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Invited Review

The membrane as the gatekeeper of infection: Cholesterol in host–pathogen interaction

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ABSTRACT

The cellular plasma membrane serves as a portal for the entry of intracellular pathogens. An essential step for an intracellular pathogen to gain entry into a host cell therefore is to be able to cross the cell membrane. In this review, we highlight the role of host membrane cholesterol in regulating the entry of intracellular pathogens using insights obtained from work on the interaction of *Leishmania* and *Mycobacterium* with host cells. The entry of these pathogens is known to be dependent on host membrane cholesterol. Importantly, pathogen entry is inhibited either upon depletion (or complexation), or enrichment of membrane cholesterol. In other words, an optimum level of host membrane cholesterol is necessary for efficient infection by pathogens. In this overall context, we propose a general mechanism, based on cholesterol-induced conformational changes, involving cholesterol binding sites in host cell surface receptors that are implicated in this process. A therapeutic strategy targeting modulation of membrane cholesterol would have the advantage of avoiding the commonly encountered problem of drug resistance in tackling infection by intracellular pathogens. Insights into the role of host membrane cholesterol in pathogen entry would be instrumental in the development of novel therapeutic strategies to effectively tackle intracellular pathogenesis.

1. The plasma membrane: the portal for entry of intracellular pathogens

Biological membranes are complex assemblies of a diverse variety of lipids and proteins that impart an identity to the cell and its organelles. The cellular plasma membrane acts as an indispensable platform for the initiation and regulation of essential cellular activities such as signal transduction, trafficking and sorting. Besides providing cellular identity, a major function of the cell membrane is to interact with the extracellular milieu and elicit appropriate responses to external cues. The cell membrane acts as the portal for the entry of intracellular pathogens in order for them to gain access into the host cell interior (Rosenberger et al., 2000; Shin and Abraham, 2001; van der Goot and Harder, 2001). Host–pathogen interaction involves several key components at the cell surface of both the host and the pathogen. In this context, host cell membrane components such as cholesterol (Rosenberger et al., 2000; Shin and Abraham, 2001; van der Goot and Harder, 2001; Goluszko and Nowicki, 2005; Hawkes and Mak, 2006; Riethmüller et al., 2006; Pucadyil and Chattopadhyay, 2007; Vieira et al., 2010; Chattopadhyay and Jafurulla, 2012; Machado et al., 2012) and the actin cytoskeleton (Rottner et al., 2004; Lodge and Descoteaux, 2008; Humphries and Way, 2013; Roy et al., 2014) are important players in the entry of intracellular pathogens.

In this review, we provide an overview of the role of host membrane cholesterol in modulating the process of entry of intracellular pathogens using insights obtained from work on the interaction of *Leishmania* and *Mycobacterium* with host cells. In spite of the available literature on cholesterol-dependent entry of pathogens into host cells, there appears to be no common mechanism that can be attributed to the role of membrane cholesterol in pathogen entry. We propose here a general mechanism, involving cholesterol binding sites in host cell surface receptors that are implicated in pathogen entry, to provide novel insight in cholesterol-mediated entry of intracellular pathogens.

2. Cholesterol as a modulator of pathogen entry

Cholesterol is an essential lipid in higher eukaryotic cell membranes and is unique in terms of the functional role it plays in cellular physiology (Simons and Ikonen, 2000; Mouritsen and

Abbreviations: CFU, colony forming unit; CRAC, cholesterol recognition/interaction amino acid consensus; GPCR, G protein-coupled receptor; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; MβCD, methyl-β-cyclodextrin.

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Zuckermann, 2004). It has important structural properties by which it can influence membrane organization and dynamics (Xu and London, 2000; Maxfield and Tabas, 2005; Lingwood and Simons, 2010; Chaudhuri and Chattopadhyay, 2011). A hallmark of cholesterol organization in biological membranes stems from its ability to nonrandomly organize into distinct domains or pools that are often characterized by different functional attributes. As a result, cholesterol is involved in signal transduction and trafficking (Simons and Toomre, 2000). Importantly, cholesterol plays a crucial role in the organization and function of membrane proteins and receptors, including G protein-coupled receptors (Burger et al., 2000; Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay, 2010; Oates and Watts, 2011; Jafurulla and Chattopadhyay, 2013; Chattopadhyay, 2014; Sengupta and Chattopadhyay, 2015).

