth in macrophages.

Effects of host cholesterol synthesis on T. gondii growth in macrophages: To confirm the effects of host cholesterol synthesis on *T. gondii* growth, we examined the expression levels of HMG-CoA reductase, which is a key enzyme in the mevalonate pathway (Figs. 2 and 3A). Real-time PCR data indicated that expression of HMG-CoA reductase in both wild-type and LDLR KO macrophages was not significantly enhanced when infected with *T. gondii* (Fig. 3A). Expression levels of HMG-CoA reductase in infected LDLR KO macrophages were higher than those in infected and control wild-type macrophages. An absence of exogenous cholesterol mediated by LDLR potentially stimulated the host mevalonate pathway in infected LDLR KO macrophages. This was confirmed by treating macrophages with lovastatin and compactin, which both inhibit HMG-CoA reductase (Figs. 2, 4A and 4B). Pre-infection treatment of wild-type macrophages with these inhibitors resulted in a dose-dependent reduction in *T. gondii* growth with IC_{50} values of 17.3 μM and 17.1 μM for lovastatin and compactin, respectively. Although lovastatin and compactin slightly reduced the viability of both types of macrophages, *T. gondii* was more sensitive to the effects of these statins. In addition, a reduction in parasite growth in the treated LDLR KO macrophages was observed (IC_{50} values of 24.3 μM and 28.4 μM for lovastatin and compactin, respectively). Higher IC_{50} values against the parasites in LDLR KO macrophages may be due to the increased expression of HMG-CoA reductase. These results indicated that the host mevalonate pathway is an important cholesterol source for intracellular *T. gondii*. Moreover, *T. gondii* infection did not significantly enhance the expression of LDLR in macrophages (Fig. 3B). These findings suggest that cholesterol synthesized via the host mevalonate pathway could be an important source for *T. gondii* growth in macrophages.

Effects of squalene synthase on T. gondii growth in macrophages: Although cholesterol is the main product of the mevalonate pathway, other products, such as ubiquinone and dolichol, are synthesized from farnesyl pyrophosphate (Fig. 2). To further examine the effects of host sterol biosynthesis on *T. gondii* growth, we focused on squalene synthase (EC 2.5.1.21). The expression levels of squalene synthase in infected and control LDLR KO macrophages were significantly higher than those in infected and control wild-type macrophages (Fig. 3C), suggesting sterol biosynthesis is upregulated in the absence of the LDLR mediated pathway. To confirm the effects of host cholesterol synthesis on *T. gondii* growth, we treated macrophages with the squalene synthase inhibitor, squalestatin (Figs. 2, 5A). The growth of both wild-type and LDLR KO macrophages, pre-treated with squalestatin in medium containing 10% FBS, was slightly enhanced by the inhibitor. Importantly, proliferation of *T. gondii* was inhibited in both types of macrophages pre-treated with squalestatin in medium containing 10% FBS. The growth inhibition was saturated around 30% at 10 μM and 1 μM squalestatin in wild-type and LDLR KO

