CHAPTER 7

Conclusions and General Discussion
Leishmaniasis is a disease caused by the protozoan parasite *Leishmania*, and it affects as many as 12 million people worldwide, with 1.5-2 million new cases each year. Transmitted by the bite of a sandfly, the clinical spectrum of leishmaniasis ranges from a self-resolving cutaneous ulcer, to a mutilating mucocutaneous disease, to a fatal systemic illness. The global incidence of this infectious disease has been increasing during recent years because of increased international travel, human alteration of vector habitats, and concomitant factors that result in increased susceptibility such as HIV infection and malnutrition.

Control of leishmaniasis has been hampered by various factors like varied reservoirs of rodents, dogs or other animals, uncertainties regarding the biology of the parasite and its vector etc. Therapy has long been a challenge for the more severe forms of the disease and is made more difficult by the emergence of drug resistance to the commonly used pentavalent antimonials. So there is an urgent need to develop alternative chemotherapeutic approaches and also vaccine strategies to combat and prevent the disease. An anti-*Leishmania* vaccine is feasible since it was found that localized skin ulcers induced by *Leishmania* parasites tend to heal spontaneously, leaving the host immune to reinfection by the same parasite. Also, strong resistance against re-infection followed drug-induced recovery from VL. So there is an urgent need to identify new drug targets and vaccine candidates.

Among the promising vaccine candidates, are the genes, which are frequently amplified, in the natural isolates, since amplification is an index of importance of these genes for the parasite’s survival. The multigene LD1 locus located on chromosome 35 of *Leishmania* is amplified in ~15% of the *Leishmania* isolates examined. The gene ORFF invariably forms a part of the amplified region, suggesting its importance in the parasite. ORFF is present in the promastigotes and amastigote stages of the parasite life-cycle. However the level of expression is higher in the amastigotes versus promastigotes. In addition, sera from patients with VL specifically recognize the purified recombinant antigen. Our preliminary studies have showed that this gene could be used as a potential vaccine candidate. Previous studies in our lab have shown that rORFF antigen when used with Complete Freund’s adjuvant (CFA) or with alum provided 40-50% protection against *L. donovani* infection in mouse model system (Dole et al., 2000) (Sukumaran et al., unpublished data). Use of alum as an adjuvant along with rORFF antigen did not significantly increase the level of protection when compared to CFA. Though alum is less toxic in nature it is known to be a Th2
promoting adjuvant. In order to confer better protection against *L. donovani* infection, the cell-mediated response has to be improved since, protection is thought to be T cell mediated and antibodies have little effect in conferring protection against leishmaniasis. So a better adjuvant that can skew the response towards cell-mediated type has to be sought. Adjuvants like immunostimulatory DNAs or CpG motifs and cytokines like IL-12 have been used to modulate the immune response towards a Th1 type for many intracellular pathogens. Recent studies have found that CpG ODN, when used as a vaccine adjuvant with either a recombinant protein or heat killed leishmanial antigen, can induce long-term protection against an intracellular infection in a CD8-dependent manner. Similar studies have been performed using IL-12 as an immune adjuvant. Therefore it was of considerable interest to study the role of these two adjuvants in conferring protective immunity against visceral leishmaniasis. Apart from this, I have also studies the efficacy of a prime boost vaccination strategy using combination of ORFF DNA and rORFF protein against experimental visceral leishmaniasis. The work presented in Part A of the thesis is summarized below:

1) **To assess the protective efficacy of Immunostimulatory oligodeoxynucleotides or CpG motifs as immune adjuvants In BALB/c mice when immunized with recombinant ORFF antigen**

   a) The immunomodulatory properties of ODN were assessed *in vitro* by studying their effect on splenocyte proliferation and cytokine production. Splenocytes, incubated with CpG-ODN 1826 (6.0 μg/ml) resulted in 2.5 fold proliferation as compared to the untreated cells. Control ODN 1911 without CpG motif was significantly less active when given alone. Cytokine analysis of *in vitro* stimulated splenocytes showed that CpG-ODN 1826 induced 4.3 fold more IFN-gamma when compared with the non-treated splenocytes. No change in IL-4 production was observed with CpG-ODN. CpG-ODN 1826 resulted in a slight but significant increase in IL-12 production when compared with untreated splenocytes.

