



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



G. Aditya Kumar^{a,1}, Saptarshi Roy^{b,1}, Md. Jafurulla^a, Chitra Mandal^{b,*}, Amitabha Chattopadhyay^{a,*}

^a CSIR-Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

^b CSIR-Indian Institute of Chemical Biology, Raja S.C. Mullick Road, Kolkata 700 032, India

ARTICLE INFO

Article history:

Received 10 January 2016

Received in revised form 8 June 2016

Accepted 15 June 2016

Available online 16 June 2016

Keywords:

Leishmania

Lovastatin

Promastigotes

Amastigotes

Optimal membrane cholesterol

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.

© 2016 Elsevier B.V. All rights reserved.

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; M β CD, 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.

* Corresponding authors.

E-mail addresses: cmandal@icb.res.in (C. Mandal), amit@ccmb.res.in

(A. Chattopadhyay).

¹ Equal contribution.

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 (M β CD). M β CD 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 M β CD resulted in the specific removal of membrane cholesterol and a concomitant reduction in the entry of *Leishmania* [16,23]. However, membrane cholesterol depletion using M β CD suffers from a number of limitations [30]. A major drawback is that cholesterol depletion using M β CD 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, M β CD, 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). Pre-coated 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 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B) solution in a humidified atmosphere with 5% CO₂ 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 μ g/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 $\sim 5 \times 10^4$ on glass coverslips and incubated at 37 °C in a humidified atmosphere with 5% CO₂. 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 μ M 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 M β CD-cholesterol complex

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

of M β CD and cholesterol in the ratio of 10:1 (5 mM:0.5 mM) were dissolved in IMDM medium without serum under constant vortexing at room temperature (~25 °C). Cells were incubated with freshly prepared complex for 20 min at 37 °C. After the treatment, cells were washed with PBS to remove excess complex.

2.2.7. MTT viability assay

Viability of macrophages upon treatment with lovastatin or M β CD-cholesterol complex was assessed using MTT assay as described earlier [24,37].

2.2.8. Lipid and protein estimation

Free cellular cholesterol and sphingomyelin were estimated from cell lysates using the Amplex Red assay kit [38]. Cholesterol and sphingomyelin content was normalized to total cellular protein levels estimated using the bicinchoninic acid (BCA) assay [39]. Total

phospholipid was assayed subsequent to total digestion by perchloric acid as described previously [29].

2.2.9. Thin layer chromatography

Total lipids were extracted from statin-treated and control macrophages according to Bligh and Dyer [40]. The lipid extracts were dried under a stream of nitrogen at 45 °C. The dried extracts were then dissolved in a mixture of chloroform/methanol (1:1, v/v). Total cellular lipids were resolved on pre-coated silica gel TLC plates using chloroform/methanol/water (65:25:4, v/v/v) as the solvent system and visualized using ultraviolet light after spraying a fluorescent solution of 0.01% (w/v) primuline [41] (Fig. S1).

2.2.10. Flow cytometric analysis of promastigote binding

Labeling of *L. donovani* promastigotes with FITC was carried out as described previously [24]. Parasites in the mid log phase were incubated

