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Dissecting the membrane cholesterol requirement for mycobacterial entry into host cells

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ABSTRACT

Mycobacteria are intracellular pathogens that can invade and survive within host macrophages, and are a major cause of mortality and morbidity worldwide. The molecular mechanism involved in the internalization of mycobacteria is poorly understood. In this work, we have explored the role of host membrane cholesterol in the entry of the avirulent surrogate mycobacterial strain *Mycobacterium smegmatis* into THP-1 macrophages. Our results show that depletion of host membrane cholesterol using methyl-β-cyclodextrin results in a significant reduction in the entry of *M. smegmatis* into host cells. More importantly, we show that the inhibition in the ability of *M. smegmatis* to enter host macrophages could be reversed upon replenishment of membrane cholesterol. To the best of our knowledge, these results constitute the first report showing that membrane cholesterol replenishment can reverse the inhibition in the entry of mycobacteria into host cells. In addition, we demonstrate that cholesterol complexation using amphotericin B (without physical depletion) is sufficient to inhibit mycobacterial entry. Importantly, we observed a significant reduction in mycobacterial entry upon enrichment of host membrane cholesterol. Taken together, our results demonstrate, for the first time, that *an optimum host plasma membrane cholesterol* is necessary for the entry of mycobacteria. These results assume relevance in the context of developing novel therapeutic strategies targeting cholesterol-mediated mycobacterial host cell entry.

1. Introduction

Mycobacterium, a genus of Actinobacteria, includes a large number of different species, which range in virulence from the non-pathogenic *Mycobacterium smegmatis* to the causative organism of tuberculosis (TB) in humans, *Mycobacterium tuberculosis* (Killick et al., 2013). TB continues to be a major cause of mortality and morbidity worldwide with an estimated 8.6 million new cases and 1.3 million deaths reported in 2012 alone (World Health Organization, 2013). The prevalence of opportunistic TB infection among HIV positive patients has further worsened this situation (Pawlowski et al., 2012; World Health Organization, 2013). In spite of the availability of approved treatment regimens, the emergence of multi and extensively drug-resistant TB has necessitated novel therapeutic strategies to combat this disease (Dye, 2009; Gandhi et al., 2006; Keshavjee and Farmer, 2012).

The most common mode of infection with mycobacteria is by inhalation or ingestion of bacilli. *M. tuberculosis* spreads through aerosols generated during coughing and sneezing by patients with active infection and is believed to be internalized primarily by alveolar macrophages in the lungs (Russell, 2007). The inability of these macrophages to kill TB bacilli often leads to recruitment of immune cells to the site of infection and the formation of a granuloma, an organized immune cell aggregate (Ramakrishnan, 2012). Although this serves to limit the infection, it results in clinical 'latency', a state in which bacilli are viable but do not produce symptoms of their presence. Reactivation of the bacilli could occur if latently infected individuals are exposed to immunosuppressive conditions.

The entry of intracellular pathogens in general and mycobacteria in particular involves interaction with the plasma membrane of host cells (Chattopadhyay and Jafurulla, 2012; Gatfield and

Abbreviations: AmB, amphotericin B; BCA, bicinchoninic acid; CFU, colony forming unit; CRAC, cholesterol recognition/interaction amino acid consensus; DMSO, dimethyl sulfoxide; HIV, human immunodeficiency virus; MβCD, methyl-β-cyclodextrin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PMA, phorbol 12-myristate 13-acetate; TB, tuberculosis.

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Pieters, 2000; Pucadyil and Chattopadhyay, 2007). However, the molecular mechanism involved in the entry of mycobacteria is poorly characterized. Studies aimed at understanding the molecular events in the entry of mycobacteria into host cells have resulted in the identification of a number of candidate receptors facilitating multiple routes of entry, thereby highlighting the redundancy in the entry process. These include receptors on the host macrophage cell surface such as the mannose receptor, scavenger receptors, CD-14, dectin-1, DC-SIGN, and complement receptors (Ernst, 1998; Tailleux et al., 2003; Yadav and Schorey, 2006). Due to the large variety of receptors responsible for mycobacterial entry into host macrophages, no panacea is available for the treatment of TB.

A number of studies have indicated the crucial requirement of membrane cholesterol in host–pathogen interaction (Chattopadhyay and Jafurulla, 2012; Goluszko and Nowicki, 2005; Hawkes and Mak, 2006; Pucadyil and Chattopadhyay, 2007; Riethmüller et al., 2006; Rosenberger et al., 2000; Shin and Abraham, 2001; Simons and Ehehalt, 2002; van der Goot and Harder, 2001). Cholesterol is an essential and representative membrane lipid in higher eukaryotes and is crucial in membrane organization, dynamics, function, and sorting (Chaudhuri and Chattopadhyay, 2011; Mouritsen and Zuckermann, 2004; Simons and Ikonen, 2000). A hallmark of membrane cholesterol is its nonrandom distribution in domains (or pools) in biological and model membranes (Chaudhuri and Chattopadhyay, 2011; Lingwood and Simons, 2010; Mukherjee and Maxfield, 2004; Xu and London, 2000). These domains are believed to be crucial since various cellular processes such as membrane sorting/trafficking (Simons and van Meer, 1988), and signal transduction (Simons and Toomre, 2000) have been attributed to these types of domains. Another emerging and interesting area is the role of cholesterol in the function and organization of membrane proteins and receptors. Membrane cholesterol plays a vital role in the function and organization of membrane proteins and receptors, including G protein-coupled receptors (GPCRs) (Burger et al., 2000; Jafurulla and Chattopadhyay, 2013; Oates and Watts, 2011; Paila and Chattopadhyay, 2010; Pucadyil and Chattopadhyay, 2006).