   b) *In vitro* studies were performed to analyze the effect of ODN and rORFF on nitrite production by activated macrophages. There was a dose dependent increase of NO production by cells that were incubated with the CpG-ODN 1826. When cells were incubated with a combination of rORFF (5.0 μg/ml) and varying concentrations of CpG-ODN 1826, a synergistic effect was observed. Interestingly cells that were treated with a combination of rORFF (5.0 μg/ml) and ODN 1911 showed an increase in the
NO production. The increase in NO levels in this case which was comparable to the NO levels produced when cells were incubated with the rORFF alone.

c) The splenocytes from BALB/c mice immunized with rORFF antigen with or without CpG ODN showed strong proliferation when incubated in vitro with rORFF antigen. Co-administration of CpG-ODN 1826 and rORFF augmented splenocyte proliferation in vitro by 1.6 fold when compared with group treated with rORFF alone. Co-administration of the control ODN 1911 resulted in 1.25 fold increase in splenocyte proliferation over the group treated with rORFF. This effect might be due to the non specific proliferation conferred by the phosphorothioate backbone.

d) In order to examine whether there was a preferential induction of Th1 response after immunization with rORFF and CpG ODN, RT-PCR was done using IFN-γ and IL-4 primers with spleen cells isolated from immunized mice. It was observed that both CpG-ODN 1826, and rORFF induced preferentially the IFN-γ mRNA when compared to alum immunized mice. Co-administration of CpG-ODN 1826 and rORFF resulted in enhanced expression of IFN-γ when compared to groups where injections were given individually. However, there was no significant difference in the expression of IL-4 mRNA in mice injected with either rORFF or CpG-ODN 1826. Thus it appears that this immunization schedule appears to preferentially induce IFN-γ dominant response.

e) Immunization with rORFF antigen or rORFF antigen plus CpG ODN 1826 showed high titers of anti-ORFF antibodies before and after challenge infection as estimated by ELISA. The Western blot data confirmed the ELISA results in demonstrating that co-administration of rORFF with CpG-ODN results in enhanced antibody titres against rORFF protein. IgG isotype were also compared in nonprotective vaccinated mice. Immunization with rORFF alone resulted in a Th response with more anti-rORFF antibodies of IgG1 than IgG2a isotype. Control ODN without CpG motifs did not affect the isotype profile of sera from mice injected with ODN-1911 plus rORFF. In contrast, mice injected with the rORFF plus CpG-ODN 1826, the majority of the isotypes were IgG2a.

f) Immunization of BALB/c mice with rORFF with or without CpG ODN provided considerable protection against challenge with L. donovani AG83 promastigotes. BALB/c mice vaccinated with rORFF, CpG-ODN 1826 alone or rORFF plus CpG-ODN 1826 resulted in 56%, 36%, 84% inhibition of parasite load in liver after 4 weeks respectively. CpG-ODN alone also gave partial protection at 4 weeks and 8 weeks compared with control-unvaccinated mice. A comparison of the protective efficacy of
rORFF vaccinated and rORFF plus CpG-ODN 1826 vaccinated mice shows a modifying role of the ODN on the immune response to rORFF antigen. Parasite load was also checked in the spleen of mice. The parasite load decreased by 54%, 32%, 84% in BALB/c mice vaccinated with either rORFF, CpG-ODN 1826 alone or rORFF plus CpG-ODN 1826 respectively.

