Studies on Generation and Characterization of Live Attenuated *Leishmania* Parasites as Vaccine Candidates of Visceral Leishmaniasis

Synopsis

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Synopsis

Chapter 1: Introduction

Leishmaniasis constitutes a globally widespread group of neglected diseases caused by an obligatory, intracellular, protozoan parasite of genus *Leishmania* and is transmitted by the bite of female sand fly. It results in a variety of different clinical syndromes ranging from the self-healing cutaneous lesions to the more serious, potentially fatal visceralizing form, the metastasizing muco-cutaneous form and the post kala-azar dermal leishmaniasis (PKDL). Leishmaniasis is endemic in 98 countries with more than 1 billion people living in endemic areas are at risk. An estimated 0.2–0.4 million new cases of visceral leishmaniasis (VL) causing 20,000- 40,000 deaths and 0.7–1.3 million new cases of cutaneous leishmaniasis (CL) occurs each year worldwide. At present, there is no licensed vaccine available against any form of human leishmaniasis, although, development of an anti-*Leishmania* vaccine has been a goal for nearly a century. Attempts to develop a vaccine for humans using different strategies such as heat killed, subunit or DNA vaccines have not been successful. Studies have shown that the parasite persistence may be an important factor to develop long lasting protective immunity and it can be achieved by immunization with live attenuated parasites with known irreversible gene defect. In the digenetic life cycle of *Leishmania* parasite, several genes undergo the process of differential regulation to survive into two different host milieus. Amastigote specific/over-expressing genes are likely to be involved in the pathogenesis and intracellular survival of the *Leishmania* parasite. Further, deletion of such gene can also lead to the generation of amastigote stage attenuated *Leishmania* parasites that can be used as potential vaccine candidate.

In the present study, we have targeted an amastigote stage over-expressing LdA1 gene for generation of live attenuated parasite. Furthermore, LdA1 gene was functionally characterized to understand its role in *Leishmania* life cycle. Besides, we have also carried out studies using human blood samples to evaluate the vaccine potential of two live attenuated *Leishmania* parasites for human use: i) lacking centrin1gene (*Ld*cen1$^{-/-}$), an amastigote growth regulating gene and ii) lacking p27 gene (*Ld*p27$^{-/-}$), an essential component of cytochrome c oxidase complex, involved in oxidative phosphorylation. Both parasites specifically get attenuated at the mammalian infecting amastigote stage and are safe, immunogenic and protective in animal models.

Chapter 2: Review of Literature

This chapter present detailed information related to the topic of study under headings: - history of leishmaniasis, systemic position of *Leishmania*, morphology and life cycle of *Leishmania*, epidemiology, types of leishmaniasis, diagnosis, drug treatment, *Leishmania* macrophage interaction, immune responses in leishmaniasis, vaccines for leishmaniasis and gaps in research.

Chapter 3: Aims and Objectives

Aim of the proposed research

The aim of the present study is to generate live attenuated *Leishmania* parasites as vaccine candidates against leishmaniasis.

Objectives

1. **Characterization of *Leishmania* amastigote specific gene (LdA1) by gene deletion/episomal expression study.**

In order to investigate the potential applicability of LdA1 for generation of live attenuated vaccine candidate and its biological role in the life cycle of *Leishmania* parasites following studies will be carried out:

- Confirmation of upregulated expression of LdA1 in amastigote stage at protein level
- *In silico* analysis to predict structure and function of LdA1
- Immunofluorescence analysis to ascertain the subcellular localization
- Overexpression of LdA1 in *Leishmania* parasite with the help of expression plasmid and analysis of its effect on parasite growth/attenuation, phenotype and infectivity
- Deletion of LdA1 from *Leishmania* genome by homologous recombination method and its effect on parasite growth/attenuation, phenotype and macrophage infectivity

2. Evaluation of immune responses in human PBMCs elicited by live attenuated *Leishmania* parasites as vaccine candidates.