As mentioned above, a number of studies have indicated the importance of membrane cholesterol in host–pathogen interaction. We highlight here results from our previous work on the role of membrane cholesterol in the entry of two potent intracellular pathogens, *Leishmania* and *Mycobacterium*.

2.1. Cholesterol in leishmaniasis

Leishmaniasis, caused by intracellular protozoan parasites of the genus *Leishmania*, is a disease of the tropics and subtropics and is usually fatal, if left untreated. This vector-borne disease is transmitted by the bite of the infected female sandfly (*Phlebotomus* spp.) while taking a blood meal from the host. Leishmaniasis threatens about 350 million people across 98 countries around the globe, with ~1.3 million new cases reported annually (Herwaldt, 1999; Alvar et al., 2012; World Health Organization website). The correlation between leishmaniasis and poverty (Alvar et al., 2006), and its emergence as an opportunistic infection among HIV-1 infected patients (Wolday et al., 1999), significantly contribute to the morbidity and mortality associated with the disease. *Leishmania* exists in two distinct forms through its lifecycle. The flagellated extracellular promastigote forms, found in the midgut of sandflies, are internalized by dendritic cells and macrophages once they gain access to the host bloodstream. The parasites subsequently transform into aflagellar amastigote forms within host cells (Handman and Bullen, 2002; Chappuis et al., 2007; Chattopadhyay and Jafurulla, 2012). Several receptors on the host cell membrane that recognize specific ligands on the parasite cell surface have been identified and implicated in the process of entry of *Leishmania* into macrophages. These include the mannose-fucose receptor, receptor for advanced glycosylation end products, the fibronectin receptor, the Fc receptor and complement receptors such as CR1 and CR3 (Rittig and Bogdan, 2000; Sacks and Kamhawi, 2001; Podinovskaia and Descoteaux, 2015).

We utilized several complementary approaches to study the role of host membrane cholesterol in the entry of *Leishmania donovani*, the causative organism of visceral leishmaniasis (Pucadyil et al., 2004; Tewary et al., 2006; Chattopadhyay and Madhubala, 2007, 2010; Paila et al., 2010; Chattopadhyay and Jafurulla, 2011). In this context, we were the first to demonstrate that the depletion of cholesterol from host macrophages using the soluble carrier methyl-β-cyclodextrin (MβCD) results in a substantial reduction in the entry of the parasite (Pucadyil et al., 2004; see Fig. 1). Importantly, upon replenishing the cholesterol content of host cells, the number of *Leishmania* promastigotes associated with macrophages was restored to control levels (Pucadyil et al., 2004). In agreement with our observations, it was later reported that membrane cholesterol is necessary for the entry of *Leishmania chagasi* into host cells (Rodríguez et al., 2006).

Keeping in mind the possible mechanism involved, we tested whether modulating cholesterol availability by other means could affect leishmanial entry. As shown in Fig. 1, upon treatment of host macrophages with cholesterol complexing agents, such as sterol-binding antifungal polyene antibiotics nystatin (Tewary et al., 2006) or amphotericin B (Paila et al., 2010), a similar reduction in the number of *Leishmania* promastigotes bound to macrophages was observed. These agents sequester host membrane cholesterol (Bolard, 1986; Hartsel and Bolard, 1996) and reduce its ability to interact with membrane components responsible for parasite entry. Importantly, chronic depletion of host membrane cholesterol using statins (Sirtori, 2014), which are competitive inhibitors of HMG-CoA reductase (the rate-limiting enzyme in the

