CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Nematode parasitic diseases are time immemorial and are even attributed with immortality in Hades for their destroying work on sinning people with the adage in scriptures that states, “Their worm will not die, nor will their fire be quenched” (Avraham Gileadi 1994). Interestingly, human lymphatic filariasis is also caused by parasitic worms namely, Wuchereria bancrofti, Brugia malayi and Brugia timori that reach the definitive human host and reside in the lymphatic tissues causing dysregulation of lymph vessels and immune function resulting in the disfiguring appearance popularly known as “Elephantiasis”. These parasites are transmitted via infected mosquitoes, primarily by the Culex and Anopheles that act as intermediate host. Apart from the disfiguring and agonizing social stigma of the disease to the chronic patients, the economic impact due to loss of young labor force is enormous (Melrose 2002).

The infection cycle starts when infective-stage larvae (L3) of filarial worms are dropped on the skin of the humans by infected mosquitoes during their feed for blood meal. These L3 migrate to lymphatic tissues and take 3–5 months to mature and become adult worms that mate and release embryonic motile larvae known as microfilariae (mf). Later, the microfilariae (mf) of the parasitic nematodes are picked up by mosquitoes from an infected human during blood meal and they mature in the gut and flight muscles of the
mosquitoes to become infective larvae (L3) to complete the cycle. The approximate life-span of adult worms in human hosts ranges between 8–16 years and that of microfilaria between 8–10 months. This amazing longevity of parasitic nematodes in human definitive hosts together with the utilization of wide range of mosquito species as intermediate hosts ensures spread and sustenance of infection in endemic areas (Ravindran et al 2002).

The pathologic progression of lymphatic filariasis is intriguing and gruesome. Once inside the human hosts, these tissue dwelling filarial adult worms and mf cause lymphatic dysregulation and evoke parasite specific immune suppression for its survival in the otherwise hostile immune system. Patients who harbor fecund adult worms are asymptomatic except for the presence of mf or circulating antigens in the peripheral circulation detected in a nocturnal or diurnal fashion depending on the nematode species (Thomas and Rajan 2002). The overall immune status remains less characteristic other than the presence of parasite specific immune suppression, which can continue for several years. This initial trend of active immune evasion by adult worm nests wanes off gradually as the adult worms die triggering acute or chronic host-damaging inflammatory response resulting in the fibrosis of lymphatic vessels leading to chronic pathologic manifestations of the disease. The acute symptoms of the disease are characterized by fever, chills, headaches and skin lesions. These early-stage symptoms if untreated, can progress to elephantiasis marked by gross enlargement of the limbs and genitalia. In crux, LF is a “Disease of Disability” that causes a major health drain on the economies, well-being, and development of the 83 mostly poor endemic nations.

### 1.1.1 Global Elimination of Lymphatic Filariasis (GELF)

Fearfully, the global burden of lymphatic filariasis is 128 million people with infection, 43 million with active symptoms and 1.2 billion people
at the risk of acquiring infection (Hotez 2009). Alarmingly, India contributes about 74% of endemic population and 81% of the disease burden in the region (Agrawal and Sashindran 2006). As a response for curbing lymphatic filariasis, a unified global initiative was launched by WHO known as Global Elimination of Lymphatic Filariasis initiative in 1998 with a goal to eliminate LF as a public health problem. It tends to focus on two primary objectives namely, (1) Interrupting transmission of the parasites across hosts that cause LF, (2) Morbidity alleviation and prevention of further infection.

Primarily for interrupting transmission, the currently proposed strategies include (i) Annual, community-wide dual-drug distribution of Albendazole with either DEC or Ivermectin in endemic areas, also known as Mass Drug Administration (MDA) until the criteria to stop distribution are met (expected after 4–6 rounds of effective MDA coverage) or (ii) Specific use of table/cooking salt fortified with DEC for 1–2 years by populations living in endemic areas. Secondly for morbidity alleviation, patient awareness and community education are implemented. This tends to focus on the necessary benefits of intensive local hygienic practices, which makes LF patients to develop measures for prevention of debilitating and painful episodes of inflammation. However, these recommended interventions do not solve the core problem of complete parasite clearance neither restore pathologic manifestation. Hence, this dissertation looks into other possibilities of long-term solution to the eradication of LF by means of prophylactic support and along with the use of targeted drugs against adult worms.

