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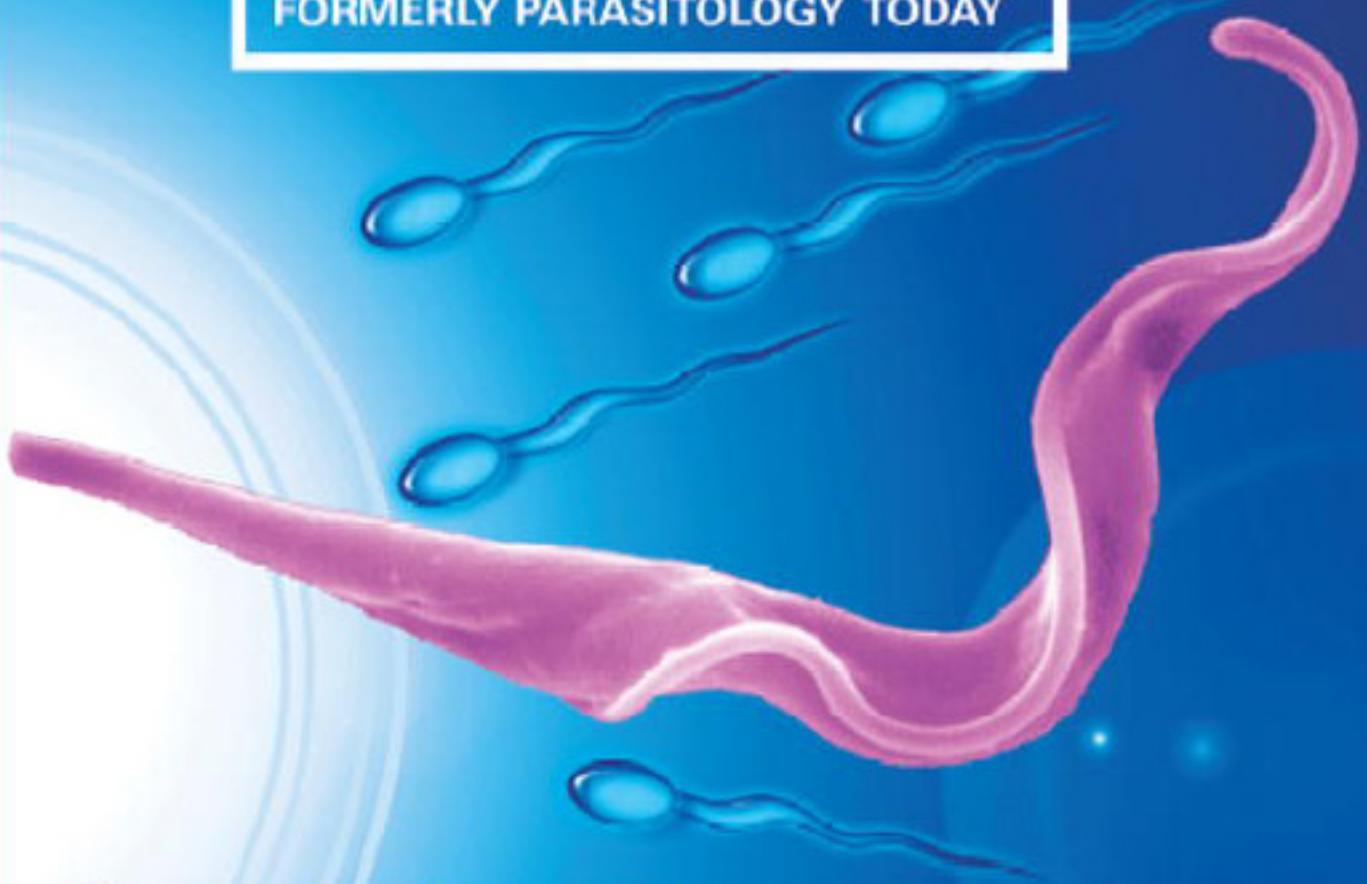

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Cholesterol: a potential therapeutic target in *Leishmania* infection?

