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Statin-induced chronic cholesterol depletion inhibits *Leishmania donovani* infection: Relevance of optimum host membrane cholesterol

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ABSTRACT

Leishmania are obligate intracellular protozoan parasites that invade and survive within host macrophages leading to leishmaniasis, a major cause of mortality and morbidity worldwide, particularly among economically weaker sections in tropical and subtropical regions. Visceral leishmaniasis is a potent disease caused by *Leishmania donovani*. The detailed mechanism of internalization of *Leishmania* is poorly understood. A basic step in the entry of *Leishmania* involves interaction of the parasite with the host plasma membrane. In this work, we have explored the effect of chronic metabolic cholesterol depletion using lovastatin on the entry and survival of *Leishmania donovani* in host macrophages. We show here that chronic cholesterol depletion of host macrophages results in reduction in the attachment of *Leishmania* promastigotes, along with a concomitant reduction in the intracellular amastigote load. These results assume further relevance since chronic cholesterol depletion is believed to mimic physiological cholesterol modulation. Interestingly, the reduction in the ability of *Leishmania* to enter host macrophages could be reversed upon metabolic replenishment of cholesterol. Importantly, enrichment of host membrane cholesterol resulted in reduction in the entry and survival of *Leishmania* in host macrophages. As a control, the binding of *Escherichia coli* to host macrophages remained invariant under these conditions, thereby implying specificity of cholesterol requirement for effective leishmanial infection. To the best of our knowledge, these results constitute the first comprehensive demonstration that *an optimum content of host membrane cholesterol* is necessary for leishmanial infection. Our results assume relevance in the context of developing novel therapeutic strategies targeting cholesterol-mediated leishmanial infection.

1. Introduction

Leishmania are obligate protozoan parasites that are responsible for substantial public health problems in 98 countries around the world, especially in tropical and subtropical regions [1]. The parasite is the causative organism for the disease leishmaniasis which is usually fatal, if left untreated [2–4]. It is estimated that there are 1.3 million new cases reported annually, and the yearly toll of human fatality is between 20,000 and 30,000 [5]. The magnitude of morbidity and mortality associated with leishmaniasis has been correlated to a strong link of the disease with poverty [6]. Human visceral leishmaniasis (VL), one of the four types of leishmaniasis which affects liver, spleen, and bone marrow, is a potent disease caused by *Leishmania donovani* [2]. The recent worldwide increase in leishmaniasis to epidemic proportions, and the emergence of VL as an important opportunistic infection among people with HIV-1 infection [7] have contributed to an urgency to provide treatment for leishmaniasis.

Entry of *Leishmania* is facilitated by the bite of the infected female sandfly (*Phlebotomus* spp.) vector while taking a blood meal from a host [8]. *Leishmania* exists in two distinct forms through its lifecycle; the flagellated extracellular promastigote form that subsequently transforms into aflagellated amastigote form within host cells [4,9]. Entry of *Leishmania* into host macrophages involves multiple parasite-host interactions, and recognition of specific ligands on the parasite cell surface by receptors on the macrophage cell surface [8,10,11]. A number of studies toward understanding the molecular mechanisms of parasite entry have led to the identification of several candidate receptors (such as the mannose-fucose receptor, receptor for advanced glycosylation end products, the fibronectin receptor, the Fc receptor and

Abbreviations: FCS, fetal calf serum; FITC, fluorescein isothiocyanate; MβCD, methyl-β-cyclodextrin; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; MTT, 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl-tetrazolium bromide; TLC, thin layer chromatography; VL, visceral leishmaniasis.

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complement receptors CR1 and CR3) that facilitate multiple routes of entry into host macrophages [12,13]. The large number of distinct receptors responsible for the entry of *Leishmania* into host macrophages highlights the redundancy in the entry process, thereby making it extremely challenging to establish a unique therapeutic target for the treatment of leishmaniasis.

Leishmanial entry involves interaction of the parasite with the plasma membrane of host cells. The host plasma membrane is complex, and patchy, and exhibits lateral heterogeneity, collectively termed as membrane domains [14]. These specialized regions in the plasma membrane are believed to be enriched in specific lipids and proteins, which enable them to facilitate processes such as trafficking, sorting, and signal transduction over a range of spatiotemporal scale [15]. Does the lipid composition of the plasma membrane control parasite entry into host cells, and if the answer is yes, could this principle be utilized to inhibit parasite entry? In order to address this question, we previously demonstrated the requirement of host membrane cholesterol in the binding and internalization of *Leishmania donovani* into macrophages using complementary approaches [16–22]. In support of these results, Rodríguez et al. showed that plasma membrane cholesterol is necessary for the entry of *Leishmania chagasi* into host macrophages [23]. In addition, we have recently shown that destabilization of the actin cytoskeleton of macrophages results in reduction in the entry of *Leishmania donovani* into host cells, due to a possible cross-talk between membrane cholesterol and the actin cytoskeleton [24]. This observation is relevant in light of the emerging relationship between membrane cholesterol and the actin cytoskeleton [25–28; P. Sarkar, G.A. Kumar, S. Shrivastava, A. Chattopadhyay, unpublished observations].

