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Toxoplasma gondii modulates neutral lipid metabolism in macrophage J774 cells

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ABSTRACT

The intracellular protozoan *Toxoplasma gondii* scavenges cholesterol from host cells for its growth. Here, we demonstrated that *T. gondii* modified neutral lipid metabolism in macrophage cell line J774A.1 cells. Cell-surface expression of low-density lipoprotein receptor (LDLR) and the scavenger receptor SR-A were increased upon *T. gondii* infection at 40 hours post infection (hpi). In addition, RT-PCR analyses showed that the infection induced the upregulation of hydroxymethylglutaryl-CoA (HMG-CoA) reductase at 20 hpi and ATP-binding cassette, sub-family A (ABC1), member 1 (ABCA1) at 40 hpi. On the other hand, the downregulation of acyl-CoA:cholesterol acyltransferase 1 (ACAT1) and hormone sensitive lipase (HSL) was observed at 40 hpi. Acyl-CoA: diacylglycerol acyltransferase 1 (DGAT1) expression increased in both infected and uninfected cells at 40 hpi. Accumulation of lipid bodies and high levels of cellular cholesterol and triacylglycerols (TAG) were observed in J774A.1 cells following *T. gondii* infection. These results suggest that intracellular cholesterol may be used for *T. gondii* replication, not for lipid body formation. Our findings support the notion that modulation of the lipid metabolism in host cells is a potential strategy for the treatment and prevention of toxoplasmosis.

Keywords: *Toxoplasma gondii*; cholesterol; triacylglycerols; lipid bodies.

INTRODUCTION

An obligate intracellular parasite *Toxoplasma gondii* replicates only inside a host cell in a specialized nonfusogenic vacuole, parasitophorous vacuole (PV) (Mordue *et al.*, 1999). Successful replication of *T. gondii* within the PV requires considerable amounts of selected lipids for membrane biogenesis. *T. gondii* has the autonomous capacity to synthesize phospholipids, but it also readily scavenges precursors of these lipids from host cells (Charron and Sibley, 2002; Gupta *et al*., 2005). Previous study has shown that *T. gondii* is auxotrophic from low-density lipoproteins (LDL)-derived cholesterol and that interfering with host cholesterol acquisition by *T. gondii* impairs parasite growth (Coppens *et al*., 2000). Although *T. gondii* has no capability to synthesize sterol, sterol esterification has been detected in this parasite (Nishikawa *et al*., 2005). Characterization of cholesterol esters (CE) synthetic enzymes, CE synthesis (Charron and Sibley, 2002; Sonda *et al*., 2001) and acyl-CoA: cholesterol acyltransferase (ACAT) enzymatic activity (Sonda *et al*., 2001) have been described in *T. gondii*. Moreover, two isoforms of ACAT-related enzymes designated TgACAT1α and TgACAT1β have been identified in *T. gondii* (Nishikawa *et al*., 2005). Regarding other esterification in *T. gondii*, one study indicated triacylglycerol (TAG) formation in this parasite occurs through an acyl-CoA:diacylglycerol acyltransferase (DGAT)-mediated pathway and cloning of *Tg*DGAT1 (Quittnat *et al.*, 2004).

Lipid bodies are found in many kinds of cells. Their surface is composed of a phospholipid monolayer containing free cholesterol, and the core is a mixture of lipid esters, mostly TAG and CE (Murphy and Vance, 1999; Tauchi-Sato *et al*., 2002). TAG and CE stored in lipid bodies are used for energy production and synthesis of steroid hormones, respectively. Lipid bodies are thought to be formed in

microdomains of the endoplasmic reticulum (ER), where ACAT and DGAT, the enzymes synthesizing CE and TAG, respectively, are located (Murphy, 2001). *T. gondii* can acquire lipids from the host and modify them to TAG and CE by TgDGAT1 and TgACAT1, respectively, resulting in the formation of lipid bodies in the parasite (Nishikawa *et al*., 2005; Quittnat *et al*., 2004). Currently, their principal function is thought to be as a reservoir of lipids required for membrane biogenesis and energy storage.

