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Research Article

RESEARCH ARTICLE

Chlamydia trachomatis growth depends on eukaryotic cholesterol esterification and is affected by Acyl-CoA:cholesterol acyltransferase inhibition

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One sentence summary: This manuscript shows that cholesterol esterification by the important eukaryotic enzyme Acyl-CoA:cholesterol acyltransferase (ACAT) is necessary for intracellular *Chlamydia* growth.

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ABSTRACT

Chlamydia trachomatis is auxotrophic for a variety of essential metabolites. Inhibitors that interrupt host cell catabolism may inhibit chlamydial growth and reveal *Chlamydia* metabolite requirements. We used the known indoleamine-2,3-dioxygenase (IDO)-inhibitor 4-phenyl imidazole (4-PI) to reverse Interferon (IFN)-γ-induced chlamydial growth inhibition. However, at elevated inhibitor concentrations chlamydial growth was arrested even in the absence of IFN-γ. Since 4-PI is known to interfere with cholesterol metabolism, the effect of cholesterol add-back was tested. *Chlamydia* growth was restored in the presence of cholesterol in serum-containing, but not serum-free medium suggesting that cholesterol and other serum components are required for growth recovery. When serum factors were tested, either cholesteryl linoleate or the combination of cholesterol and linoleic acid restored chlamydial growth. However, growth was not restored when either cholesterol or linoleic acid were added alone, suggesting that the production of cholesteryl esters from cholesterol and fatty acids was affected by 4-PI treatment. In eukaryotic cells, the enzyme Acyl-CoA:cholesterol acyltransferase (ACAT) catalyzes the production of cholesteryl esters. When HeLa cells were treated with the ACAT-specific inhibitor 4-hydroxycinnamicacid amide *C. trachomatis* growth was interrupted, but was restored by the addition of cholesteryl linoleate, suggesting that ACAT activity is necessary for intracellular *Chlamydia* growth.

Keywords: cholesteryl esters; sterol o-acyltransferase

INTRODUCTION

The obligate intracellular bacterial pathogen *Chlamydia trachomatis* is responsible for ocular or genital infections in humans. The *C. trachomatis* serovars D–K are the worldwide leading cause of bacterial sexually transmitted diseases with more than 1.4 million newly reported cases in the USA alone in 2012 (STD surveillance report 2012, CDC: http://www.cdc.gov/std/stats12/default.htm, 13 April, date last accessed)

The unique biphasic developmental cycle with two morphologically distinguishable forms is characteristic for *Chlamydia*. The extracellular elementary bodies (EBs) exhibit limited metabolic activity and are the infectious form of *Chlamydia*. Once EBs invade the host cell they are found in a membrane-bound

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compartment called the inclusion. Here, the EBs transform into metabolically active, but non-infectious reticulate bodies (RBs). After multiple rounds of replication RBs convert back to the infectious EBs, which are released either by cell lysis or by extrusion and can invade new eukaryotic cells (Hybiske and Stephens 2007).

The *Chlamydia* genome is small (~1 Mbp) compared to most other bacteria and reflects the adaptation to an obligate intracellular life style with an auxotrophy for many metabolites (Stephens *et al.* 1998; Kalman *et al.* 1999; Read *et al.* 2000; Read *et al.* 2003; Horn *et al.* 2004; Azuma *et al.* 2006; Thomson *et al.* 2008) that are obtained from the eukaryotic host during infection. One important group of metabolites obtained from the host cell is lipids. Although *Chlamydia* produce some lipids on their own (phosphatidylethanolamine, phosphatidylglycerol and phosphatidylserine), others (phosphatidylcholine, phosphatidylinositol, sphingomyelin, cardiolipin and cholesterol), are transported from various sources within the host cell into the inclusion and are acquired by RBs (reviewed in Elwell and Engel 2012). For example, it is known that exogenous lipids may be obtained via receptor-mediated uptake (e.g. low-density lipoprotein (LDL), CD36 scavenger receptor), which are re-routed to the inclusion (Kalayoglu *et al.* 1999). Other eukaryotic *de novo* synthesized lipids are acquired by *Chlamydia* by manipulating exocytic pathways and re-routing lipid-containing vesicles to the inclusion. Beatty (2006) demonstrated that multivesicular bodies are re-directed from the host cytoplasm to the inclusion. In addition, a Brefeldin A-independent transport of Golgi particles has also been reported (Wylie, Hatch and McClarty 1997; Heuer *et al.* 2009). Lipid droplets can be found in the lumen of inclusions (Kumar, Cocchiaro and Valdivia 2006; Cocchiaro *et al.* 2008), which are transported to the inclusion in association with peroxisomes (Boncompain *et al.* 2014). The lipid transporter ABCA1 and the lipid acceptor protein ApoA1 also are recruited to the inclusion (Cox *et al.* 2012).

