CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Helminths are multicellular pathogens which infect vast numbers of human and animal hosts, causing widespread chronic disease and morbidity. Parasitic worm (helminth) infections are the most common infections in poor people living in the developing world known as the neglected tropical diseases (NTDs). In both Asia and sub-Saharan Africa, lymphatic filariasis and ocular trachoma are high-prevalence NTDs. Lymphatic filariasis causes millions of cases of lymphedema and hydrocele. The three parasites, *Wuchereria bancrofti, Brugia malayi* and *Brugia timori*, all of which produce disease in the lymphatic vessels, are the most widespread and abundant of all the human filarial worms. As global elimination of human lymphatic filariasis is based on Mass Drug Administration of DEC and Albendazole, the control of this disease relies heavily on availability of the drugs. The parasites are transmitted via the bite of infected mosquitoes, primarily by the night-biting *Culex* and *Anopheles* mosquitoes. The filarial parasites infect about 128 million people (Hotez 2009), in 83 countries (WHO 2006) of the world causing lymphatic filariasis and over 40 million of them are seriously incapacitated and disfigured by the disease. Currently there may be up to 31 million microfilaraemics, 23 million cases of symptomatic filariasis, and about 473 million individuals potentially at risk of infection in India (Agrawal and Sashindran 2006). It is the second leading cause of permanent and long-term disability in the world. Ninety percent of these infections are caused by
Wuchereria bancrofti, and most of the remainder by Brugia malayi. The World Health Organization (WHO) has named filariasis as one of only six "potentially eradicable" infectious diseases and has embarked upon a 20-year campaign to eradicate the disease. Almost 25 million men suffer from genital disease (most commonly hydrocoele); an estimated 15 million people — the majority of them women — have lymphoedema or elephantiasis of the leg. Approximately 20% of the world's population lives in areas at risk of infection with lymphatic filarial parasites which is endemic in tropical regions of Asia, Africa, Central and South America. WHO initiated the ‘Global Program to Eliminate Lymphatic Filariasis’ (GPELF) by the year 2020 and it has been successfully implemented in China, Malaysia, Korea and in certain islands of the Pacific (Ottesen 2000; Burkot et al 2002; Molyneux and Zagaria 2002). 

Human infections are initiated by the bite of infected mosquitoes releasing infective larvae which home into the lymphatics and develop over a period of approximately 80–100 days into adult male and female worms measuring a few centimeters in length. These worms mate in the lymphatics and the females release several million embryos (microfilariae, Mf), which appear in blood circulation. The extraordinary life-span of adult filarial nematodes within the mammalian host (estimated to be 8-16 years), combined with an estimated microfilarial life-span of up to 300 days, ensure the spread of disease. Filarial worms have evolved immune evasion strategies causing widespread chronic disease and morbidity in human host. Existence of protective immunity in human filariasis has been a subject of intense debate (Ravindran et al 2003). The majority of individuals appear to remain clinically asymptomatic for years with only a minority progressing to the acute and chronic stages of infection. In an endemic area a spectrum of responses to disease is seen.
One of the most pressing issues in human parasitic diseases is the need to develop new drugs and effective vaccines. Traditional vaccines have involved the use of killed microorganisms, live attenuated cultures or antigenic extracts. In spite of extensive research, there have been very few newly developed vaccines for humans for the past 20 years. Vaccine studies have not advanced appreciably for lymphatic filariasis which is considered to be among the most immunologically complex infections of humans (Nutman and Kumaraswami 2001), despite the possible existence of protective immunity in humans (Ravindran et al 2003). Several proteins have been identified and characterized from the parasites and their potential use as vaccines have been tested. Parasite anti-oxidant enzymes offer an advance over existing vaccine candidates as they target adult worms also (LoVerde 1998; LoVerde et al 2004). Several of these antioxidant enzymes are known to require the thioredoxin system as a source of reducing equivalents to remain active (Kunchithapautham et al 2003). *B. malayi* Thioredoxin (BmTRX-1), a small ubiquitous 12kDa multifunctional protein with antioxidant properties, distributed in prokaryotes and eukaryotes and Thioredoxin Peroxidase (BmTPX-1), a major detoxification enzyme for the removal of oxygen free radical produced by the parasite were reported to possess protective properties. Another important vaccine target among the crucial parasitic proteins is the enzyme Transglutaminase (TGA) that catalyze the formation of polyamine linkages between or within the proteins of the exoskeleton during parasite development.