Macrophages are phagocytic cells of vertebrates that are widely distributed throughout the body. Macrophages play an important role in neutralizing and removing foreign agents, such as pathogens and cellular debris. The presence of extensive deposits of lipid bodies in macrophages has long been recognized in both normal individuals (Dvorak *et al*., 1983) and those in various pathological states (Schlesinger *et al*., 1982). While some macrophages may contain relatively few lipid bodies, they are characteristically abundant in macrophages that are involved in inflammation, atherosclerosis and neoplasia (Galli *et al*., 1985). Macrophages are the main source of lipid-laden foam cells that are one of the earliest manifestations of atherosclerosis (Small, 1988). Foam cell formation is mediated by the cholesterol imported from LDL that has become oxidized by free radicals. For an intracellular pathogen, exposure of macrophages to *Chlamydia pneumoniae* specifically increases the LDL uptake and foam cell formation by accumulation of CE by a mechanism not involving the LDL receptor (LDLR) (Kalayoglu *et al*., 1999). *T. gondii* might regulate acquisition and synthesis of lipids in macrophages since this parasite uptakes cholesterol via LDL-mediated endocytosis in fibroblasts (Coppens *et al*., 2000). Therefore, our objective is to investigate the lipid metabolism in macrophages following *T. gondii* infection.

MATERIALS AND METHODS

Parasite and cell cultures

The *Toxoplasma gondii* strain, RH, was maintained in monkey kidney adherent fibroblasts (Vero cells) cultured in Eagle's minimum essential medium (EMEM, Sigma, St. Louis, MO) supplemented with 8% heat-inactivated fetal bovine serum (FBS). For the purification of tachyzoites, parasites and host-cell debris were washed in cold PBS, and the final pellet was resuspended in cold medium and passed through a 27-gauge needle and a 5.0-μm-pore filter (Millipore, Bedford, MA). The mouse macrophage cell line J774A.1 (ATCC no. TIB 67), was cultured in Dulbecco's modified Eagle's Medium (DMEM, Sigma) supplemented with 10% heat-inactivated FBS.

Reagents

Purified rat anti-mouse CD16/CD32 (Fcγ III/II receptor) mAb (FcBlock^{TM}) was purchased from BD Pharmingen (San Diego, CA). Anti-LDLR mouse mAb (15C8) and anti-SR-A rat mAb (2F8) were purchased from Calbiochem (Darmstadt, Germany) and Cell Sciences (Canton, MA), respectively. Alexa Fluor® 488 goat anti-mouse or rat IgG were obtained from Molecular Probes (Eugene, OR).

RT-PCR analysis

RNA of the cells was extracted using the TRIzol reagent (Gibco BRL, Grand Island, NY). Reverse transcription of 4 μg RNA was performed using Superscript II Reverse Transcriptase (Gibco BRL) in a final volume of 25 μl. PCR was performed in 50 μl of reaction mixture containing 1 μl of the reverse-transcribed RNA diluted in a buffer consisting of 0.2 mM deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, 2 μM of each primer, and 1 U *Taq* DNA Polymerase (Perkin-Elmer, Boston, MA). After initial incubation for 10 min at 95^{o}C, samples were subjected to cycles of denaturing at 94^{o}C for 1 min, annealing at 55^{o}C for 1 min, and extension at 72^{o}C for 2 min. After 25 cycles, the program executed a final extension of 10 min at 72^{o}C. The final PCR products were electrophoresed on 1.5% agarose gels and visualized using ultra violet (UV) light illumination after ethidium bromide staining. Band intensities were quantified by a