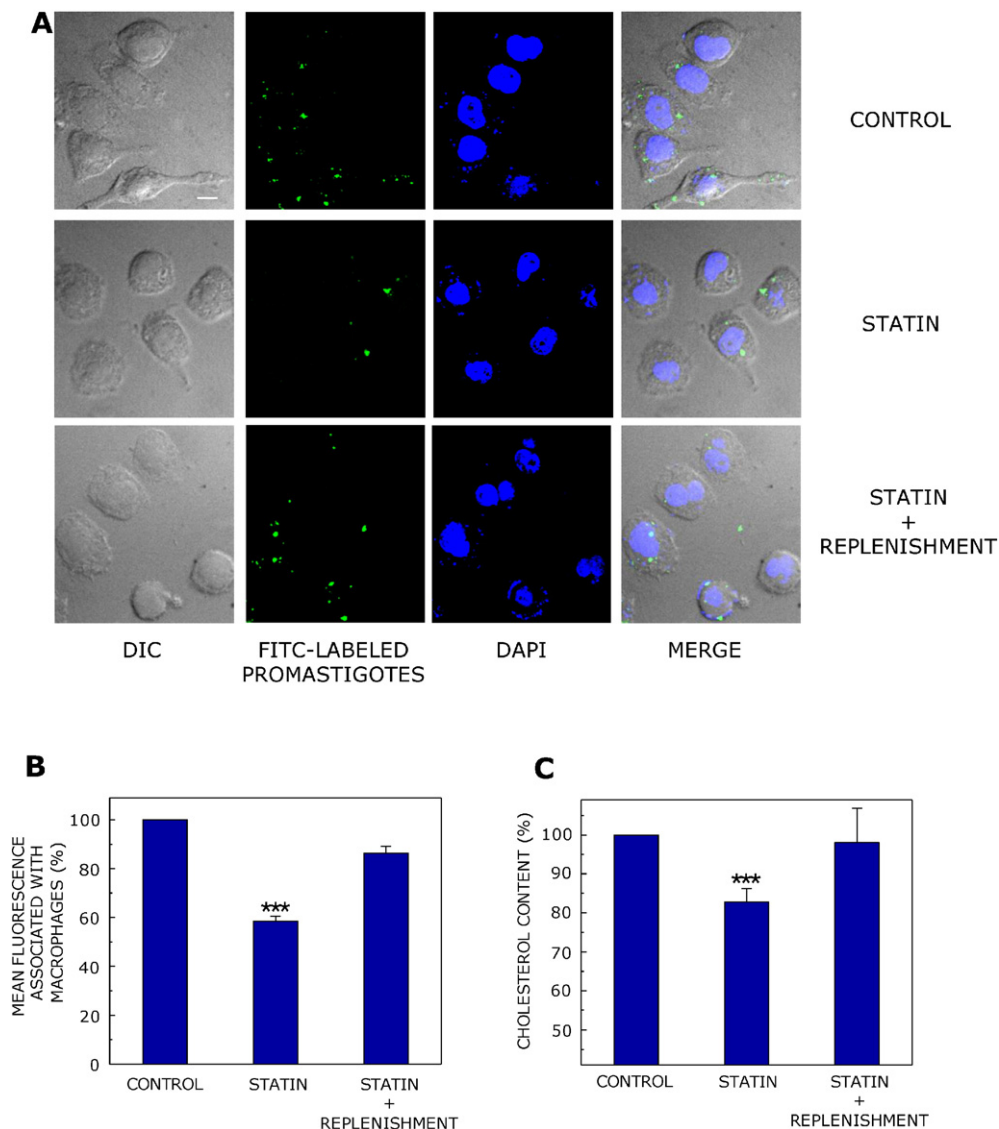


Fig. 1. Chronic cholesterol depletion inhibits binding of *Leishmania donovani* promastigotes to host macrophages. J774A.1 macrophages treated with lovastatin were infected with *Leishmania donovani* promastigotes at a multiplicity of infection of 10:1 (parasite to macrophage). Panel A shows representative confocal microscopic images of promastigotes bound to untreated (control), statin-treated and cholesterol-replenished macrophages. The figure shows macrophages (DIC images) infected with FITC-labeled *Leishmania* promastigotes (green). The macrophage nuclei were stained with DAPI (blue). Merged images are shown in the panel on the extreme right. The scale bar represents 10 μ m. Panel B shows quantitative flow cytometric estimates of FITC-labeled promastigotes bound to untreated (control), statin-treated and cholesterol-replenished macrophages. Values are normalized to mean fluorescence associated with control macrophages. Panel C shows cholesterol content in host cells under control, statin-treated and cholesterol-replenished conditions. Values are normalized to cholesterol content in control (untreated) macrophages. Data represent means \pm S.E. of at least three independent experiments (***) corresponds to significant ($p < 0.001$) difference in mean fluorescence associated with macrophages (panel B) and cholesterol content in macrophages (panel C) treated with statin relative to control cells. See Section 2 for other details.

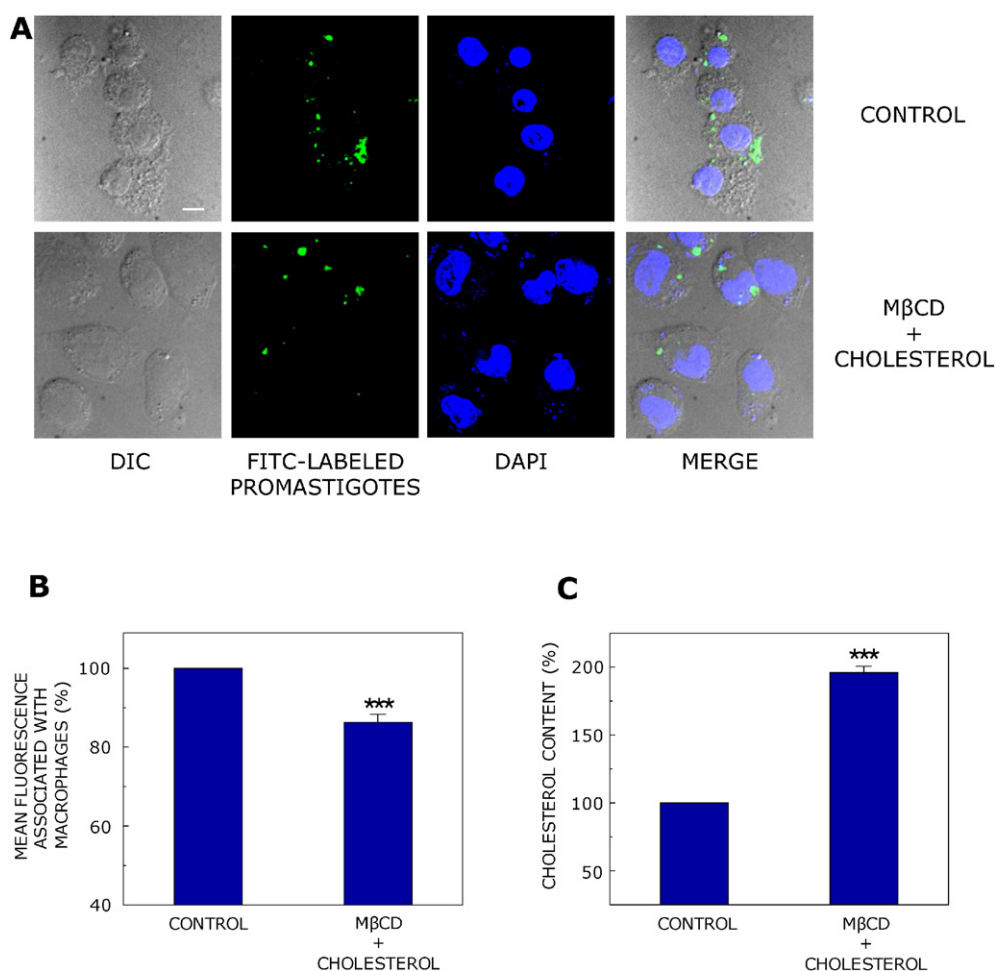


Fig. 2. Enrichment of host membrane cholesterol inhibits binding of *Leishmania donovani* promastigotes to host macrophages. Macrophages treated with MβCD-cholesterol complex were infected with *Leishmania donovani* promastigotes at a multiplicity of infection of 10:1 (parasite to macrophage). Panel A shows representative confocal microscopic images of promastigotes bound to untreated (control) macrophages and cholesterol-enriched macrophages. The figure shows macrophages (DIC images) infected with FITC-labeled *Leishmania* promastigotes (green). The macrophage nuclei were stained with DAPI (blue). Merged images are shown in the panel on the extreme right. The scale bar represents 10 μm. Panel B shows quantitative flow cytometric estimates of FITC-labeled promastigotes bound to untreated (control) and cholesterol-enriched macrophages. Values are normalized to mean fluorescence associated with control macrophages. Panel C shows cholesterol content in host cells under control and cholesterol-enriched conditions. Values are normalized to cholesterol content in control macrophages. Data represent means ± S.E. of at least three independent experiments (***) corresponds to significant ($p < 0.001$) difference in mean fluorescence associated with macrophages (panel B) and cholesterol content in macrophages (panel C) treated with MβCD-cholesterol complex relative to control cells). See Section 2 for other details.

in 0.1% FITC in buffer A (50 mM carbonate buffer, pH 8.0) at 22 °C for 1 h. Parasites were thoroughly washed in PBS and suspended in IMDM medium containing 2% heat-inactivated FCS. Flow cytometry was used to confirm uniform labeling of promastigotes with FITC. J774A.1 macrophages were infected with FITC-labeled promastigotes at a multiplicity of infection of 10:1 (parasite to macrophage) and incubated at 37 °C for 4 h. Macrophages were then collected, washed and suspended in PBS. The binding of *Leishmania* promastigotes to J774A.1 cells was monitored using FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). The mean fluorescence of FITC-labeled promastigotes associated with 10,000 macrophages was monitored and subsequently analyzed using the CellQuest Pro analysis software.