In human cell lines Interferon (IFN)-γ-induced persistence in *Chlamydia* is mainly a result of the depletion of tryptophan due to the induction of the tryptophan de-cyclizing, heme-containing enzyme indoleamine-2,3-dioxygenase (IDO) (Byrne, Lehmann and Landry 1986). To study IDO-independent effects of IFN-γ on *Chlamydia* growth, we used the IDO-inhibitor 4-phenyl imidazole (4-PI), but at higher concentrations the inhibitor blocked chlamydial growth independent of IFN-γ treatment. 4-PI is known to target enzymes other than IDO, including members of the cytochrome P450 family (McLean *et al.* 2002). In addition, derivatives of 4-PI inhibit the eukaryotic enzyme Acyl:CoA: cholesterol o-acetyltransferase and these chemical inhibitors are used as treatment for hypercholesterolemia (Kimura *et al.* 1993). We found that chlamydial growth inhibition by 4-PI was reversed by cholesterol addition in serum-containing, but not in serum-free medium. We identified phosphocholine and cholesteryl linoleate, as essential components in serum for *Chlamydia* growth recovery. Interestingly, cholesterol and linoleic acid reversed the inhibitory effect of 4-PI only when added together to the medium. When we inhibited the enzyme that catalyzes cholesterol esterification in eukaryotic cells, Acyl-CoA:cholesterol acyltransferase (ACAT), with the specific ACAT-inhibitor 4-hydroxycinnamicacid amide (4-HCAA), we observed growth reduction of three different *C. trachomatis* strains representing each *C. trachomatis* biovar. Taken together, our data demonstrate that *C. trachomatis* requires cholesterol esterification by eukaryotic ACAT before it is available for chlamydial growth.

MATERIALS AND METHODS

Chemicals

IFN-γ was purchased from R&D Systems (Minneapolis, MN) and the ACAT-inhibitor 4-HCAA (chemical name: N-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]-L-phenylalanine, methyl ester) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Unless stated otherwise all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All lipids were dissolved at a concentration of 1 mg ml^{-1} in medium containing 200 μg ml^{-1} methyl-β-cyclodextrin.

Cell culture and bacterial strains

The human epithelial cell line HeLa 229 (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco's modified Eagle medium (DMEM, Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum, 0.01 mg ml^{-1} gentamicin (Gibco, Grand Island, NY), 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM Hepes and 0.055 mM β-mercaptoethanol at 37°C with 5% CO_2 in a humidified atmosphere. For some experiments, the cell culture medium was used without the addition of FBS. Two different media were used in the course of this work: DMEM (full) refers to serum (FBS) containing; and DMEM (-serum) for FBS-free medium. *Chlamydia trachomatis* serovar D/UW-3Cx and *C. trachomatis* serovar A/HAR-13 were routinely grown in HeLa cells at 37°C, 7% CO_2 and stocks were purified as previously described (Schachter and Wyrick 1994). *Chlamydia trachomatis* serovar L2/434/Bu was kindly provided by R. Belland (UTHSC Memphis, TN).

Inhibitor treatment and *C. trachomatis* infection of HeLa cells

HeLa cells were seeded at a density of 3.75 × 10^4 cells per well in 48 well plates 48 h prior to infection. Twenty four hours before infection, the medium was removed and replaced with DMEM containing 50 ng ml^{-1} IFN-γ or inhibitor solution. Unless stated otherwise experiments were done with inhibitor concentrations of 1 mM 4-PI or 200 μM 4-HCAA. At the day of infection, the medium was transferred to a tube (maintained medium), the cells were washed in HBSS (Gibco, Grand Island, NY) and incubated with 1 × DEAE-Dextran in HBSS for 10 min at 37°C. DEAE-Dextran was removed, the cells were washed in HBSS, and infected with *C. trachomatis* diluted in sucrose-phosphate-glutamic acid buffer (SPG, 0.22 M sucrose, 3.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 5 mM L-glutamic acid; pH7.4). Cells were infected with an MOI of 0.5 for immunofluorescence or an MOI of 1 for titration experiments, respectively. Infected cells were centrifuged at 1200 rpm for 1 h at 30°C and incubated for 30 min at 37°C. The inoculum was removed (start of infection), and the maintained medium was returned. The infected cells were further incubated at 37°C with 7% CO_2 until either fixed in methanol for immunofluorescence or harvested for titration.

Immunofluorescence microscopy and EB titration

For fixation the cells were washed in PBS at the indicated times post infection (p.i.), and fixed in methanol for 10 min at RT. *Chlamydia trachomatis* was identified by immunofluorescence microscopy using a *C. trachomatis* specific staining kit (Argene, North Massapequa, NY) as described by the manufacturer's protocol. HeLa cells were counterstained with 2 μg ml^{-1} Evans blue.

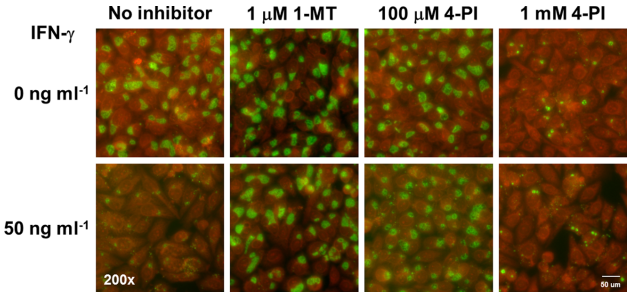

Figure 1. The IDO-inhibitor 4-PI inhibits *C. trachomatis* growth independent of IFN-γ. Cell were left untreated (upper panel) or pre-treated with 50 ng ml^{-1} IFN-γ treated (lower panel) and incubated for 24 h in the absence or presence of either 1 μM L-1-methyl tryptophan (1-MT), 100 μM or 1 mM 4-Phenyl imidazole (4-PI). The cells were infected after 24 h with an MOI of 1 and were fixed in methanol after 24 h p.i. The fixed cells were stained with an FITC-conjugated antibody against *C. trachomatis* and counterstained with Evans blue. Representative images (200× magnification fluorescence) are shown.

