# Chapter 14
# Role of Membrane Cholesterol in Leishmanial Infection

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## Abbreviations

| | |
|---|---|
| FITC | Fluorescein isothiocyanate |
| GPCR | G-protein coupled receptor |
| MβCD | Methyl-β-cyclodextrin |

## Cholesterol: An Essential Lipid in Organization and Function of Eukaryotic Membranes

Cholesterol is a major and essential constituent in higher eukaryotic cellular membranes and is crucial in membrane organization, dynamics, function, and sorting (Liscum and Underwood 1995; Simons and Ikonen 2000; Mouritsen and Zuckermann 2004). Cholesterol is a predominantly hydrophobic molecule comprising a near planar tetracyclic fused steroid ring and a flexible isooctyl hydrocarbon tail (see Fig. 14.1). The tetracyclic nucleus and isooctyl side chain create the bulky wedge-type shape of the molecule. The polar 3β-hydroxyl group provides cholesterol its amphiphilic character and helps it to orient and anchor in the membrane (Villalaín 1996).

A unique characteristic of organization of membrane cholesterol is its nonrandom distribution in domains (Mouritsen and Zuckermann 2004; Mukherjee and Maxfield 2004; Chaudhuri and Chattopadhyay 2011). Many of these domains [sometimes termed as "lipid rafts" (Lingwood and Simons 2010)] are thought to be important for the maintenance of membrane structure and function, although characterizing the

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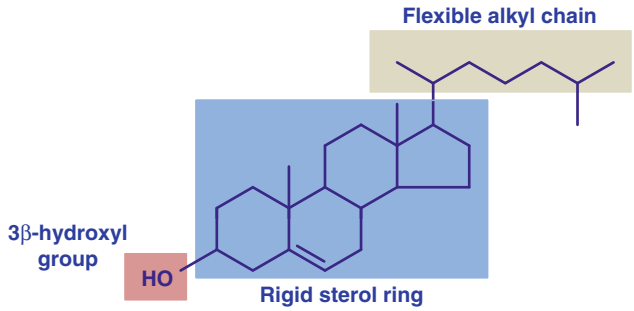


**Fig. 14.1** Chemical structure of cholesterol with the three structurally distinct regions (shown in *different color boxes*): the 3β-hydroxyl group, the rigid steroid ring and the flexible alkyl chain. The only polar group in cholesterol is the 3β-hydroxyl moiety which provides the molecule its amphiphilic character and serves to anchor and orient cholesterol in the membrane bilayer. The rest of the molecule is hydrophobic which comprises of a planar tetracyclic fused steroid ring and a flexible isooctyl hydrocarbon tail

spatiotemporal resolution of these domains has proven to be challenging (Jacobson et al. 2007). A unique property of cholesterol which contributes to its capacity to form membrane domains is its ability to form liquid-ordered-like phase in higher eukaryotic plasma membranes (Mouritsen 2010). The concept of such specialized membrane domains gains relevance in cell biology, since important functions such as signal transduction (Simons and Toomre 2000) have been implicated to these putative domains. Importantly, cholesterol plays a crucial role in the function and organization of membrane proteins and receptors (Pucadyil and Chattopadhyay 2006; Gimpl 2010; Paila and Chattopadhyay 2010).

## Membrane Cholesterol and Host–Pathogen Interaction

A number of studies have indicated the crucial requirement of membrane cholesterol in host–pathogen interaction [reviewed in (Rosenberger et al. 2000; van der Goot and Harder 2001; Shin and Abraham 2001; Simons and Ehehalt 2002; Goluszko and Nowicki 2005; Bansal et al. 2005; Riethmüller et al. 2006; Hawkes and Mak 2006; Pucadyil and Chattopadhyay 2007; Vieira et al. 2010)]. The ability to manipulate levels of membrane cholesterol with a reasonable degree of specificity has contributed to our understanding of its role in host–pathogen interaction and subsequent infection. Cholesterol content in the membrane can be modulated using a number of approaches. Such approaches include the use of water soluble carriers that efficiently remove cholesterol from membranes, cholesterol-binding compounds that sequester it in the membrane, cholesterol-modifying enzymes, and biosynthetic inhibitors of cholesterol (Pucadyil and Chattopadhyay 2006).