As mentioned above, we [16] and others [23] have previously demonstrated the requirement of host membrane cholesterol in the entry of *Leishmania* into host cells. This was achieved by the use of methyl-β-cyclodextrin (MβCD). MβCD is a water-soluble polymer that has earlier been shown to selectively and efficiently extract cholesterol from cellular membranes by including it in a central nonpolar cavity [29]. Treatment of macrophages with MβCD resulted in the specific removal of membrane cholesterol and a concomitant reduction in the entry of *Leishmania* [16,23]. However, membrane cholesterol depletion using MβCD suffers from a number of limitations [30]. A major drawback is that cholesterol depletion using MβCD is an *acute* process due to the relatively short time of treatment. An alternative and more physiological approach is *chronic* cholesterol depletion using statins [31]. Statins are competitive inhibitors of HMG-CoA reductase, the crucial enzyme that catalyzes the rate-limiting step in the cholesterol biosynthetic pathway. This specific step involves the conversion of HMG-CoA into mevalonate, the precursor of cholesterol and other isoprenoids, further downstream in the biosynthetic pathway. Statins represent one of the best selling drugs globally and in clinical history [32]. They are extensively used as oral cholesterol lowering drugs to treat hypercholesterolaemia and dyslipidaemia [33,34].

In this work, we have explored the effect of chronic cholesterol depletion using lovastatin on the entry of *Leishmania donovani* into host macrophages. Our results show that chronic cholesterol depletion of host macrophages results in reduction in the attachment of *Leishmania* promastigotes, along with a concomitant reduction in the intracellular amastigote load. More importantly, we demonstrate that the reduction in the ability of *Leishmania* to enter host macrophages could be reversed upon metabolic replenishment of host cell membrane cholesterol. In order to further explore the role of membrane cholesterol in the entry of *Leishmania*, we enriched macrophages with excess cholesterol. *Interestingly*, our results show that the entry of *Leishmania* is *reduced* upon enrichment of host membrane cholesterol. To the best of our knowledge, these novel results constitute the first comprehensive demonstration that an optimum host plasma membrane cholesterol is necessary for the entry of *Leishmania* into host cells.

2. Materials and methods

2.1. Materials

Cholesterol, MβCD, antibiotic antimycotic solution, gentamicin sulfate, IMDM (Iscove's Modified Dulbecco's Medium), M-199 (Medium-199), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), FITC (Fluorescein isothiocyanate) and Giemsa stain were obtained from Sigma (St. Louis, MO). Fetal calf serum (FCS) was from Gibco/Life Technologies (Grand Island, NY). Lovastatin was obtained from Calbiochem (San Diego, CA). Amplex Red cholesterol assay kit was purchased from Molecular Probes/Invitrogen (Eugene, OR). Precoated silica gel 60 thin layer chromatography plates were from Merck (Darmstadt, Germany). All other chemicals and solvents 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. Cell culture

Murine macrophage cell line J774A.1 (American Type Culture Collection) was cultured as described previously [24]. Briefly, cells were maintained in IMDM medium supplemented with 2.4 g/l sodium bicarbonate, 10% heat-inactivated FCS, and antibiotic antimycotic (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) solution in a humidified atmosphere with 5% CO_2 at 37 °C.

2.2.2. Parasite culture

Leishmania donovani strain AG83 (MHOM/IN/1983/AG83) promastigotes were maintained in M-199 medium as described previously [35], supplemented with 200 μg/ml gentamicin sulfate and 10% heat-inactivated FCS at 22 °C. The capacity of promastigotes to infect hamsters was routinely checked to ensure the virulence property of promastigotes.

2.2.3. Isolation of murine primary peritoneal macrophages

Peritoneal macrophages were isolated from 8 to 10 week old BALB/c mice as described earlier with some modifications [21]. Resident peritoneal macrophages were obtained by injecting 10 ml of chilled PBS into the peritoneal cavity of BALB/c mice. Buffer containing the peritoneal exudates were centrifuged and washed with PBS. Cells were then suspended in IMDM medium containing 10% FCS, plated at a density of ~5×10^4 on glass coverslips and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Non-adherent cells were washed off after 24 h and adhered macrophages were incubated for 24 h prior to further experiments.

2.2.4. Chronic cholesterol depletion with lovastatin

Lovastatin was used for chronic depletion of cholesterol from J774A.1 cells and primary peritoneal macrophages. Lovastatin stock solution was prepared as described previously [36]. Cells were grown for 24 h and subsequently treated with 5 μM lovastatin for 24 h in IMDM medium with serum. Following the treatment, medium containing lovastatin was removed and cells were washed with PBS to remove excess statin.

2.2.5. Metabolic cholesterol replenishment

Metabolic replenishment of cholesterol was carried out with serum as described previously [36]. For this, cells were incubated in IMDM medium containing 20% serum for 24 h after treatment with statin.

2.2.6. Cholesterol enrichment using MβCD-cholesterol complex

Macrophage membrane cholesterol was enriched over its basal levels using the water-soluble MβCD-cholesterol complex as described previously [37] with some modifications. Briefly, the required amount