The stained cells were examined with a Nikon Eclipse TE200-U fluorescence microscope using a 200× magnification. Images were obtained with a Retiga 1300 cooled 12-bit camera (QImaging, Surrey, Canada) using the IPLab software version 3.9.2 (Becton, Dickinson and Company, Franklin Lakes, NJ). For determination of inclusion forming units (IFU) cells were harvested at indicated time points, sonicated, pelleted by centrifugation at 14 000 rpm at 4°C for 20 min and resuspended in SPG buffer. Fresh HeLa cells were infected with dilutions as described above. After 24 h, the cells were fixed in methanol and stained with Giemsa stain for IFU determination as described in (Conn *et al.* 1960).

Statistics

All experiments were done with three biologically independent replicate samples and experimental data is represented as mean ± standard deviation (SD). All statistic calculations were done in Excel using a one-tailed paired Student's *t*-test to compare two groups of data or ANOVA to compare more than two groups. Only *P* values ≤ 0.05 were considered significant.

RESULTS

The IDO-inhibitor 4-PI, but not L-1-methyl tryptophan, blocks chlamydial growth

The original intent of the study was to study IDO-independent effects of IFN-γ induced persistence. We therefore treated HeLa cells with IFN-γ with or without the addition of IDO inhibitors at concentrations of 10 nM, 100 nM and 1 μM (1-MT); or 100 μM and 1 mM (4-PI), and infected the cells 24 h post-treatment with *C. trachomatis* serovar D as described in the section 'Material and Methods'. Treatment with IFN-γ resulted in growth inhibition of *C. trachomatis* D with reduced and abnormal inclusion size (as seen in Fig. 1) consistent with persistent *Chlamydia* morphology (Beatty, Byrne and Morrison 1993). Cotreatment with 1-MT at 1 μM and 4-PI at 100 μM led to large inclusion with normal morphology suggesting that addition of the inhibitors blocked the effect of IDO. Interestingly, growth of the chlamydiae was significantly reduced at higher concentration of 4-PI (1 mM) and showed smaller inclusions compared to untreated cells. Growth reduction was observed with or without IFN-γ demonstrating an IFN-γ and IDO independent growth inhibition in the presence of this inhibitor.

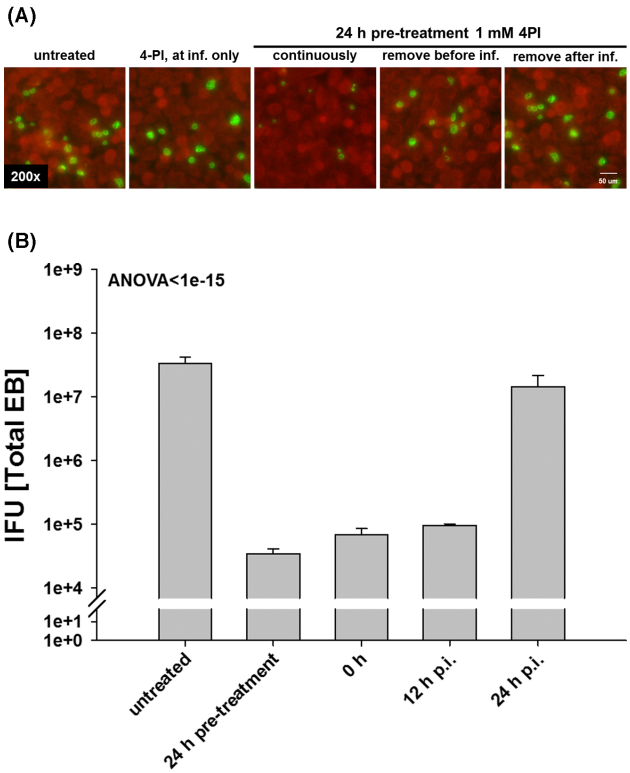

Figure 2. 4-PI inhibits *C. trachomatis* growth when added before EB to RB transition. HeLa cells were treated at different time points prior to or after infection with *C. trachomatis* D. (**A**) HeLa cells were left untreated, pre-treated for 24 h with 1 mM 4-PI, inhibitor added only during or directly after infection. The cells were infected with *C. trachomatis* with an MOI of 0.5 and were methanol fixed 48 h p.i. *Chlamydia trachomatis* was stained with an FITC-conjugated antibody to *C. trachomatis* and cells counterstained with Evans blue. Representative images (200× magnification fluorescence) are shown. (**B**) HeLa cells were left untreated, pre-treated for 24 h with 1 mM 4-PI, or the inhibitor added directly after infection (0 h), 12 h or 24 h p.i. The cells were infected with an MOI of 1 and harvested after 48 h p.i. Fresh HeLa cells were infected with dilutions to titrate the *C. trachomatis* IFU. All titrations are the mean of three biologically independent replicate samples and error bars represent SD.

Inhibition of 4-PI reduces chlamydial growth during the metabolically active phase

To identify at which stage during the chlamydial developmental cycle chlamydiae are affected by 4-PI, we added 4-PI at different time points during the infection and varied the length of treatment time. We first analyzed if 4-PI affected chlamydial attachment or invasion. For some samples, we pre-treated the cells for 24 h before the infection and either removed 4-PI before or after the infection. For another set of samples the inhibitor was only present during the time of infection. We infected the cells, fixed the cells at 24 h p.i., and stained for *C. trachomatis*. As Fig. 2A shows, only cells pre-treated with 4-PI in the continuous presence of the inhibitor after infection showed inhibited growth. We next tested if 4-PI inhibited the chlamydiae at later stages of the developmental cycle. We pre-treated cells for 24 h with 4-PI, or added the inhibitor after 0, 12 or 24 h p.i. and harvested EBs after 48 h p.i. Fig. 2B shows that the amount of recovered EBs was only reduced when the inhibitor was added at 12 h p.i. or earlier. Treatment after 24 h p.i. showed similar IFU production as did infections of untreated cells. It is known that *C. trachomatis* converts from EBs to RBs at around 12 h p.i. (Miyairi *et al.* 2006) and