Cyclodextrins are efficient carriers of membrane cholesterol and have been utilized to achieve acute modulation of cholesterol content in membranes (Härtel et al. 1998). In addition to their capacity to extract cholesterol, a variety of cyclodextrins are used in

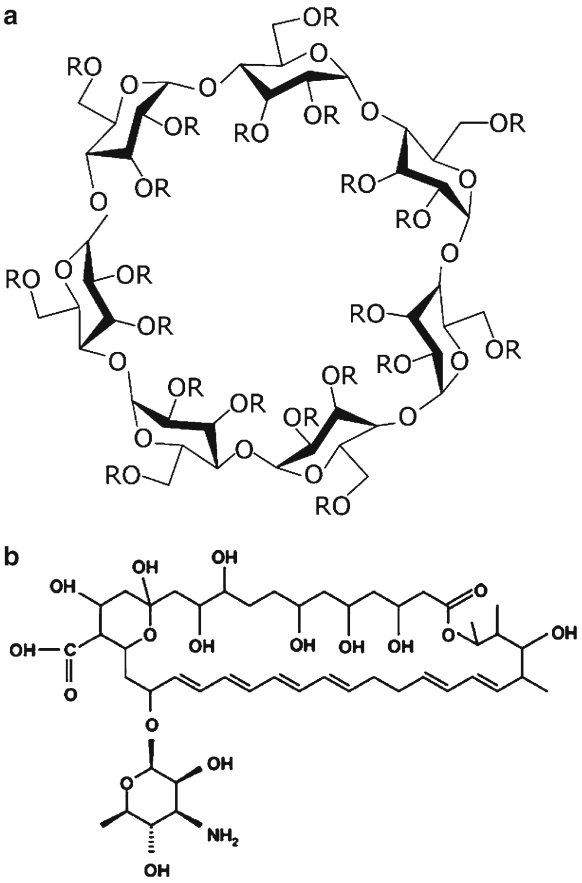


**Fig. 14.2** Agents that modulate cholesterol availability in the membrane. (**a**) Chemical structure of β-cyclodextrin (containing seven glucose residues) molecule. Cyclodextrins can solubilize a variety of hydrophobic compounds by trapping them in their inner cavity. The resultant complex has a characteristic stoichiometry. The specificity of this process depends on the structure of the inner cavity, which can be modified by substitution of the hydrogen atom of hydroxyl groups (indicated as R in the figure) in each glucose residue. The commonly used cholesterol-depleting agent is methyl-β-cyclodextrin (MβCD), in which R is a methyl group. Adapted and modified from Davis and Brewster (2004). (**b**) Chemical structure of nystatin, a sterol-binding antifungal polyene antibiotic. While MβCD physically depletes cholesterol from membranes, nystatin specifically interacts with membrane cholesterol to sequester it. Both MβCD and nystatin modulate the availability of membrane cholesterol, thereby effectively reducing the ability of cholesterol to interact with and exert its effects on other membrane components

pharmaceutical, agrochemical, food, and cosmetic industries (Hawkes and Mak 2006; Davis and Brewster 2004). Trace amounts of natural cyclodextrins are present in certain microorganisms and plants that express the enzyme cyclodextrin glucosyltransferases (CGTs), that degrade starch into cyclodextrins (Hawkes and Mak 2006). Water-soluble carriers such as β-cyclodextrins (see Fig. 14.2a) have been effectively used to modulate cholesterol levels in cell membranes. Methyl-β-cyclodextrin (MβCD),

the oligomer with seven residues (β-cyclodextrin) of methylated-glucose, has been extensively used to selectively and efficiently extract cholesterol from membranes by incorporating it in a central nonpolar cavity (Zidovetzki and Levitan 2007). The stoichiometry of the cholesterol–cyclodextrin complex thus formed has been reported to be 1:2 (mol/mol) (Tsamaloukas et al. 2005).

In addition, compounds that physically bind to cholesterol and sequester it in the membrane have been utilized to effectively reduce the availability of cholesterol (Pucadyil and Chattopadhyay 2006). The sterol-binding antifungal polyene antibiotic nystatin (see Fig. 14.2b) is a typical example of this class of molecules and has been used to sequester membrane sterols (Holz 1974; Marty and Finkelstein 1975; Bolard 1986). It has been proposed that nystatin forms a 1:1 (mol/mol) complex with membrane cholesterol and forms channels in the membrane (de Kruijff and Demel 1974). Nystatin specifically interacts with cholesterol to sequester it in the membrane, thereby effectively reducing the ability of cholesterol to interact with and exert its effects on other membrane components such as receptors (Pucadyil et al. 2004a). Approaches using cholesterol-modifying enzymes (Pucadyil et al. 2005) and biosynthetic inhibitors of cholesterol (Paila et al. 2008; Shrivastava et al. 2010) have also been used to monitor cholesterol sensitivity of receptor function.