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Leishmania **are obligate intracellular parasites that invade and survive within host macrophages and can result in visceral leishmaniasis, a major public health problem worldwide. The entry of intracellular parasites, in general, involves interaction with the plasma membrane of host cells. Cholesterol in host cell membranes was recently shown to be necessary for binding and internalization of *Leishmania* and for the efficient presentation of leishmanial antigens in infected macrophages. This article describes the need to explore cyclodextrin-based compounds, which modulate host membrane cholesterol levels, as a possible therapeutic strategy against leishmaniasis in addition to other intracellular parasites.**

Role of cholesterol in membrane function and organization

Cholesterol is an essential component of eukaryotic membranes and has a crucial role in membrane organization and function [1]. It is a predominantly hydrophobic molecule (Figure 1), comprising of a near planar tetracyclic fused sterol ring and a flexible isooctyl hydrocarbon tail. The 3β-hydroxyl moiety gives cholesterol its amphiphilic character causing it to orient in a bilayer with its long axis perpendicular to the plane of the membrane. Cholesterol has a variety of effects on the physical properties of membranes [2]. When present at high concentrations in model membranes, cholesterol induces the formation of the liquid-ordered phase characterized by high packing density and high mobility of membrane constituents [2].

Cholesterol is often found distributed non-randomly in domains or pools in biological membranes [3,4]. For example, caveolae represent morphologically distinct, cholesterol-enriched structures in membranes of cells expressing isoforms of the cholesterol-binding protein caveolin [3]. The existence of a type of membrane domain enriched in cholesterol (known as lipid rafts) has been proposed [5], and is believed to represent the liquid-ordered phase observed in model membranes [6]. Recent studies focused on monitoring membrane dynamics and distribution of membrane components, on a scale far lower than the resolution of visible light microscopy, have indicated the presence of small and dynamic lipid-based heterogeneities under native conditions in cell membranes [7–9]. Cholesterol is believed to be the principal constituent of these domains, and, therefore, its removal from cell membranes would disrupt their integrity [10], thereby leading to a loss of putative functions mediated by these domains. Because of their unique membrane environment and their potential to organize receptors in the plane of the membrane, domains such as lipid rafts and caveolae are believed to represent portals for the entry of pathogens [11–13]. In addition, cholesterol has been implicated in maintaining the structure and function of integral membrane proteins and receptors [14].

Role of cholesterol in infection by intracellular pathogens

Several studies have indicated the crucial requirement of cholesterol in host–pathogen interactions [reviewed in Refs 11–13]. The ability to manipulate levels of cholesterol in the membrane with a reasonable degree of specificity has contributed to our understanding of its role in host–pathogen interactions and subsequent infection. Cholesterol content in the membrane can either be lowered by using specific carriers, such as cyclodextrins (Figure 2), or be made unavailable by using cholesterol-sequestering agents (e.g. nystatin and filipin) [14]. Cyclodextrins, in general, exhibit a broad specificity for extracting membrane lipids. However, when used under low concentrations, the oligomer with seven residues (β-cyclodextrin) of methylated-glucose, methyl-β-cyclodextrin (MβCD), displays a relatively higher specificity for cholesterol and other steroids compared with phospholipids [14,15]. Several studies, using cultured cells as hosts, indicate that the use of these compounds reduces infectivity of intracellular pathogens, an effect that often correlates with the extent of reduction in the membrane cholesterol content [11–13]. The role of cholesterol in pathogen infection can be categorized as a requirement either at the stage of pathogen binding to cell surface receptors or during their internalization into cells (or both) [13], or for their subsequent survival within host cells [16]. Although a classification of this kind provides mechanistic insights into the role of cholesterol in pathogen infection, the situation could be a combination of several factors that eventually manifests in a reduction in infectivity of intracellular pathogens in cells with reduced cholesterol content.

