

# CHAPTER X

*Conclusions*

A key strategy that is used by intracellular survival of pathogens to survive in a hostile host environment is to interfere with normal cell signalling to disable the defences that are aimed at controlling and eliminating foreign invaders. *Leishmania* are obligate intracellular protozoa that reside within mononuclear phagocytes, cause a wide range of human diseases from the localized self-healing cutaneous lesions to fatal visceral infections. The intra- and extracellular signal transduction have to become activated in order to kill infectious pathogens, indicating the need for signal transduction pathways that can transmit the information from the cell membrane to the nucleus. An effective mean of cellular coordination in complex regulatory mechanisms such as immune response is required in this “marriage of inconvenience”. *L. donovani* infected macrophages are known to have an impaired oxidative burst, which is a primary defense mechanism of the cell during infections and also impairing the ability of macrophage to produce gene products like IL-1 $\beta$  or MHC, which are essential for induction of T cell dependent immune responses.

The outcome of *Leishmania* infections depends not only on the species initiating disease but also on the immunological competence of the individual to combat parasite growth. Studies in mice and man have shown that multiple genetic loci influence the success of infection, affecting both acquired and innate immune responses against the parasite. The susceptibility or resistance to the disease is determined by the ratio of Th1 or Th2 cells. The susceptibility is associated with expansion of subsets of CD4<sup>+</sup> cells expressing Th2 phenotype and producing IL-4, IL-5, IL-6 and IL-10 and the resistance is associated with the expansion of Th1 cells producing IL-12, IL-2, IFN- $\gamma$  and TNF- $\beta$ . Recent studies suggest that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are required for immunity against the parasite. Once the balance between Th1 or Th2 becomes skewed, it results in

progression of disease. The mechanism of signal transduction that leads to this polarized response is not known.

The mechanism of signal transduction as a result of parasite interacting with the host is a very important area to be studied and this would basically decide if the parasite is going to survive or not in the host macrophages. One of the key determinants of parasite infectivity and survival in the host macrophages is a novel glycoconjugate, lipophosphoglycan (LPG). This is the major macromolecule on the surface of *Leishmania*.

The effect of *Leishmania donovani* and its surface molecule lipophosphoglycan (LPG) on signal transduction mechanism in macrophages has been explored.

## **1.0 A rapid and sensitive method to quantify *Leishmania* infection in macrophages**

- i) Firefly luciferase was used as a reporter for rapid detection and quantitation of host-parasite interaction over manual and tedious Giemsa staining, by studying episomal expression of luciferase reporter gene.
- ii) pGL- $\alpha$ NEO $\alpha$ LUC vector, containing the neomycin phosphotransferase gene (NEO) and two  $\alpha$ -tubulin intergenic regions were electroporated in *Leishmania* and transfectants were selected for resistance to G418.
- iii) Growth profile of *L. donovani* promastigotes *in vitro* transfected with LUC (luciferase) gene containing, episomal vector pGL- $\alpha$ NEO $\alpha$ LUC, at different time intervals was studied. A good correlation between the number of *L. donovani* promastigotes and luciferase activity was observed which indicated the sensitivity of this assay.
- iv) Resistance of leishmanial isolates to pentavalent antimonial compound (Sb<sup>V</sup>-Cl<sub>2</sub>) *in*

*vitro* by luciferase assay was also determined. Strain 41-R, CK2-R and AG83-W were found to be resistant to pentavalent antimonial compound ( $\text{Sb}^{\text{V}}\text{-Cl}_2$ ). Whereas, clinical isolate NS2-R strain which was supposedly resistant was found to be sensitive in these studies. On the other hand AG83-W that had been passaged in the laboratory for several years was found to be resistant to antimony. It is well known that several passages of a strain in the laboratory result in the loss of virulence of the parasites. It is possible that strain AG83 has undergone some changes at the genetic level in the laboratory and these changes need to be looked into. These changes may have resulted in the development of resistance to antimony.

- v) Northern blots analysis of *Leishmania* LUC-transfectants, examined the stable expression of episomal vector pGL- $\alpha$ NEO $\alpha$ LUC.