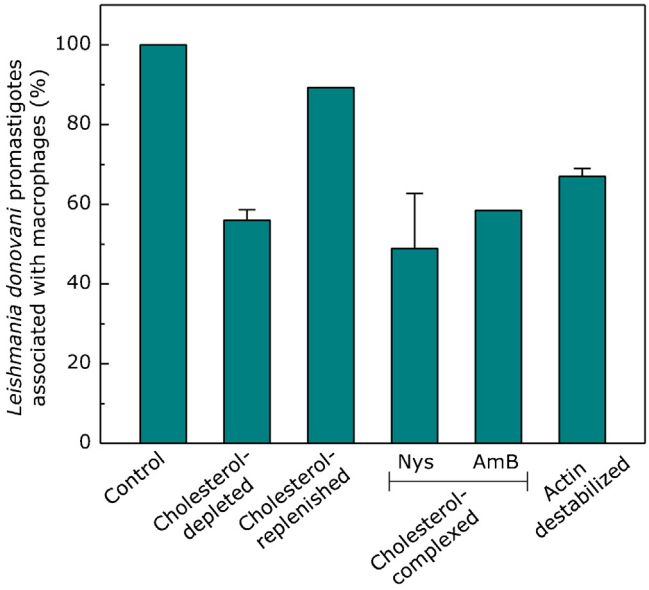

Fig. 1. Role of host membrane cholesterol and the underlying actin cytoskeleton in the entry of *Leishmania donovani*. Depletion of host membrane cholesterol using MβCD resulted in reduction in the number of *Leishmania* promastigotes associated with macrophages. Replenishment of cholesterol using cholesterol-MβCD complex restored the entry of promastigotes to control levels (data from Pucadyil et al., 2004). A similar reduction in the entry of the parasite was observed upon restricting membrane cholesterol availability by sequestration of host membrane cholesterol using complexing agents such as nystatin (Nys; data from Tewary et al., 2006) or amphotericin B (AmB; data from Paila et al., 2010). Cytochalasin D-mediated destabilization of the macrophage actin cytoskeleton inhibited the entry of *Leishmania* into host cells, paralleling the observations from cholesterol depletion and sequestration (data from Roy et al., 2014).

cholesterol biosynthetic pathway) results in reduced entry of *Leishmania donovani* into host cells (Kumar, G.A., Roy, S., Jafurulla, M., Mandal, C., Chattopadhyay, A., unpublished observations). These results therefore demonstrate that the availability of host membrane cholesterol is a crucial prerequisite for leishmanial entry, regardless of the *modus operandi* of modulation of its availability. Interestingly, enrichment of host membrane cholesterol over normal levels also resulted in reduction in the entry and survival of the parasite (Kumar, G.A., Roy, S., Jafurulla, M., Mandal, C., Chattopadhyay, A., unpublished observations). These results comprehensively demonstrate the requirement of an optimum level of host membrane cholesterol for efficient leishmanial infection.

An emerging molecular player in this overall scenario is the host actin cytoskeleton. The interplay between membrane cholesterol and the actin cytoskeleton has been reported to be a key regulatory mechanism governing membrane organization and dynamics (Kwik et al., 2003; Maxfield and Tabas, 2005; Sun et al., 2007; Ganguly and Chattopadhyay, 2010; Sarkar, P., Kumar, G.A., Shrivastava, S., Chattopadhyay, A., unpublished observations). It is worth noting here that we recently demonstrated that destabilization of the actin cytoskeleton in host macrophages leads to reduction in leishmanial entry (Roy et al., 2014; see Fig. 1).

2.2. Cholesterol in mycobacterial infection

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a major cause of mortality and morbidity worldwide, with an estimated 9.6 million new cases in 2014 alone (World Health Organization, 2015). Infection by *M. tuberculosis* is propagated *via* aerosols generated by patients with active infection during coughing and sneezing, and is internalized primarily by alveolar macrophages in the lungs (Russell, 2007). TB is a prevalent opportunistic infection among HIV positive patients (Pawlowski et al., 2012; World Health Organization, 2015). The emergence of multi- and extremely drug-resistant TB calls for novel therapeutic strategies to tackle mycobacterial infection (Gandhi et al., 2006; Dye, 2009; Keshavjee and Farmer, 2012). *Mycobacterium* gains access to the interior of host cells *via* receptors on the 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) in a fashion analogous to *Leishmania* (see Section 2.1). Interestingly, some of the receptors implicated in the process of pathogen entry are shared by *Leishmania* and *Mycobacterium*, thereby highlighting common pathways of infection and reinforcing the importance of host membrane cholesterol in pathogenesis.