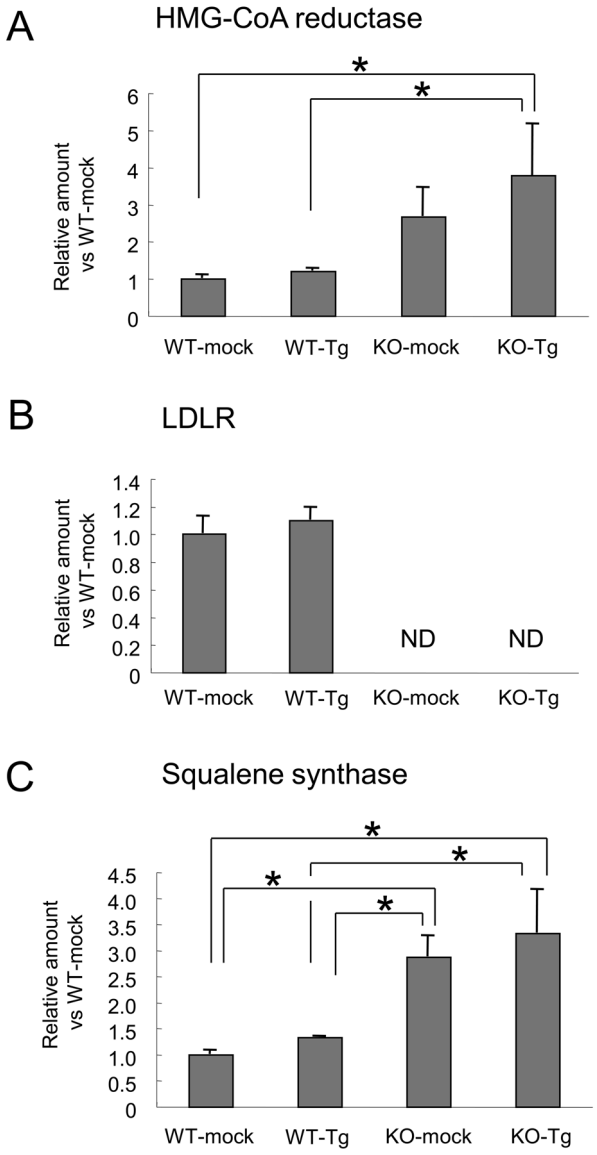

Fig. 3. Real-time PCR analyses of (A) HMG-CoA reductase, (B) LDLR and (C) squalene synthase. Macrophages infected with RH strain of *T. gondii* tachyzoites were cultured in RPMI-1640 supplemented with 10% FBS. At 20 hr after infection, total RNA was isolated from parasitized (Tg), control wild-type (WT) or LDLR KO (KO) macrophages and used for real-time RT-PCR. Values represent the amount of mRNA relative to those in the mock-infected wild-type macrophages, which are arbitrarily assigned a value of 1. Each value represents the mean ± the standard deviation of three independent experiments. Statistical analysis of the data was carried out using one-way ANOVA followed by Tukey's multiple comparison tests. (*) Values of $P<0.05$ were considered significant.

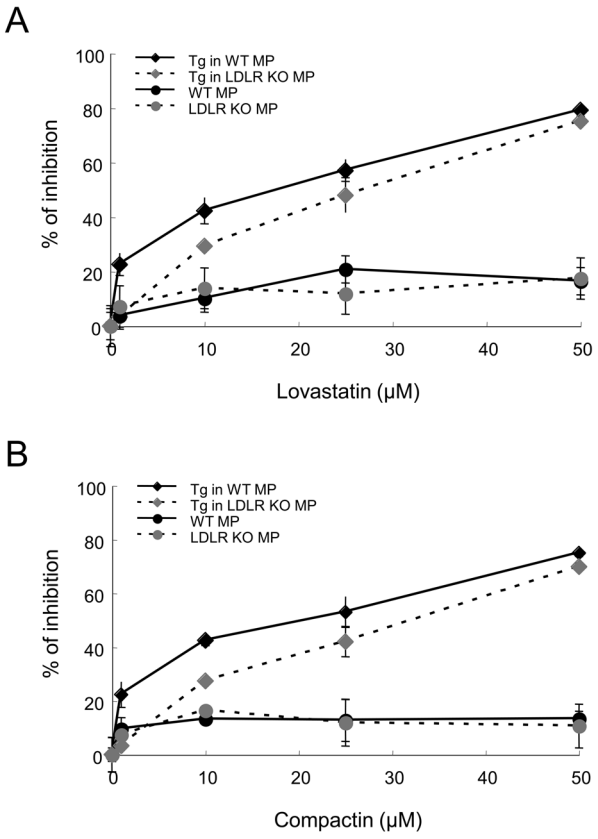

Fig. 4. Effects of lovastatin (A) and compactin (B) on growth of *T. gondii* RH strain in wild-type or LDLR KO macrophages. Macrophages in medium containing 10% FBS were pre-treated with lovastatin or compactin for 1 hr, and then infected or not with *T. gondii* for an additional 20 hr in the presence of inhibitors. Uracil incorporation was assayed to measure *T. gondii* growth. Tg in WT MP; % of inhibition on *T. gondii* in wild-type macrophages, Tg in LDLR KO MP; % of inhibition on *T. gondii* in LDLR KO macrophages, WT MP; % of inhibition on wild-type macrophages, LDLR KO MP; % of inhibition on LDLR KO macrophages. Results were expressed as the percentage of inhibition in relation to the control (in the absence of the inhibitors) used in the experiment as follows: The percentage of inhibition = [(mean value of control) − (value of test sample)] / (mean value of control) × 100. Value of parasite growth, parasite radioactivity; value of macrophage viability, absorbance at 450 nm (See Materials and methods). These experiments were repeated three times with similar results.

