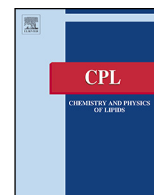
Chemistry and Physics of Lipids 189 (2015) 19–27

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip



Dissecting the membrane cholesterol requirement for mycobacterial entry into host cells

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ARTICLE INFO

Article history:
Received 14 April 2015
Received in revised form 19 May 2015
Accepted 21 May 2015
Available online 26 May 2015

Keywords:
Mycobacterium
Membrane cholesterol
MβCD
Amphotericin B

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.

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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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http://dx.doi.org/10.1016/j.chemphyslip.2015.05.006
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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 $mc^2 6$ 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

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