## **2.0 Signal transduction in macrophages by *Leishmania donovani* lipophosphoglycan**

### **2.1 Role of protein kinase C in LPG mediated signal transduction in murine macrophage**

- i) Signal transduction events mediated by the surface glycoconjugate of *Leishmania*, LPG, were studied in J774A.1 murine macrophages, using ODC as a marker of macrophage activation.
- ii) LPG was found to stimulate ODC activity in macrophages in a dose and time dependent manner, confirming the possible role of ODC as an early marker of macrophage activation. LPG stimulated ODC activity within 30 min of macrophage treatment, suggesting that interaction of LPG with its receptor stimulated a specific signal transduction pathway within the macrophages. This induction of ODC was a

transient event since the ODC levels reached basal levels at 3 h after exposure of LPG.

- iii) LPG stimulated ODC through protein kinase signal transduction as judged by the use of a broad-spectrum inhibitor of protein kinase, staurosporine.
- iv) ODC activity appeared to be coupled to the activation of protein kinase C in macrophages as activators of PKC caused a rapid increase in the ODC activity.
- v) The use of okadaic acid (OKA) along with staurosporine showed that a protein serine-threonine kinase might not be the relevant target for staurosporine within macrophages. However, it may also be possible that the treatment time with OKA may not have been sufficient to cause changes in the degree of protein phosphorylation. Specific inhibitors of individual protein kinases may help to sort out which of the many protein kinases present in the macrophage could be the relevant target for staurosporine.
- vi) Role of PKC in activation of ODC by LPG in macrophages was confirmed by using H7, an inhibitor of PKC.
- vii) LPG did not inhibit the induction of ODC activity by dibutyryl cyclic AMP ruling out the role of PKA in LPG stimulation of ODC activity.
- viii) These kinases can therefore be used as potential target not only for the development of novel strategies to combat intracellular pathogens but also for the therapeutic immunomodulation.

## **2.2 Role of Mitogen activated protein kinases in LPG mediated signal transduction in macrophages**

LPG stimulated all three classes of MAP kinases, the ERKs, JNKs and p38 with different kinetics in macrophages. However, the kinetics of induction of MAP kinases by LPS was different from that with LPG.

## **2.3 Molecular mechanism involved in the signal transduction by activation/down regulation of transcription factors by *Leishmania* LPG in murine macrophages: Activating Protein-1 (AP-1), NF- $\kappa$ B and Interferon regulatory factor (IRF)**

- i) *L. donovani* and LPG resulted in a dose and time dependent increase in DNA-binding activity of AP-1, NF- $\kappa$ B and IRF.
- ii) Unlabeled probe competition experiment were performed to detect the binding specificity of the complex formed. The corresponding unlabeled probe strongly competes for complex formation of AP-1, NF- $\kappa$ B and IRF respectively. A low level of predominant DNA oligomer-protein complex was present in untreated nuclei. Thus the proteins activated by LPG and detected by EMSA were highly specific for AP-1, NF- $\kappa$ B and IRF respectively.
- iii) Supershift assay were performed to characterize the protein composition of DNA oligomer-protein complex formed, using antibody against AP-1 (c-Jun and c-Fos), NF- $\kappa$ B (p65, p50 and c-rel) subunits and IRF-1. c-Fos but not c-Jun subunit was one of the components of *L. donovani* activated AP-1. In case of NF- $\kappa$ B, the results suggested that both p65 and c-rel subunits constitute the *L. donovani* activated NF- $\kappa$ B-DNA complex and not p50 subunit. Whereas, in case of IRF, the identity of the