Cholesterol has been found to be required for the internalization of several species of *Mycobacteria* into macrophages [17,18]. Depletion or sequestration of cholesterol in mouse macrophages results in a reduction in the population of infected macrophages. Similar results have

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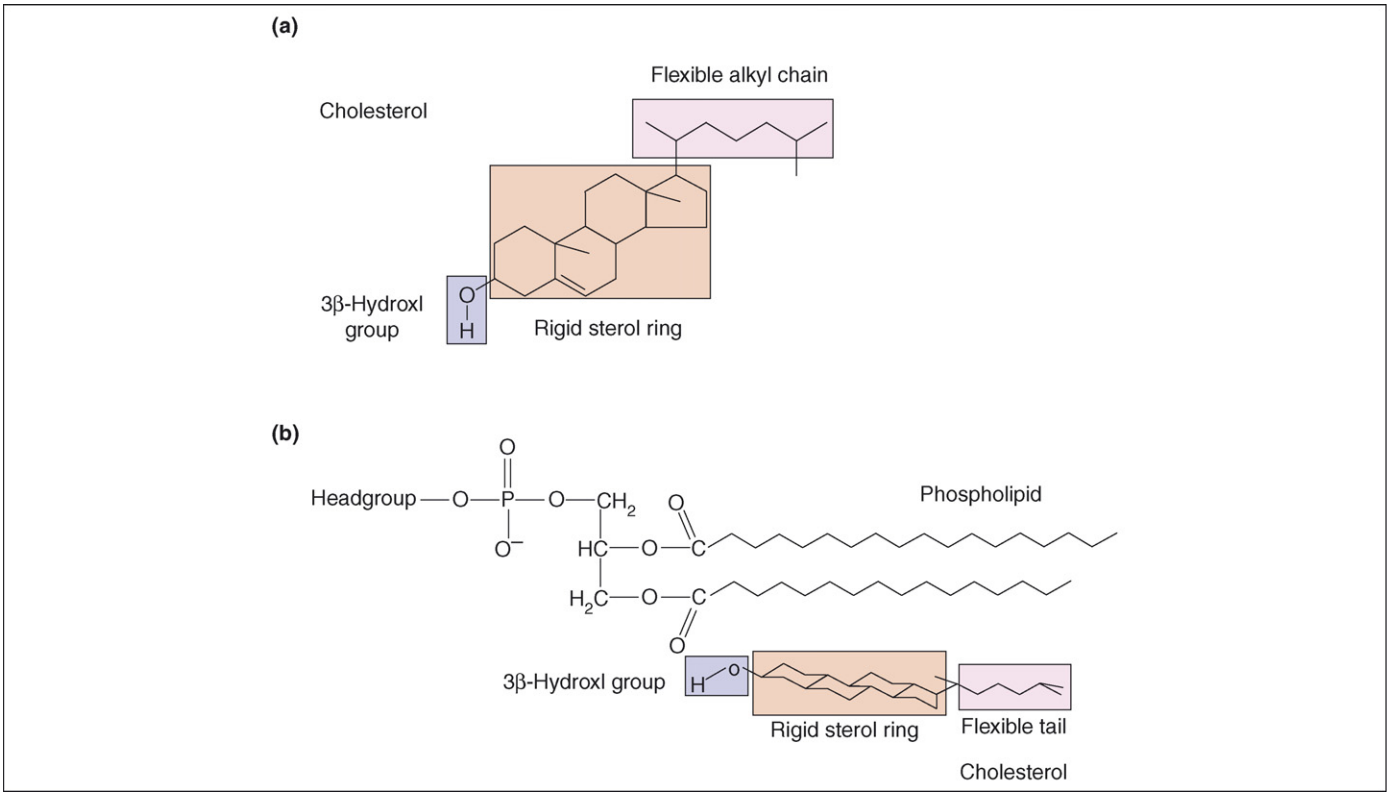

Figure 1. Structure and membrane orientation of cholesterol. **(a)** There are three structurally distinct regions of cholesterol (shaded boxes): the 3β-hydroxyl group, the rigid sterol ring and the flexible alkyl chain. The 3β-hydroxyl moiety is the only polar group in cholesterol and serves to anchor cholesterol in the membrane. **(b)** Orientation of cholesterol in relation to a phospholipid molecule in a lipid bilayer. The rigid and near-planar sterol ring contributes to the ordering effect of cholesterol in phospholipid bilayers as a result of the restriction in motion imposed on it by adjacent phospholipid fatty acyl chains. The flexible alkyl chain extends into the hydrophobic core of the membrane. Adapted and modified from Ref. [14].