density meter (Luminous Imager version 2.0, Aisin cosmos, Tokyo, Japan). Data represents the relative amount of amplified target mRNA normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample. The primer sequences (sense and antisense sequences) used and the PCR product size in cDNA amplification were as follows: mouse natural cholesterol esterase (NCEase), 5' CTC CTC ATG GCT CAA CTC CTT TCC 3' and 5' AGG GGT TCT TGA CTA TGG GTG 3', 434 bp; mouse hormone sensitive lipase (HSL), 5' GCT GGT GCA GAG AGA CAC 3' and 5' GAA AGC AGC GCG CAC GCG 3', 408 bp; mouse hydroxymethylglutaryl-CoA (HMG-CoA) reductase, 5' GGG ACG GTG ACA CTT ACC ATC TGT ATG ATG 3' and 5' ATC ATC TTG GAG AGA TAA AAC TGC CA 3', 882 bp; mouse acid cholesterol esterase (ACEase), 5' GGC GGA AGA ACC ATT TTG G 3' and 5' ATT GAG AGA CAA CAC GGG AG 3', 415 bp; mouse ACAT1, 5' GGA CAA TGG TGG GTG TGC AC 3' and 5' AGA GTT CCA CCA GTC CTT AT 3', 1 kbp; mouse DGAT1, 5' CTC CTA CTT TGT GTT ATG AAC 3' and 5' GAA TCG GCC CAC AAT CCA 3', 569 bp; mouse HMG CoA reductase, 5' GGG ACG GTG ACA CTT ACC ATC TGT ATG ATG 3' and 5' ATC ATC TTG GAG AGA TAA AAC TGC CA 3', 882 bp; mouse GAPDH, 5' GAG AAC GGG AAG CTT GTC ATC AAT GG 3' and 5' ATG TGA GTC CTT CCA CGA TAC CAA AG 3', 339 bp; mouse ATP-binding cassette, sub-family A (ABC1), member 1 (ABCA1), 5' CAA CTA CAA AGC CCT CTT TG 3' and 5' CTT GGC TGT TCT CCA TGA AG 3', 310 bp.

Flow cytometry

After washing harvested cells (1×10^6) with cold PBS, the cells were suspended in cold PBS containing 0.5% bovine serum albumin treated with FcBlock™ to avoid non-specific adherence of mAb to Fc receptors and subsequently incubated with primary antibodies, followed by Alexa Fluor® 488-conjugated secondary antibody. Labeled cells (1×10^4) were examined using an EPICS ® XL flow cytometer (Beckman Coulter, Hialeah, FL).

Oil Red O staining

Cells were cultured in 4-chamber plates at 1×10^6 cells/chamber. The cells were then washed twice with PBS, fixed with 4% (w/v) paraformaldehyde in PBS, stained with Oil Red O in 60% (v/v) isopropanol.

Measurement of cellular neutral lipid

To measure cellular cholesterol and cholesteryl ester, cellular lipids were extracted using chloroform-methanol (2:1), evaporated and dissolved in isopropanol with 10% Triton®X-100. The cellular cholesterol and cholesteryl ester were quantified by a cholesterol/cholesteryl ester quantitation kit (Calbiochem). Cellular triacylglycerol was measured using a method from Fletcher (1968) with minor modification. The amount of cellular protein was quantified using Lowry Protein Assay Kit (Pierce, Rockford, IL).

RESULTS

T. gondii infection upregulates the expression of LDLR and SR-A

Previous studies have shown that *T. gondii* exploits host LDLR-mediated endocytosis for cholesterol acquisition (Coppens *et al*., 2000; Robibaro *et al*., 2002; Yang *et al*., 2004). Furthermore, *T. gondii* contains CE and TAG synthetic enzymes, following the cloning of *Tg*ACAT1 (Nishikawa *et al*., 2005) and *Tg*DGAT1 (Quittnat *et al*., 2004), respectively. Thus, *T. gondii* infection might modulate the metabolism of neutral lipids in host cells. Acquisition of cholesterol is mediated by LDL internalized by LDLR. In addition, modified LDL promotes its own uptake into macrophages by upregulating the scavenger receptor such as SR-A (Shiffman *et al*., 2000). We therefore hypothesized that *T. gondii*

infection might affect expression of these receptors. To test this idea, flow cytometry analyses were carried out (Fig. 1). As expected, the parasite infection upregulated the expression of LDLR and SR-A in J774A.1 cells at 40 hours-post infection (hpi). These results showed modulation of the expression of host receptors upon *T. gondii* infection.