1.1.2 Lacunae in the Implementation of GELF

Vector control strategies have been reported to pose more long-term environmental toxicity, financial burden, development of resistance and fails as a sole means for disease control (WHO 2006). Unfortunately,
chemotherapeutic choice in filariasis is limited to only two drugs (Diethylcarbamazine and Albendazole) that does not cure chronic conditions and is limited for reducing the motile embryo forms in circulation transiently (microfilariae burden) and also faces drug resistance due to its current increase in the use by WHO’s Mass Drug Administration programmes (Taylor et al 2010). LF parasitic worms have complex life stages and require multiple hosts to complete their life cycle. Strikingly, the parasite burden accumulated from continuous transmission determines the severity of disease. This is directly reflected in the chronic nature of LF, with typically low mortality but high morbidity.

However, since disease is caused by the accumulation of parasites, methods to develop immunity against parasite will have lasting effect for parasite clearance. This can be achieved through research in parasite vaccine efforts that will fill the void in GELF operations. These proposed vaccines need not possess sterilizing immunity as compared to viral or bacterial vaccines. In this context, an 'incomplete protection' vaccine may still significantly reduce both morbidity and transmission.

Further, the current choice of LF drugs used for MDA only temporarily clears microfilariae (mf) without killing all the adult worms (Gyapong et al 2005). This suggests that MDA must be administered for the reproductive life of the adult lymphatic filarial parasites which could be longer than 5 years. Additionally, there have been also reports of serious adverse events associated with MDA for lymphatic filariasis using current drugs in areas in Africa where LF coexists with Loa loa. These include progressive neurologic decline and encephalopathy within a few days of taking ivermectin (Kamgno et al 2007, Kamgno et al 2009), that has caused great concern. Hence, alternative strategies like development of new antifilarial drugs that cover all the stages of the parasite life and effective
MDA regimens will be needed if GPELF is to achieve the goals of global elimination of lymphatic filariasis by 2020.

1.1.3 Research Findings to Uphold GELF Cause

Implementation of GELF has initiated in parallel a flurry of research activities (Lazaro et al 2004). This includes efforts to develop more antifilarial drugs as limitations of existing drugs are revealed when their abilities are more closely evaluated during MDA programmes. This has also rekindled the old problem of lack of vaccines for parasitic disease in general (Hoerauf et al 2004). Apart from the known benefits of vaccine against parasitic nematodes that totally prevent infection, even a vaccine that could block patency (microfilaremia) would indeed be enormously useful in epidemiologic point of view to interrupt transmission in the eradication of LF (Bockarie et al 2010).

Moreover, the foresight of research in LF is even beyond the vision of GELF because the reduction of LF transmission will eventually lower parasite exposure for individuals living in endemic areas, which will reduce populations’ naturally acquired immunity to filariasis. This change could have unintended consequences for global elimination efforts, such as increased susceptibility to infection or to patency, increased disease burden in children, or increased morbidity in both filariasis and other concurrent infections (Hoerauf et al 2004). Hence, considering these grave lacunae in GELF, there is a dire need to develop new drugs and effective vaccines.

Although LF is one among the most immunologically complex infections of humans (Nutman and Kumaraswami 2001), yet extensive research in the immunology of filariasis has revealed the existence of protective immunity (Ravindran et al 2003) against these nematodes in humans that can be effectively tapped for vaccine development to augment
the efforts of GELF. Despite the limited availability of parasites and animal models, a number of strategies have emerged to identify targets for protective and transmission-blocking immune responses.

Among the strategies of identifying the right filarial vaccine candidates, differential antibody recognition of specific filarial proteins, particularly larval stage proteins, by putatively immune (Endemic Normals) sera versus infected patients’ sera has been a popular method (Freedman et al 1989). Candidate antigens were also screened from native purified parasite antigens using sera obtained from permissive filarial animal models that had been conferred with protective immunity by vaccination with radiation-attenuated filarial larvae (Wong et al 1969). This was due to the fact that radiation increased expression and exposure of potentially protective antigens from parasites in animal hosts. Subsequently, several antigens like Paramyosin (Li et al 1993), Tropomyosin (Taylor et al 1996) and Chitinase (Wang et al 1997) were identified using the above strategies and have varied responses but have not been very successful due to the limitations like, lack of availability of parasite material, reproducibility, undesired toxic effects, ambiguous immune responses, problems in purification methods. This suggests the need for characterizing more antigens by exploiting genomic information to develop more defined vaccines.