The present study will evaluate the vaccine potential of *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$ for humans by assessing the infectivity and correlates of protection, induced by them in human blood samples obtained from different clinical groups. Towards this goal, following studies will be carried out:

- Determination of infectivity of *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$in human PBMCs differentiated macrophage
- Evaluation of immune responses induced by *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$ in the human PBMCs obtained from healthy, healed VL and active cases of VL and PKDL subjects

- Analysis of phenotype of cytokine producing cells in response to *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$ exposure

Chapter 4: Characterization of *Leishmania* Amastigote Specific Gene (LdA1) by Gene Deletion/Episomal Expression Study for Vaccine Development

The morphological and biochemical alteration between the two life stages of *Leishmania* parasites are regulated by stage specific expression of several genes. Amastigote specific/over-expressing genes are believed to be responsible for the survival and replication of the parasite in the hostile environment of mammalian hosts and could be targeted to block new infection. Further, they can also serve as a target to generate live parasite vaccine candidates, specifically attenuated at the mammalian infecting amastigote stage. In the present study we describe characterization of a single copy, amastigote stage over-expressing LdA1 gene to evaluate its potential for the generation of live attenuated parasites and its role in *Leishmania* life cycle.

To gain some insight into the probable molecular function of LdA1 and its role in pathogenesis and survival of parasites, *in silico* analysis was performed. Sequence homology analysis revealed that LdA1 protein is highly conserved in the genus *Leishmania,* as no homologs are present in any other organism. Since LdA1 did not show homology with any other protein of the existing database, we used *ab intio* modelling and molecular dynamics simulations to propose the first 3D structure of the LdA1. The predicted 3D structure enhanced our understanding in predicting potential function of LdA1 and it can be considered as a critical step towards the characterization of the novel LdA1 gene. Both sequence and structure level function prediction suggested the essential role of LdA1 in *Leishmania* parasite life cycle.

Previous studies have shown up-regulated expression of LdA1 at RNA level in amastigote stage. In the present study, we investigated the expression profile of LdA1

between the two life stages of the parasites at protein level. We observed that expression of LdA1 in amastigote stage was also up regulated at protein level. Further, Immunofluorescence analysis revealed that LdA1 is primarily localized near kinetoplast of *Leishmania* parasite. To determine the effect of over-expression on phenotype changes associated with growth of the parasite, LdA1 was episomally expressed to generate stage independent LdA1 overexpressing *Leishmania* parasites. There was no significant difference on the growth and morphology of the LdA1 overexpressing parasites in comparison to the wild type, indicating an unaffected metabolic rate. Further, we examined the effect of LdA1 gene deletion on growth and attenuation of the parasites. To replace both the alleles of LdA1, two gene knockout constructs were prepared with two different antibiotic resistance markers. LdA1 single allele deleted parasites ($LdA1^{+/-}$) were generated using homologous recombination method, however, its double allele deleted mutants ($LdA1^{-/-}$) failed to survive. Inability to create null mutant of LdA1 was suggestive of gene essentiality. $LdA1^{+/-}$ parasites showed reduced size and sluggish movement compared with the wild type parasites. In addition to the altered morphology, they also exhibited slower growth kinetics compared to the wild type, indicating the effect of deletion on metabolic processes of the parasite. Since deletion of single allele of LdA1 affected both growth and phenotype of the parasite, next we examined its effect on the macrophage infectivity. We observed that infectivity of $LdA1^{+/-}$ mutants was not significantly reduced, as initially, percentage of infected macrophages was comparable to the wild type. However, the number of $LdA1^{+/-}$ amastigotes inside macrophages significantly decreased in comparison to the wild type, which indicated that their capacity to survive inside the macrophages is reduced and they were not able to multiply as efficiently as the wild type parasite.