We explored the role of host membrane cholesterol in the process of mycobacterial entry using *M. smegmatis* as the model system. *M. smegmatis* is a relevant model to examine the entry of *Mycobacterium* into host cells, since *M. smegmatis* and *M. tuberculosis* share similar architecture of the cell envelope and exhibit similar bacterial loads in the macrophage model upon infection (Sani et al., 2010). We observed that the depletion of membrane cholesterol from host macrophages using MβCD resulted in a significant reduction in the entry of *M. smegmatis* into host cells (Viswanathan et al., 2015; see Fig. 2). Mycobacterial entry could be restored to normal levels upon replenishment of host membrane cholesterol. These results confirmed and extended the observations from other groups highlighting the importance of host membrane cholesterol in mycobacterial infection (Gatfield and Pieters, 2000; Martens et al., 2008; Miner et al., 2009; Muñoz et al., 2009). In line with our observations on the entry of *Leishmania* into host cells (see Section 2.1), inhibiting the availability of membrane cholesterol using the complexing agent amphotericin B (without physical depletion) was sufficient to inhibit the entry of *Mycobacterium* into host macrophages (see Fig. 2). Importantly, a significant reduction in mycobacterial entry was observed upon enrichment of host membrane cholesterol. Taken together, these observations, along with the findings on leishmanial entry outlined in Section 2.1, provide compelling evidence in favor of the requirement of optimum host membrane cholesterol for the entry of intracellular pathogens. We propose a common mechanism, based on cholesterol binding sites in the receptors implicated in parasite entry, to address cholesterol-mediated entry of intracellular pathogens into host cells (see below).

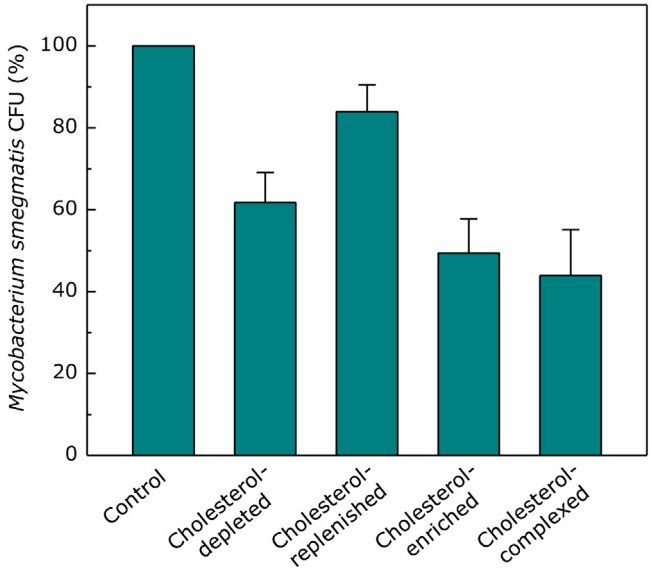

Fig. 2. Role of host membrane cholesterol in the entry of *Mycobacterium*. Depletion of host membrane cholesterol using MβCD led to reduction in the entry of *Mycobacterium smegmatis* into macrophages as scored by counting bacterial CFUs. Mycobacterial entry was restored to control levels upon replenishment of cholesterol using cholesterol-MβCD complex. Interestingly, enrichment of membrane cholesterol over control levels or sequestration of cholesterol using amphotericin B, resulted in significant inhibition of mycobacterial entry into host macrophages. Data from Viswanathan et al., 2015.