In the present study, we have explored the role of host membrane cholesterol in the entry of the avirulent mycobacterial strain *M. smegmatis* into THP-1 macrophages. *M. smegmatis* has been extensively used as a surrogate model to understand the physiology of *M. tuberculosis*. Since cell wall associated factors are involved in mediating bacillary entry into host cells, our approach of using *M. smegmatis* as a model to examine the entry process is relevant. This is due to the fact that *M. smegmatis* and *M. tuberculosis* have similar architecture of their cell envelope and therefore components involved in maintaining envelope integrity are conserved among these species (Sani et al., 2010). In addition, it has been observed that at a high multiplicity of infection, bacterial loads of *M. bovis* BCG, *M. tuberculosis* and *M. smegmatis* in the macrophage model of infection are comparable, thereby pointing to the conservation in entry mechanisms for both pathogenic and non-pathogenic mycobacteria (Zhang, 2013).

Our results show that depletion of host membrane cholesterol using methyl-β-cyclodextrin (MβCD) results in a significant reduction in the entry of *M. smegmatis* into macrophages. More importantly, we show that the reduction in the ability of the *Mycobacterium* to enter host macrophages can be reversed upon replenishment of cell membrane cholesterol. In addition, we demonstrate using amphotericin B (AmB; a cholesterol-sequestering agent) that sequestration of membrane cholesterol, without physical depletion, is sufficient to inhibit mycobacterial entry. To further explore the role of membrane cholesterol in the entry of *M. smegmatis*, we enriched macrophages with additional cholesterol. Our results show that mycobacterial entry exhibits significant reduction upon enrichment of host membrane cholesterol. Taken together, our results demonstrate, for the first time, that an optimum host plasma membrane cholesterol is necessary for the entry of mycobacteria.

2. Materials and methods

2.1. Materials

Penicillin, streptomycin, gentamicin sulfate, cholesterol, methyl-β-cyclodextrin, amphotericin B, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Triton X-100, DMSO, NaCl, Tween 80 and Phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). Middlebrook 7H9 broth and 7H10 agar were obtained from Becton, Dickinson Difco (Sparks, MD). Amplex Red cholesterol assay kit was from Molecular Probes/Invitrogen (Eugene, OR). Bicinchoninic acid (BCA) assay reagent was obtained from Pierce (Rockford, IL). RPMI-1640 medium and fetal bovine serum were obtained from Gibco/Life Technologies (Grand Island, NY). All other chemicals used were of the highest available purity. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

2.2. Methods

2.2.1. THP-1 monocyte culture and differentiation

Human monocyte cell line THP-1 (American Type Culture Collection) was maintained and differentiated, as described previously (Tiwari et al., 2012, 2014). Briefly, cells were grown in RPMI-1640 medium supplemented with 2 g/l of sodium bicarbonate, 10% fetal bovine serum, 60 μg/ml penicillin, 50 μg/ml streptomycin and 30 μg/ml gentamicin sulfate in a humidified atmosphere with 5% CO_2 at 37 °C. THP-1 cells were seeded in 6-well plates at a density of $\sim 2 \times 10^6$/well and were differentiated into macrophages by treating with 5 ng/ml PMA for 24 h, followed by incubation in PMA free medium for 48 h.

2.2.2. Bacterial strains and culture conditions

M. smegmatis mc^26 was cultured as described previously (Tiwari et al., 2012, 2014). Briefly, *M. smegmatis* was cultured in Middlebrook 7H9 broth and 7H10 agar supplemented with albumin dextrose complex (5 g/l BSA, 2 g/l glucose and 0.85 g/l NaCl), 0.5% (v/v) glycerol and 0.05% (v/v) Tween 80. *E. coli* DH5α was cultured in Luria Bertani broth. *M. smegmatis* and *E. coli* were grown at 37 °C under shaking conditions.

2.2.3. Modulation and estimation of cellular cholesterol content

Cholesterol levels of THP-1 macrophages were modulated as described previously (Pucadyil et al., 2004) with some modifications. THP-1 macrophages were incubated in RPMI-1640 medium without serum and antibiotic supplements for 3 h prior to further treatment. Cholesterol depletion was carried out by incubating macrophages with 10 mM MβCD in serum-free medium for 30 min at 37 °C. Cholesterol-depleted macrophages were replenished with cholesterol by incubating with cholesterol-MβCD complex at 37 °C for 10 min. The complex was prepared by dissolving required amounts of cholesterol and MβCD in a ratio of 1:10 (mol/mol) in water by constant vortexing at room temperature (~23 °C). Stock solution (of 2× concentration) of cholesterol-MβCD complex was freshly prepared prior to each experiment and diluted with equal volume of 2× serum-free RPMI-1640 medium to yield a final concentration of 1 mM cholesterol and 10 mM MβCD. Enrichment of membrane cholesterol over normal levels in macrophages was carried out as described previously (Chattopadhyay et al., 2006; Saxena and Chattopadhyay, 2012) with some modifications. Cholesterol enrichment was achieved by incubating cells with cholesterol–MβCD complex (prepared as described above) at 37 °C for 30 min. Cell

monolayers were washed twice with PBS after each treatment. Total free cellular cholesterol was estimated in the cell lysates using the Amplex Red cholesterol assay kit (Amundson and Zhou, 1999). Cholesterol values were normalized to total protein levels estimated using the bicinchoninic acid (BCA) assay reagent (Smith et al., 1985).