2) **To assess the protective efficacy of Immunostimulatory oligodeoxynucleotides or CpG motifs as immune adjuvant in BALB/c mice when immunized with soluble antigen of *Leishmania donovani*.**

a) Relative antibody titers in groups immunized with soluble antigen were nearly 7,659 and CpG-ODN 1826 combined with soluble antigen had a relative titer of 7,968. Relative antibody titers in animals receiving ODN without CpG motifs (1911) alone or with alum were not significantly different from those in respective control groups. Soluble antigen specific antibody titer showed a significant increase 4 weeks after parasite challenge, with relative titer of 7953 in sera from mice immunized with SA and the titer of 8893 in sera from mice injected with a combination of SA plus CpG-ODN 1826. Thus, antibody titers were still maintained even after 8 weeks of infection. A comparison of the SA vaccinated and SA plus CpG-ODN 1826 vaccinated mice shows a modifying role of the ODN on the immune response to SA. IgG isotypes were compared in nonprotective vaccinated mice, after the second immunization. Immunization with SA antigen alone gave a mixed Th1/Th2 response with more anti-SA antibodies of IgG1 (Th2) than IgG2a (Th1) isotype. Control ODN without CpG motifs (1911) did not affect the isotype profile of sera from mice injected with ODN 1911 and SA antigen. In contrast, mice injected with SA antigen plus CpG-ODN 1826, the majority of antibodies were IgG2a.

b) **Cell Mediated response was analyzed by splenocyte proliferation and cytokine analysis.** The splenocytes from BALB/c mice immunized with soluble antigen with or without CpG ODN showed strong proliferation when incubated in vitro with soluble antigen. Co-administration of CpG-ODN 1826 and rORFF augmented splenocyte proliferation in vitro by 1.76 fold when compared with group treated with soluble antigen alone. Co-administration of the control ODN 1911 (which has composition matched to 1826 but lacks CpG dinucleotide motif) resulted in only 1.03 fold increase in splenocyte proliferation over the group treated with soluble antigen. The levels of IFN gamma, IL-12 and IL-4 were measured in splenocytes isolated from immunized mice. There was an enhanced production of IFN-γ (3.5 fold) and IL-12 (2.1 fold) in
mice injected with soluble antigen and CpG-ODN 1826 when compared to groups where injections were given individually. However there was no significant difference in the IL-4 levels in mice injected with either SA or CpG-ODN-1826. Non CpG-ODN did not result in enhanced production of IFN-γ.

c) Immunization of BALB/c mice with SA with or without CpG ODN provided considerable protection against challenge with *L. donovani* AG83 promastigotes. BALB/c mice vaccinated with soluble antigen, CpG-ODN 1826 alone or soluble antigen plus CpG-ODN 1826 resulted in 42%, 33%, 55% inhibition respectively of parasite load in liver after 4 weeks. Parasite load was also checked in the spleen. The parasite load decreased by 40%, 31%, 60% in BALB/c mice vaccinated with either soluble antigen, CpG-ODN 1826 alone or soluble antigen plus CpG-ODN 1826 respectively. The inhibition of parasite load after 8 weeks was 40%, 35% and 59% in liver and 46%, 34% and 61% in spleen in BALB/c mice vaccinated with either soluble antigen, CpG-ODN 1826 alone or soluble antigen plus CpG-ODN 1826 respectively.

3) To assess the efficacy of a prime boost vaccination strategy using ORFF DNA and rORFF protein against experimental visceral leishmaniasis.

a) Relative antibody titers in groups immunized with rORFF antigen alone were 27,300, 25800, 18,300 after 2, 4 and 6 weeks of immunization respectively. As expected titres against the ORFF DNA vaccine were relatively low. However the relative titres of the prime boost group were 23,280, 20,400 and 17,700 respectively. These were not different from the rORFF treated groups. However analysis of the IgG isotypes indicated that priming with F/pcDNA 3.1 induced a humoral response with Th1 characteristics. DNA vaccine group and the Prime boost group reflected high IgG2a/IgG1 ratio. In contrast to the prime boost group, mice immunized with rORFF alone reflected high levels of IgG1 and comparatively much lower levels of IgG2a.