The advent of filarial genomic projects combined with recombinant DNA technology has opened a new avenue for developing novel therapeutic solutions in the control of LF. Accordingly, the most promising strategy for vaccine candidate identification is based on post-genomic data to select antigens that are up-regulated or unique to a particular parasite stage of interest (Ghedin et al 2007). Proteins expressed at specific larval stages might be critical to the establishment of infection and dissecting appropriate immune responses will enable the identification of potential candidates (Lizotte-
Based on genome sequence data, filarial gene products were targeted with critical roles in parasite development (e.g. L3 specific abundant proteins, antioxidant enzymes, key metabolites etc.). This led to the identification of some important vaccine candidates from the cDNA library of B. malayi such as Abundant Larval Transcript (ALT-2) (Ramachandran et al 2004), Thioredoxin Peroxidase (TPX) (Vanam et al 2009a), Venom Allergen Homologue (VAH) (Anand et al 2007, 2011), Thioredoxin (TRX-1) (Kunchithapautham et al. 2003, Madhumathi et al 2010a) and Transglutaminase (TGA) (Devarajan et al 2004, Vanam et al 2009b) and were further characterized by Prof.Kaliraj’s group. Their protective efficacy in animal models has been investigated. Some of these infective larval stage-specific candidates have shown promising results like ALT-2 and VAH (>60% reduction in worm burden). In order to pull the immune response to Th1 pathway and thereby restore the Th1/Th2 balance, our group also investigated DNA vaccine strategies (Vanam et al 2009b). However, this strategy for the same antigens has not been very effective like recombinant protein vaccines, which may be due to the profound extracellular nature of parasitic infections.

Recently, with the inclusion of multiple antigen vaccination strategy for recombinant protein vaccines, there has been tremendous improvement in the protective efficacy (Eg: ALT+VAH conferred 80% protection (Anand et al 2011)). Further to improve the protective efficacy, advanced strategies like epitope peptides derived from non-homologous regions of key metabolic enzymes of parasites like TRX, TPX and TGA have been reported by our group (Madhumathi et al 2010a). Additionally, strategies like lipid modification of vaccine antigens viz. ALT-2, were studied to analyze the adjuvant effect of lipid moiety attached to the protein. These experiences in parasite vaccine studies have prompted us to identify and characterize more infective-stage-specific targets and explore antigen
enhancement strategies like the use of eukaryotic expression systems in multivalent vaccination mode. In this regard, the current dissertation characterizes the vaccine potential of GP29 for the first time and also utilizes two approaches for vaccination – i) Multiple antigen mode using GP29 along with other L3 antigens, ALT-2/VAH and ii) utilization of eukaryotic expression system for vaccine antigens (Pichia pastoris).

Additionally, for LF the armamentarium choice of drugs is gravely narrow, there being few alternatives if resistance develops or drug availability becomes a problem (Ottesen et al 1994, Schwab et al 2007). Given the economics of drug development and the limited resources currently available for the identification and development of new parasitic drugs, it is important to clarify and prioritise the approaches to suit immediate needs in drug discovery process. However, the priority for LF is to find a macrofilaricide (Pink et al 2005). Many biochemical and molecular targets have been identified for LF (Gupta et al 2005), and the imperative has been to define their active sites for inhibitor design, which again has been elusive due to lack of experimentally solved 3-D structures. One irony of rational drug design approaches is that crystallization methods are still essentially empirical. Hence, the immediate choice would be to identify and structurally characterize more therapeutic targets that are present in all the stages of the parasite life cycle and vital for its survival to initiate rational drug development strategies.

In this regard, the current study has selected filarial glutathione S-transferase (Wb-GST) for X-ray crystallographic structural studies. Wb-GST has been reported earlier to be a potential vaccine candidate against LF and has demonstrated to nearly 61% protection against B.malayi challenge infection in a jird model (Veerapathran et al 2009). This is based on the fact that filarial worms utilize glutathione-S-transferase (GST) as part of the
antioxidant system that use glutathione (GSH) as a major substrate for quenching the free radicals. This enables them to stay for years in mammalian hosts and protects them against reactive oxygen species produced by normal metabolism and also by immune cells of the host (Gupta et al 2005). GSTs also perform functions ranging from catalysing the detoxification of electrophilic compounds to protecting against peroxidative damage (Armstrong 1991). Further, the protective role of GSTs as vaccine candidate has been reported in several helminth parasites including schistosomes, fasciola and also in the filarial parasite Seteria cervi (Grezel et al 1993, Morrison et al 1996). The mechanisms underlying the protection conferred following immunization with S. mansoni GST appears to be due to an inactivation of the GST enzymatic activity (Grzych et al 1993). Thus, GST appears to be a critical protein for the survival of the parasite in the host and their inhibition can deprive the parasite of its major defence against oxidative stress and impairs its ability to survive. Hence the knowledge of both the structure of the mammalian GST and the filarial GST, along with the comparative active site configuration will be useful in synthesising specific inhibitors by which biochemical pathways utilizing GST can be targeted for therapeutic intervention.