2.2.4. Complexation of membrane cholesterol

Cholesterol complexation using AmB was carried out as described previously (Paila et al., 2010) with some modifications. Macrophages were incubated in serum-free RPMI-1640 medium for 3 h followed by incubation with 5 μM AmB in serum-free RPMI-1640 medium at 37 °C for 30 min. Macrophages were washed twice with PBS before being exposed to bacteria.

2.2.5. Quantitation of bacterial entry into THP-1 macrophages

Bacterial entry into THP-1 macrophages was quantitated as described previously (Tiwari et al., 2012, 2014). Exponentially growing *M. smegmatis* and *E. coli* strains were pelleted, washed and resuspended in RPMI-1640 medium (without serum and antibiotics) to an optical density (A_{600}) of 1. *M. smegmatis* culture was passed through 261/2 gauge needles 5–6 times to obtain single cell suspension. Colony forming units (CFUs) were counted at each step to assess bacillary viability. A multiplicity of infection (MOI) of 100:1 (bacteria to macrophage) was used to perform infections. After incubation for 2 h, macrophages were washed with PBS and treated with gentamicin (50 μg/ml) in serum-free RPMI-1640 medium for 30 min at 37 °C to kill extracellular bacteria. Macrophages were lysed using 0.1% (v/v) Triton X-100 and appropriate dilutions of *M. smegmatis* were made in Middlebrook 7H9 media and were plated on Middlebrook 7H10 agar. In case of *E. coli*, appropriate dilutions were made in Luria Bertani broth and were plated on Luria Bertani agar. Plates were incubated at 37 °C till visible colonies appeared for counting CFUs.

2.2.6. Fluorescence imaging of M. smegmatis *entry into THP-1 macrophages*

THP-1 cells were seeded on glass cover slips in 6-well plates at a density of ~2×10^6/well and were differentiated into macrophages as described above. After modulation or complexation of membrane cholesterol, macrophages were infected with *M. smegmatis* stably expressing fluorescent protein dsRed2 (strain transformed with pMSP12::*dsRed2* (Cosma et al., 2004)). After infection, macrophages were washed with PBS, fixed with 4% (v/v) formaldehyde and mounted in media containing DAPI. Images were acquired on a Zeiss LSM 510 Meta confocal microscope (Jena, Germany) with a 63×/1.4 NA oil immersion objective.

2.2.7. MTT viability assay

MTT assay was carried out to assess the viability of THP-1 macrophages as described earlier (Roy et al., 2014; Vistica et al., 1991)

