CHAPTER 11

Conclusions and General Discussion
Human visceral leishmaniasis is an opportunistic disease caused by the protozoan parasite *Leishmania donovani*. These digenetic protozoan parasites inhabit two highly specific hosts, the sandfly and the mammalian macrophage. Entry into macrophages involves recognition of specific parasite ligands by receptors on the macrophage cell surface and eventual internalization of the parasite by the macrophage. Numerous studies have identified macrophage cell surface receptors involved in the internalization of *Leishmania* promastigotes. These include, the mannose–fucose receptor (MFR). Advanced glycosylation end products (AGE), the fibronectin receptor, the Fc receptor (FcR) and complement receptors such as the CR1 and CR3. All or most of these receptors are an integral part of the plasma membrane of the host macrophages. Thus the entry of *Leishmania donovani* in particular and other intracellular parasites in general involves interaction with the plasma membrane of host cells.

Cholesterol is a major constituent of eukaryotic membrane and plays a crucial role in cell membrane organization, dynamics, function and sorting. Apart from this, cholesterol also plays important role as receptor on plasma membrane for various molecules and microbes, which include, HIV, *Mycobacterium*, *Influenza* virus. Since cholesterol is the main component of lipid rafts, we hypothesise the involvement of lipid rafts in the attachment and internalization of *Leishmania*. The recognition of the involvement of lipid rafts in various infectious and non-infectious pathologies can offer new possibilities of therapeutic interventions.

The work presented in part B of this thesis deals with identification of molecular mechanisms of host parasite interaction. The main aim was to evaluate the role of cholesterol in the entry of *L. donovani* into the host macrophages. We have examined the role of cholesterol in *Leishmania* infectivity by treating the macrophages with the sterol-depleting agent MβCD or sterol binding agent nystatin. Nystatin is known to specifically sequester cholesterol from the membranes thereby effectively reducing the ability of cholesterol to interact with receptors. We have also assessed the involvement of CR3 receptor and its role in association with GPI-anchored proteins for the internalization of *Leishmania*. The results obtained are summarized below.

1. Cholesterol depletion was carried out using 5 and 10 mM MβCD. Analysis of the total cellular cholesterol level indicated that treatment with MβCD led to an acute depletion
of cholesterol, which was concentration dependent. This treatment resulted in a ~ 40% reduction of total cellular cholesterol when 10 mM MβCD was used for 30 min.

2. To analyze the role of cholesterol in phagocytosis of *L. donovani* in host macrophages we treated cells with either cholesterol-depleting molecules like MβCD or binding molecules like nystatin. Pretreatment of J774A.1 cells by these molecules followed by infection with tritiated or FITC labeled parasites resulted in a time dependent reduction in macrophage parasite interaction. Around 45% reduction in binding/attachment of the *Leishmania* promastigotes was observed in cholesterol depleted/sequestered cells when compared to control macrophages.

3. To assess whether entry through opsonic receptors is dependent on cholesterol, we treated macrophages with MβCD and nystatin respectively and analyzed the macrophage parasite interaction of serum opsonized parasites by either tritium labeling or by flow cytometry. It was observed that cholesterol depletion/sequestration had no effect on attachment/attachment of the opsonized promastigotes indicating entry through opsonic receptors is independent of cholesterol.

4. Chelation of cholesterol by MβCD or sequestration by nystatin did not affect binding of control *E. coli* cells to host macrophages when analyzed by flow cytometry indicating entry of these bacteria is independent of membrane cholesterol.

5. Since cholesterol depletion/sequestration led to a reduction in the ability of non-opsonic promastigotes to interact and bind to host macrophages, we wanted to analyze whether the reduction in binding of the promastigotes resulted in reduction in the intracellular load of amastigotes. This was carried out by Giemsa staining of infected macrophages after 3 hours of infection. It was observed that treatment of macrophages with MβCD (5 and 10 mM) caused a concomitant reduction in the number of amastigotes present (compared to control cells) in the macrophages with only 50% amastigotes present when depletion was carried out with 10 mM MβCD. Similar reduction was observed when infection was carried out after treatment with nystatin.

6. To analyze whether replenishment of cholesterol would lead to a reversal in the reduction of macrophage parasite interaction, binding assays were performed after replenishment of cellular cholesterol. Interestingly, the increase in cellular cholesterol content led to a concomitant increase in the ability of the parasite to attach to the macrophage cell surface.
7. To analyze the involvement of CR3 in attachment and subsequent internalization of *L. donovani* promastigotes, macrophages were treated with anti-CR3 antibodies. It was observed that, in the presence of anti-CR3 antibodies, attachment of both non-opsonized and serum treated *L. donovani* was inhibited by 56 and 60 % respectively.

8. To assess the importance of association of CR3 and GPI linked proteins in phagocytosis of *Leishmania*, macrophages were pretreated with NADG followed by interaction with [³H] thymidine labeled parasites. A reduction in the ability of *Leishmania* promastigotes to interact with host macrophages was observed. Interestingly, binding of opsonized promastigotes was not inhibited. This data suggested that the interaction of CR3 with GPI-linked receptors might be crucial for binding and internalization of non-opsonic *Leishmania*.

Thus the present work explores the importance of membrane cholesterol and association of CR3 and GPI linked proteins in the entry of *Leishmania*. We demonstrate that cholesterol acts as a potent receptor on the membrane of macrophages and that depletion or sequestration of cholesterol from the membrane with the help of depleting or sequestering agents like MβCD or nystatin results in decrease in the infectivity. Since cholesterol is an integral part of lipid rafts we propose that these membrane microdomains are important for the attachment and subsequent internalization of *Leishmania*.

It is known that CR3 is associated with other receptors in the membrane, especially with GPI anchored proteins and this association enhances CR3 activity. Most of the members of the GPI linked proteins are present in cholesterol rich regions called rafts. We demonstrate that association of CR3 with GPI-linked proteins aids in internalization of non-opsonic *L. donovani*. Thus it would be interesting to study the functional relationship between these receptors and whether they cooperate in promoting attachment of promastigotes to macrophages.