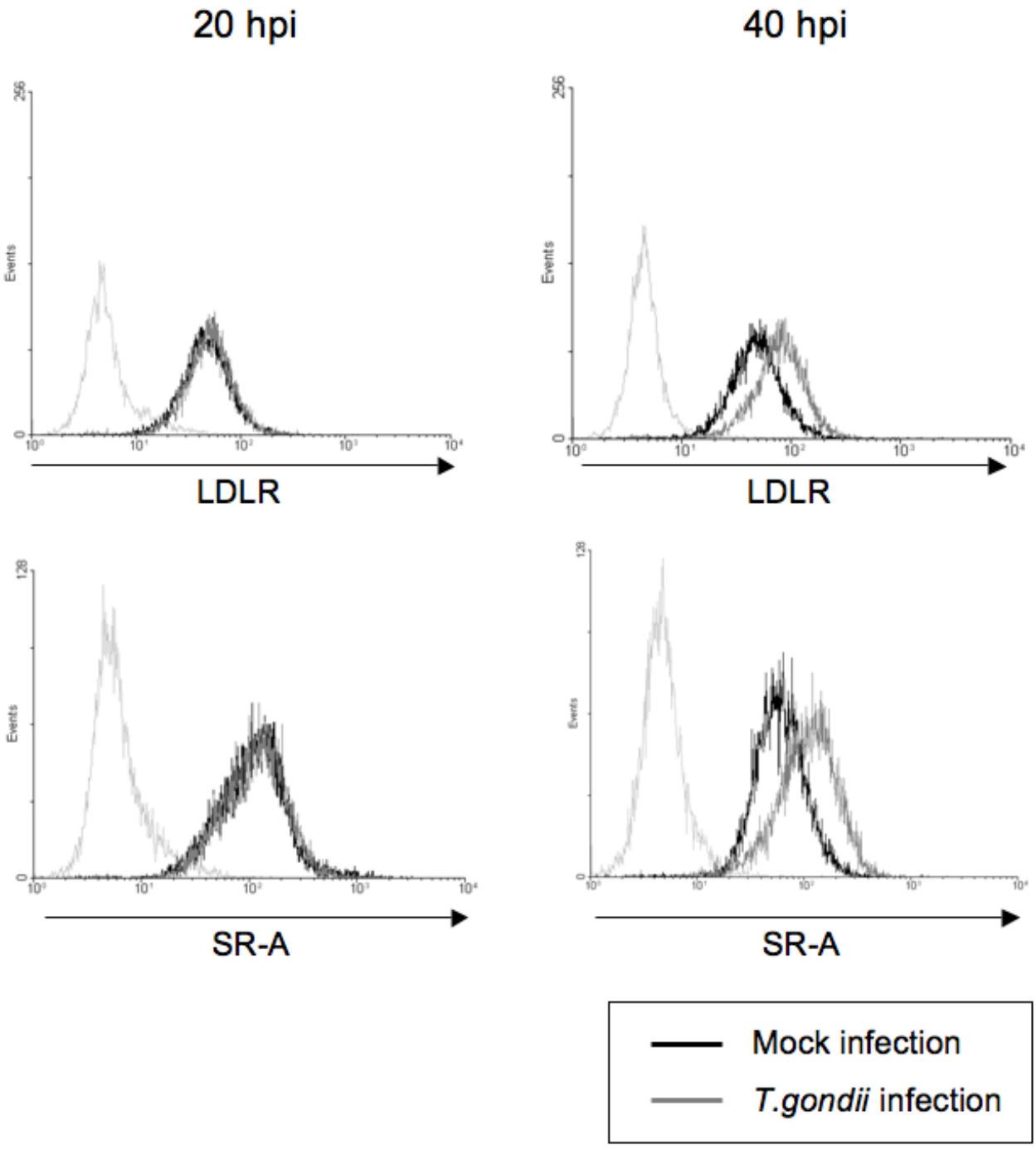

Figure 1. Flow cytometry analysis of J774A.1 cells following *T. gondii* infection (MOI of 1). The cells were collected at 20 and 40 hpi and analyzed using anti-LDLR mAb and anti-SR-A mAb.