been seen in the internalization of uropathogenic *Escherichia coli* [19] and caveolae-mediated endocytosis of fimbriated *E. coli* [20]. Cholesterol has also been found to be required for phagocytosis of *Brucella suis* [21]. In the case of *Chlamydia trachomatis*, cholesterol depletion seems to inhibit phagocytosis of the parasite but not its binding to cell surface receptors [22]. A cholesterol-dependent entry process into host cells has been reported for *Listeria monocytogenes* [23]. In this situation, depletion of membrane cholesterol from the bacterium was found to diminish binding of the pathogen to cellular receptors and also to reduce endocytic uptake of the pathogen into host cells. Interestingly, when *Mycobacteria* and *B. suis* are coated with serum components, a process called opsonization, the effect of cholesterol depletion or sequestration on parasite entry is no longer observed [18,21].

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 [24]. The protozoan parasite *Toxoplasma gondii* is particularly interesting in this context. The growth and survival of *T. gondii* in the host depend on efficient acquisition of cholesterol from host membranes by a process involving the endocytosis of low-density lipoprotein (LDL) into host cells [16,25]. Direct lipid transfer from host membranes to the parasite, mediated by specific transporters in the parasite, has been proposed as a mechanism for the acquisition of host cholesterol, thereby representing strong candidates for therapeutic drugs [16]. In contrast to *T. gondii*, the kinetoplast parasite *Trypanosoma cruzi* can synthesize specific sterols, such as ergosterol, which are required for its survival and growth. This specific requirement is met by the enzyme squalene synthase, which catalyzes the first committed step in sterol biosynthesis, not only in the parasite but also in humans. Although similar to its mammalian homologue, in the parasite this enzyme is emerging as a potential target for antiparasite chemotherapy [26]. Interestingly, although sterol synthesis inhibitors can induce growth arrest of *T. cruzi*, perhaps as a result of a reduction in its endogenous sterols, a concomitant increase in externally derived cholesterol in the parasite is observed, implying the existence of an adaptive mechanism [26]. Nevertheless, this compensatory mechanism does not contribute to the survival of the parasite possibly as a result of the inability of cholesterol to fully mimic ergosterol, which is required for the growth of the parasite. Taken together, these examples indicate an intricate relationship that has evolved between host cholesterol metabolism and infection by intracellular parasites.

Leishmania infection

Leishmania cause leishmaniasis, which, under conditions of overt visceral infection, is usually fatal if untreated [27,28]. Leishmaniasis is now considered endemic in 88 countries in Africa, Asia, Europe, North America and South America, with a total of 12 million people infected worldwide and 350 million people at risk of infection (WHO Factsheet, 2000: http://www.who.int/mediacentre/factsheets/fs116/en/index.html). The estimated annual