2.2.11. Estimation of intracellular amastigote load

The number of intracellular amastigotes was estimated by microscopic analysis of infected mouse peritoneal macrophages as described previously [16,24] with some modifications. Briefly, peritoneal macrophages grown on coverslips were infected with *L. donovani* promastigotes at a multiplicity of infection of 10:1 (parasite to macrophage) for 4 h at 37 °C in IMDM medium. Unbound parasites were washed off and macrophages were further incubated for 20 h under

similar conditions. Coverslips were then washed with PBS, air dried and fixed with methanol prior to Giemsa staining. The intracellular amastigote load was visually scored using a Zeiss microscope with a 100× oil objective, and the number of amastigotes was normalized to 100 macrophages.

2.2.12. Flow cytometric analysis of *Escherichia coli* binding to macrophages

Labeling of *E. coli* DH5α cells with FITC was carried out as described previously [24]. Briefly, bacteria grown overnight in Luria broth at 37 °C under shaking were incubated in 0.1% FITC in buffer A for 30 min at the same temperature. Bacteria were pelleted down and washed thoroughly in PBS to remove unbound stain. J774A.1 macrophages were infected with FITC-labeled *E. coli* at a multiplicity of infection of 100:1 (bacteria to macrophage) and incubated at 37 °C for 30 min. Cells were processed for flow cytometric analysis as described in Section 2.2.10.

2.2.13. Fluorescence imaging of promastigote binding to macrophages

J774A.1 macrophages were plated on glass coverslips at a density of $\sim 2 \times 10^4$ and grown in IMDM medium for 24 h. After modulation of cholesterol content, macrophages were infected with FITC-labeled *L. donovani* promastigotes for 4 h as described in Section 2.2.10. Cells

were washed with PBS to remove unbound parasites prior to fixation with 3.5% (v/v) formaldehyde. Coverslips were mounted in media containing DAPI and images were acquired on an Andor Spinning Disc Confocal microscope (Belfast, U.K.) with a 60 \times /1.42 NA oil immersion objective.

2.2.14. Statistical analysis

Student's two-tailed unpaired *t*-test was performed to estimate significance levels using Graphpad Prism software, version 4.0 (San Diego, CA). Plots were generated using OriginPro software, version 8.0 (OriginLab, Northampton, MA).

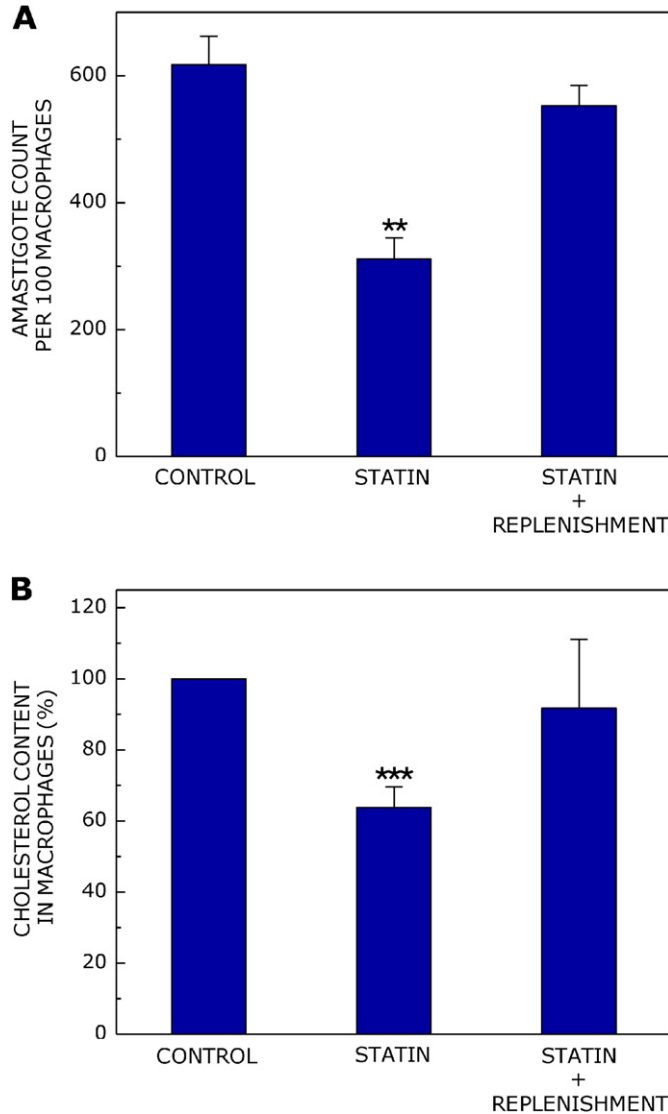


Fig. 3. Chronic depletion of host membrane cholesterol results in reduced intracellular amastigote load. Peritoneal macrophages obtained from BALB/c mice were treated with statin and subsequently infected with *Leishmania donovani* at a multiplicity of infection of 10:1 (parasite to macrophage). Panel A shows the number of intracellular amastigotes in control (untreated), statin-treated and cholesterol-replenished macrophages upon Giemsa staining. Data represent means \pm S.E. of at least three independent experiments (** corresponds to significant ($p < 0.01$) difference in intracellular amastigote counts in statin-treated cells relative to control macrophages). Panel B shows cholesterol content in host macrophages under control, statin-treated and cholesterol-replenished conditions. Values are normalized to cholesterol content in control (untreated) macrophages. Data represent means \pm S.E. of at least three independent experiments (*** corresponds to significant ($p < 0.001$) difference in cholesterol content in macrophages treated with statin relative to control cells). See Section 2 for other details.

3. Results

3.1. Statin-induced chronic cholesterol depletion in host macrophages inhibits binding of *Leishmania donovani*

Cholesterol is an essential membrane lipid in higher eukaryotes and plays a vital role in several pathophysiological conditions owing to its crucial role in the organization, dynamics and function of membrane constituents [42–45]. The biosynthesis of cholesterol involves a long, multi-step enzymatic pathway which parallels the evolution of sterols [42]. Statins are a class of molecules that act as competitive inhibitors of HMG-CoA reductase, the rate-limiting enzyme in the cholesterol

