Aims and Objectives
Host defense against infection and malignancy is critically controlled by the presence and functional integrity of mononuclear phagocytes (Mφs). In particular, these cells perform as the central components in both the early, innate response and the acquired immune response to microbial invasion. Ironically, monocytes and macrophages are also target cells for a range of microorganisms including that of bacteria, viruses, fungi, and protozoa which share a common, obligate requirement for an intracellular environment in which to replicate. This is a curious paradox of mammalian host defense and still need to be deciphered. *Leishmania donovani*, the causative agent of visceral leishmaniasis, exemplifies a diverse group of microorganisms that induces an immune silencing mechanism for its intracellular survival inside the hostile environment of macrophages. The toll-like receptors (TLRs), presence on the surface of macrophages, are the sentinel factor of the innate immunity, which are essential for host defense. These receptors detect the presence of conserved molecular patterns of potentially pathogenic microorganisms and contribute in both, cellular as well as humoral immune responses. *Leishmania*, being a very intelligent parasite, silently invades host immune system by manipulating TLR mediated macrophage signaling pathway, during various phases of infection to ensure their successful survival and replication. Beside TLR mediated host protective immune response, another vital mechanism by which host cells defend themselves against intracellular pathogens is the induction of apoptosis. Therefore, apart from tampering TLR signaling pathway, another vital strategy taken by *Leishmania* is to inhibit host cell apoptosis, in order to get enough time to replicate within macrophages.

In the present study, we attempted to provide a mechanistic insight into the novel regulatory signaling mechanism of establishing *Leishmania* infection in macrophages or in a broader sense host-parasite interaction.

**Chapter I**

The complicated interactions between *Leishmania* and the host phagocytic cells have fundamental effects on the final outcome of the disease. Macrophages play critical roles in mediating resistance and susceptibility during *Leishmania* infection. Recognition of invading microorganisms by the innate immune system is a first and essential step in their successful elimination. Macrophages sense the pathogens by the specific surface protein of pathogen named
pathogen-associated molecular pattern (PAMP). This is mediated by interaction between PAMP and macrophage surface receptor TLR. All TLRs possess a leucine-rich extracellular domain that is supposed to mediate ligand binding, as well as a conserved cytoplasmic Toll/IL-1 receptor (TIR) domain that upon activation recruits adaptor proteins, such as MyD88, via homomeric interaction of TIR domains. The N-terminal death domain of MyD88 subsequently binds to and activates the interleukin receptor-associated kinase 1/4 (IRAK1/4), TNF receptor-associated factor 6 (TRAF6), ubiquitin-conjugating enzyme 13 (Ubc13), and cellular inhibitor of apoptosis 1/2 (cIAP1/2). The entire protein complex formed by this transient interaction is termed as Signalosome complex. This complex formation is followed by a Complicated signaling events comprised of number of ubiquitination steps which critically regulate the onset of downstream process. In the presence of the active TLR inducers the perfect ubiquitination process leads to subsequent release and translocation of the entire complex to the cytosol resulting in activation of TGF-β-associated kinase 1 (TAK1) and downstream mitogen activated protein kinases (MAPKs) ultimately resulting in IKK/NF-κB activation. To protect cells from constant pro-inflammatory cytokines synthesis mediated endotoxin shock cells recruit different regulators that negatively control TLR signaling pathway and a TRAF family protein TRAF3, major component of TLR signaling pathway, is one such example. TRAF 3 degradation is a necessary prerequisite for perfect translocation of entire signalosome complex from membrane to cytosol. We found, during infection TRAF3 degradation is prevented. Since improper signalosome complex translocation may lead to suppression of TLR mediated immune response and Leishmania also prevent TLR mediated proinflammatory cytokines synthesis, we hypothesized that TRAF3 may be exploited by Leishmania. In the present study we aim to determine the mechanism used by Leishmania to modulate TLR signaling cascade for its own favour using TRAF 3 as a negative regulator.

Specific objectives:

i) To study whether L. donovani infection prevents pro-inflammatory cytokine response even in presence of Lipopolysaccharide (LPS), a strong TLR4 inducer.

ii) To study the status of signalosome complex during infection and compare it with LPS treated cells.
iii) To measure the level of TRAF3 protein during infection and compare that with other components of signalosome complex.

iv) To determine the involvement of TRAF3 in *L. donovani* mediated suppression of TLR signaling pathway using siRNA mediated knock down system.

v) To determine the ubiquitination status of TRAF3 as well as other major E3 ligases involved in TLR anchored complex formation and their interdependency.