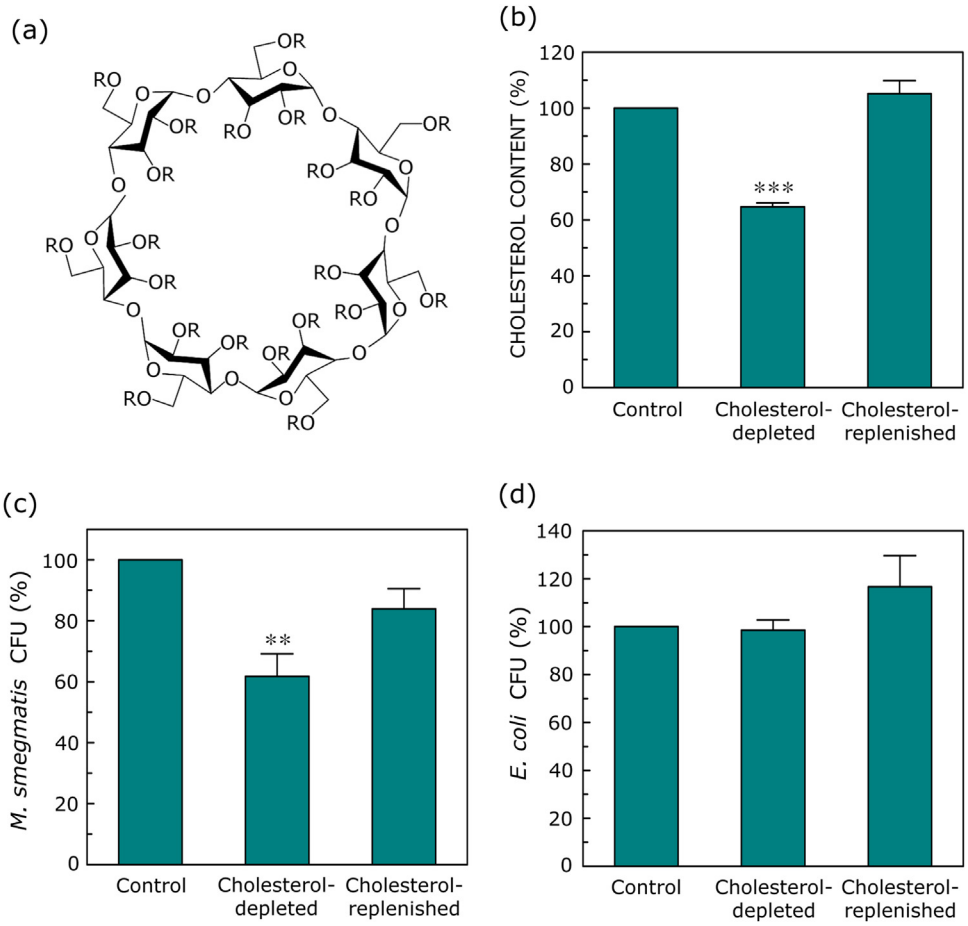

Fig. 1. Membrane cholesterol content modulates entry of *M. smegmatis* into THP-1 macrophages. (a) Chemical structure of MβCD. (b) Cellular cholesterol content of THP-1 macrophages upon depletion with MβCD and subsequent replenishment of MβCD-treated cells with cholesterol:MβCD (1:10 mM) complex. Values are normalized to the cholesterol content of untreated (control) macrophages. Macrophages (control, cholesterol-depleted, and cholesterol-replenished) were exposed to (c) *M. smegmatis* or (d) *E. coli* DH5α at a multiplicity of infection of 100:1 (bacteria to macrophage) for 2 h. After lysing macrophages, intracellular bacilli were cultured and the colony forming units (CFUs) were counted. Values are normalized to CFU counts obtained from untreated (control) macrophages. Data represent means ± S.E. of triplicate points from two independent experiments (** and *** correspond to significant ($p < 0.01$ and $p < 0.0001$) difference in cholesterol content or CFU counts of cholesterol-depleted macrophages relative to control macrophages). See Section 2 for more details.

with some modifications. Macrophages were plated at a density of $\sim 2 \times 10^5$ in 24-well plates and treatments with MβCD and AmB were carried out as described above. MTT was dissolved in serum-free RPMI-1640 medium and added to macrophages at a final concentration of 0.4 mg/ml, followed by incubation at 37 °C for 2.5 h. Formazan crystals formed upon reduction of MTT salt by mitochondrial enzymes in live cells (Vistica et al., 1991) are insoluble in aqueous medium. Formazan crystals formed were subsequently dissolved in DMSO after discarding the medium. The color obtained was measured by absorbance at 540 nm in a PowerWave XS2 microplate spectrophotometer (BioTek Winooski, VT).

2.2.8. Statistical analysis

Significance levels were estimated using Student's two-tailed paired *t*-test using Graphpad Prism software version 4.0 (San Diego, CA). Plots were generated using GRAFIT program, version 3.09b (Erithacus Software, Surrey, UK).

3. Results

3.1. Depletion of membrane cholesterol inhibits entry of M. smegmatis, but not E. coli, into host macrophages

Membrane cholesterol was depleted using MβCD. MβCD is a water-soluble polymer with seven residues (β-cyclodextrin) of methylated-glucose, and has been extensively used to selectively and efficiently extract cholesterol from membranes by incorporating it in a central nonpolar cavity (see Fig. 1a; (Zidovetzki and Levitan, 2007)). The stoichiometry of the cholesterol:cyclodextrin complex has been reported to be 1:2 (mol/mol) (Tsamaloukas et al., 2005). Acute depletion of cholesterol using MβCD resulted in ~35% reduction in total cellular cholesterol content in THP-1 macrophages (see Fig. 1b). The entry of *M. smegmatis* into host macrophages exhibited a significant reduction (~38%) upon cholesterol depletion from host macrophages (Fig. 1c). This clearly demonstrates the requirement of host membrane cholesterol in the entry of *Mycobacterium* into host cells. As a control, we monitored the entry of *E. coli* DH5α into cholesterol-depleted host macrophages. Interestingly, the entry of *E. coli* is not inhibited upon cholesterol depletion (see Fig. 1d), in contrast to what was observed with *M. smegmatis*. These results indicate that while the entry of *M. smegmatis* is dependent on membrane cholesterol content, the entry of *E. coli* is not (*i.e.*, independent of membrane cholesterol content). Our results therefore point to the specificity of cholesterol-dependent interaction between *M. smegmatis* and the macrophage cell membrane.

3.2. Replenishment of membrane cholesterol restores entry of M. smegmatis into host macrophages

In order to ascertain the specificity of membrane cholesterol content in mycobacterial entry, we replenished cholesterol-depleted macrophages using cholesterol–MβCD complex. Fig. 1b shows that treatment with cholesterol–MβCD complex was able to replenish cholesterol to ~105% of control. Interestingly, this resulted in restoration of mycobacterial entry into host macrophages to ~84% (from ~62% observed in cholesterol-depleted macrophages) of control macrophages (Fig. 1c). Importantly, the entry of *E. coli* remains unaltered (does not show any significant change) upon cholesterol replenishment (Fig. 1d). These results conclusively demonstrate that the reduced ability of *M. smegmatis*