b) In order to examine the cellular immune response elicited by the prime boost vaccination regimen, splenocyte proliferation and cytokine response was analyzed after immunization. Splenocytes from mice immunized with rORFF alone did not exhibit increased proliferation of cells when compared with the F/pcDNA vaccine group. However priming with a single dose of DNA vaccine augmented the proliferative response by 1.5-1.6 fold as compared to the protein vaccine alone at different intervals of vaccine studies. Concomitantly, cells from mice immunized with the prime boost regimen secreted much higher levels of IFN gamma and IL-12 levels as compared to the protein vaccine group. In contrast IL-4 levels were comparatively lower in the prime
boost when compared to the other groups, thereby indicating development of a Th1 response following this prime boost protocol.

c) As a further measure of the vaccine efficacy we evaluated the parasite burden in liver and spleen after 4 and 8 weeks of challenge with *L. donovani*. BALB/c mice vaccinated with rORFF alone resulted in 45 to 60% inhibition in parasite burden in spleen and liver after 4 and 8 weeks of challenge respectively. Reduction in parasite burden was around 75-80% in the DNA vaccine group. Similar reduction in LDU was observed in the prime boost vaccinated mice in both liver and spleen.

d) Antibody titres were also measured after 4 and 8 weeks of challenge. Analysis of the antibody titres after challenge indicated that high levels of anti-ORFF antibodies were still maintained even after 4 and 8 weeks of challenge in the rORFF and Prime boost immunized mice. The titres in DNA vaccine immunized group were comparatively much lower that the other groups.

4) To assess the protective efficacy of IL-12 as immune adjuvant in BALB/c mice when immunized with rORFF of *Leishmania donovani*.

a) The splenocytes from BALB/c mice immunized with rORFF antigen with or without IL-12 showed strong proliferation when incubated *in vitro* with rORFF antigen. Splenocytes from mice injected with IL-12 alone resulted in a significant 1.8 fold increase in proliferation when compared to alum immunized mice. However, splenocytes from mice injected with IL-12 alone did not exhibit increased proliferation of cells when compared with rORFF group. Interestingly, co-administration of IL-12 and rORFF augmented splenocyte proliferation by 3.1 fold when compared to proliferation by spleen cells from groups immunized with rORFF alone.

b) The levels of IFN gamma, IL-12 and IL-4 were measured in splenocytes isolated from immunized mice. Analysis of culture supernatants by ELISA revealed that splenocytes from mice immunized with IL-12 alone did not induce increased levels of IFN-gamma when compared to rORFF-immunized mice. However co-administration of IL-12 with rORFF augmented the IFN gamma levels by 3.7 folds as compared to groups immunized with rORFF alone. There was a 1.5 fold increase in IL-12 levels in culture supernatants of spleen cells from group immunized with IL-12 DNA and the recombinant ORFF protein. However there was no significant difference in the IL-4 levels in mice injected with either rORFF alone with or without IL-12.

c) The isotype profile was checked after immunization. Sera from mice immunized with rORFF alone resulted in a mixed response with higher titres of IgG1. Interestingly
in the group immunized with combination of IL-12 and rORFF the majority of the antibodies were of IgG2a isotype.

d) Parasite burden was calculated after 4 and 8 weeks of challenge in liver and spleen. BALB/c mice vaccinated with rORFF, IL-12 DNA alone or rORFF plus IL-12 resulted in 55%, 37%, 82% inhibition of parasite load in liver after 4 weeks respectively. Similar protection levels were observed after 8 weeks of challenge. IL-12 alone also gave partial protection at 4 weeks and 8 weeks compared with control-unvaccinated mice. A comparison of the protective efficacy of rORFF vaccinated and rORFF plus IL-12 vaccinated mice shows a reduction in parasite burden in an IL-12 dependent manner indicating the role of IL-12 in sustaining long-term immunity. Parasite load was also checked in the spleen of mice. The parasite load decreased by 48%, 40%, 83% in BALB/c mice vaccinated with either rORFF, IL-12 alone or rORFF plus IL-12 respectively.

e) We also characterized the changes in cytokine levels following challenge in animals previously immunized with rORFF, IL-12 alone and combination of IL-12 and rORFF. Consistent with the results obtained from pre-challenge samples, elevated levels of IFN-γ and IL-12 were observed in mice immunized with combination of rORFF and IL-12 as compared to IL-12 alone indicating significant correlation between cytokine levels and reduction in parasite burden.