macrophages, respectively. Next, the effects of serum growth factors on *T. gondii* growth were reduced by treating macrophages with 5% FBS or 5% LPDS. In wild-type macrophages, the parasite growth was inhibited by the treatment with squalestatin. There was no significant difference on the parasite growth between FBS- and LPDS-cultured cells, suggesting that serum lipoprotein did not affect on the parasite growth in macrophages. In addition, treatment of the infected cells with squalestatin for 44 hr resulted in the higher levels of the growth inhibition compared with the drug treatment for 20 hr. These data suggested that host cholesterol synthesis is important for *T. gondii* growth in macrophages.

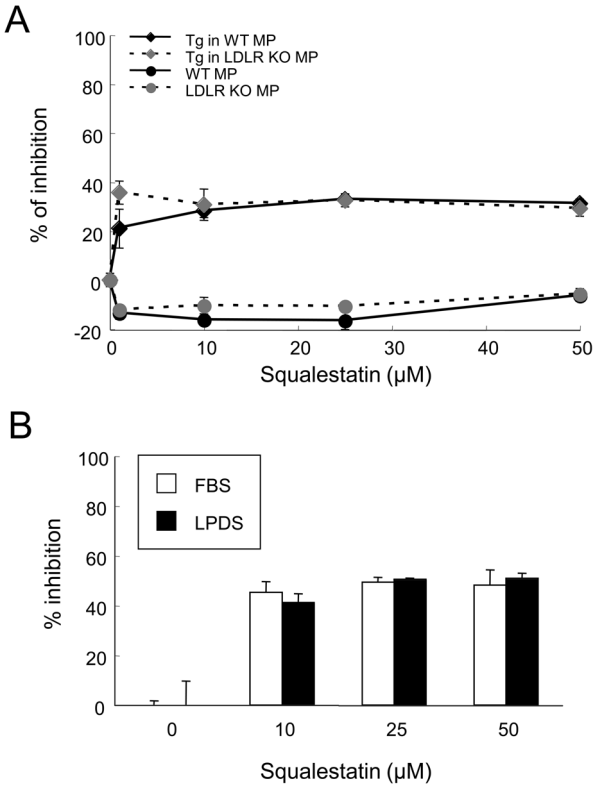

Fig. 5. Effects of squalestatin on growth of *T. gondii* RH strain in wild-type or LDLR KO macrophages. (A) Macrophages in medium containing 10% FBS were pre-treated with squalestatin for 1 hr, and then infected or not with *T. gondii* for an additional 20 hr in the presence of inhibitors. Tg in WT MP; % of inhibition on *T. gondii* in wild-type macrophages, Tg in LDLR KO MP; % of inhibition on *T. gondii* in LDLR KO macrophages, WT MP; % of inhibition on wild-type macrophages, LDLR KO MP; % of inhibition on LDLR KO macrophages. (B) Uracil incorporation was assayed using wild-type macrophages infected with the RH strain of *T. gondii* for 44 hr in medium containing 5% LPDS or 5% FBS treated with squalestatin. Results were expressed as the percentage of inhibition in relation to the control (in the absence of the inhibitors) used in the experiment as follows: The percentage of inhibition = [(mean value of control) − (value of test sample)] / (mean value of control) × 100. Value of parasite growth, parasite radioactivity; value of macrophage viability, absorbance at 450 nm (See Materials and methods). These experiments were repeated twice with similar results.