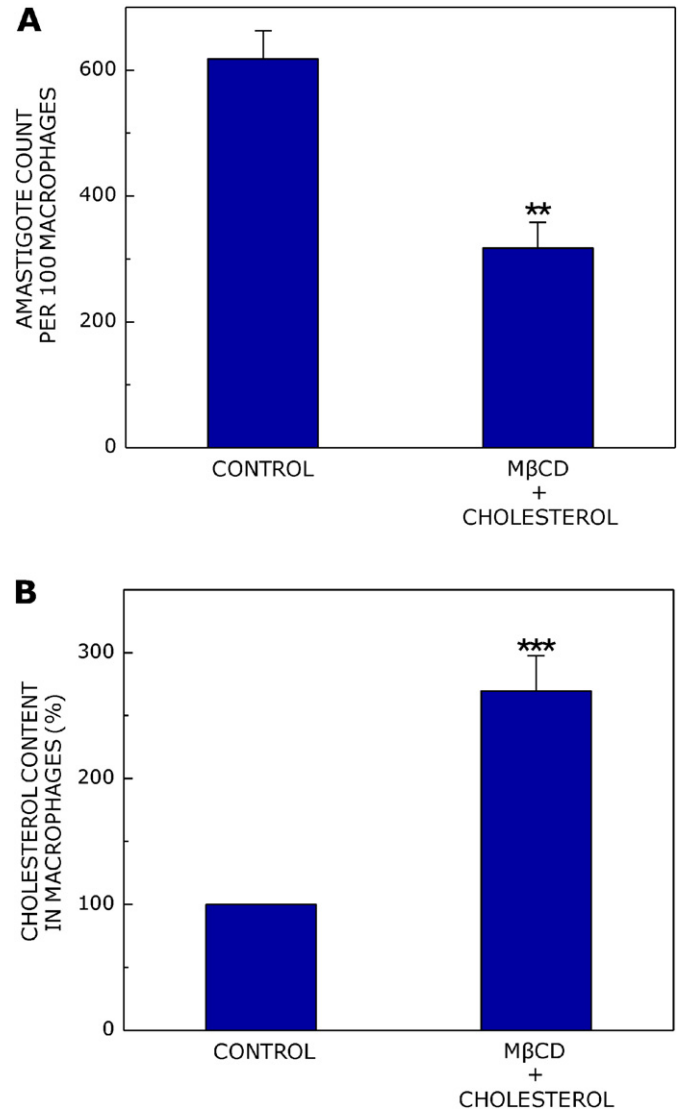


Fig. 4. Reduction in intracellular amastigote load upon enrichment of host membrane cholesterol. Peritoneal macrophages from BALB/c mice treated with M β CD-cholesterol complex were infected with *Leishmania donovani* promastigotes at a multiplicity of infection of 10:1 (parasite to macrophage). Panel A shows the number of intracellular amastigotes in control (untreated) and cholesterol-enriched macrophages upon Giemsa staining. Data represent means \pm S.E. of at least three independent experiments (** corresponds to significant ($p < 0.01$) difference in intracellular amastigote counts in cholesterol-enriched cells relative to control macrophages). Panel B shows cholesterol content in host macrophages under control and cholesterol-enriched conditions. Values are normalized to cholesterol content in control (untreated) macrophages. Data represent means \pm S.E. of at least three independent experiments (*** corresponds to significant ($p < 0.001$) difference in cholesterol content in macrophages treated with M β CD-cholesterol complex relative to control cells). See Section 2 for other details.

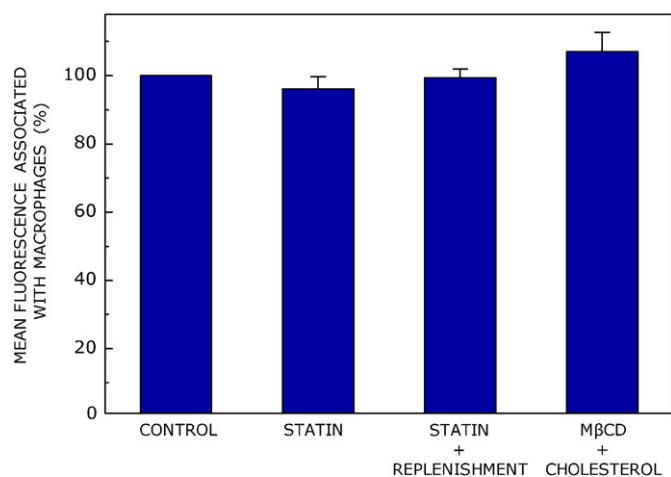


Fig. 5. Modulation of host membrane cholesterol does not affect the binding of *E. coli* to macrophages. J774A.1 macrophages were infected with FITC-labeled *E. coli* at a multiplicity of infection of 100:1 (bacteria to macrophage). The figure shows quantitative estimates of the number of *E. coli* bound to control (untreated), statin-treated, cholesterol-replenished and cholesterol-enriched macrophages. Values are normalized to mean fluorescence associated with control macrophages. Data represent means \pm S.E. of at least three independent experiments. See Section 2 for other details.

biosynthetic pathway [34]. HMG-CoA reductase catalyzes the conversion of HMG-CoA into mevalonate, which is a precursor of cholesterol. Statins are the top selling drugs globally and in clinical history [32].

We explored the effect of chronic depletion of host membrane cholesterol on the binding of *Leishmania donovani* promastigotes upon treating J774A.1 macrophages with lovastatin. The association of fluorescently (FITC)-labeled promastigotes with macrophages was monitored using confocal microscopic and flow cytometric approaches. Fig. 1A shows fluorescence microscopic images of *Leishmania donovani* promastigotes bound to macrophages upon chronic depletion and metabolic replenishment of cholesterol. The figure shows reduction in the number of FITC-labeled promastigotes bound to macrophages treated with lovastatin compared to control macrophages. Quantitative flow cytometric analysis indicated ~41% reduction in fluorescence associated with statin-treated macrophages relative to control macrophages upon infection with FITC-labeled promastigotes (see Fig. 1B). Interestingly, metabolic replenishment of membrane cholesterol in statin-treated macrophages could restore binding of promastigotes to host cells to control levels (see Fig. 1A and B). The corresponding changes in cholesterol under these conditions are shown in Fig. 1C. These results show that chronic cholesterol depletion by lovastatin affects the ability of *Leishmania* promastigotes to interact with the host macrophage cell surface, and metabolic replenishment of cholesterol restores the binding of promastigotes to normal levels. Interestingly, we found that the reduction in promastigote binding is more sensitive to host cell membrane cholesterol content in case of statin (chronic) treatment relative to acute depletion of cholesterol using M β CD reported previously by us [16]. The manner in which cholesterol depletion is carried out (acute vs. chronic) is therefore important rather than merely cholesterol content. The total lipid contents of statin-treated and control macrophages were analyzed by TLC (see Fig. S1). It may be noted here that the total phospholipid and sphingomyelin contents remained invariant in macrophages upon statin treatment (Fig. S2).