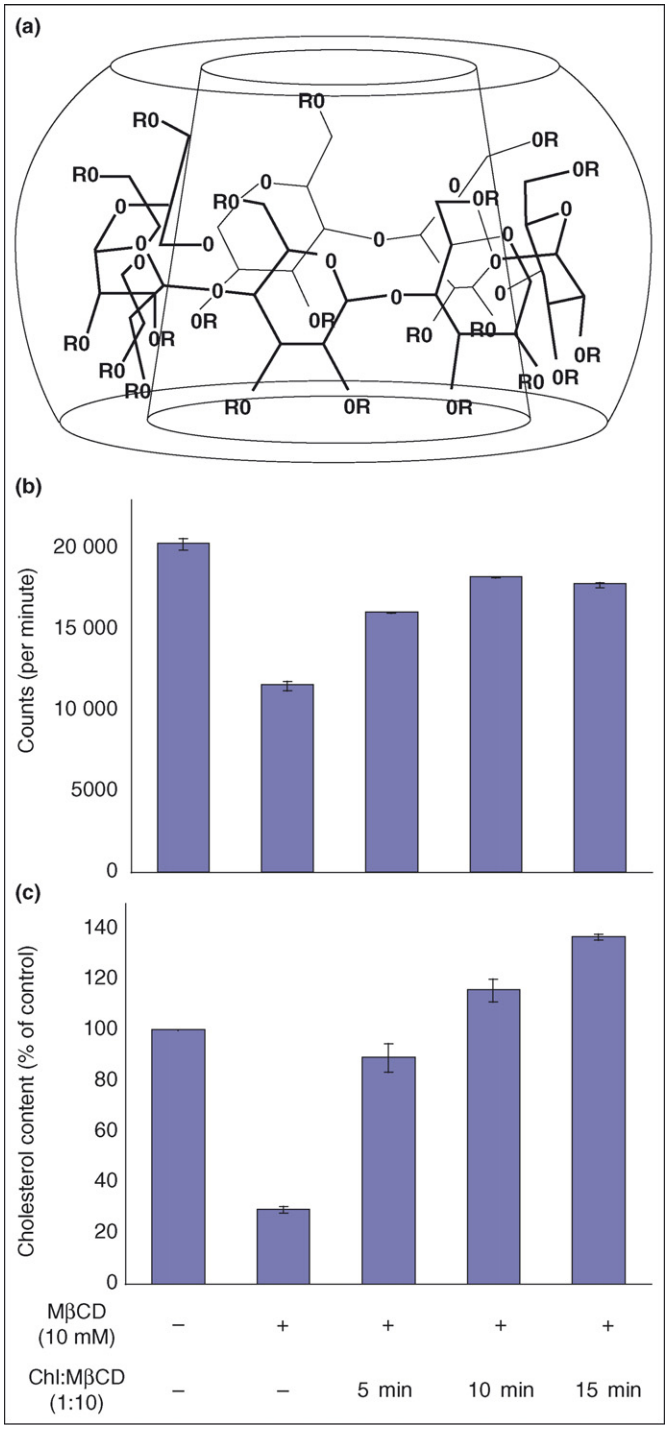

Figure 2. Role of cholesterol in *Leishmania donovani* infection. Cholesterol levels in J774A.1 mouse macrophages were modulated using a specific carrier, methyl-β-cyclodextrin (MβCD). **(a)** Chemical structure of a β-cyclodextrin (containing seven glucose residues) molecule. Adapted and modified from Ref. [14]. Cyclodextrins can solubilize a variety of hydrophobic compounds by trapping them in their inner cavity. The specificity of this process depends on the structure of their inner cavity, which can be modified by substitution of the hydroxyl groups (indicated as R in the figure) in each glucose residue. The most commonly used cholesterol-depleting agent is MβCD in which R is a methyl group. **(b)** The role of cholesterol in *Leishmania*–macrophage interactions was monitored using radiolabeled binding assays using *L. donovani* promastigotes on mouse macrophages depleted of cholesterol using MβCD. Replenishment of cholesterol into cholesterol-depleted macrophages using cholesterol complexed with MβCD demonstrated the specificity of the role of cholesterol in host–pathogen interactions. Cholesterol depletion with 10 mM MβCD results in a reduction in binding and uptake of radiolabeled *Leishmania* into host macrophages. Replenishment of cholesterol into MβCD-treated cells increasingly supports radiolabeled parasite-binding ability. **(c)** The membrane cholesterol content in MβCD-treated macrophages before and after treatment of cholesterol-depleted macrophages with cholesterol–MβCD complex for varying time periods (5, 10 and 15 min). Figures 2b and c are adapted and modified from Ref. [32].

number of new cases of leishmaniasis is thought to be two million [29] and of visceral leishmaniasis is ~500 000 [27]. The current worldwide increase in leishmaniasis to epidemic proportions, and the emergence of visceral leishmaniasis as an important opportunistic infection among people with human immunodeficiency virus-1 (HIV-1) infection [29], have resulted in an urgent requirement for treatment.