- L. donovani*, LPG and LPS-induced IRF-like complexes is unclear since the antibody supershift studies showed that they do not contain IRF-1.
- iv) Low and high virulent strains of *L. donovani* caused an increase in DNA-binding activity of AP-1. The low virulent strain was passaged in the laboratory for several months and high-virulent strains was maintained in BALB/c mice. These results show that the ability of *L. donovani* to activate AP-1 was independent of its virulence.
  - v) The viability of the parasite on NF- $\kappa$ B activation was also determined. Live parasite resulted in induction of DNA-binding activity as early as 5 min whereas heat killed parasites resulted in delayed activation by 30 min. Moreover, cytoplasmic degradation of I $\kappa$ B- $\alpha$  was also noted at 15 min by live *L. donovani* infection and with heat-killed *L. donovani* was found at 60 min. These results confirm that the viability of the parasite to some extent contributes to the activation of NF- $\kappa$ B and I $\kappa$ B- $\alpha$  degradation.
  - vi) Cytochalasin B, an actin-depolymerizing drug which inhibits parasite invasion and phagocytosis did not inhibit NF- $\kappa$ B, AP-1 and IRF-DNA-binding activity in macrophage suggesting that the cellular uptake of *L. donovani* is not required for NF- $\kappa$ B, AP-1 and IRF activation.
  - vii) *L. donovani* induced NF- $\kappa$ B activation is mediated through reactive oxygen species was evaluated by using different antioxidants like BHA, BHT, and GSH. Pretreatment of J774A.1 cells with BHT, BHA and GSH followed by treatment with *L. donovani*. These antioxidants resulted in dose dependent inhibition of *L. donovani* stimulated NF- $\kappa$ B-DNA-binding activity. These oxidants can therefore be used as possible therapeutic modulators.

- viii) Role of MAP kinases in regulation of the transcription factors AP-1, NF- $\kappa$ B and IRF was evaluated by using specific inhibitors of ERK1/2 or p38 MAPK pathway. These results indicate that in J774A.1 cells, LPG involves both p38 and ERK1 and ERK2 pathway in AP-1 and NF- $\kappa$ B activation. Whereas, preincubation of cells with PD98059 and SB203580, followed by treatment with *L. donovani*, did not affect the binding activity of IRF suggesting that both p38 and ERK MAP kinase activation are not involved in the IRF activation. At these concentrations the inhibitors did not cause any cellular damage.
- ix) Polymyxin B (PB), binds to LPS and abrogates its effect. Use of PB showed that activation of transcription factors AP-1, NF- $\kappa$ B and IRFs was clearly due to *L. donovani* and LPG, and not due to LPS contamination.

#### **2.4 Regulation of nitric oxide (NO) production in murine macrophages**

- i) LPG was found to induce nitrite levels in J774A.1, macrophage. Both dose and time dependent increase in nitrite levels was observed with LPG.
- ii) LPG induced nitrite levels through extracellular signal regulated kinase (ERK1/2) and p38 mitogen activation protein kinase (p38 MAPK). This was assessed by the use of selective inhibitor of ERK1/2 kinase and p38 MAPK namely PD98059 and SB203580 respectively.
- iii) *Leishmania* lipophosphoglycan induced nitrite levels in macrophage through protein kinase signal transduction was judged by the use of a broad-spectrum inhibitor of protein kinase, staurosporine.
- iv) Role of protein kinase C (PKC) in LPG-induced nitrite levels in macrophage was confirmed by using H7 and PMA, inhibitors of PKC.
- v) Role of calmodulin kinase (CAM-kinase) on LPG-induced nitrite levels in

macrophage was confirmed by using W7, an inhibitor of CAM-kinase.

It has been reported earlier that ceramide induces the suppression of ERK activation, AP-1 and NF- $\kappa$ B activity and NO generation leading to intracellular survival of the parasite in BALB/c susceptible mice (Ghosh et al., 2002). However in C.D2 resistant mice, protection against *Leishmania* pathogenesis in *Leishmania*-resistant strain involved upregulation by ceramide of ERK, AP-1 and NF- $\kappa$ B activity and NO generation. The present study showing activation of protein kinases, ERK1/2, JNK and p38, NF- $\kappa$ B, AP-1 and IRF in J774A.1 cells is analogous to the macrophages from the resistant mouse model.

Thus, the present study shows the role of *Leishmania* lipophosphoglycan in the signal transduction events in macrophages. Selective impairment of signal transduction may therefore represent a new strategy for identifying targets for treating *Leishmania* infection.