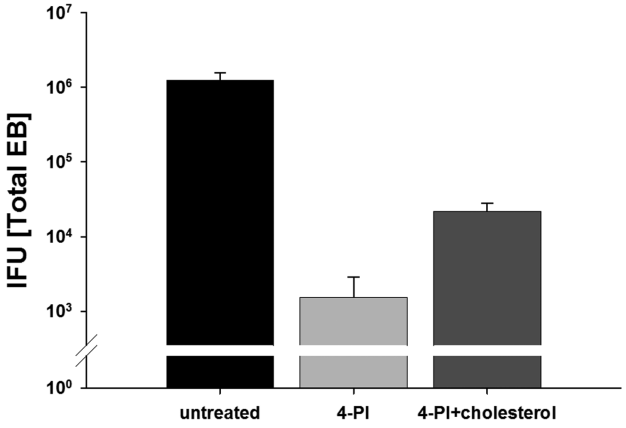

Figure 3. 4-PI induced growth inhibition can be restored after removing the inhibitor or adding exogenous cholesterol. *Chlamydia trachomatis* infected cells with or without 1 mM 4-PI were incubated for 24 h and the medium exchanged with inhibitor, with inhibitor and 25 μg ml^{-1} cholesterol, or medium without inhibitor and further incubated for additional 24 h. Cells were harvested 48 h p.i. and HeLa cells fresh infected with dilutions of the harvested samples to titrate the *C. trachomatis* IFU. All titrations are the mean of three biologically independent replicate samples. Error bars represent SD.

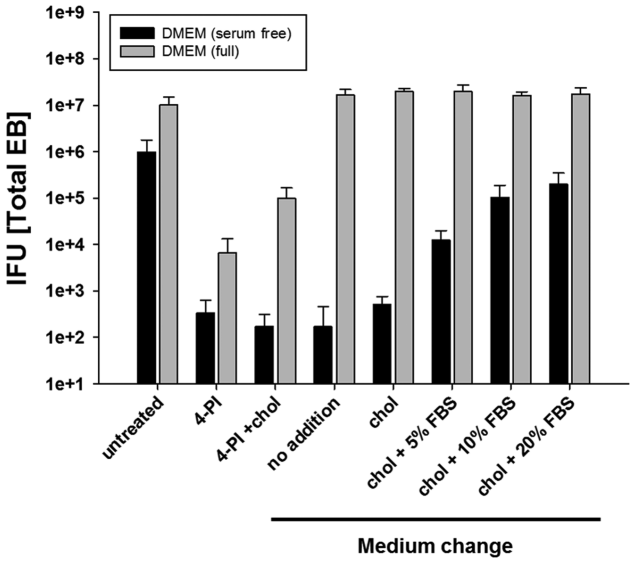

Figure 4. Serum is required to reverse the 4-PI-induced *C. trachomatis* growth inhibition. HeLa cells were left untreated or pre-treated for 24 h with 1 mM 4-PI in serum-free or serum-containing complete medium. The cells were infected with *C. trachomatis* with an MOI of 1 and after 24 h p.i. cells either maintained in medium or the medium replaced. Cholesterol without or with different concentrations of FBS in the medium was added as supplement. Cells were harvested 48 h p.i. and fresh HeLa cells infected with dilutions of the harvested samples to titrate the *C. trachomatis* IFU. All titrations are the mean of three biologically independent replicate samples and error bars represent SD.

that many chlamydiae convert back to EBs by 24 h p.i. These data indicate that the 4-PI induced growth inhibition affects *Chlamydia* during the metabolically active phase of RB development.

4-PI affects cholesterol-dependent metabolic pathways during chlamydial infections

It is known that 4-PI and its derivatives affect enzymes that are part of the catabolism of cholesterol and other sterols. To test if cholesterol depletion is a limiting factor for chlamydial growth after 4-PI treatment, we first inhibited chlamydial growth with 4-PI. The medium was then removed from the cells after 24 h p.i. and either 25 μg ml^{-1} cholesterol was added directly to inhibitor containing medium or replaced with fresh cell culture medium without inhibitor. We harvested the cells 24 h after rescue and determined the recovered EBs by IFU measurement. The addition of cholesterol to the inhibitor-containing medium partially restored chlamydial growth as shown in Fig. 3. These results demonstrated that cholesterol depletion is one limiting factor for chlamydial growth inhibition during 4-PI treatment.

Fetal bovine serum contains components that are required for *Chlamydia* recovery after 4-PI inhibition

To further characterize the nature of the 4-PI inhibition and to identify a potential enzyme target for the inhibitor, we used serum-free medium for better defined growth conditions. Surprisingly, chlamydial growth did not resume after 4-PI induced inhibition for infected cells cultured in serum-free medium, while the addition of cholesterol in complete medium resulted in an increase of infectious EBs (data not shown). To test if serum was required for cholesterol mediated recovery, we pre-treated cells with 4-PI in complete or serum-free medium. For recovery, we either replaced the medium completely or added cholesterol directly to the inhibitor containing medium (Fig. 4). The replaced medium had either no additional supplements or contained cholesterol with final concentrations of FBS at 0%, 5%, 10% or 20% (v/v). When cholesterol in serum-free medium alone was used to recover the growth inhibition, no recovery was observed.