Leishmaniasis is transmitted by the female sand fly, *Phlebotomous* spp., infected with *Leishmania* when taking a bloodmeal from a host [30,31]. Once in the bloodstream, promastigotes are efficiently phagocytosed by host macrophages. Entry of promastigotes into macrophages involves recognition of specific ligands on the parasite cell surface by macrophage receptors and subsequent internalization of the promastigotes. Complement receptors CR1 and CR3 on the macrophage cell surface have major roles in the entry processes. The interaction of the parasite with complement receptors occurs through either a serum-dependent or serum-independent pathway. In the presence of serum, *Leishmania* promastigotes undergo opsonization, resulting in their surface being covered with the C3bi fragment of the host complement system. Because C3bi binds to CR3 present on the macrophage cell surface, opsonization enhances the entry of the parasite into macrophages. The entry of *Leishmania* parasites can also occur through a serum-independent process, which involves either binding of the parasite surface protease gp63 to CR3, or binding of the parasite lipophosphoglycan (LPG) to the lectin-like site on CR3 and to CR1. The CR4, fibronectin receptor, mannose receptor and the advanced glycosylation end-product receptor have also been implicated in the entry process [30]. Once inside the macrophage, promastigotes undergo biochemical and metabolic changes, thereby converting into the obligatory intracellular amastigote form of the parasite. Amastigotes are released from macrophages and can re-invade dendritic cells and fibroblasts and other macrophages. The re-entry of amastigotes into host cells is assumed to be mediated by the Fc receptor for immunoglobulin G, in addition to CR3 and the mannose receptor [30]. Taken together, the redundant nature (i.e. multiple receptor–ligand interactions) of the parasite entry process makes it difficult to identify a unique therapeutic target [28]. Host cells harboring amastigotes subsequently mediate the spread of infection from its initial site in the skin to the lymph nodes, in which the parasites persist indefinitely.

Novel role of cholesterol in *Leishmania* infection

The requirement of host membrane cholesterol in the binding and internalization of *Leishmania donovani* into macrophage cells has recently been demonstrated using complementary approaches [32,33]. Cholesterol depletion from macrophages by cyclodextrins [32], or its sequestration by nystatin [33], results in a reduction in the extent of *Leishmania* infection by promastigotes, probably by affecting the binding of the parasite to the cell surface (Figure 2). When hosts are examined for a longer period of time,

post-infection, a reduction in the number of the intracellular amastigote form of the parasite is observed [32]. Importantly, the reduction in binding of *L. donovani* promastigotes to cholesterol-depleted macrophages can be reversed by replenishment of cholesterol (Figure 2), thereby reinforcing the specific requirement of cholesterol in the infection process [32]. As seen with *Mycobacteria* [18] and *B. suis* [21], entry of serum-opsonized *L. donovani* promastigotes into host macrophages seems to be unaffected by cholesterol depletion or sequestration [32,33]. This points toward the essential role of cholesterol in supporting entry of *L. donovani* promastigotes via the non-opsonic pathway into macrophages. More recently, cholesterol has been found to be necessary for the entry of *Leishmania chagasi* into host bone marrow macrophages through cholesterol-enriched caveolar domains [34]. However, unlike *L. donovani* [32], cholesterol depletion of bone marrow macrophages also seems to inhibit the entry of opsonized *L. chagasi* [34]. Because studies with *L. donovani* [32,33] and *L. chagasi* [34] were performed with virulent strains of the parasite, these differences could therefore reflect a unique mode of interaction between cholesterol and the cognate receptor for opsonized *L. donovani* and *L. chagasi* on the host cell surface.

Because amastigotes of *Leishmania* set up secondary sites of infection *in vivo*, the role of cholesterol in their entry into host tissues represents an important aspect in *Leishmania* infection that needs to be explored. Nevertheless, the entry of promastigotes into host cells represents a crucial event in the establishment of primary infection and cholesterol depletion of host macrophages has been shown to effectively inhibit this process [32,34]. Consequently, cholesterol depletion has been found to result in a concomitant reduction in the intracellular amastigote form of the parasite in host macrophages [32]. Whether such a reduction in the intracellular load of amastigotes could serve to inhibit further infection *in vivo* would require research trials at the preclinical stage.