3.2. Binding of *Leishmania* promastigotes to macrophages is inhibited by enrichment of host membrane cholesterol

In order to monitor the effect of enhancement of host membrane cholesterol over control levels on the binding of *Leishmania* promastigotes, we enriched macrophage cholesterol using M β CD-cholesterol complex. Treatment with the M β CD-cholesterol complex augmented the

cholesterol content in J774A.1 macrophages to ~196% of control levels (see Fig. 2C). As shown in the representative confocal images in Fig. 2A, we observed a reduction in the number of promastigotes bound to macrophages upon cholesterol enrichment. Flow cytometric analysis of the fluorescence associated with macrophages infected with FITC-labeled promastigotes showed ~14% reduction in the binding of *Leishmania* to macrophages under cholesterol enrichment condition (Fig. 2B). These results, combined with our observation on the reduction in binding of *Leishmania* promastigotes to host macrophages upon chronic cholesterol depletion (Fig. 1), demonstrate the requirement of optimal levels of cholesterol in host cells for parasite binding. In other words, the entry of *Leishmania donovani* is inhibited both above and below the optimum level of membrane cholesterol. Control experiments using MTT assay showed that macrophage viability remained invariant under these conditions (see Fig. S3).

3.3. Chronic cholesterol depletion results in reduced intracellular amastigote load

Leishmania promastigotes transform into aflagellar amastigotes (the intracellular form of the parasite) upon invading host cells. To further assess the effect of modulation of host membrane cholesterol content on *Leishmania* amastigotes, we infected primary peritoneal macrophages from BALB/c mice with the parasite under above conditions. Fig. 3B shows ~36% reduction in the cholesterol content in primary macrophages upon treatment with lovastatin. The intracellular amastigote load (i.e., the number of amastigotes within primary macrophages) was visually scored upon Giemsa staining of the infected macrophages. Fig. 3A shows that statin-induced chronic reduction in cholesterol content was associated with a concomitant decrease in the number of *Leishmania* amastigotes by ~50% compared to control cells. Importantly, metabolic replenishment of cholesterol was able to restore the cholesterol content as well as the amastigote count to control levels (see Fig. 3A and B).

3.4. Enrichment of host membrane cholesterol leads to reduction in the number of amastigotes within host cells

The effect of cholesterol enrichment on the number of intracellular amastigotes within host cells was checked. Fig. 4B shows that treatment of macrophages with M β CD-cholesterol complex enhanced the cholesterol content by ~270% over control. Under these conditions, we observed ~49% reduction in the number of amastigotes within macrophages, as shown in Fig. 4A. These results, taken together with our results shown in Fig. 3, point toward a stringent dependence of intracellular amastigote load on host membrane cholesterol with the underlying message that an optimum host membrane cholesterol is necessary for efficient parasite infection (see Discussion and Fig. 6).

3.5. Binding of *E. coli* to host cells remains invariant with modulation of membrane cholesterol

The specificity of the requirement of optimal membrane cholesterol for *Leishmania* infection was addressed by monitoring the effect of modulation of host membrane cholesterol on the binding of *E. coli* DH5 α in a control experiment. Fig. 5 shows that the binding of *E. coli* to host macrophages did not exhibit any significant variation, regardless of the level of host membrane cholesterol. These results therefore support specificity of the requirement of cholesterol for effective interaction between *Leishmania donovani* and the macrophage cell membrane.

4. Discussion

A number of studies have indicated the crucial requirement of membrane cholesterol in host-pathogen interaction (recently reviewed in [9]). In this overall scenario, we previously demonstrated the

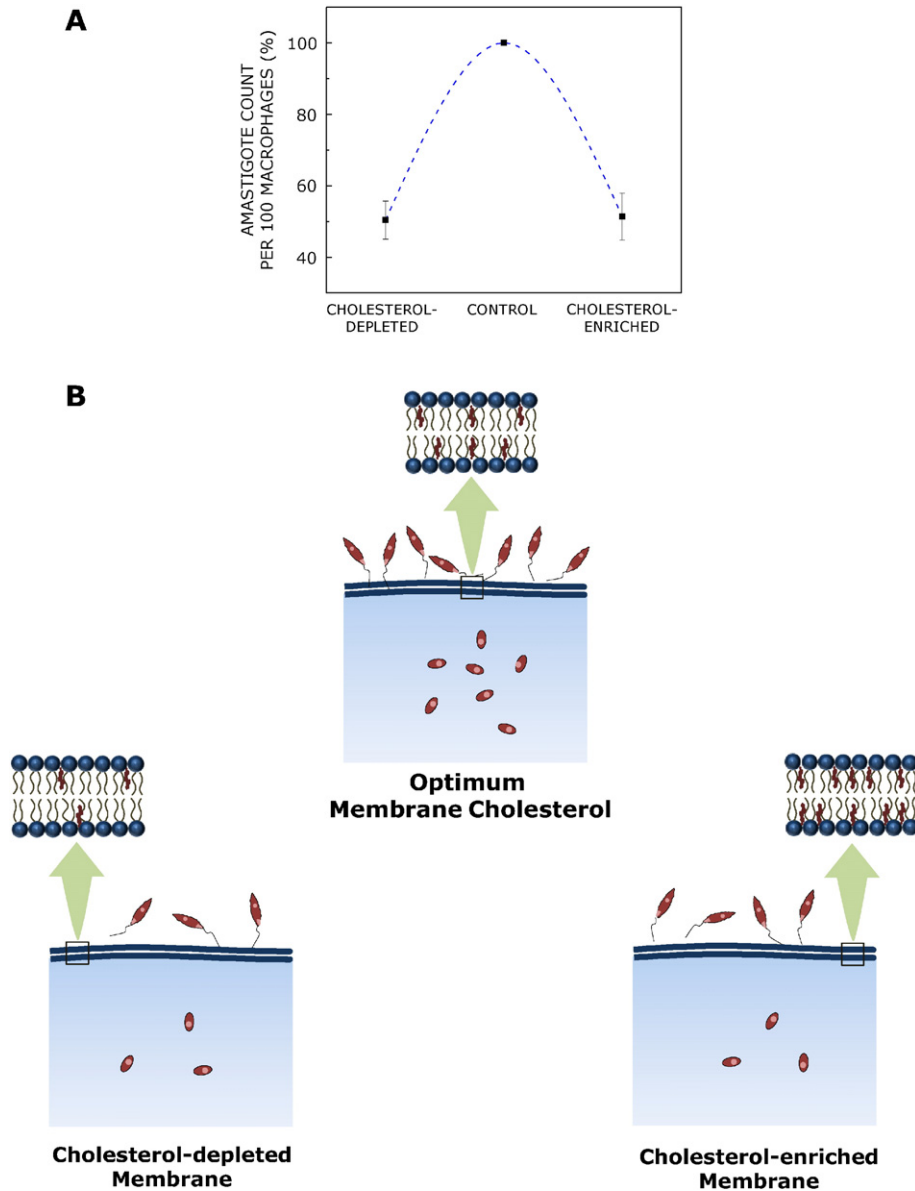


Fig. 6. An optimum host membrane cholesterol is necessary for leishmanial infection. Panel A shows the number of amastigotes within macrophages under conditions of chronic cholesterol depletion and enrichment. Data plotted are taken from Figs. 3A and 4A, and have been represented as a percentage of amastigotes within macrophages visually scored normalized to respective control conditions. Panel B shows a schematic representation of the requirement of optimum levels of host membrane cholesterol for leishmanial infection in host macrophages. Statin-induced chronic cholesterol depletion, as well as enrichment of host membrane cholesterol, result in reduction in the number of promastigote and amastigote forms of the parasite associated with macrophages, thereby pointing to the requirement of an optimum membrane cholesterol for infection.