A number of studies on host–pathogen interaction show that the use of cholesterol carriers (such as MβCD) and cholesterol-sequestering agents (such as nystatin or amphotericin B) result in reduction in infectivity of several intracellular pathogens, an effect that correlates with the extent of reduction in the effective concentration of membrane cholesterol (Rosenberger et al. 2000; van der Goot and Harder 2001; Shin and Abraham 2001; Goluszko and Nowicki 2005; Bansal et al. 2005; Riethmüller et al. 2006; Hawkes and Mak 2006; Pucadyil and Chattopadhyay 2007; Vieira et al. 2010). The role of cholesterol in pathogen infection can be classified as a requirement either at the stage of pathogen binding to cell surface receptors or in their internalization into cells (or both) (Goluszko and Nowicki 2005). Membrane cholesterol has been shown to be necessary for the internalization of several species of *Mycobacteria* into macrophages (Gatfield and Pieters 2000; Peyron et al. 2000). Similar results have been found for the internalization of fimbriated *E. coli* (Shin et al. 2000). In addition to bacterial pathogens, cholesterol depletion has been found to inhibit the entry and sustained infection of the protozoan malaria parasite *Plasmodium falciparum* in erythrocytes (Lauer et al. 2000; Samuel et al. 2001). Interestingly, membrane cholesterol has been reported to be essential for human immunodeficiency virus-1 (HIV-1) infection (Liao et al. 2001; Campbell et al. 2001; Carter et al. 2009), and topical application of cyclodextrins has previously been shown to block the transmission of cell-associated HIV-1 in mice (Khanna et al. 2002). In addition, the entry of several other viruses such as poliovirus (Danthi and Chow 2004), flavivirus (Lee et al. 2008), gastroenteritis virus (Ren et al. 2008), borna disease virus (Clemente et al. 2009) have been shown to require membrane cholesterol. Taken together, the cellular entry and survival of pathogens of a diverse variety, *with no similarity in their biology*, appear to be dependent on membrane cholesterol. This points to a generalized mechanism underlying these observations (see later).

## Role of Membrane Cholesterol in Leishmaniasis

*Leishmania* are protozoan parasites that are responsible for substantial public health problems, especially in tropical and subtropical regions. The parasite is responsible for the disease leishmaniasis which is usually fatal if left untreated (Herwaldt 1999; Alexander et al. 1999; Chappuis et al. 2007). Leishmaniasis threatens about 350 million men, women, and children in 88 countries around the world. As many as 12 million people are believed to be currently infected, with about one to two million estimated new cases occurring every year (World Health Organization Web site). The current worldwide increase in leishmaniasis to epidemic proportions, and the emergence of visceral leishmaniasis as an important opportunistic infection among people with HIV-1 infection (Wolday et al. 1999) have given rise to an urgency to provide treatment for leishmaniasis.

Leishmaniasis is transmitted by the bite of the infected female sandfly (*Phlebotomus* spp.) when taking a bloodmeal from a host (Handman and Bullen 2002; Sacks and Noben-Trauth 2002). The lifecycle of *Leishmania* has two distinct forms: an extracellular promastigote flagellar form found in the midgut of sandflies and an intracellular amastigote form that resides in phagolysosomes of mammalian (host) macrophages. Once in the bloodstream, promastigotes are internalized by dendritic cells and macrophages that subsequently transform into amastigotes by losing their flagella (Chappuis et al. 2007). Entry of promastigotes into host macrophages involves multiple parasite–host interactions such as recognition of specific ligands on the parasite cell surface by receptors on the macrophage cell surface. A number of studies toward understanding the molecular mechanisms of parasite entry have led to the identification of several candidate receptors facilitating multiple routes of entry thereby highlighting the redundancy in the entry process (Alexander et al. 1999; Rittig and Bogdan 2000). These include membrane proteins present on the macrophage cell surface such as the mannose–fucose receptor, receptor for advanced glycosylation end products, the fibronectin receptor, the Fc receptor, and complement receptors such as CR1 and CR3. The large number of different receptors responsible for the entry of the parasite into host macrophages makes it difficult to establish a unique therapeutic target for the treatment of leishmaniasis.