Chapter 5: Evaluation of Immune Responses in Human PBMCs Elicited by Live Attenuated *Leishmania* Parasites as Vaccine Candidates

It has been reported that centrin1 (growth regulating gene) and p27 gene (component of cytochrome c oxidase complex) deleted live attenuated *Leishmania* parasites (*Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$), specifically get attenuated at the human infecting amastigote stage and have good potential as live attenuated vaccine candidates in animal models. However, as the animal models do not fully recapitulate the full spectrum of human-parasite interactions, translation of results obtained from studies in the animal models remains a major challenge. Therefore, it became important to assess the vaccine potential of *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$ for human use. This study was undertaken to evaluate the vaccine potential of *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$ live attenuated parasites using human blood samples obtained from different clinical groups including, active VL, healed VL (HVL), PKDL and healthy individuals. We found that macrophage infectivity of both *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$ was comparable to that of the wild type parasite, indicating that the attenuation did not limit their ability to infect human macrophages, Further, both *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$ strongly stimulated production of pro-inflammatory cytokines including, IL-12, IFN-γ, TNF-α, IL-2, IL-6 and IL-17 in the PBMCs culture supernatant obtained from individuals pre-exposed to *Leishmania* parasites (HVL and PKDL). There was no significant stimulation of anti-inflammatory cytokines (IL-4 and IL-10) between parasite exposed and control-uninfected cells in any study group.

In order to assess the intracellular cytokines producing cells after exposure to the parasites, production of IFN-γ, IL-17 and IL- 10 by $CD4^+$ and $CD8^+$ T cells was analyzed. We found that PBMCs infected with live attenuated and wild type parasite displayed higher frequency of $CD4^+$ T cells expressing IL-17 and both $CD4^+$ as well

as $CD8^+$ T cells expressing IFN-γ in HVL with no increase in IL-10 secreting T cells. The increase in percentage of pro-inflammatory cytokine producing $CD4^+$ and $CD8^+$ T cells upon stimulation with live attenuated parasites in HVL group corroborates with our cytokine profile data. An increased ratio of IFN-γ/IL-10 has been correlated with the parasite clearance in VL. Hence, we determined IFN-γ/IL-10 ratio for both $CD4^+$ and $CD8^+$ T-cells. Parasite exposed PBMCs of HVL group showed an elevated ratio of IFN-γ/IL-10 producing $CD4^+$ and $CD8^+$ T-cells, suggesting that exposure of PBMCs to *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$ live attenuated parasites induced a host- protective cell-mediated pro-inflammatory cytokines producing $CD4^+$ and $CD8^+$ T cells.

Chapter 6: Conclusions and Future Scope of Work

Conclusions

The study on LdA1 gene demonstrated that it is a amastigote stage over-expressing gene and unique to *Leishmania* genus as no homologs are present in any other organism. It might play some essential role in life cycle of *Leishmania* parasite, as was evident from the change in phenotype and growth kinetics of LdA1 single allele deleted mutant parasites at both the life stages and their inability to multiply efficiently as amastogotes inside the macrophages. Essentiality of LdA1 was further supported by *in silico* predictions as well as inability to delete both the alleles.

Evaluation of vaccine potential of *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ by assessing their ability to induce cellular immune responses in human PBMCs demonstrated that both parasites induce protective immune response comparable to the wild type parasite, indicating that both parasites have great potential as live attenuated vaccine against human VL.

Future Scope of Work

Demonstration of attenuation and difference in size and motility of LdA1$^{+/-}$ offers possibilities to explore the mechanism behind it. *In vivo* studies in rodent animal models are needed to evaluate safety, immunogenicity and protective efficacy conferred by LdA1$^{+/-}$ against virulent *Leishmania* parasites challenge to explore its potential as a vaccine candidate.

The other objective of the study evaluated vaccine potential of *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ in the blood PBMCs of active cases of VL and PKDL, HVL and naïve individuals. However, future studies are needed to evaluate the immune responses induced in the PBMCs of asymptomatic carriers. To establish *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ as a vaccine for humans, toxicological studies followed by clinical trials are needed.