requirement of membrane cholesterol in infection by *Leishmania donovani* [16,17,21] and *Mycobacterium smegmatis* [37]. These results are supported by work from other groups [23,46]. In the present work, we have further dissected the membrane cholesterol requirement in the binding and internalization of *Leishmania* into host macrophages. We show here that chronic cholesterol depletion of host cell membranes using lovastatin results in inhibition of leishmanial infection. Although the effect of host membrane cholesterol on the entry of *Leishmania* has previously been examined by us [16] and others [23], depletion of cholesterol was achieved in these cases by acute treatment, i.e., by using M β CD as a cholesterol carrier. To the best of our knowledge, the present work constitutes the first comprehensive demonstration that leishmanial entry is inhibited by chronic (metabolic) cholesterol depletion. As mentioned above, chronic cholesterol depletion enjoys certain advantages over acute cholesterol depletion, and is believed to be closer to cholesterol changes observed in physiological conditions. Another novel finding is the observation that leishmanial

entry showed considerable reduction upon enrichment of host membrane cholesterol. Taken together, our results show, for the first time, that an optimum level of host membrane cholesterol is necessary for efficient entry of *Leishmania donovani* (see Fig. 6). We have recently shown that such dependence on optimal host membrane cholesterol could be a hallmark of mycobacterial entry into host cells [37]. Whether this holds good for other intracellular pathogens whose entry is regulated by host membrane cholesterol requires careful examination.

Besides their major use as cholesterol-lowering agents, statins are a class of molecules with multiple functions [47]. For example, they have been implicated in neuroprotection [48], cancer [49] and cell cycle arrest [36]. A common theme emerging from a number of studies is the role of statins in treating infections [50–55], although there appears to be a lack of consensus on a common mechanism. Our present results of optimal host membrane cholesterol requirement in leishmanial infection using statin treatment of host cells extend these observations,

and could have potential implications in the control and therapy of leishmaniasis.

As mentioned above, a number of receptors on the host cell membrane have been implicated in the entry of *Leishmania* [12,13]. Since these receptors reside in the host cell membrane, their regulation by membrane cholesterol offers an attractive mechanism for their role in leishmanial entry into host cells. Previous results from our and other laboratories have shown that the organization and function of many membrane receptors depend on membrane cholesterol [56–60]. Based on this, we propose that the conformation of host membrane receptors necessary for leishmanial entry could be dependent on an optimal level of membrane cholesterol. This could imply that the local conformation of these receptors may not support leishmanial entry at cholesterol concentrations above and below the optimum level [61]. In agreement with this proposition, there are examples of membrane proteins (such as the Na⁺, K⁺-ATPase [62] and GABA_A receptor [63]) whose function has been shown to be optimal within a range of membrane cholesterol (i.e., optimal membrane cholesterol). The function of these proteins is inhibited both above and below the optimum level of cholesterol. It is interesting to speculate that receptor(s) necessary for leishmanial entry could share such a property of cholesterol-dependent conformation and function. This proposal awaits experimental validation.

In this context, it is worth noting here that several membrane proteins that interact with cholesterol have been identified with a characteristic amino acid sequence, termed the cholesterol recognition/interaction amino acid consensus (CRAC) motif. The CRAC motif is denoted by the pattern –L/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 [64,65]. CRAC motifs have been shown to be present in a number of membrane proteins such as caveolin-1 [66], the peripheral-type benzodiazepine receptor [65], the HIV-1 transmembrane protein gp41 [67], and G protein-coupled receptors [68,69]. An encouraging feature is the presence of multiple CRAC motifs in membrane receptors (such as complement receptors, mannose receptor, Fc receptor and fibronectin receptor) responsible for the entry of *Leishmania* into host macrophages (not shown). This could offer a possible mechanism for interaction of these crucial receptors with membrane cholesterol.

Taken together, these results demonstrate that leishmanial infection is stringently controlled by host membrane cholesterol, and an optimum content of host membrane cholesterol is necessary for effective interaction between the parasite and the host cell membrane. Our results could be significant in developing future therapeutic strategies to tackle leishmaniasis in particular, and diseases caused by other intracellular pathogens in general, whose entry is dependent on host membrane cholesterol.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by the Council of Scientific and Industrial Research (Govt. of India) Network project HOPE (BSC0114), BIOCERAM (ESC0103) and the Indian Council of Medical Research (Govt. of India) (GAP 294). G.A.K. and S.R. thank the Council of Scientific and Industrial Research for the award of Senior Research Fellowships. A.C. and C.M. gratefully acknowledge support from J.C. Bose Fellowship (Department of Science and Technology, Govt. of India). A.C. is an Adjunct Professor of Tata Institute of Fundamental Research (Mumbai), RMIT University (Melbourne, Australia), Indian Institute of Technology (Kanpur), and Indian Institute of Science Education and Research (Mohali). We thank members of the Chattopadhyay laboratory for their comments and discussions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbmem.2016.06.010>.