The entry of *Leishmania* in particular and other intracellular parasites in general involves interaction with the plasma membrane of host cells. As mentioned earlier, cholesterol is an essential component of higher eukaryotic cellular membranes and plays an important role in the function and organization of membrane proteins and receptors (Pucadyil and Chattopadhyay 2006; Gimpl 2010; Paila and Chattopadhyay 2010), some of which may be necessary for parasite entry (Harrison et al. 2003). Our group was the first to demonstrate the requirement of host membrane cholesterol in the binding and internalization of *Leishmania donovani* into macrophages using complementary approaches (Pucadyil et al. 2004b; Tewary et al. 2006; Paila et al. 2010). In our previous work, we showed that treatment of macrophages in culture with the cholesterol carrier MβCD resulted in specific removal of membrane cholesterol and a concomitant reduction in binding and subsequent infection by

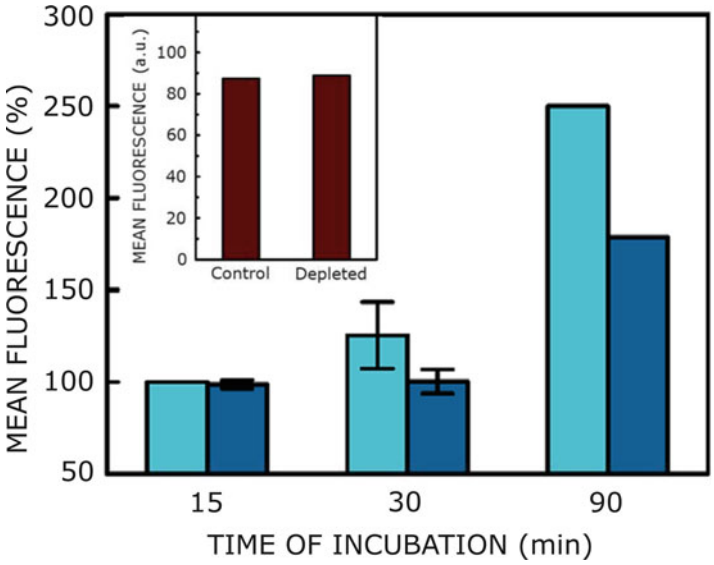


**Fig. 14.3** Effect of cholesterol depletion on binding kinetics of FITC-labeled *Leishmania* promastigotes to J774A.1 macrophages monitored by flow cytometry. Data show a time-dependent reduction in fluorescence associated with MβCD-treated macrophages (*blue bars*) compared to control macrophages (*cyan bars*). Values are normalized to the fluorescence associated with control macrophages after 15 min exposure to the parasite. The reduction in binding upon cholesterol depletion appears to be specific (see *inset*). *Inset* shows the effect of MβCD-mediated cholesterol depletion of J774A.1 cells on binding of FITC-labeled *E. coli* DH5α studied using flow cytometry. Representative data shown in the figure indicate a lack of sensitivity of binding of *E. coli* to macrophages depleted of cholesterol, unlike what is observed with *Leishmania* promastigotes. Adapted and modified from Pucadyil et al. (2004b). See Pucadyil et al. (2004b) for other details

*Leishmania* promastigotes [(Pucadyil et al. 2004b), see Fig. 14.3]. Importantly, our results showed that the binding/attachment of *E. coli* to macrophages remain unaffected upon cholesterol depletion (see inset of Fig. 14.3), thereby rendering stringent specificity to the process of host–parasite interaction. When followed for a longer period of time postinfection, a reduction in the number of intracellular amastigote form of the parasite was observed in case of cholesterol-depleted macrophages [(Pucadyil et al. 2004b), see Fig. 14.4b]. Importantly, the reduction in binding of *L. donovani* promastigotes to cholesterol-depleted macrophages could be reversed by replenishment of cholesterol, thereby reinforcing the specific requirement of cholesterol in the infection process (Pucadyil et al. 2004b).

If membrane cholesterol is necessary for leishmanial infection, modulating cholesterol availability by other means could affect infection. We tested this proposal by treating host macrophages with the sterol-binding antifungal polyene antibiotic nystatin (Tewary et al. 2006). As shown in Fig. 14.5a, treatment of macrophages with increasing concentrations of nystatin progressively leads to a reduction in the binding of *Leishmania* promastigotes to macrophages. This was accompanied