Visceral leishmaniasis is characterized by defective cell-mediated immunity [31]. Subsequent to their entry into the host, *Leishmania* parasites prolong their survival by subverting host immunity [35]. 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 [36]. Defective antigen-presenting ability of infected macrophages correlates with alterations in the physical properties (such as dynamics of membrane-embedded molecules) of macrophage cell membranes, and has been proposed to be a mechanism employed by the parasite to evade the host immune system [37]. Because cholesterol has a wide variety of effects on membrane physical properties [2], a possible manner by which *Leishmania* parasites achieve this could be by the uptake of host cholesterol, as in the case of *T. gondii* (see above). Restoration of these physical properties by an exogenous supply of cholesterol to host cells leads to recovery in antigen-presenting ability of *Leishmania*-infected macrophages [36]. These results indicate a role for cholesterol in supporting *Leishmania* infection inside the host.

The requirement of cholesterol in supporting host–pathogen interactions and pathogen survival within host tissues seems to be fairly well established. However, the question remains: by what mechanism does membrane cholesterol influence these processes? The role of membrane cholesterol in the function and cell surface organization of integral membrane proteins has been extensively investigated [14]. It is possible that the inhibition of pathogen interaction and infectivity could be a result of loss of function of the several host receptors that pathogens use to gain entry into cells, and/or a result of reorganization of receptors that would otherwise have provided a favorable platform for multivalent interactions with ligands on the parasite cell surface.

Implications in leishmaniasis

The reduction in infection by cholesterol depletion might lead to novel therapeutic strategies against leishmaniasis. A major advantage of this approach lies in the fact that development of drug resistance, an emerging problem encountered in the treatment of leishmaniasis [38], is virtually absent because the therapeutic focus is on the host rather than the parasite. Based on the inhibitory effects of cyclodextrins on *Leishmania* infection *in vitro* [32–34], the feasibility of using cyclodextrins as a therapeutic strategy against leishmaniasis *in vivo* seems encouraging. Earlier studies have shown that cyclodextrin-like molecules are important in treating unstable atherosclerotic plaques as a result of their ability to remove cholesterol from macrophage foam cells *in vitro* [39]. In addition, cyclodextrins have been shown to cause hypolipidemic effects in genetically hypercholesterolimic rats when fed orally [40]. Furthermore, intravenous administration of cyclodextrins in normal rats has been shown to reduce plasma cholesterol levels transiently in a dose-dependent manner [41]. Whether reduction in the levels of plasma cholesterol by cyclodextrin administration also manifests in a reduction in levels of cell membrane cholesterol remains to be determined, although this has been reported to be true in the case of statin treatment [42].

As mentioned earlier, visceral leishmaniasis has emerged as an important opportunistic infection among people with HIV-1 infection [29]. Interestingly, cholesterol has been reported to be essential for HIV-1 entry into host cells [43,44], and topical application of cyclodextrins has previously been shown to block the transmission of cell-associated HIV-1 in mice [45]. The administration of compounds 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. On a broader perspective, the requirement of cholesterol in *Leishmania* infection forces re-evaluation of the mechanism behind the effectiveness of current therapeutic strategies to treat leishmaniasis. Among the popular clinically prescribed therapeutic drugs to treat visceral leishmaniasis is amphotericin B [38], which is a sterol-binding antibiotic [46]. Although amphotericin B is a potent leishmanicidal agent [47], it is possible that its effectiveness *in vivo* is based partly on its ability to sequester cholesterol in the host membrane, thereby reducing macrophage–parasite interactions, similar to what has been observed for a related sterol-binding agent, nystatin, *in vitro* [33].

Concluding remarks

In summary, the role of membrane cholesterol in diseases caused by widely divergent intracellular pathogens is being increasingly recognized. It is, therefore, timely to explore compounds that modulate host membrane cholesterol levels as a possible therapeutic strategy against such diseases.

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