3. Sensors of cholesterol in pathogen entry: cholesterol interaction motifs

As mentioned above, cholesterol acts as a crucial regulator of membrane protein organization and function (Burger et al., 2000; Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay, 2010; Oates and Watts, 2011; Jafurulla and Chattopadhyay, 2013; Chattopadhyay, 2014; Sengupta and Chattopadhyay, 2015). Although the role of cholesterol in membrane protein function is well established, the mechanism underlying such interplay lacks consensus. It has been proposed that cholesterol could modulate the function of membrane proteins either by specific (direct) interaction, or by altering physical properties of the membrane, or by a combination of both the effects (Paila and Chattopadhyay, 2009). Results from structural, biochemical and computational studies on several membrane proteins including GPCRs offer evidence of specific interaction (preferential close association) of cholesterol with membrane proteins (Cherezov et al., 2007; Hanson et al., 2008; Paila et al., 2008; Sengupta and Chattopadhyay, 2012; Jafurulla et al., 2014).

The unique structural and stereochemical features of cholesterol contribute to its specific interaction with membrane proteins and receptors. Cholesterol is a polycyclic amphiphile with a polar 3β-hydroxyl group and a hydrophobic near-planar tetracyclic fused sterol ring. The hydroxyl group in cholesterol provides it the ability to form hydrogen bonds with polar residues of membrane proteins. The sterol ring and the isooctyl side chain constitute the apolar part of cholesterol, with an inherent asymmetry about the ring plane owing to methyl substitutions on one of its faces. The protruding methyl groups on the rough side (β face) of cholesterol are believed to intercalate with the side chains of branched amino acids such as Ile, Val and Leu *via* van der Waals interactions. On the other hand, side chains of aromatic amino acids stack onto the smooth side (α face) through CH–π interactions (Paila and Chattopadhyay, 2010; Chaudhuri and Chattopadhyay, 2011; Fantini and Barrantes, 2013). We have previously shown that the interaction between membrane cholesterol with GPCRs is stringent since immediate biosynthetic precursors of cholesterol, differing with cholesterol merely in a double bond, were not able to maintain receptor function (Paila et al., 2008). In addition, the interaction of GPCRs with cholesterol was shown to be stereospecific (Jafurulla et al., 2014). Taken together, these results point out the extent of stringency cholesterol-receptor interactions enjoy.

A number of structural features of proteins that are believed to result in their preferential association with cholesterol have been suggested (Epand, 2006). As a result, the existence of sequence motifs (a characteristic stretch of amino acids) on membrane proteins that show specificity toward cholesterol has been postulated (Epand, 2006; Fantini and Barrantes, 2013). Among these, cholesterol recognition/interaction amino acid consensus (CRAC) is one of the most studied motifs in membrane proteins that interact with cholesterol. The CRAC motif is characterized by the presence of the sequence pattern -L/V-$(X)_{1-5}$-Y-$(X)_{1-5}$-R/K- in the N-terminus to C-terminus direction, where $(X)_{1-5}$ represents one to five residues of any amino acid (Li and Papadopoulos, 1998; Epand, 2006; Fantini and Barrantes, 2013). Subsequent to initial reports on the identification of the CRAC motif in peripheral-type benzodiazepine receptors (Li and Papadopoulos, 1998), several membrane proteins including representative GPCRs such as rhodopsin, the β_2-adrenergic receptor, the serotonin$_{1A}$ receptor (Jafurulla et al., 2011) and the human type I cannabinoid receptor (Oddi et al., 2011) have been reported to possess CRAC motifs. In