1.2 OVERVIEW OF THE THESIS

The wholesome solution to LF eradication based on available literature heavily rests on two vital approaches namely,

- **Preventive Vaccination Approach** – Prevention in the spread of filarial infection within and across endemic areas and between hosts through filarial-stage-specific/transmission blocking vaccines. The first part of the dissertation incorporates this approach under two objectives. This involved in the characterization of recombinant filarial infective-stage antigens
(VAH and GP29) expressed from eukaryotic (P.pastoris) systems as vaccine targets. This approach also investigated the use of multiple antigen combination in a comparative fashion for recombinant antigens expressed from both prokaryotic (E.coli) and eukaryotic systems (P.pastoris) for vaccine development.

➢ **Broad spectrum Drug Therapy Approach** – Arresting acquired filarial infection at any given stage of parasite development (mf to adult) in the human host through filarial adult – killing/sterilizing drugs. The second part of the dissertation incorporates this approach under two objectives. This involved utilisation X-ray crystallographic methods to determine the 3-D structure of filarial enzymes (GST and TRX). The structural details in atomic resolution of these therapeutic targets were explored for target validation towards therapeutic intervention.

Therefore the thesis objectives were conceived for active contribution to the growing knowledge of vaccine and drug targets in LF and primarily imbibe these two approaches.

1.2.1  **Preventive Vaccination Approach**

The vaccination strategy for LF will directly undermine any chances of escalating morbidity whether the human host is naïve in terms of parasite infection or is in a state of active/progressive/established infections. Where else conventional viral or bacterial vaccine attempts a converse approach of building terminal immunity right from the beginning as mortality drastically supersedes morbidity. Further, nematode vaccination when administered early before infection can create massive host resistance for
parasite propagation in endemic areas. Hence, the research objectives under purview were planned within this special requirement of filarial vaccinology and tend to include recombinant multiple antigen combinations of GP29 and VAH for vaccination to increase the spectrum of immune response. The study also includes the use of structured antigens by expressing the same in eukaryotic systems.

1.2.1.1 Parasite infective-stage-specific proteins as vaccine targets

Infective stage specific secretory and surface proteins of the parasite are attractive candidates for vaccine development (Maizels et al 1989a). Earlier studies have shown that recombinant L3 antigens have been found to induce protective immunity in rodent models of filarial disease, and have been proposed potential vaccine candidates (Ramachandran et al 2004, Anand et al 2007, Vanam et al 2009, Madhumathi et al 2010b).

Among such antigens, the current research study has focused on Bm-VAH and Bm-GP29 from B.malayi expressed in the infective larval stage (L3) (Rao et al 1999). Earlier studies show that Bm-GP29 is the most abundant glycoprotein (29 kDa) present on the surface of B.malayi parasites and is homologous with glutathione peroxidases (GSHPxs). It has been suggested to have a role in the local immune and inflammatory responses of the host towards the parasites. Similarly VAH belongs to the family of ancylostoma like proteins. Preliminary studies on VAH from B.malayi have clearly demonstrated their role as effective vaccine candidates (Murray et al 2001). Hence, the dissertation also attempts to show for the first time, the protective efficacy of these recombinant antigens expressed in Pichia pastoris in the permissive Mastomys model.
Accordingly in this study, recombinant GP29 was expressed and purified using E.coli expression system. The humoral and cell mediated immune responses were evaluated in mice (Anand et al 2011). Other L3-specific candidate antigens like ALT and VAH purified from E.coli system were immunized in multivalent mode along with GP29 in mice model. The best antigen combination for GP29 was selected based on immune profile in mice. The same antigens (GP29 and VAH) were also purified from Pichia expression system to ensure better folding of antigens. The humoral and cell mediated immune responses were studied in a comparative manner for Pichia and E.coli expressed antigens in clinical filarial samples (EN, MF, CP). The protective efficacy of Pichia and E.coli expressed GP29 and VAH either alone or in combination were studied in permissible Mastomys model to further ascertain the usefulness of multiple antigen strategy and the usefulness of higher expression systems enhance immunogenicity of filarial antigens.