Thus the present work explores the role of immunostimulatory oligodeoxynucleotides and IL-12 as vaccine adjuvants against experimental visceral leishmaniasis. An attempt has also been made to analyze the efficacy of a heterologous prime boost vaccination strategy using ORFF gene against L. donovani infection. Table.1 summarizes the protective efficacy conferred by the above mentioned vaccination strategies.

Our findings for the first time report that the immunostimulatory effect of CpG-ODN can be utilized to create a protective vaccine when given in combination with specific antigen from L. donovani in mice model system. We show that a CpG-ODN drives the production of higher levels of antigen-specific antibodies (predominantly Th1) when given along with Leishmania specific antigen (rORFF) or total soluble antigen (SA). There is strong synergistic response when ODN is used together with the vaccinating antigen.

We also demonstrate here that IL-12 plays a crucial role in resolution of visceral leishmaniasis in mice, initiating a polarized Th1 response required to mount a
protective host immune response. However the efficacy is further enhanced when combined with a strong antigen like ORFF. Co-administration of IL-12 with rORFF has an overall additive effect over the cellular immune responses generated by rORFF protein alone. Our current data suggest that both the adjuvant and the cellular source of the protein are likely to play a role in displaying antiparasitic responses and control of the disease.

Our results in murine *L. donovani* model, shows promising role of ODN and IL-12 as adjuvants in combination with *Leishmania* specific antigen. Additionally, both CpG-ODNs and IL-12 may be very useful as vaccine adjuvants for eliciting an enhanced cellular and humoral response and increasing the efficacy of individual antigens that induce partial but incomplete protection.

The vaccination strategy utilized also plays an important role in the development of a protective immune response against *Leishmania* infection. One promising approach towards development and establishment of strong cellular immunity is the heterologous prime-boost strategy, which includes sequential administration of vaccines that use different antigen-delivery systems. In the work presented here we have established that a regimen based on ORFF DNA priming followed by a booster with rORFF is effective in triggering protection against experimental visceral leishmaniasis in the murine model. The protective efficacy correlated with a skewing towards a Th1 type of immune response. Thus, this strategy, in combination with other Leishmanial antigens can be very useful for eliciting an enhanced cellular and humoral response and increasing the efficacy of individual antigens towards controlling active *Leishmania* infections.
### Table 1

<table>
<thead>
<tr>
<th>% Protection</th>
<th>Spleen</th>
<th>Liver</th>
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<tbody>
<tr>
<td>Group</td>
<td>4 Weeks</td>
<td>8 Weeks</td>
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<tr>
<td>(rORFF)</td>
<td>44</td>
<td>53</td>
</tr>
<tr>
<td>(F/pcDNA3.1)</td>
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<td>73</td>
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<tr>
<td>F/pcDNA3.1 + rORFF</td>
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<td>SA + CpG DNA 1826</td>
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<td>61</td>
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<tr>
<td>rORFF + CpG DNA 1826</td>
<td>84</td>
<td>86</td>
</tr>
<tr>
<td>rORFF + IL-12 DNA</td>
<td>82</td>
<td>83</td>
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</tbody>
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Concentration of recombinant ORFF (rORFF) adsorbed on alum per injection - 50 µg in 100 µl PBS

Concentration of ORFF DNA (F/pcDNA3.1) vaccine per injection - 100 µg in 100µl of PBS.

Concentration of Soluble antigen (SA) adsorbed on alum per injection - 50 µg in 100 µl PBS

Concentration of ODNs per injection - 10 µg in 100 µl PBS

Concentration of IL-12 DNA per injection - 100 µg in 100µl of PBS