However, fresh serum-containing complete medium without the addition of cholesterol also restored chlamydial growth, demonstrating that FBS contains the necessary components to reverse the effect of 4-PI inhibition. In addition, we found that 4-PI induced growth inhibition could be reversed when cholesterol was added to the inhibitor in complete medium in an FBS-dose-dependent manner verifying that FBS contains components that are required to restore chlamydial growth after 4-PI inhibition. Collectively, the data suggest that additional components in serum are necessary for cholesterol dependent rescue.

Vitamins and lipids in serum contribute to chlamydial recovery after 4-PI inhibition

FBS contains many different possible components such as proteins, hormones, salts, lipids and vitamins. We concentrated on nutrition groups known to be required by *Chlamydia* (salts, lipids, vitamins) although we are aware that other components in serum may be also required for recovery after 4-PI induced growth inhibition. We first used pools of the tested supplements. For the pool of salts, we used $CaCl_2$, $FeCl_2$, $FeCl_3$, $CuSO_4$ and $MgCl_2$ at final concentration of 25 μg ml^{-1} for each individual salt. For vitamins, we used a commercially available mix of all vitamins required by eukaryotic cells (RPMI 1640 100 $\times$ vitamins, Sigma). The lipids tested were a mix of phosphocholine, cholesterol, linoleic acid, cholesteryl linoleate and glyceride trilinoleate at a final concentration of 25 μg ml^{-1}. These additions represent lipids typically found in serum. HeLa cells were pre-treated with 4-PI in serum-free medium and the medium replaced after 24 h p.i. with fresh serum-free medium containing the different nutrition pools or medium with 10% FBS final concentration. Fig. 5A shows that FBS, vitamins and lipids, but not the mix of salts, reversed the 4-PI induced inhibition. We focused on the groups of lipids and repeated the experiment using single

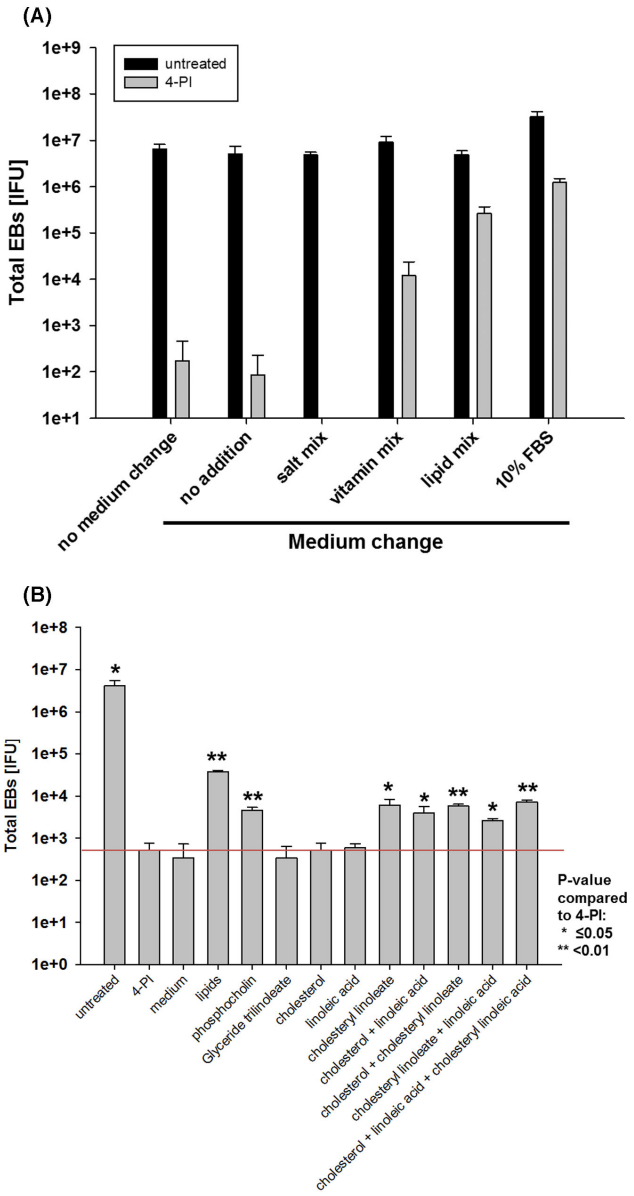

Figure 5. Lipids in serum are the main contributing components during recovery of *C. trachomatis* after 4-PI growth inhibition. HeLa cells were left untreated or pre-treated with 1 mM 4-PI in serum-free medium and infected with an MOI of 1. (**A**) After 24 h, the medium was replaced with fresh medium with or without supplements of cholesterol, or pools of vitamins, salts or lipids, respectively. (**B**) After 24 h the medium was replaced with fresh medium with or without supplements of glyceride trilinoleate, phosphocholine, cholesterol, linoleic acid, cholesteryl linoleate or combinations of the latter three. Cells were harvested 48 h p.i. and HeLa cells fresh infected with dilutions the harvested samples to titrate the *C. trachomatis* IFU. All titrations are the mean of three biologically independent replicate samples and error bars represent SD.

lipids. The main source of lipids in serum is from LDL. The LDL lipids break down as 10% triacylglycerols, 11% free cholesterol, 29% phospholipids and 50% cholesteryl esters (from Christy 2014): http://lipidlibrary.aocs.org/Lipids/lipoprot/index.htm, 13 April 2015, date last accessed). When we added the single lipids to serum-free medium (Fig. 5B), only phosphocholine and cholesteryl linoleate, but not cholesterol, linoleic acid or glyceride trilinoleate resulted in recovery of chlamydial growth. However, the combination of cholesterol and linoleic acid also reversed the effect of 4-PI indicating that this esterified cholesterol compound may be required for the rescue of *Chlamydia* after 4-PI induced growth inhibition.