T. gondii infection regulates the expression of molecules related to lipid metabolism

Next, we investigated the expression levels of HMG-CoA reductase, ACEase, ACAT-1, DGAT-1, NCEase, HSL and ABCA1 in J774A.1 cells by RT-PCR (Fig. 2). In mammalian cells, LDL internalized by LDLR is delivered to late endosomes/lysosomes for hydrolysis by ACEase. When cholesterol is effluxed from lysosomes, the bulk of cholesterol is transported to the plasma membrane by a Golgi-dependent pathway, while a portion is delivered to the endoplasmic reticulum (ER) by vesicular transport. Deposition of excess cellular cholesterol in the form of CE is catalyzed by ACAT, leading to the biogenesis of lipid bodies. On the other hand, NCEase is responsible for the hydrolytic phase of CE to free cholesterol. ABC proteins such as ABCA transport various molecules across extra- and intracellular membranes. ABCA1, a member of the ABCA subfamily, mediates the efflux of cholesterol and phospholipids to lipid-poor apolipoproteins (apo-A1 and apoE), which then form high density lipoprotein (HDL). In addition, cholesterol is synthesized in the ER by mevalonate pathway via HMG-CoA reductase. Furthermore, TAG synthesized by DGAT in ER is also stored on cytosolic lipid bodies. Translocation of HSL from the cytosol to the surface of the lipid bodies triggers hydrolysis of TAG. Infection with *T. gondii* at multiplicity of infection (MOI, ratio of parasites to cells) of 1 did not show any significant changes in the expression of these molecules (data not shown). To examine the effect of infection dose on the expression levels, J774A.1 cells were infected with the parasite at MOI of 4 for 20 and 40 hours and analyzed (Fig. 2). The expression of HMG-CoA reductase was increased by *T. gondii* infection at 20 hpi, and then decreased at 40 hpi. The infection of *T. gondii* decreased the ACAT1 expression. These results suggest that *T. gondii* infection triggered cholesterol synthesis and that free cholesterol for CE formation might be at a low level. Increased ABCA1 expression suggested the higher levels of cellular cholesterol. The DGAT1 expression was upregulated at 40 hpi in both of the parasite and mock-infected cells. According to the downregulated HSL level in *T. gondii*-infected cells at 40 hpi, excess cellular TAG might exist.

T. gondii infection induces the formation of lipid bodies in J774A.1 cells

Neutral lipids are stored in cytoplasmic lipid bodies, which comprise CE and TAG. When J774A.1 cells were infected with *T. gondii*, the number and size of intracellular lipid bodies were markedly increased in the host cells (Fig. 3A). Heat-inactivated or UV-eradicated parasites did not induce the formation of lipid bodies in J774A.1 cells (data not shown). Therefore, the active infection plays a crucial role for acquisition and synthesis of neutral lipid in host cells. We next examined cellular lipid content in J774A.1 cells following *T. gondii* infection (Fig. 3B). *T. gondii* infection significantly increased intracellular TAG. Unexpectedly, CE level was lower than 1.0 ng/μg cellular protein in both the infected and uninfected cells. In contrast, intracellular cholesterol was increased upon infection with the parasite. Together with the results of RT-PCR (Fig. 2), the high level of intracellular cholesterol in *T. gondii*-infected J774A.1 cells, was caused by acquisition and synthesis of cholesterol via the upregulation of specific receptors and HMG-CoA reductase. Moreover, accumulation of lipid bodies mainly comprised of TAG might regulate the higher level of DGAT1 and lower level of HSL in the parasite-infected cells. Hence, increased intracellular cholesterol might be used for *T. gondii* replication, not for lipid body formation.