Some of the important vaccine candidates such as Abundant Larval Transcript (ALT-2), Thioredoxin Peroxidase (TPX), Venom Allergen Homologue (VAH), Thioredoxin (TRX-1) (Kunchithapautham et al. 2003) and Transglutaminase (TGA) (Devarajan et al 2004), have been isolated from the cDNA library of *B. malayi* and characterized in our laboratory by
Dr. Kaliraj’s group (Gnanasekhar et. al 2004). All these recombinant proteins have been thoroughly investigated in animal models.

ALT-2 is a L3 stage specific, abundantly expressed filarial antigen that is well established to be a potential vaccine by many groups. Immunization with recombinant ALT-1 conferred 76% protection (Gregory et al. 2000). In our previous studies, the protective efficacy of ALT proteins has been thoroughly tested as DNA vaccine, protein vaccine, Phage displayed antigen and bimodal vaccine strategy. The protein vaccine conferred ~64-75% protection consistently in mice as well as in the permissive animal model, gerbils (Ramachandran et al. 2004). The ALT-2 DNA vaccine construct showed poor response with 30% reduction in worm burden in mice (Ramachandran et al. 2004) and 57% in jirds (Thirugnanam et al. 2007). The bimodal vaccination using the prime boost strategy where DNA prime and protein boost is administered was less effective with 64% protection compared to protein alone that showed 75% (Thirugnanam et al. 2007). Bm TGA and Bm TPX conferred 30% and 43% protection respectively in jirds (Vanam et al. 2009a) whereas VAH showed ~60% protection (unpublished observation). Also, multiple antigen strategy using a cocktail of proteins showed better results than single antigen in some combinations. The combination of ALT-2 and TPX conferred ~78-80% protection in animal models (Anand et al. 2008). Similarly, the protective efficacy of BmTGA was enhanced significantly (74%) by immunizing the jirds in multiple antigen vaccination mode along with BmTPX.

However, when combined with ALT-2, TGA showed 47% protection which was much less than ALT-2 as single antigen (69-75%) (Vanam et al. 2009a). The DNA vaccine strategy has not proved to be successful so far in filariasis which could be due to the induction of Th1 biased response that does not clear the parasite efficiently (Thirugnanam
et al 2007). Prime boost strategy also has not enhanced the efficacy appreciably compared to protein immunization.

Although multiple antigen strategy shows promising results, the response appears to be unique with each antigen combination and thus is unpredictable. Some combinations works better with a synergistic effect whereas some antigenic combinations seem to have an opposite or inhibitory response. Multiple antigen combinations can be used to target all the stages of the parasite if the cocktail antigens do not show inhibitory effect. Thus, a large number of combinations need to be screened experimentally, in order to find the right combination, as it varies from antigen to antigen and theoretical prediction does not work. This is also one of the reasons for the failure of crude undefined mixture of antigens that are often toxic or allergic. One explanation for this could be the presence of immunosuppressive and toxic domains in some antigens that reduce the immunogenic effect induced by another antigen. Thus, although ALT-2 is immunogenic individually its combination with TGA reduces its prophylactic potential (Vanam et al 2009). However this is not the case with the combination of TGA and TPX or ALT-2 and TGA (Anand et al 2008; Vanam et al 2009).