Chlamydial growth is reduced by an ACAT-specific inhibitor in a dose-dependent manner and is restored by cholesteryl linoleate

In eukaryotic cells, the main enzyme that catalyzes the production of cholesteryl esters from cholesterol and fatty acids is ACAT also called sterol o-acyltransferase. The previous result suggests that ACAT is a possible target of 4-PI and that cholesteryl ester production may be required for the transport of cholesterol into *Chlamydia*. To test if ACAT activity is necessary for chlamydial growth, we used the specific ACAT-inhibitor 4-HCAA (Lee *et al.* 2004).

We first tested if the inhibitor affected HeLa cell growth by doing a dose response of 4-HCAA on uninfected cells and incubated for 24 h p.i. We found that ACAT-inhibitor concentrations $\geq$250 μM reduced HeLa cell growth (data not shown). Therefore, only 4-HCAA concentrations of 100, 150 and 200 μM were used for further analysis, which did not affect growth of the HeLa cells even after 48 h of treatment (upper panel in Fig. 6A). HeLa cells were infected, fixed after 24 h p.i. and stained for IFA as

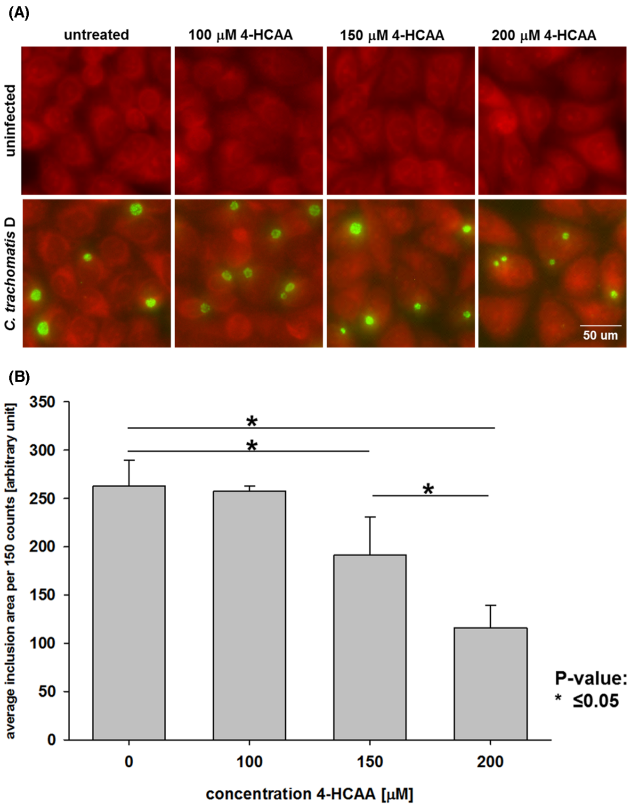

Figure 6. Chlamydial growth reduction by the specific ACAT inhibitor is concentration dependent. HeLa cells were left untreated or pre-treated for 24 h p.i. with ACAT-inhibitor concentrations between 100–200 μM. Cells were infected with an MOI of 0.5 and fixed after 24 h p.i. *Chlamydia trachomatis* was stained with an FITC-conjugated antibody to *C. trachomatis* and cells counterstained with Evans blue. (**A**) Representative images of *C. trachomatis* inclusions are shown (200× magnification) (**B**) Images were randomly taken and the area size in the green channel determined with ImageJ. The graph shows the mean of 150 different inclusion areas and error bars represent SD.

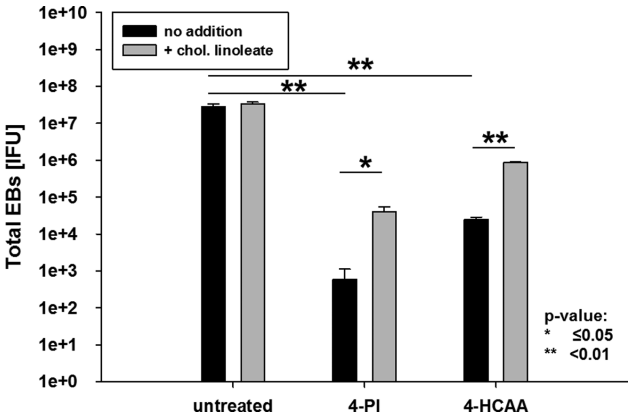

Figure 7. Chlamydial growth is reduced by the specific ACAT inhibitor and can be restored with cholesteryl linoleate. HeLa cells were left untreated or pre-treated with either 1 mM 4-PI or 200 μM ACAT inhibitor. HeLa cells were infected with an MOI of 1 and after 24 h p.i. the inhibitor containing medium replace with 25 μg ml^{-1} cholesteryl linoleate. The cells were harvested after 48 h p.i. and fresh HeLa cells were infected with dilutions of harvested samples to titrate the *C. trachomatis* IFU. All titrations are the mean of three biologically independent replicate samples and error bars represent SD.