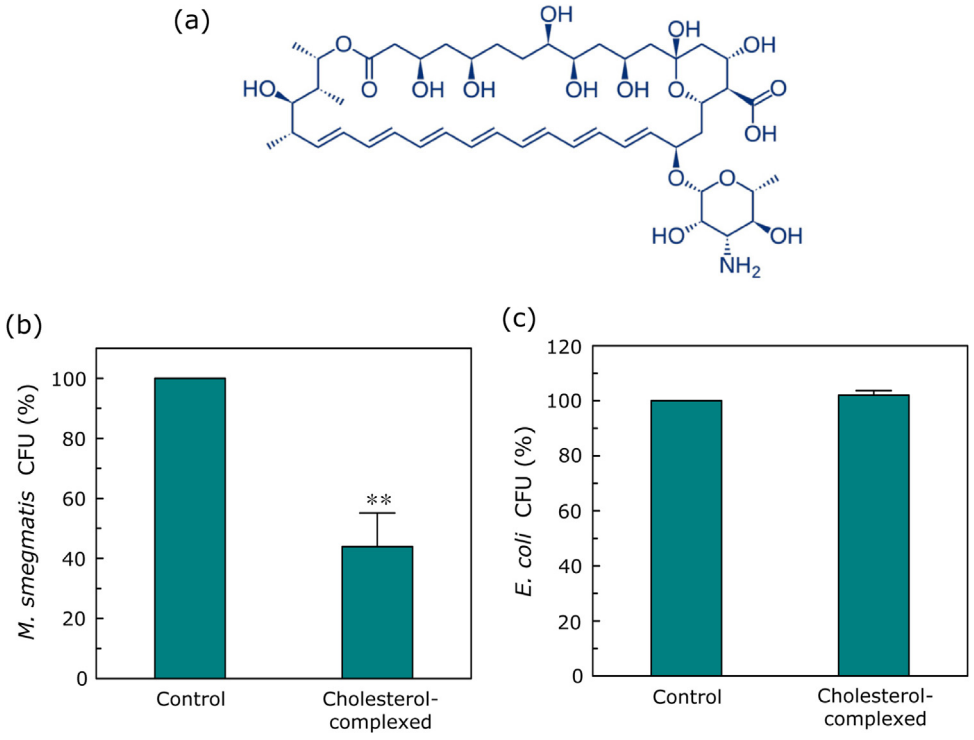

Fig. 2. Cholesterol complexation by amphotericin B (AmB) inhibits entry of *M. smegmatis* into THP-1 macrophages. (a) Chemical structure of AmB. Macrophages treated with AmB were exposed to (b) *M. smegmatis* or (c) *E. coli* DH5α at a multiplicity of infection of 100:1 (bacteria to macrophage) for 2 h. CFUs were counted after lysing macrophages, as described in Fig. 1. The concentration of AmB used was 5 μM. Values are normalized to CFU counts obtained from untreated (control) macrophages. Data represent means ± S.E. of triplicate points from two independent experiments (** corresponds to significant ($p < 0.01$) difference in CFU counts of AmB-treated macrophages relative to control macrophages). See Section 2 for more details.

to enter cholesterol-depleted macrophages is a specific effect that could be reversed upon cholesterol replenishment.

3.3. Cholesterol sequestration by amphotericin B inhibits entry of *M. smegmatis*, but not *E. coli*, into macrophages

If membrane cholesterol is necessary for the entry of *M. smegmatis*, modulating membrane cholesterol availability by other means could affect its entry. We tested this proposal by treating host macrophages with the sterol-binding polyene antibiotic AmB (see Fig. 2a). AmB is a broad antimycotic agent and highly antiparasitic (Chattopadhyay and Jafurulla, 2011). AmB specifically interacts with membrane cholesterol and sequesters it, thereby limiting its availability to interact with other membrane components. Fig. 2b shows that treatment of macrophages with AmB resulted in a significant reduction (~56%) in the entry of *M. smegmatis* into host cells. In other words, AmB-treated macrophages displayed ~44% entry of *M. smegmatis* compared to control macrophages. These results further highlight the role of *accessible* membrane cholesterol in the entry of *M. smegmatis* into host cells. Our results demonstrate that sequestration of membrane cholesterol, without physical depletion, is sufficient to inhibit mycobacterial entry. The entry of *E. coli* DH5α did not exhibit any significant change between control and AmB-treated macrophages (see Fig. 2c).

3.4. Enrichment of membrane cholesterol inhibits entry of *M. smegmatis* into macrophages

To further explore whether the entry of *M. smegmatis* into host cells exhibits a requirement for an optimum level of membrane cholesterol, we enriched macrophages with cholesterol using cholesterol–MβCD complex. Fig. 3a shows that treatment of control macrophages with cholesterol–MβCD complex was able to enrich cholesterol level to ~212% of control. Interestingly, this led to a reduction in mycobacterial entry into host macrophages to ~49% (*i.e.*, a 51% reduction relative to control macrophages; see Fig. 3b). These results point toward the requirement of an optimal level of host membrane cholesterol for efficient entry of *M. smegmatis*. In other words, the entry of *M. smegmatis* is inhibited both above and below the optimum level of membrane cholesterol (see Section 4 and Fig. 6).

3.5. Fluorescence imaging reinforces the requirement of optimum host membrane cholesterol for the entry of *M. smegmatis*

To validate entry phenotypes observed above, we performed confocal microscopy of macrophages infected with *M. smegmatis* (stably expressing dsRed2) upon modulation or complexation of membrane cholesterol. Confocal images in Fig. 4 show that the entry of *M. smegmatis* into macrophages was reduced upon cholesterol depletion, which upon cholesterol replenishment was restored back to control levels. This is consistent with our results showing quantitation of mycobacterial entry under these conditions (see Fig. 1c). Importantly, Fig. 4 also shows that complexation and enrichment of membrane cholesterol resulted in reduction in the entry of *M. smegmatis* into macrophages, in agreement with our results shown in Figs. 2b and 3b, respectively. These results reinforce the requirement of optimum host membrane cholesterol levels for the entry of *M. smegmatis*.

3.6. Membrane cholesterol depletion or sequestration does not affect macrophage viability

In order to assess the effect of depletion and sequestration of membrane cholesterol (using MβCD and AmB, respectively) on the viability of macrophages, we carried out MTT viability assay under these conditions. This is a cell proliferation assay and provides an estimate of the cell growth rate and viability of the cells. Fig. 5 shows that there was no significant cell death upon cholesterol depletion or sequestration.