References

- [1] J. Alvar, I.D. Vélez, C. Bern, M. Herrero, P. Desjeux, J. Cano, J. Jannin, M. den Boer, The WHO Leishmaniasis Control Team, leishmaniasis worldwide and global estimates of its incidence, *PLoS One* 7 (2012), e35671.
- [2] B.L. Herwaldt, Leishmaniasis, *Lancet* 354 (1999) 1191–1199.
- [3] J. Alexander, A.R. Satoskar, D.G. Russell, *Leishmania* species: models of intracellular parasitism, *J. Cell Sci.* 112 (1999) 2993–3002.
- [4] F. Chappuis, S. Sundar, A. Hailu, H. Ghalib, S. Rijal, R.W. Peeling, J. Alvar, M. Boelaert, Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat. Rev. Microbiol.* 5 (2007) 873–882.
- [5] World health organization, (website) <http://www.who.int/leishmaniasis/en/>.
- [6] J. Alvar, S. Yactayo, C. Bern, Leishmaniasis and poverty, *Trends Parasitol.* 22 (2006) 552–557.
- [7] D. Wolday, N. Berhe, H. Akuffo, S. Britton, *Leishmania*-HIV interaction: immunopathogenic mechanisms, *Parasitol. Today* 15 (1999) 182–187.
- [8] E. Handman, D.V.R. Bullen, Interaction of *Leishmania* with the host macrophage, *Trends Parasitol.* 18 (2002) 332–334.
- [9] A. Chattopadhyay, M. Jafurulla, Role of membrane cholesterol in leishmanial infection, *Adv. Exp. Med. Biol.* 749 (2012) 201–213.
- [10] P. Kaye, P. Scott, Leishmaniasis: complexity at the host-pathogen interface, *Nat. Rev. Microbiol.* 9 (2011) 604–615.
- [11] M. Podinovskaia, A. Descoteaux, *Leishmania* and the macrophage: a multifaceted interaction, *Future Microbiol.* 10 (2015) 111–129.
- [12] M.G. Rittig, C. Bogdan, *Leishmania*-host-cell interaction: complexities and alternative views, *Parasitol. Today* 16 (2000) 292–297.
- [13] D. Sacks, S. Kamhawi, Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis, *Annu. Rev. Microbiol.* 55 (2001) 453–483.
- [14] D.M. Engelman, Membranes are more mosaic than fluid, *Nature* 438 (2005) 578–580.
- [15] K. Jacobson, O.G. Mouritsen, R.G.W. Anderson, Lipid rafts: at a crossroad between cell biology and physics, *Nat. Cell Biol.* 9 (2007) 7–14.
- [16] T.J. Pucadyil, P. Tewary, R. Madhubala, A. Chattopadhyay, Cholesterol is required for *Leishmania donovani* infection: implications in leishmaniasis, *Mol. Biochem. Parasitol.* 133 (2004) 145–152.
- [17] P. Tewary, K. Veena, T.J. Pucadyil, A. Chattopadhyay, R. Madhubala, The sterol-binding antibiotic nystatin inhibits entry of non-opsonized *Leishmania donovani* into macrophages, *Biochem. Biophys. Res. Commun.* 339 (2006) 661–666.
- [18] T.J. Pucadyil, A. Chattopadhyay, Cholesterol: a potential therapeutic target in *Leishmania* infection? *Trends Parasitol.* 23 (2007) 49–53.
- [19] A. Chattopadhyay, R. Madhubala, Method of treating leishmaniasis using methyl-beta-cyclodextrin, U.S. patent # 7186702 (2007).
- [20] A. Chattopadhyay, R. Madhubala, A composition useful for the treatment of leishmaniasis, Indian patent # 242180 (2010).
- [21] Y.D. Paila, B. Saha, A. Chattopadhyay, Amphotericin B inhibits entry of *Leishmania donovani* into primary macrophages, *Biochem. Biophys. Res. Commun.* 399 (2010) 429–433.
- [22] A. Chattopadhyay, M. Jafurulla, A novel mechanism for an old drug: amphotericin B in the treatment of visceral leishmaniasis, *Biochem. Biophys. Res. Commun.* 416 (2011) 7–12.
- [23] N.E. Rodríguez, U. Gaur, M.E. Wilson, Role of caveolae in *Leishmania chagasi* phagocytosis and intracellular survival in macrophages, *Cell. Microbiol.* 8 (2006) 1106–1120.
- [24] S. Roy, G.A. Kumar, M. Jafurulla, C. Mandal, A. Chattopadhyay, Integrity of the actin cytoskeleton of host macrophages is essential for *Leishmania donovani* infection, *Biochim. Biophys. Acta* 1838 (2014) 2011–2018.
- [25] J. Kwik, S. Boyle, D. Fooksman, L. Margolis, M.P. Sheetz, M. Edidin, Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 13964–13969.
- [26] F.R. Maxfield, I. Tabas, Role of cholesterol and lipid organization in disease, *Nature* 438 (2005) 612–621.
- [27] M. Sun, N. Northup, F. Marga, T. Huber, F.J. Byfield, I. Levitan, G. Forgacs, The effect of cellular cholesterol on membrane-cytoskeleton adhesion, *J. Cell Sci.* 120 (2007) 2223–2231.
- [28] S. Ganguly, A. Chattopadhyay, Cholesterol depletion mimics the effect of cytoskeletal destabilization on membrane dynamics of the serotonin_{1A} receptor: a zFCS study, *Biophys. J.* 99 (2010) 1397–1407.
- [29] T.J. Pucadyil, A. Chattopadhyay, Cholesterol depletion induces dynamic confinement of the G-protein coupled serotonin_{1A} receptor in the plasma membrane of living cells, *Biochim. Biophys. Acta* 1768 (2007) 655–668.
- [30] R. Zidovetzki, I. Levitan, Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies, *Biochim. Biophys. Acta* 1768 (2007) 1311–1324.
- [31] S. Shrivastava, T.J. Pucadyil, Y.D. Paila, S. Ganguly, A. Chattopadhyay, Chronic cholesterol depletion using statin impairs the function and dynamics of human serotonin_{1A} receptors, *Biochemistry* 49 (2010) 5426–5435.
- [32] S. Schlyer, R. Horuk, I want a new drug: G-protein-coupled receptors in drug development, *Drug Discov. Today* 11 (2006) 481–493.
- [33] E.S. Istvan, J. Deisenhofer, Structural mechanism for statin inhibition of HMG-CoA reductase, *Science* 292 (2001) 1160–1164.