DISCUSSION

Our study has revealed that pre-infection treatment of macrophages with lovastatin and compactin effectively reduced the growth of *T. gondii*, highlighting the importance of the host mevalonate pathway for parasite replication. De novo cholesterol biosynthesis is dependent on the mevalonate pathway in which HMG-CoA reductase and squalene synthase catalyzes the production of cholesterol during sterol biosynthesis [1]. HMG-CoA reductase is the target for the widely available statin drugs that have cell proliferation and pro-apoptotic effects [21]. A previous study suggested that proliferation of *T. gondii* tachyzoites in macrophages is inhibited by simvastatin [7]. As *T. gondii* lacks the ability to synthesize sterols via the mevalonate pathway, it consequently must scavenge cholesterol from host cells for the synthesis of membranes, phospholipids and fatty acids [5, 24]. Mevalonate is also used for protein isoprenylation and the synthesis of non-sterol products. Statins may therefore affect the production of the non-sterol products dolichol and ubiquinone. Our study focused on the inhibition of squalene synthase in sterol synthesis and revealed that squalestatin inhibited *T. gondii* growth in the presence and absence of lipoproteins or in the absence of LDLR pathway in macrophages. This indicated that cholesterol derived from the host mevalonate pathway plays a crucial role in the intracellular growth.

Previous microarray analysis using *T. gondii*-infected human foreskin fibroblasts (HFF) revealed up-regulation of key genes involved in the mevalonate pathway and cholesterol synthesis but not those involved with dolichol metabolism [2]. This included a 4.3-fold increase in expression level of HMG-CoA reductase [2]. In contrast, it has also been reported that HMG-CoA reductase activity was unchanged in CHO cells infected with *T. gondii*, and that the distribution of PV size was similar between wild-type CHO cells and squalene synthase-deficient mutant cells, indicating that host cholesterol synthesis did not affect *T. gondii* growth [5]. Differences in the cell type used, namely, macrophages, fibroblasts and epithelial cells, may account for the variation in results observed.

Our results suggested that *T. gondii* infection did not significantly upregulate the mRNA expression of HMG-CoA reductase and squalene synthase in both wild-type and LDLR KO macrophages, while the expression levels of the two enzymes were higher in LDLR KO macrophages. *T. gondii* growth was not enhanced in LDLR KO macrophages compared with wild-type macrophages, suggesting that increased transcription levels of the two enzymes did not affect parasite growth. Lovastatin, compactin and squalestatin inhibited *T. gondii* growth between wild-type and LDL KO macrophages and suggested that inhibition of these enzymes decreases parasite growth. Although there still remain the possibility lovastatin, compactin and squalestatin directly affected parasites via unknown mechanism, our findings further suggest that *T. gondii* in macrophages use cholesterol via the host mevalonate pathway for parasite replication.

Previous studies have shown that *T. gondii* exploits host LDLR-mediated endocytosis for cholesterol acquisition from endo-lysosomes [5, 20, 27]. This dependence on exogenous sources of cholesterol suggests the parasite has developed mechanisms for acquiring, transporting and sorting this lipid. The hydrolysis of CE from LDL represents an important step in the internalization of host endo-lysosomes into the PV. Host endo-lysosomes are translocated to the PV membranes and stabilized by secreted parasite coat pro-

teins [6]. Cholesterol generated by the mevalonate pathway exists in the ER. While the mechanisms underlying this translocation of cholesterol from the ER to PVs are unknown, cholesterol scavenging by intravacuolar parasites may result from the PV membrane being in close proximity to the host ER and mitochondria [22]. Transportation of lipids may then occur between host organelles and parasite vacuole membrane [23] and suggests that the presence of cholesterol transporters would be necessary to mediate this transfer. Recently, the ATP-binding cassette G family transporters related to lipid redistribution were identified in *T. gondii* [10] and further characterization of these transporters will allow for a better understanding of *Toxoplasma* biology and the development of novel therapeutic targets.

Currently the standard drug therapies for toxoplasmosis are pyrimethamine and sulfadiazine, and while effective, these produce serious side effects that include bone marrow suppression. Thus, the need for alternative anti-*Toxoplasma* therapies remains. Our study has provided further evidence that inhibitors of host sterol biosynthesis have a selective effect on *T. gondii* growth. However, the lower effects of squalestatin (Fig. 5A) may be due to the cholesterol metabolism in *T. gondii* for the parasite survival. *T. gondii* can storage cholesterol in parasite lipid bodies and uses the stored lipids under lipoprotein starvation [19]. Therefore, a combination of inhibitor to host cholesterol metabolism and antifolates such as pyrimethamine and sulfadiazine will be a potential new approach for the treatment of toxoplasmosis.

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