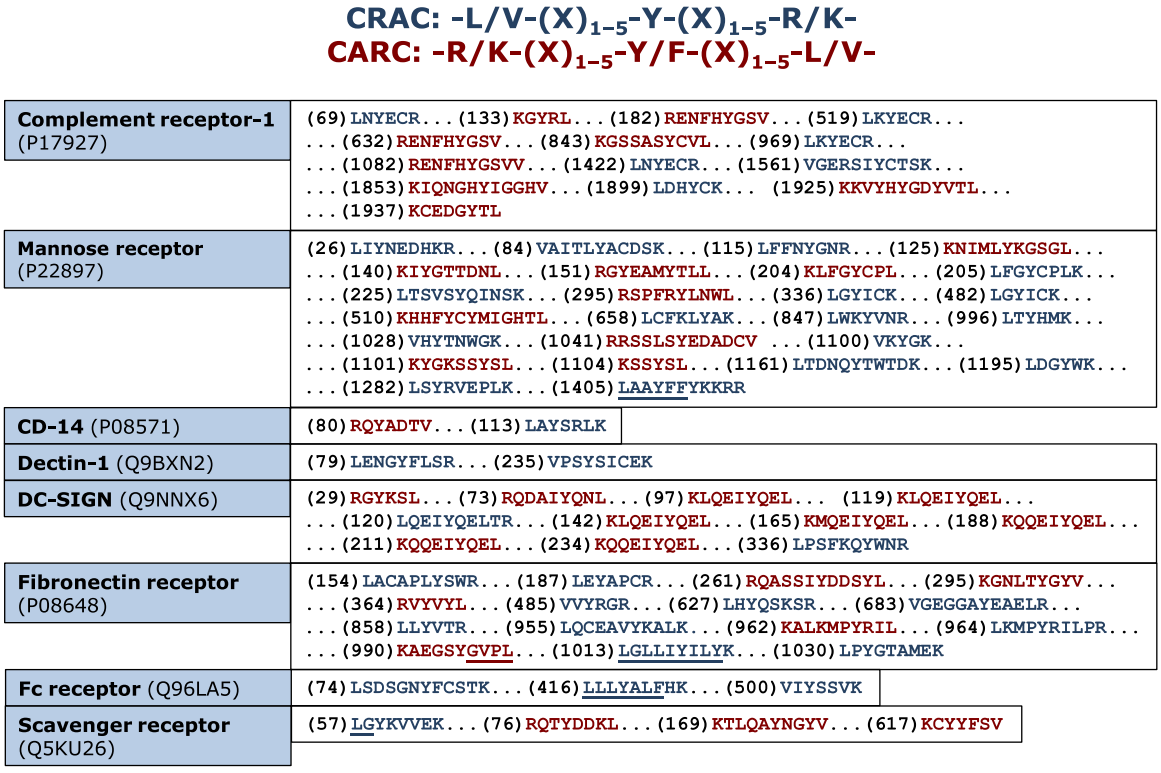

Fig. 3. Cholesterol binding motifs (CRAC and CARC) in representative host cell membrane receptors implicated in pathogen entry. Characteristic residues of CRAC and inverted CRAC (CARC) motifs are indicated. Putative cholesterol binding motifs, CRAC (blue) and CARC (maroon), are shown. The underlined residues correspond to transmembrane regions of the receptors. The protein accession numbers are indicated in parentheses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

addition, membrane proteins such as caveolin-1 (Epand et al., 2005) and the HIV-1 transmembrane protein gp41 (Vincent et al., 2002) have also been shown to possess CRAC motifs. Recent studies on the nicotinic acetylcholine receptor revealed the presence of the CRAC motif oriented in the opposite direction along the polypeptide chain, *i.e.*, -K/R-$(X)_{1-5}$-Y/F-$(X)_{1-5}$-L/V-sequence pattern in the N-terminus to C-terminus direction (Baier et al., 2011; Fantini and Barrantes, 2013). Apart from the nicotinic acetylcholine receptor, such 'inverted CRAC' motifs, intuitively termed CARC, are present in several GPCRs, including receptors implicated in the pathophysiology of Alzheimer's disease (Barrantes et al., 2010; Baier et al., 2011). In addition to its reverse orientation, CARC differs from CRAC in that the central aromatic amino acid could be either tyrosine or phenylalanine (Fantini and Barrantes, 2013). However, it should be noted here that the mere presence of a CRAC (or CARC) motif does not necessarily imply that this site preferentially associates with cholesterol. Presence of these motifs is indicative in nature and further experimental validation is required to establish their interaction with cholesterol.