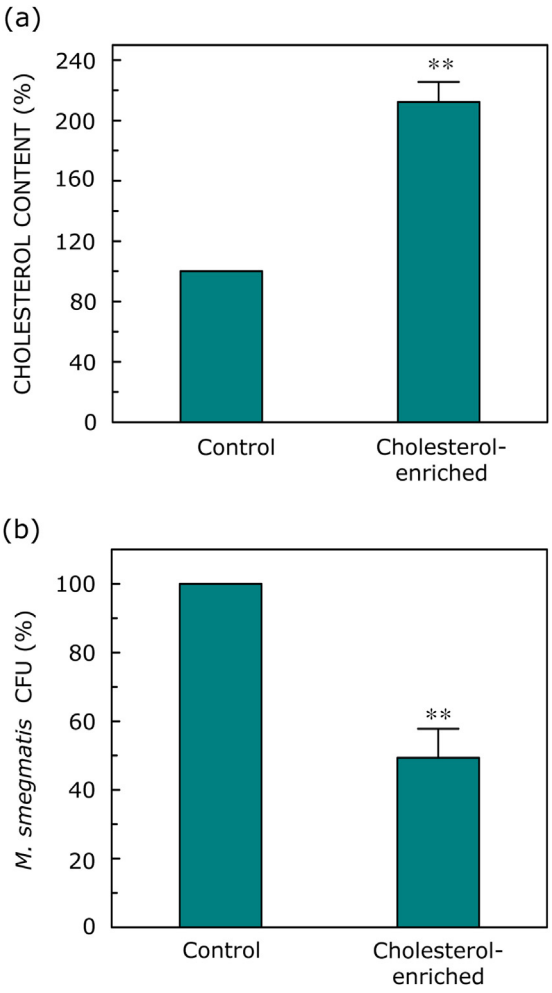

Fig. 3. Cholesterol enrichment inhibits entry of *M. smegmatis* into THP-1 macrophages. (a) Cellular cholesterol content of THP-1 macrophages upon cholesterol enrichment by treatment with cholesterol:MβCD (1:10 mM) complex. Values are normalized to the cholesterol content of untreated (control) macrophages. (b) Cholesterol-enriched macrophages were exposed to *M. smegmatis* at a multiplicity of infection of 100:1 (bacteria to macrophage) for 2 h. CFUs were counted after lysing macrophages, as described in Fig. 1. Values are normalized to CFU counts obtained from untreated (control) macrophages. Data represent means ± S.E. of triplicate points from two independent experiments (** corresponds to significant ($p < 0.01$) difference in cholesterol content or CFU counts of cholesterol-enriched macrophages relative to control macrophages). See Section 2 for more details.

4. Discussion

The role of cholesterol in mycobacterial infection is an active area of research (Gatfield and Pieters, 2000; Han, 2009; Lobato et al., 2014; Martens et al., 2008; Miner et al., 2009; Muñoz et al., 2009; Parihar et al., 2014). It was previously shown that mycobacterial entry into host cells involved cholesterol-rich membrane microdomains ('rafts') and depletion of plasma membrane cholesterol from host cells led to a reduction in mycobacterial entry (Gatfield and Pieters, 2000; Muñoz et al., 2009). Our results confirm and extend these observations using *M. smegmatis* as a surrogate model to examine mycobacterial entry into macrophages. In this work, we have explored the specificity of