- [34] C.R. Sirtori, The pharmacology of statins, *Pharmacol. Res.* 88 (2014) 3–11.
- [35] S. Bandyopadhyay, M. Chatterjee, T. Das, S. Bandyopadhyay, S. Sundar, C. Mandal, Antibodies directed against O-acetylated sialoglycoconjugates accelerate complement activation in *Leishmania donovani* promastigotes, *J. Infect. Dis.* 190 (2004) 2010–2019.
- [36] P. Singh, R. Saxena, G. Srinivas, G. Pande, A. Chattopadhyay, Cholesterol biosynthesis and homeostasis in regulation of the cell cycle, *PLoS One* 8 (2013), e58833.
- [37] G. Viswanathan, M. Jafurulla, G.A. Kumar, T.R. Raghunand, A. Chattopadhyay, Dissecting the membrane cholesterol requirement for mycobacterial entry into host cells, *Chem. Phys. Lipids* 189 (2015) 19–27.
- [38] D.M. Amundson, M. Zhou, Fluorometric method for the enzymatic determination of cholesterol, *J. Biochem. Biophys. Methods* 38 (1999) 43–52.
- [39] P.K. Smith, R.L. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, *Anal. Biochem.* 150 (1985) 76–85.
- [40] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol.* 37 (1959) 911–917.
- [41] G. van Echten-Deckert, Sphingolipid extraction and analysis by thin-layer chromatography, *Methods Enzymol.* 312 (2000) 64–79.
- [42] K.E. Bloch, Sterol structure and membrane function, *CRC Crit. Rev. Biochem.* 14 (1983) 47–92.
- [43] O.G. Mouritsen, M.J. Zuckermann, What's so special about cholesterol? *Lipids* 39 (2004) 1101–1113.
- [44] D. Lingwood, K. Simons, Lipid rafts as a membrane-organizing principle, *Science* 327 (2010) 46–50.
- [45] A. Chaudhuri, A. Chattopadhyay, Transbilayer organization of membrane cholesterol at low concentrations: implications in health and disease, *Biochim. Biophys. Acta* 1808 (2011) 19–25.
- [46] J. Gatfield, J. Pieters, Essential role for cholesterol in entry of mycobacteria into macrophages, *Science* 288 (2000) 1647–1650.
- [47] B. Haslinger-Löffler, Multiple effects of HMG-CoA reductase inhibitors (statins) besides their lipid-lowering function, *Kidney Int.* 74 (2008) 553–555.
- [48] P.J. van der Most, A.M. Dolga, I.M. Nijholt, P.G.M. Luiten, U.L.M. Eisel, Statins: mechanisms of neuroprotection, *Prog. Neurobiol.* 88 (2009) 64–75.
- [49] A.J. Brown, Cholesterol, statins and cancer, *Clin. Exp. Pharmacol. Physiol.* 34 (2007) 135–141.
- [50] G. del Real, S. Jiménez-Baranda, E. Mira, R.A. Lacalle, P. Lucas, C. Gómez-Moutón, M. Alegret, J.M. Peña, M. Rodríguez-Zapata, M. Alvarez-Mon, C. Martínez-A., S. Mañes, Statins inhibit HIV-1 infection by down-regulating Rho activity, *J. Exp. Med.* 200 (2004) 541–547.
- [51] S. Crunkhorn, Antimicrobials: cholesterol-biosynthesis inhibitor stops infection, *Nat. Rev. Drug Discov.* 7 (2008) 291.
- [52] I.M. Tleyjeh, T. Kashour, F.A. Hakim, V.A. Zimmerman, P.J. Erwin, A.J. Sutton, T. Ibrahim, Statins for the prevention and treatment of infections: a systematic review and meta-analysis, *Arch. Intern. Med.* 169 (2009) 1658–1667.
- [53] L. Björkhem-Bergman, P. Bergman, J. Andersson, J.D. Lindh, Statin treatment and mortality in bacterial infections – a systematic review and meta-analysis, *PLoS One* 5 (2010), e10702.
- [54] C.M. Martín-Navarro, J. Lorenzo-Morales, R.P. Machin, A. López-Arencibia, J.M. García-Castellano, I. de Fuentes, B. Loftus, S.K. Maciver, B. Valladares, J.E. Piñero, Inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and application of statins as a novel effective therapeutic approach against *Acanthamoeba* infections, *Antimicrob. Agents Chemother.* 57 (2013) 375–381.
- [55] S.P. Parihar, R. Guler, D.M. Lang, H. Suzuki, A.D. Marais, F. Brombacher, Simvastatin enhances protection against *Listeria monocytogenes* infection in mice by counteracting *Listeria*-induced phagosomal escape, *PLoS One* 8 (2013), e75490.
- [56] T.J. Pucadyil, A. Chattopadhyay, Role of cholesterol in the function and organization of G-protein coupled receptors, *Prog. Lipid Res.* 45 (2006) 295–333.
- [57] Y.D. Paila, A. Chattopadhyay, Membrane cholesterol in the function and organization of G-protein coupled receptors, *Subcell. Biochem.* 51 (2010) 439–466.
- [58] J. Oates, A. Watts, Uncovering the intimate relationship between lipids, cholesterol and GPCR activation, *Curr. Opin. Struct. Biol.* 21 (2011) 802–807.
- [59] M. Jafurulla, A. Chattopadhyay, Membrane lipids in the function of serotonin and adrenergic receptors, *Curr. Med. Chem.* 20 (2013) 47–55.
- [60] A. Chattopadhyay, GPCRs: lipid-dependent membrane receptors that act as drug targets, *Adv. Biol.* 2014 (2014) 143023.
- [61] G.A. Kumar, M. Jafurulla, A. Chattopadhyay, The membrane as the gatekeeper of infection: cholesterol in host-pathogen interaction, *Chem. Phys. Lipids* DOI: <http://dx.doi.org/10.1016/j.chemphyslip.2016.02.007> (in press).
- [62] F. Cornelius, Cholesterol modulation of molecular activity of reconstituted shark Na⁺, K⁺-ATPase, *Biochim. Biophys. Acta* 1235 (1995) 205–212.
- [63] T. Sooksawat, M.A. Simmonds, Effects of membrane cholesterol on the sensitivity of the GABA_A receptor to GABA in acutely dissociated rat hippocampal neurons, *Neuropharmacology* 40 (2001) 178–184.
- [64] R.M. Epand, Cholesterol and the interaction of proteins with membrane domains, *Prog. Lipid Res.* 45 (2006) 279–294.
- [65] H. Li, V. Papadopoulos, Peripheral-type benzodiazepine receptor function in cholesterol transport. Identification of a putative cholesterol recognition/interaction amino acid sequence and consensus pattern, *Endocrinology* 139 (1998) 4991–4997.
- [66] R.M. Epand, B.G. Sayer, R.F. Epand, Caveolin scaffolding region and cholesterol-rich domains in membranes, *J. Mol. Biol.* 345 (2005) 339–350.
- [67] N. Vincent, C. Genin, E. Malvoisin, Identification of a conserved domain of the HIV-1 transmembrane protein gp41 which interacts with cholesterol groups, *Biochim. Biophys. Acta* 1567 (2002) 157–164.
- [68] M. Jafurulla, S. Tiwari, A. Chattopadhyay, Identification of cholesterol recognition amino acid consensus (CRAC) motif in G-protein coupled receptors, *Biochem. Biophys. Res. Commun.* 404 (2011) 569–573.
- [69] D. Sengupta, A. Chattopadhyay, Identification of cholesterol binding sites in the serotonin_{1A} receptor, *J. Phys. Chem. B* 116 (2012) 12991–12996.