4. A working mechanism for cholesterol-mediated pathogen entry

The entry of intracellular pathogens involves receptors on the host plasma membrane that recognize cognate ligands on the pathogen cell surface. In fact, several receptors, such as mannose and complement receptors, implicated in pathogen entry are shared by *Leishmania* and *Mycobacterium* as common hubs to gain access to the host cell interior. Signaling by a representative GPCR, the β_2-adrenergic receptor, has been shown to be responsible for the entry of malaria parasite, *Plasmodium falciparum*, into host cells (Harrison et al., 2003). In order to understand the mechanism underlying cholesterol-mediated pathogen entry, we examined the presence of cholesterol binding motifs in the sequence of these receptors. Fig. 3 shows that receptors responsible for pathogen entry display typical cholesterol binding motifs such as CRAC and CARC. More importantly, some of these CRAC/CARC motifs are present in transmembrane domains of these receptors (highlighted in Fig. 3) that could enable them to sense membrane cholesterol. The presence of cholesterol binding motifs on receptors implicated in pathogen entry are indicative of a possible mechanism in cholesterol-mediated entry of intracellular pathogens into host cells (see Fig. 4).

Cholesterol-mediated pathogen entry appears to be independent of the type and complexity of the pathogen. *Leishmania* and *Mycobacterium*, for example, have very few common features that they share in terms of their genome size and composition, and evolutionary biology. Yet, entry of both pathogens exhibits sensitivity to host membrane cholesterol. In fact, pathogens belonging to diverse groups, such as HIV (Carter et al., 2009) and *Plasmodium falciparum* (Lauer et al., 2000), display