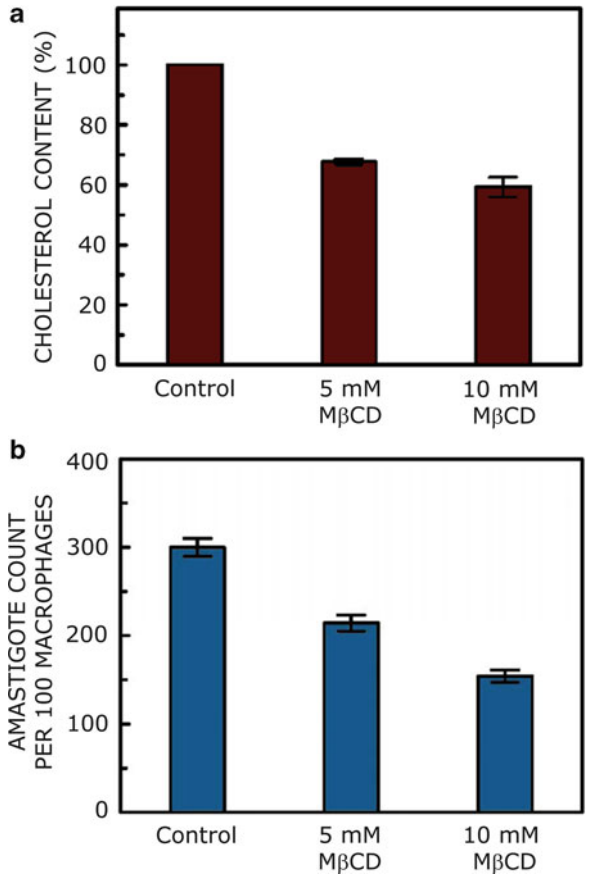


**Fig. 14.4** Cholesterol content in control and cholesterol-depleted macrophages and the effect of cholesterol depletion on internalization of the parasite assessed by the amastigote load in infected J774A.1 macrophages. (**a**) Total cellular cholesterol of control and cholesterol-depleted macrophages shows a concentration-dependent reduction of cholesterol upon treatment with MβCD. (**b**) Cholesterol-depleted macrophages using increasing concentrations of MβCD show considerable reduction in the number of intracellular amastigotes as revealed by Giemsa staining. Adapted and modified from Pucadyil et al. (2004b). See Pucadyil et al. (2004b) for other details

by a similar concentration-dependent reduction in intracellular amastigote load (see Fig. 14.5b). These results demonstrate that mere sequestration of host plasma membrane cholesterol (rather than physical depletion) is sufficient to inhibit leishmanial infection. In other words, the nonavailability of membrane cholesterol, *rather than the manner in which its availability is modulated*, is crucial for leishmanial infection. These results were recently reinforced by the observation that treatment of host macrophages with the sterol-binding antifungal polyene antibiotic

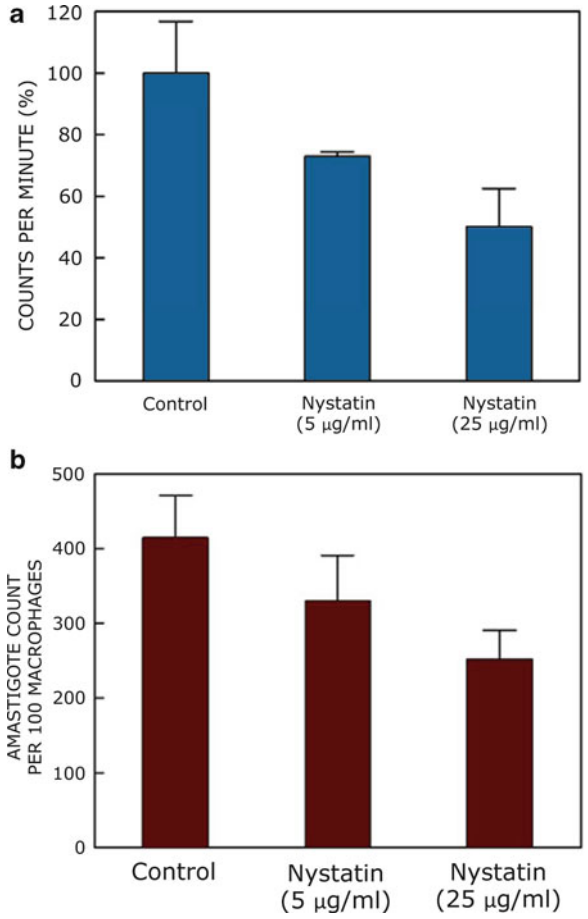


**Fig. 14.5** Effect of cholesterol sequestration by nystatin on the extent of promastigote binding and intracellular amastigote load of *Leishmania* parasite in host macrophages. (**a**) Treatment of macrophages with increasing concentrations of nystatin results in reduction in the binding of radiolabeled *Leishmania* promastigotes to macrophages in a concentration-dependent manner. Values are normalized with respect to the mean counts per minute obtained for untreated macrophages (control). (**b**) The count of the intracellular amastigote form of the parasite is shown in macrophages either untreated (control) or treated with increasing concentrations of nystatin. Macrophages pretreated with nystatin show considerable reduction in the number of intracellular amastigotes as revealed by Giemsa staining. Adapted and modified from Tewary et al. (2006). See Tewary et al. (2006) for other details