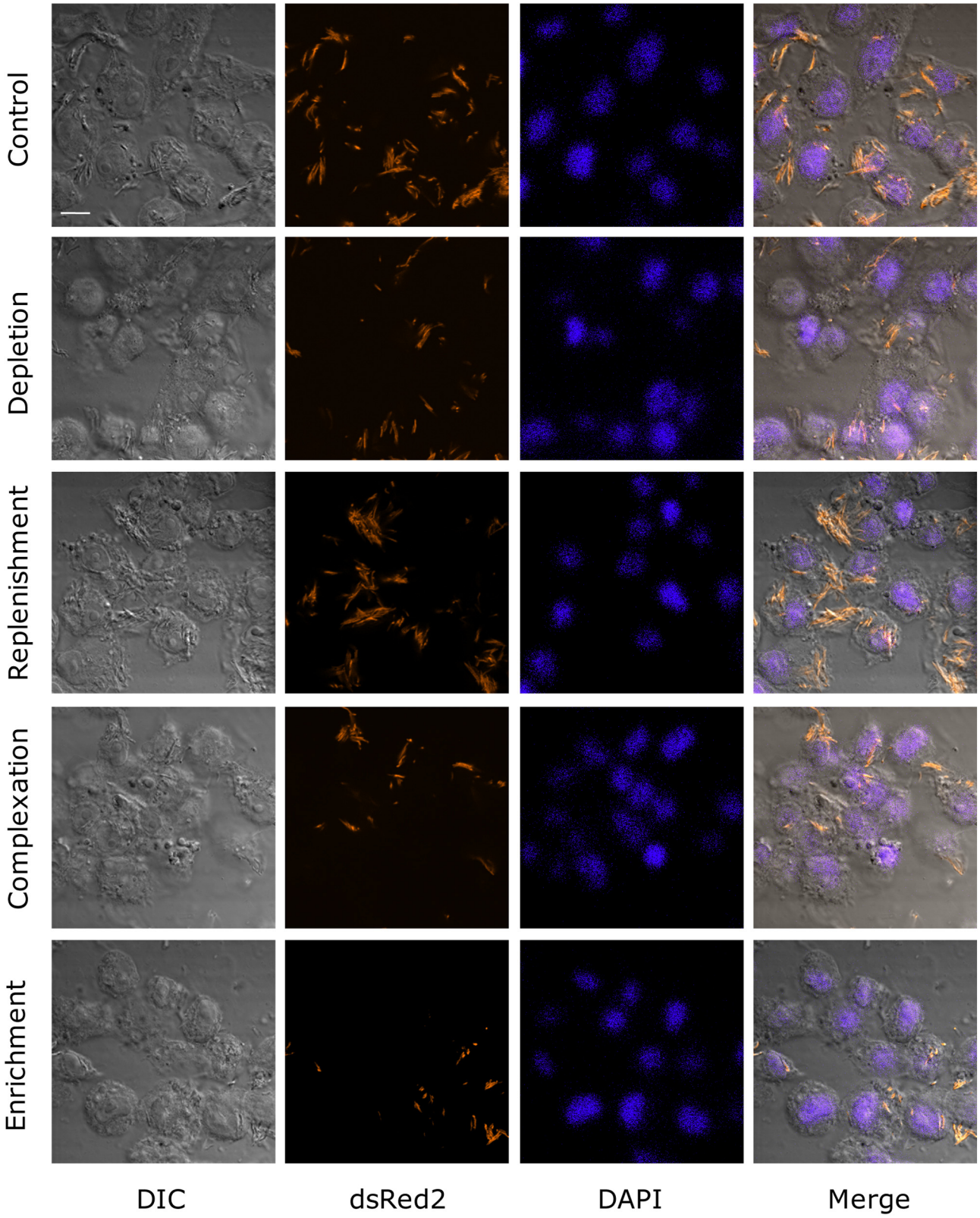

Fig. 4. Entry of *M. smegmatis* into macrophages, monitored by confocal microscopic imaging, confirms the requirement of optimum host membrane cholesterol. Representative confocal microscopic images showing entry of *M. smegmatis* (expressing dsRed2) into untreated (control), and in macrophages depleted, replenished, complexed or enriched with cholesterol. The figure shows macrophages (DIC images) infected with *M. smegmatis* expressing dsRed2 (orange). The macrophage nucleus was stained with DAPI (blue). The last panel shows merged images. The scale bar represents 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

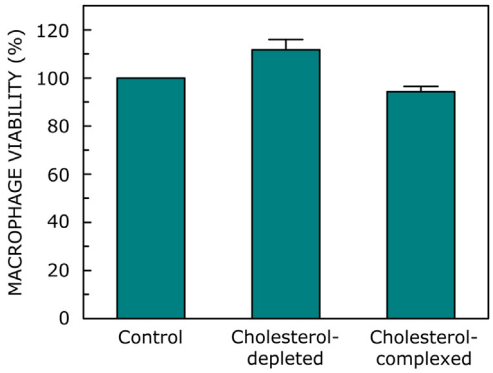

Fig. 5. Macrophage viability is not affected upon depletion or complexation of membrane cholesterol. THP-1 macrophages were assayed for viability by MTT assay after treating cells either with MβCD (10 mM) or AmB (5 μM). Values are expressed as percentages of viability of treated macrophages normalized to control macrophages. Data represent means ± S.E. of triplicate points from two independent experiments. See Section 2 for more details.

the requirement of membrane cholesterol in mycobacterial entry. Importantly, our results show that the inhibition of mycobacterial entry into host macrophages could be reversed upon replenishment of cell membrane cholesterol. To the best of our knowledge, these results constitute the first report showing that membrane cholesterol replenishment could reverse the inhibition in the entry of mycobacteria into host cells. Another novelty of our results lies in the observation that mycobacterial entry exhibited significant reduction upon enrichment of host membrane cholesterol.

In order to further confirm the specific role of membrane cholesterol in mycobacterial entry, we carried out experiments in which cholesterol availability was modulated by complexation with AmB, without physical depletion of cholesterol. Our results show that sequestration of membrane cholesterol is sufficient to inhibit mycobacterial entry. An important message that emerges from these results is that *mere non-availability of free cholesterol in the membrane due to complexation with AmB, rather than physical depletion*, is enough to significantly reduce mycobacterial entry into host cells. Since AmB does not physically deplete cholesterol from membranes (unlike agents such as MβCD), our results serve to delineate the mechanism by which cholesterol exerts its influence on mycobacterial entry. Sequestration of membrane cholesterol with AmB would effectively reduce the ability of cholesterol to interact with and exert its effects on membrane receptors that could be responsible for the entry of mycobacteria (see below). These results also open up the novel possibility of utilizing AmB formulations (Wasan et al., 2009) for the treatment of mycobacterial infections.

The possible mechanism underlying cholesterol-dependent entry of mycobacteria merits comment. As mentioned above, a number of host membrane receptors have been implicated in mycobacterial entry (Ernst, 1998; Killick et al., 2013; Tailleux et al., 2003; Yadav and Schorey, 2006). Interestingly, earlier work from us and other groups has shown that the organization and function of many membrane receptors depend on membrane cholesterol content (Burger et al., 2000; Jafurulla and Chattopadhyay, 2013; Oates and Watts, 2011; Paila and Chattopadhyay, 2010; Pucadyil and Chattopadhyay, 2006). Based on these reports, we envisage that the conformation of membrane receptors necessary for mycobacterial entry into host cells could be dependent on an optimal level of membrane cholesterol. At cholesterol concentrations above and below the optimum level, these receptors could assume a conformation that does not support entry of mycobacteria into host cells. It is encouraging to note here that there are membrane proteins such as the Na^+,K^+-ATPase (Cornelius, 1995) and $GABA_A$ receptor (Sooksawate and Simmonds, 2001) whose function has been shown to be maximum within a narrow range of membrane cholesterol, and their function is inhibited both above and below the optimum range of cholesterol concentration. It is possible that receptor(s) necessary for mycobacterial entry could share such a property of cholesterol-dependent function.