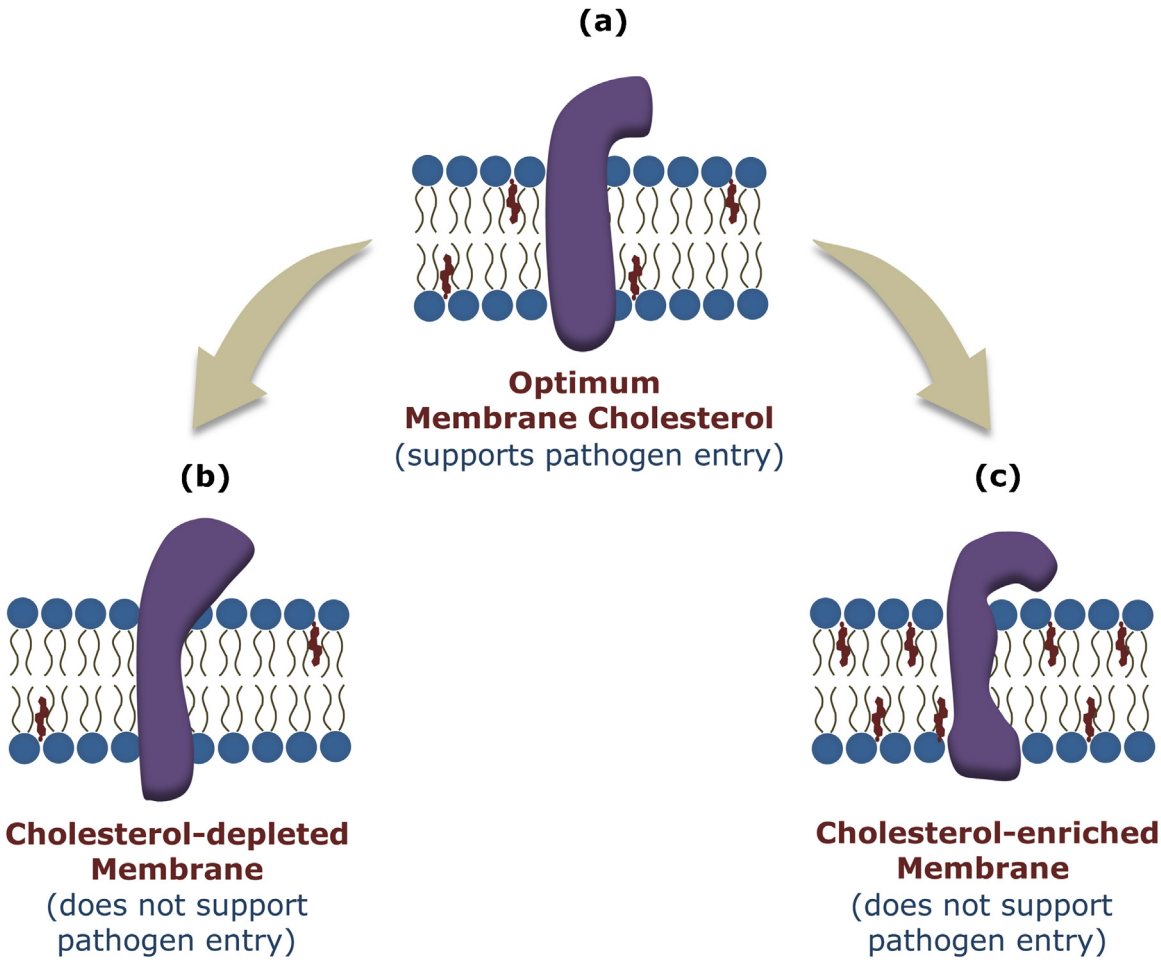

Fig. 4. A schematic representation of a possible cholesterol-mediated mechanism for pathogen entry. Host membrane cholesterol content could modulate entry of intracellular pathogens by rendering multiple functional conformations of cell surface receptors involved in this process. Insights from our studies suggest that (a) optimum level of host membrane cholesterol is necessary for supporting receptor conformation(s) that allow pathogen entry. Either (b) depletion or (c) enrichment of membrane cholesterol induces receptor conformation(s) that does not support the entry of pathogens into host cells.

cholesterol-dependent entry into host cells. A therapeutic approach involving host membrane cholesterol offers the advantage that the development of drug resistance, a common problem associated with treatment of diseases caused by these pathogens (Berman, 2003; Keshavjee and Farmer, 2012), would be absent since the therapeutic focus is on the host membrane cholesterol, rather than the pathogen. Interestingly, the administration of compounds that modulate membrane cholesterol levels could prove to be a powerful approach in tackling the combination of leishmaniasis (or tuberculosis) associated with HIV-1 infection. This is due to the fact that cholesterol has been reported to be essential for HIV-1 infection (Carter et al., 2009) and topical application of cyclodextrins (cholesterol-lowering compounds) has previously been shown to block the transmission of cell-associated HIV-1 in mice (Khanna et al., 2002).

The mechanistic basis for such a pathogen-independent process therefore must originate from the host plasma membrane, the common portal for pathogen entry. A possible mechanism underlying such a process would involve interaction of host membrane cholesterol with cell surface receptors responsible for parasite entry. Previous work from our and other groups has demonstrated the significance of membrane cholesterol in the organization, dynamics and function of several transmembrane receptors, including GPCRs, thereby showing a close correlation between membrane cholesterol and receptor function (for reviews, see Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay, 2010; Oates and Watts, 2011; Jafurulla and Chattopadhyay, 2013). Recent results from molecular dynamics simulations have shown that interaction of cholesterol with membrane proteins such as GPCRs is associated with subtle structural changes in the transmembrane regions of receptors (Khelashvili et al., 2009; Shan et al., 2012; Lee et al., 2013; Prasanna et al., 2014).

In light of the above observations, we propose that the conformation of host membrane receptors implicated in the entry of intracellular pathogens could be dependent on an optimal level of host membrane cholesterol. A schematic representation of a possible mechanism of regulation of pathogen entry by optimum host cell membrane cholesterol *via* modulation of receptor conformation is shown in Fig. 4. As shown in the figure, reduced as well as enriched levels of host membrane cholesterol could induce local conformational changes in these receptors that may not support efficient pathogen entry. Interestingly, such optimal cholesterol concentration-dependent function of membrane proteins has been previously reported. For example, the function of membrane proteins such as the Na^+,K^+-ATPase (Cornelius, 1995) and $GABA_A$ receptor (Sooksawate and Simmonds, 2001) has been shown to be optimum within a range of membrane cholesterol. Taken together, optimum levels of cholesterol stabilize receptor local conformations that support efficient pathogen entry into host cells. We envision that probing deeper into the role of host membrane components in pathogen entry would enhance our ability to design better therapeutic strategies to effectively tackle intracellular pathogenesis.

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