amphotericin B results in reduction in leishmanial infection (Paila et al. 2010). In addition, membrane cholesterol has been shown to be necessary for the entry of *Leishmania chagasi* into host bone marrow macrophages through cholesterol-enriched caveolar domains (Rodríguez et al. 2006). Subsequent to their entry into the host cells, *Leishmania* parasites prolong their survival by subverting host immunity (Olivier et al. 2005). For example, *L. donovani* infection results in a reduction in the

ability of macrophages harboring the parasite to efficiently present parasite antigens to T cells (Chakraborty et al. 2005). Interestingly, it has been recently reported that cholesterol depletion might represent an immune-evasion strategy used by *Leishmania major* (Rub et al. 2009).

## Conclusion and the Road Ahead: Mechanism of Pathogen Entry

The reduction in leishmanial infection by cholesterol depletion/sequestration may lead to novel therapeutic strategies against leishmaniasis. A major advantage of this approach is that development of drug resistance, a major problem in the treatment of leishmaniasis (Berman 2003), is absent, since the therapeutic focus is on the host membrane lipid, rather than the parasite. Based on the inhibitory effects of cholesterol carriers and sequestering agents on leishmanial infection in vitro (Pucadyil et al. 2004b; Tewary et al. 2006; Paila et al. 2010; Rodríguez et al. 2006), the potential of using cyclodextrin-like molecules as a therapeutic strategy against leishmaniasis *in vivo* appears encouraging. Cyclodextrins have previously been shown to be important in treating unstable atherosclerotic plaques due to their ability to remove cholesterol from macrophage foam cells *in vitro* (Atger et al. 1997). As mentioned earlier, visceral leishmaniasis has emerged as an important opportunistic infection among people with HIV-1 infection (Wolday et al. 1999). Interestingly, cholesterol has been reported to be essential for HIV-1 infection (Liao et al. 2001; Campbell et al. 2001; Carter et al. 2009), 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 agents that modulate membrane cholesterol levels can therefore prove to be a powerful approach in tackling the *combined* infection of leishmaniasis associated with HIV-1 infection.

The crucial role played by membrane cholesterol in host–pathogen interactions is emerging to be an important area of pathogen biology (Rosenberger et al. 2000; van der Goot and Harder 2001; Shin and Abraham 2001; Simons and Ehehalt 2002; Goluszko and Nowicki 2005; Bansal et al. 2005; Riethmüller et al. 2006; Hawkes and Mak 2006; Pucadyil and Chattopadhyay 2007; Vieira et al. 2010). The mechanism of such inhibition in pathogen entry upon depletion of membrane cholesterol remains elusive. Interestingly, G-protein coupled receptors (GPCRs) are implicated for the entry of pathogens to host cells. For example, the $\beta_2$-adrenergic receptor has been shown to be responsible for the entry of malaria parasite *P. falciparum* in host cells (Harrison et al. 2003). Based on our and others work on role of membrane cholesterol in maintaining receptor function (Pucadyil and Chattopadhyay 2006; Gimpl 2010; Paila and Chattopadhyay 2010), we propose that the conformation of membrane receptors necessary for pathogen entry into cells could be dependent on membrane cholesterol. Due to lack of membrane cholesterol availability, these receptors assume a conformation(s) that does not support pathogen entry leading to inhibition in the entry of pathogens to host cells. We plan to check this hypothesis in our future work.

Interestingly, it has been very recently reported that the host membrane machinery (host lipidome) is exploited and modulated by pathogens for their entry, survival, and replication (Cossart and Roy 2010; van der Meer-Janssen et al. 2010). This is achieved by pathogens by modulating the lipid homeostasis machinery of the host cell and inducing multiple changes in host cell signaling and trafficking. A comprehensive lipidomic analysis of host–pathogen interaction, therefore, will provide novel insight that could lead to more specific drugs against pathogens.

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