**Chapter II**

Manipulation of host TLR4 signaling pathway by *Leishmania* is crucial for successful entry into macrophages. After entry into host cells, *Leishmania* might represent a stress signal sufficient to initiate an apoptotic process in the host, thus providing an altruistic mechanism to eliminate infected cells and limit parasite dissemination. As *Leishmania* parasite and hosts live and evolve together, it is not surprising that the former had developed strategies to circumvent this apoptotic immunity of the host. Inside the macrophages, *Leishmania* parasites are housed in the parasitophorous vacuoles which are fused with lysosomes to form phagolysosomes, wherein the promastigotes transform into and replicate as amastigotes. Eventually, the parasite burden increases, physically disrupting infected host macrophages and delivering extracellular amastigotes into surrounding tissue where they are engulfed by uninfected macrophages. The fact that parasitized cells establish themselves and persist in this rich milieu of immunologic activity suggests that there are parasite-controlled mechanisms that prevent both parasites and infected cells from being killed. This hypothesis is further supported by the observation that infection of bone marrow derived macrophages (BMM) by *L. donovani* promastigotes inhibits apoptosis of the macrophage in the absence of growth factor, allowing the pathogen to silently persist and remain invisible to the immune system. Therefore, precise elucidation of the mechanism of induction of cytoprotection in macrophages, a potential vehicle for pathogen spreading in the host, is of high importance.

Many proteins of the Bcl-2 family with either anti-apoptotic (e.g. Bcl-2, Bcl-xl, A1, Mcl-1) or pro-apoptotic function (e.g. Bax, Bak or Bik) reside in the outer mitochondrial membrane. In recent years studies on anti-apoptotic effect conferred by *Leishmania* sp. have shown the involvement of some signaling pathways that lead to induction or activation of anti-apoptotic
proteins (e.g. Bcl-xl and Bad). But the role of another major anti-apoptotic protein Mcl-1 in host–parasite interaction has not been elucidated till date. The involvement and identification of Mcl-1 activation cascade and the following events which ultimately accomplish the parasite survival should provide an analytical insight into disease pathogenesis that might lend cues for devising novel therapeutic strategies.

The present research work is aimed to carry out a comprehensive study of the regulation of Mcl-1 expression, activation, localization and transport during *L. donovani* infection. Thorough and complete understanding of the pivotal pathway not only will improve our basic knowledge about intracellular parasite survival mechanism, but also will help us to identify effective drug targets against this deadly disease.

**Specific objectives**

i) To study the expression and activation of Mcl-1 in different stages of infection-induced survival of macrophages in *in vitro* culture (both cell line and primary cell) and *in vivo* mouse model.

ii) To screen various transcription factors associated with Mcl-1 and to identify the one crucial for Mcl-1 upregulation during infection.

iii) To determine the localization of MCL-1 and involvement of TOM family proteins that cumulatively facilitates translocation of Mcl-1 during infection.

iv) To elucidate the specific mechanism by which Mcl-1 exerts its anti-apoptotic effect.

**Chapter III**

When macrophages come to the end of the battle with intracellular infections and intend to be defeated, it induce apoptosis of self-cells to eradicate pathogens; thus safe the host. In our previous studies, we showed that *Leishmania* induces cytoprotection in murine macrophages, allowing the pathogen to silently persist and remain invisible to the immune system. In order to do so, this parasite tampered mitochondria dependent host apoptotic machinery which consists of pro- and anti-apoptotic members of the BCL-2 family. Macrophage apoptosis is regulated by the critical balance between these two classes of proteins. In our earlier observations we found rapid induction of anti-apoptotic protein MCL-1 upon *L. donovani* infection, which actively
participates in apoptosis prevention. Though we studied *Leishmania* mediated cytoprotection of host cells upto 24 h post infection, *Leishmania* can suppress macrophage immune response at late hours of infection (upto 48-72 h). Therefore, it is crucial for *Leishmania* to prevent apoptosis at late hours of infection. In different disease conditions MCL-1 prevents apoptosis at late time point of infection, suggesting the persistency of this particular protein even late hour infection. MCL-1 is very much labile protein. So, in a specific cellular condition, considerable level of this protein depends on its constant synthesis rate as well as its stability. That is transcriptional and post-translational regulation of this protein is a considering factor for its active participation in any apoptosis regulatory process. Since its mRNA and protein is rapidly degraded after removal of MCL-1 inducible factor, various workers reported different stabilization factors that stabilize and protect this protein from rapid degradation. In our present study we tried to elucidate the role of MCL-1 in *L. donovani* mediated cytoprotection of host cell at late hours of infection.

**Specific objectives:**

i) To measure the transcriptional and translational level of MCL-1 at late hours of infection.

ii) To study the stability of MCL-1 in different cellular conditions removing MCL-1 inducible factors.

iii) To compare the expression level of MCL-1 in *L. donovani* infected cells with MCL-1 inducing factors removing cells.

iv) To study the involvement of MCL-1 specific E3 ligases during infection.

v) To find out MCL-1 interacting partner responsible for protection of MCL-1 from proteasomal degradation.