1.2.2 Broad Spectrum Drug Therapy Approach

Structural characterization is a prerequisite for validation of therapeutic targets. Although, over one-third of all humans, mainly in the developing world, carry a nematode infection yet only a handful of experimentally solved structures are available for drug development efforts. For LF, the case is even worse because among ~ 7500 protein 3-D structures deposited in PDB (Worldwide Protein Data Bank) only six PDBs are available for Brugia malayi and none for Wucherria bancrofti till date. Hence, the current study was envisaged to structurally characterize crucial targets that will provide a foundation for rational drug design in lymphatic filariasis (Bockarie et al 2010).
1.2.2.1 Parasite anti-oxidant enzymes as therapeutic targets

Antioxidant enzymes of filarial nematodes provides protection from radical-mediated damage from both endogenous and exogenous sources and also serves as detoxifying detoxification enzymes as they lack oxygen dependent P-450 systems. Earlier studies have shown that parasite glutathione S-transferases, serving as detoxifying enzymes to be good targets for drug development in schistosomiasis and malaria (Gupta et al 2005). Hence, the current study has used X-ray crystallography methods to resolve the structure of W.bancrofti GST for gaining insights on the active sites for designing inhibitors.

Hence in this study, the recombinant Wb-GST was expressed and purified using E.coli expression system. The integrity of purified proteins was determined by DLS and CD spectra studies. The protein was crystallized and X-ray diffraction data were collected at atomic resolution from synchrotron sources. The phase was solved and the 3-D structure was determined. The structure was analyzed in comparison with human GST and Onchocerca GST to determine variations in active site. The mechanism of action for enzyme activation was studied from electron density map data. The crucial structural details that will be vital in developing inhibitors were elucidated.

Further, filarial parasite antioxidant enzymes are known to require the thioredoxin system as a source of reducing equivalents to keep vital enzymes active. It was earlier identified that B.malayi/W.bancrofti thioredoxin (TRX) is a new subclass of 16kDa thioredoxins that occur widely in nematodes (Kunchithapautham et al 2003). Hence their role in protecting filarial worms from radical-mediated damage prompts further study on thioredoxin as a vaccine candidate. In this study, recombinant Wb-TRX was cloned, expressed and purified without fusion tags. The integrity of purified
proteins was determined by DLS and CD spectra studies. Two protective peptides determined from certain host non-homologous regions of Wb-TRX, which were identified in silico and experimentally confirmed from earlier studies (Madhumathi et al 2010a) were used in this study to evaluate their role in enzyme inhibition. The current study used activity assays to determine the correlation of immune response and enzyme inhibition in mice models of these host non-homologous regions of Wb-TRX. The study also extended in putatively identifying the location of discontinuous epitopes in the host non-homologous regions of experimentally solved Wb-TRX structure as a rational basis for epitope-vaccine design.

1.3 OBJECTIVES

Considering these grave lacunae in the control and treatment measures for LF, the current research study was designed with the following multipronged objectives namely:

Part I Immunoprophylactic Research for LF Control

1. Determination of multiple antigen combination for Bm-GP29 with L3 stage specific antigens (Bm-VAH, Bm-ALT) in mice model towards vaccine study.

Humoral and cellular immune response of recombinant Bm-GP29 either alone or in combination with Bm-ALT and Bm-VAH were studied in mice model.

2. Evaluation of immune responses of E. coli and Pichia expressed Bm-GP29 and Bm-VAH in clinical samples and their immunoprophylactic efficacy in Mastomys model.

Clinical immune responses of Bm-GP29 and Bm-VAH expressed from E.coli and Pichia systems were evaluated. Their protective efficacy in Mastomys model was studied.
Part II  Drug Targeting Research for LF Control

1. Structural elucidation and characterization of Glutathione S-transferase (Wb-GST) by X-ray crystallography.

   The recombinant Wb-GST was purified and crystallized. The structure was solved by X-ray diffraction methods and the features of active site were analyzed against human host for developing parasite specific inhibitors.

2. Analysis of conformational epitopes from host non-homologous regions of filarial Wb-TRX through structure studies and activity assay.

   The inhibitory effects of sera against host-non-homologous regions of Wb-TRX were evaluated by insulin reduction assay. The conformational epitopes of Wb-TRX were also identified through 3-D structure analysis.