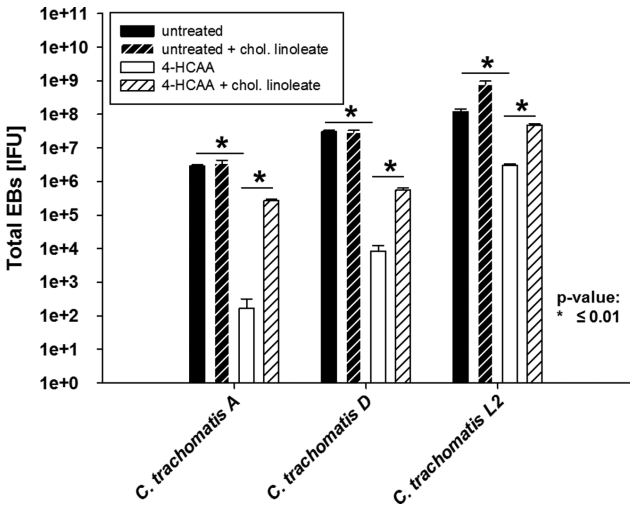

Figure 8. Growth of *C. trachomatis* serovar A and L2 is affected by ACAT inhibition in a manner similar to *C. trachomatis* D. HeLa cells were left untreated or pre-treated with 200 μM 4-HCAA. HeLa cells were infected with an MOI of 1 using *C. trachomatis* serovars A, D and L2. After 24 h p.i. the inhibitor containing medium was replaced with 25 μg ml^{-1} cholesteryl linoleate. The cells were harvested after 48 h p.i. and fresh HeLa cells were infected with dilutions of harvested samples to titrate the *C. trachomatis* IFU. All titrations are the mean of three biologically independent replicate samples and error bars represent SD.

described above. Inclusion size was measured as an indicator for growth. Images were randomly taken and the inclusion size calculated by ImageJ. The average size of 150 chlamydial inclusions with inhibitor was plotted against untreated cells. Fig. 6A shows IFA of untreated or 4-HCAA treated cells in uninfected or infected cells. Fig. 6B illustrates the graph of the average size of 150 chlamydial inclusions. As seen in the graph, the inclusion size is reduced in a dose-dependent manner with increasing amounts of 4-HCAA. These data suggest that ACAT activity is required for chlamydial growth in eukaryotic cells.

We then treated HeLa cells for 24 h with either 4-PI, 4-HCAA or left the cells untreated in serum-free medium. HeLa cells were infected with *C. trachomatis* for EB titration and incubated for 24 h p.i. To test if *C. trachomatis* D growth inhibition was reversed, we removed the inhibitor-containing medium and added fresh medium with 25 μg ml^{-1} cholesteryl linoleate to some samples for rescue. The cells were harvested after an additional 24 h for titration of the total amount of infectious EBs in the samples. Fig. 7 shows the total amount of EBs during inhibition and recovery in the presence of cholesteryl linoleate compared to untreated samples. The graph illustrates that each inhibitor reduced the amount of IFUs significantly and that growth can be partially reversed by adding cholesteryl linoleate to the medium, although the inhibitory effect of 4-PI was more dramatic compared to 4-HCAA. It is possible that ACAT is only one potential target of 4-PI and other, not yet identified, enzymes are also inhibited during 4-PI treatment. A previous publication demonstrated in *in vitro* assays that even at a concentration of 200 μM ACAT-specific inhibitor still retained some residual activity (Lee *et al.* 2004). Therefore, it is possible that the produced amount of cholesteryl esters in the cells even with low ACAT activity is sufficient for minimal *Chlamydia* growth. To test if other *C. trachomatis* strains are similarly affected by ACAT inhibition, we repeated the experiment with 4-HCAA using the ocular *C. trachomatis* serovar A strain and the lymphogranuloma venereum *C. trachomatis* serovar L2 strain. As seen in Fig. 8, both strains showed statistically significant reduced amounts of EBs recovered after ACAT inhibition. However, while the total amount of recovered EBs for *C. trachomatis* serovar A and serovar D were reduced 3–4 logs compared to untreated cells, *Chlamydia trachomatis* serovar L2 showed 1 log reduction compared to the untreated sample, likely due to the faster growth of serovar L2 strain that can 'out-compete' the cell for the lipid.

DISCUSSION

Chlamydia has a minimal genome reflecting extreme auxotrophy and dependence on host-cell derived metabolites. Lipids, including phospholipids, cholesterol and cholesteryl esters, are among the most imported eukaryotic metabolites by *Chlamydia*. Despite the current knowledge of many pathways by which *Chlamydia* transport lipids to the inclusion (Wylie, Hatch and McClarty 1997; Beatty 2006; Kumar, Cocchiaro and Valdivia 2006; Cocchiaro *et al.* 2008; Heuer *et al.* 2009; Cox *et al.* 2012; Boncompain *et al.* 2014), many steps of the lipids transport and uptake processes during the chlamydial developmental cycle are not fully understood.

We used the IDO-inhibitor 4-PI to investigate tryptophan depletion independent effects on *C. trachomatis*, and coincidentally found that 4-PI inhibited chlamydial growth even in the absence of IFN-γ suggesting that the activity of enzymes other than IDO were responsible for the inhibitory effect. Most of the known targets of 4-PI and its derivatives bind to enzymes catabolizing either cholesterol or other steroids. Chlamydiae were able to recover after 4-PI induced growth inhibition once exogenous cholesterol was added indicating that cholesterol metabolism was affected. We found that in serum-free medium phosphocholine and cholesteryl linoleate restored *Chlamydia* growth, while cholesterol, linoleic acid and glyceride trilinoleate did not reverse the inhibition. Interestingly, cholesterol and linoleic acid in combination also reversed the effect of 4-PI suggesting that the production of cholesteryl linoleate in the host cells is blocked by 4-PI. The enzyme in eukaryotic cells that is largely responsible for cholesteryl ester synthesis is ACAT and derivatives of 4-PI were used to target the eukaryotic enzyme previously (Kimura *et al.* 1993). We found a concentration-dependent growth reduction