Several proteins that interact with cholesterol have been reported to contain a characteristic amino acid sequence, termed the cholesterol recognition/interaction amino acid consensus (CRAC) motif. The CRAC sequence is represented by the pattern -L/

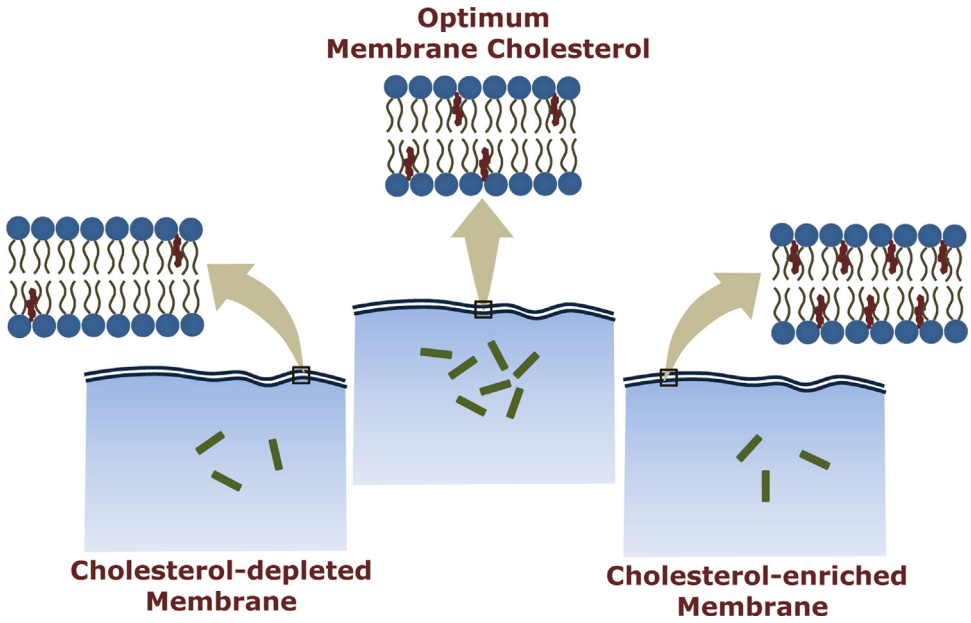

Fig. 6. An optimum level of host membrane cholesterol is necessary for mycobacterial entry into macrophages. A schematic representation depicting the entry of *M. smegmatis* into control, cholesterol-depleted and cholesterol-enriched macrophages. Mycobacterial entry is reduced when host membrane cholesterol is either depleted or enriched, thereby highlighting the requirement of an optimum cholesterol content.

V-$(X)_{1-5}$-Y-$(X)_{1-5}$-R/K-, in which $(X)_{1-5}$ represents between one and five residues of any amino acid (Epand, 2006; Li and Papadopoulos, 1998). CRAC motif has been shown to be present in a number of proteins including caveolin-1 (Epand et al., 2005), the peripheral-type benzodiazepine receptor (Li and Papadopoulos, 1998), the HIV-1 transmembrane protein gp41 (Vincenta et al., 2002), and G protein-coupled receptors such as the serotonin$_{1A}$ receptor (Jafurulla et al., 2011; Sengupta and Chattopadhyay, 2012). It is interesting to note here that some of the membrane receptors (such as complement receptors, mannose receptors and CD-14) identified to be responsible for the entry of mycobacteria into host macrophages contain multiple CRAC motifs in their amino acid sequences. This provides a possible mechanism for interaction of these receptors with membrane cholesterol.

The reduction in mycobacterial entry upon cholesterol depletion could potentially lead to novel therapeutic strategies against mycobacterial infection. A major advantage of this approach lies in the fact that development of drug resistance, an emerging problem encountered in the treatment of mycobacterial infection (Dye, 2009; Gandhi et al., 2006; Keshavjee and Farmer, 2012), would be virtually absent since the therapeutic focus is on the host rather than the parasite. As mentioned earlier, mycobacteria have emerged as an important opportunistic pathogens, among HIV-1 infected individuals (Pawlowski et al., 2012; World Health Organization, 2013). Interestingly, cholesterol has been reported to be essential for HIV-1 infection (Campbell et al., 2001; Liao et al., 2001), and topical application of cyclodextrins has previously been shown to block the transmission of cell-associated HIV-1 in mice (Khanna et al., 2002). The administration of compounds that modulate membrane cholesterol levels could therefore prove to be a powerful approach in tackling the combined infection of mycobacteria and HIV-1.

In summary, our results point toward the specific requirement of host plasma membrane cholesterol in mycobacterial entry. These results demonstrate, for the first time, that *an optimum host plasma membrane cholesterol* is necessary for the entry of mycobacteria (Fig. 6). Our present results find support from a recent report describing reduction in mycobacterial infection upon treatment with statin (Parihar et al., 2014), a competitive inhibitor of HMG-CoA reductase (a key rate-limiting enzyme in cholesterol biosynthesis) and a globally top-selling cholesterol-lowering drug (Shrivastava et al., 2010). In a broader perspective, our results are significant in developing novel therapeutic strategies targeting cholesterol-mediated mycobacterial host cell entry.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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