The use of whole recombinant antigens however has several other limitations. Apart from the toxic or suppressive domains carried by few antigens, some proteins such as the metabolically important enzymes also share a considerable portion of homology with human proteins and thus the usage of these proteins as vaccines may lead to auto immunity or immuno-tolerance. In some of the key antigens like ALT-2 it is observed that a region of the protein is highly variable among the species. Some antigens show allelic variants even among the same species making it even more complex. Also, due to the vast number of MHC alleles among the human population the same antigen that is effective in one population may not be effective in
another population and may even differ from person to person. Thus, use of a single antigen would rarely benefit any vaccine regimen for filariasis. Targeting multiple stages of the parasite, using the antigenic portions that do not share homology with host proteins and that are conserved among the species, which do not show polymorphic variations among geographic regions and which are recognized in a wide population would be the ideal strategy for an effective vaccine development. Other than the immunological limitations the recombinant proteins also pose several problems in manufacturing like the difficulty in expression of soluble proteins, purification in non denaturing conditions, cold storage and transport, expensive production system and less stability.

With advance and increased understanding of immunology, it is now clear that the immune response is induced by only a few antigenic determinants in the protein and not the whole protein. Hence, the epitope fragments which are non identical to human proteins could be exploited as vaccines. Recent developments in our understanding of the pathways of immunity required to produce protective response against parasitic diseases, allow immunological principles to be applied into the design of new and better vaccines. With advances in parasite genomics, immunoinformatics, new vaccine strategies and delivery systems, there are now excellent opportunities for new antihelminth vaccines (Maizels 1999). Synthetic peptide vaccines and peptide constructs have been widely studied and been reported for various viral, bacterial and parasitic diseases (Steward et al 1985 ;Ben-yedidia and Arnon 1997). Peptide-based vaccines have advanced from pre-clinical to human clinical studies over the last decade. A combination of peptides is used to overcome the problem of MHC restriction of T cell response in humans which is HLA specific (Ben-yedidia and Arnon 1997). The only antimalarial vaccine that has been subjected to large scale clinical trials is spf66, a synthetic peptide based vaccine (Patarroyo et al 1992).
The use of synthetic peptides for immunization is a very attractive strategy for antigen delivery, since they are relatively easy to obtain in large quantities, with high purity and offers practical advantages such as relative ease of construction and production, chemical stability, and a lack of infectious or oncogenic potential (Disis et al 1998). To circumvent the problem of autoimmunity due to homology of key metabolic enzymes like TRX, TPX and TGA with host proteins, peptide-based vaccines using the non homologous antigenic sites have been used by many researchers (Carvalho-Queiroz et al 2004). This study attempts to address the possibilities of applying epitope based peptide vaccine strategy for human lymphatic filariasis to develop effective vaccines using modern immunological techniques.

1.2 OVERVIEW OF THE THESIS

Various observations indicate the existence of protective immunity in patients. Epidemiological studies show the existence of putative immune individuals in areas endemic for filariasis (Day et al 1991; Steel et al 1996). This has been attributed to resistance of infection to the incoming L3 larvae (WHO 1992). Despite extensive research, potential vaccine for filarial parasite has been a long struggle (Nutman and Kumaraswamy 2001). DNA vaccine and prime boost strategies has not been effective for parasitic diseases (Vanam et al 2009b; Thirugnanam et al 2007). Though recombinant protein vaccines have shown some promise, the problems like homology with host, suppressive or toxic domains, allelic variation, MHC restriction in humans and difficulty in production limits their use. Also, multiple combinations are unique for each combination and may even be harmful in some cases. By careful selection and use of host non-homologous, immunological peptide regions of the antigens much of these limitations can be overcome. Peptide vaccine offers advantages like long lasting and specific immunity, ease of
immune manipulation and manufacturing over the other vaccine strategies (Demotz et al 2001). Epitope-based vaccines have been extensively shown to be promising in various diseases, capable of inducing protective immunity (Srinivasan et al 2004; Ben-yedidia and Arnon 1997). The present study attempts to develop effective filarial vaccines using modern multi-epitope peptide strategies by chemical peptide synthesis or recombinant epitope constructs.