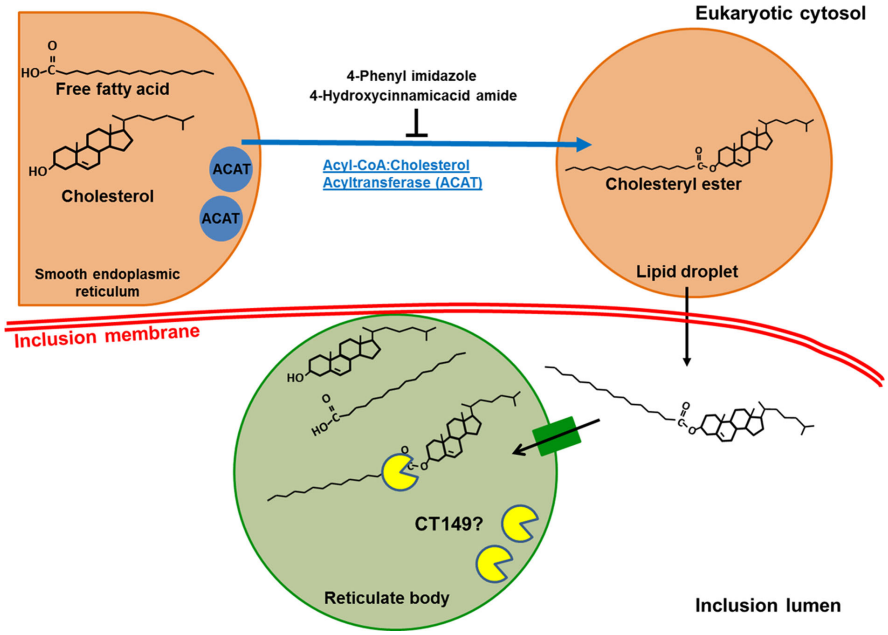

Figure 9. Model of ACAT influence on *C. trachomatis* growth. A model of the influence of ACAT activity on intracellular chlamydial growth. The ER localized eukaryotic enzyme catalyzes the esterification of cholesterol in the lumen of the smooth ER and the produced cholesteryl esters are transported to lipid droplets. Lipids in the lipid droplets are then accessible for *Chlamydia* and imported into the chlamydiae. Here, chlamydial enzymes such as CT149 can break down the esters and utilize cholesterol and the free fatty acid.

of *C. trachomatis* when using an ACAT-specific inhibitor, which we could reverse by the addition of cholesteryl esters.

There are two known paralogs of ACAT in eukaryotic cells called ACAT-1 and ACAT-2. In eukaryotic cells, ACAT-1 catalyzes the formation of cholesteryl esters, the main transport and storage form of cholesterol, while ACAT-2 is also involved in phospholipid synthesis. ACAT is essential in eukaryotic cells for the homoeostasis of cholesterol since high concentrations of cholesterol are toxic for eukaryotic cells. Excess amounts of cholesterol are esterified with fatty acids by ACAT and either stored in lipid droplets (Guo *et al.* 2009) or shuttled to the ABCA1 exporter where the lipids are transported to extracellular high-density lipoprotein (HDL) (Dove *et al.* 2005).

In our experiments, the addition of the phospholipid phosphocholine (PC) to serum-free medium also restored chlamydial growth after inhibition by 4-PI. Although it is not known if 4-PI or derivatives can also inhibit ACAT-2 and prevent esterification of phospholipids, it is possible that, similar to cholesterol, esterification of phospholipids with fatty acids is necessary before they can be transported into the inclusion. Both PC and CEs are transported by ABCA1 and Cox *et al.* (2012) showed recently that the transporter ABCA1 and its lipid-binding-associated protein ApoA1 are recruited to the inclusion. It is further known that ACAT in the ER is required for lipid droplet formation (Guo *et al.* 2009). The expression of ACAT-1 is upregulated during *C. pneumoniae* infection of macrophages and is required for foam cell formation in macrophages (He *et al.* 2009). Additionally, a previous publication demonstrated that *C. psittaci* 6BC infected MK-2 cells contained increased amounts of cholesteryl esters compared to uninfected cells (Makino *et al.* 1970), but the requirement of ACAT activity by *Chlamydia* was not known. ACAT is found in many tissues and cell types that are infected by *Chlamydia* such as macrophages and epithelial cells (Sakashita *et al.* 2000).

Our current work demonstrates that ACAT-dependent cholesterol esterification is important for *Chlamydia* and it is likely that cholesteryl esterification is an essential step before cholesterol is imported into the inclusion (illustrated in Fig. 9). The advantage of transporting cholesteryl esters into chlamydiae is that two necessary lipids, cholesterol and fatty acids, are transported in a one-step process rather than being transported separately. In chlamydiae, the cholesteryl esters can be hydrolyzed and, if required, the lipids further modified. We recently published that the *C. trachomatis ct149* gene product has esterase activity and hydrolyzes cholesteryl linoleate (Peters *et al.* 2012). This protein is therefore a possible candidate for a lipase that hydrolyzes imported eukaryotic lipids. Future research will focus on additional enzymatic activities of CT149 and other potential candidate enzymes in *Chlamydia* that can hydrolyze esters and serve as nutrient for *Chlamydia*.

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Conflict of interest. None declared.

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