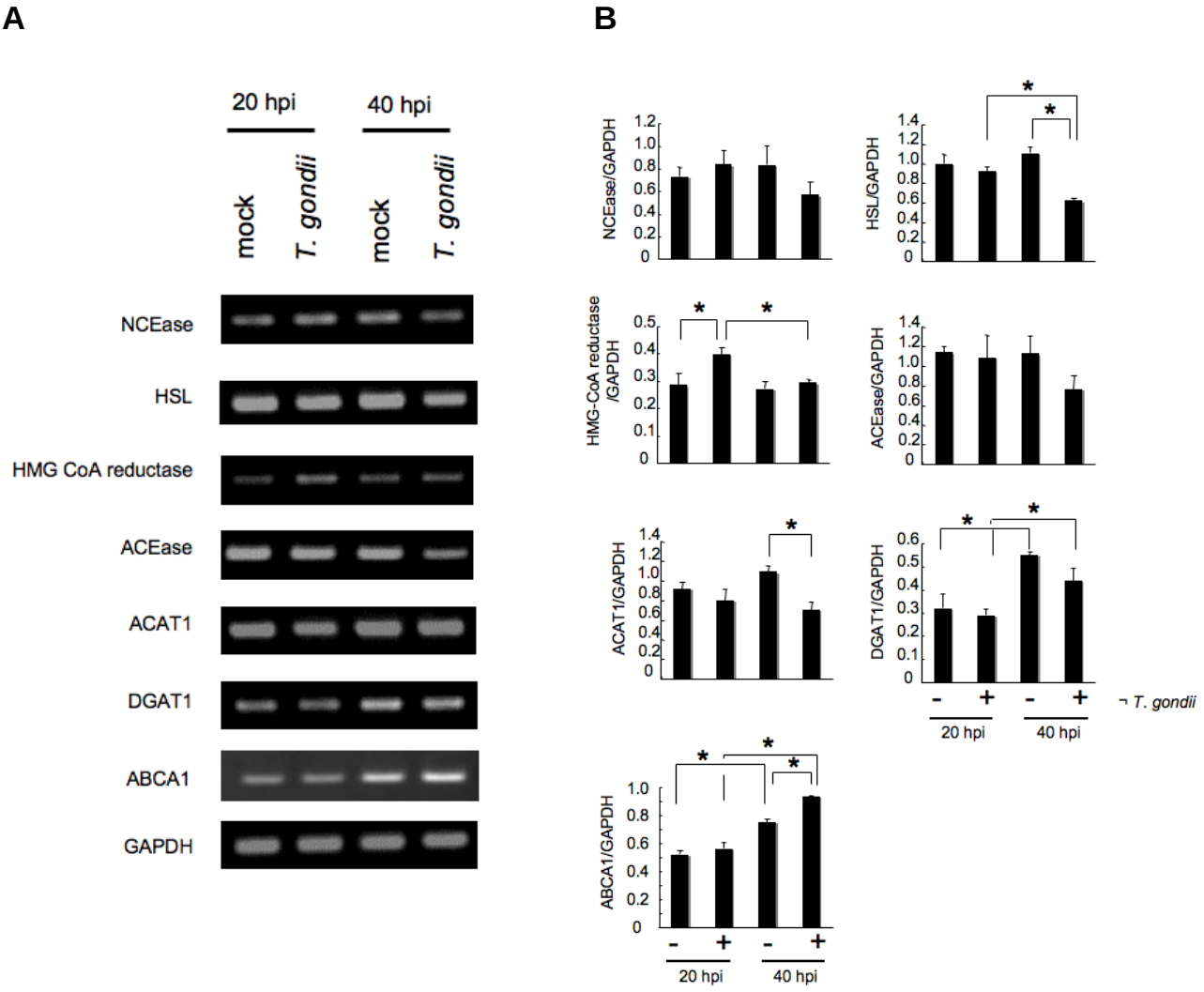

Figure 2. (A) RT-PCR analyses for NCEase, HSL, HMG-CoA reductase, ACEase, ACAT1, DGAT1, ABCA1 and GAPDH transcript levels. At 20 and 40 hpi (MOI of 4), total RNA was isolated from parasitized or mock-infected J774A.1 and used for RT-PCR. (B) Band intensities were quantified by densitometry. Bars represent the relative amount of amplified target mRNA against GAPDH mRNA in the same sample. Each value represents the mean of the target mRNA/GAPDH mRNA ± the standard deviation of triplicate samples. Statistical analysis of the data was carried out using 1-way ANOVA followed by Tukey's multiple comparison test. (*) Values of $P<0.05$ were considered significant.