Mice are semi permissive models for filariasis whereas jirds or *Meriones unguiculatus* and *Mastomys coucha* are the well established permissive hosts for *Brugia malayi* (Lok and Abraham 1992). Hence the evaluation of the prophylactic efficacy of the vaccine constructs was performed in *Mastomys/Brugia malayi* model (Sanger et al 1981). Since, large number of animals is needed for thorough investigation of immune responses, mice models are the most convenient due to the availability and also since they are immunologically well characterized. Kidd et al (2007) have identified that more *Mastomys* transcripts can be identified on mouse gene chips than on rat gene chips and that *Mastomys* has a closer phylogenetic affinity to the mouse than the rat. Hence, the initial immunological studies were carried out in BALB/c mice.

In this study, the regions that are non-homologous to human proteins were screened from the metabolic enzymes *B. malayi* TRX, TGA and TPX by multiple sequence alignment and BLAST analysis. The putative B and T cell epitopes present in these host non-homologues regions were predicted by immuno-informatic analysis using various epitope prediction tools. Five such peptides were identified and named as TRX$_{P1}$, TRP$_{P2}$, TGA$_{P1}$, TGA$_{P2}$, and TPX$_{P1}$, the peptides were synthesized by solid phase chemical synthesis and encapsulated in polymeric microspheres.
The presence of B epitopes was analyzed by the humoral responses in mice models and human samples. The antibody titer and isotype antibodies induced by the peptides in BALB/c mice, reactivity with antisera raised against whole antigens, reactivity with clinical human sera and competitive inhibition assays were carried out for experimental validation of B cell responses. This further proves the affinity of the peptides for respective antibodies and their immunogenicity. The presence of T cell epitopes was studied by *in vitro* stimulation of PBMC’s from human samples in endemic region and splenocyte proliferation assay of immunized mice.

Single epitope peptides are not known to be protective and may be less immunogenic. However, epitope based vaccines are more effective if multiple epitopes covering a region of immunological hot spots are simultaneously delivered as single chimeric construct for induction of a broad spectrum of immune responses (Srinivasan et al 2004). Hence, synthetic multi-epitope peptides were made by chemically synthesizing two peptides encompassing immunodominant B or T epitope, as a single poly peptide with two glycine linkers in between them. The efficacy of the synthetic multi-epitope conjugates as vaccines was evaluated in the permissive animal model for filariasis, *Mastomys coucha*.

Since ALT-2 is a crucial filarial antigen which is known to play a major role in establishment of infection, the entire B and T epitopes of BmALT-2 were mapped by systematic epitope mapping assays in BALB/c mice models and human samples to identify the potential domains contributing to its protective efficacy or immunomodulatory function. The epitopic domains carrying a cluster of overlapping dominant B and T epitopes were identified ALT-2. A recombinant gene containing the sequences coding for the putative epitopic domains of BmALT-2 was constructed by genetic
engineering and the immunoprophylactic potential of the recombinant protein named as ALT epitope proteins (AEP), was studied in Mastomys model.

1.3 OBJECTIVES

1. Identification of epitope peptides in the host non-homologous regions of filarial enzymes such as B. malayi Thioredoxin Peroxidase (TRX), Transglutaminase (TGA) and Thioredoxin Peroxidase (TPX).

The epitopes in the nematode specific region were predicted by immunoinformatic analysis. The predicted peptides were chemically synthesized and validated by experimental analysis in BALB/c mice for identification of B and T epitopes.

2. Synthesis of potential epitopes as chimeric peptide conjugates and their evaluation as multi-epitope vaccines.

The epitopic peptides of TRX and TGA were conjugated in different combinations by peptide synthesis and the immunoprophylactic efficacy was evaluated in Mastomys models.

3. Epitope mapping of Wb-ALT-2 to identify B and T cell epitopes.

The B and T epitopes of ALT-2 were predicted by in silico analysis and synthesized. The peptides were validated by in vitro experiments in mice models and the regions carrying dominant B or T epitopes were identified.

4. Development and evaluation of recombinant ALT multi epitope protein construct as vaccine.
A recombinant gene construct carrying epitopic regions of ALT-2 was developed by recombinant methods and its immunoprophylactic potential was evaluated in *Mastomys* models.