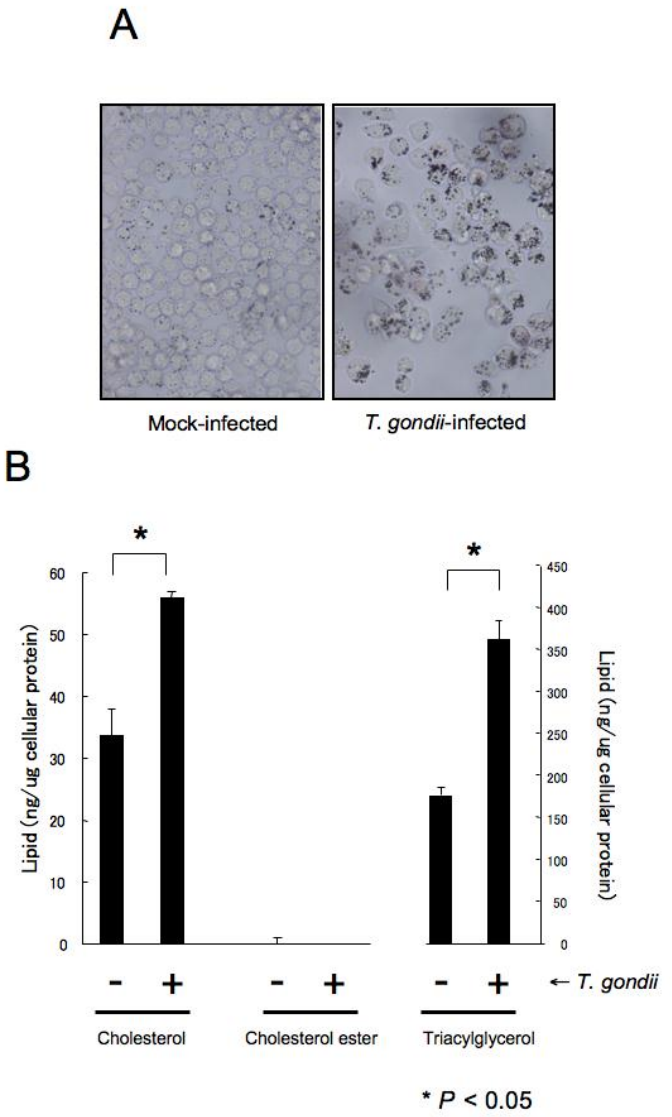

Figure 3. (A) Oil Red O staining of *T. gondii*-infected J774A.1 cells. J774A.1 cells were infected with *T. gondii* (MOI of 1) for 40 hours. Fixed cells were stained with Oil Red O. (B) Cellular lipid content following *T. gondii* infection. J774A.1 cells were infected with *T. gondii* (MOI of 1) for 40 hours. Intracellular cholesterol, CE and TAG were measured as described in Materials and Methods. Each value represents the mean ± standard deviation of triplicate samples. Statistical analysis of the data was carried out using Student's t-test. (*) Values of $P<0.05$ were considered significant.

DISCUSSION

T. gondii cannot synthesize sterols via the mevalonate pathway and, therefore, must obtain them from the host cells (Coppens *et al*., 2000). There are three mechanisms that host cells employ to replenish depleted cholesterol stores: 1) stimulation of *de novo* cholesterol biosynthesis via the mevalonate pathway; 2) enhanced LDL uptake; or 3) mobilization of intracellular CE. *De novo* cholesterol biosynthesis is dependent on the mevalonate pathway that metabolizes HMG-CoA to squalene. HMG-CoA reductase is a key enzyme in the mevalonate pathway. The level of HMG-CoA reductase mRNA was increased in macrophages 20 hours after *T. gondii* infection. This result suggests that the parasite infection might induce cholesterol synthesis in host cells. A similar result by microarray analysis was observed in human foreskin

fibroblasts infected with *T. gondii* (Blader *et al*., 2001). For enhanced LDL uptake, expression of LDLR and SR-A were upregulated upon *T. gondii* infection at 40 hpi. Our data suggest that infection with *T. gondii* induces macrophages to take up native LDL and partially modified LDL in this model. Because of the low levels of CE in the infected cells, major parts of cellular cholesterol would be used for parasite growth.

T. gondii infection induced lipid body formation in macrophages at 40 hpi. Unexpectedly, the lipid bodies mainly contained TAG. This observation could be explained by the results of RT-PCR. The balance of DGAT and HSL expression might affect the accumulation of the lipid bodies. TAG-rich cytosolic lipid bodies are accumulated following stimulation by inflammatory lipid mediators including arachidonic acid, platelet-activating factor and mitogenic phorbol esters (Triggiani *et al*., 1995; Yu *et al*., 1998). Exogenous arachidonic acid is rapidly taken up by macrophages and esterified to lipid body-TAG, which is its major long-term store (Triggiani *et al*., 1994). In fact, *T. gondii* infection triggers the production of the derivatives of arachidonic acid oxidation including eicosanoid in mouse peritoneal macrophages (Thardin *et al*., 1993). However, the effects of intracellular TAG on *T. gondii* growth are still unknown. Since extracellular *T. gondii* could incorporate DAG into TAG (Quittnat *et al*., 2004), studies using extracellular tachyzoites or compounds altering TAG synthesis may provide important insights.

In conclusion, *T. gondii* modulates neutral lipid metabolism in macrophage J774 cells. Accurate understanding of the lipid metabolism by *T. gondii* infection is useful for a potential strategy for the treatment and prevention of toxoplasmosis.

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