CHAPTER 4

RESULTS AND DISCUSSION

4.1 BACKGROUND

Lymphatic filariasis is one of the neglected parasitic diseases targeted for elimination by WHO. Human lymphatic filariasis caused by *Wuchereria bancrofti* and *Brugia malayi*, is a leading cause of permanent and long-term disability worldwide that imposes a severe physical and socioeconomic burden on 1.3 billion persons at-risk in 83 endemic countries (Chu et al 2010). These long-living helminth parasites with the highest average lifespan in the body of their host (Hoerauf et al 2005) exploit sophisticated strategies to evade host immunity and establish long-term chronic infections in the context of an antiparasite immunity. The clinical presentations in infected individuals display a spectrum of disease. Complex host parasite interaction and associated immune response is thought to be responsible for such a wide clinical spectrum (Oettesen 1984).

The causative agent *Wuchereria bancrofti* and *Brugia malayi* are closely related and show significant antigenic overlap. Sequence similarity in genes like *BmALT-1* and *BmALT-2* showed 79% amino acid identity (Gregory et al 1997), tetraspanin showed 99% identity with *W.bancrofti* (Gnanasekar et al 2008). This high sequence identity is not surprising as genes cloned from *B.malayi* and *W.bancrofti* is shown to be highly conserved between these two species (Gnanasekar et al 2002, Rathaur et al 2003). Thus,
antibodies generated against *W. bancrofti* in infected or immune individuals show significant cross reactivity with *B. malayi* antigens. Chemotherapeutic agents like diethylcarbamazine (DEC), ivermectin and albendazole are used to treat lymphatic filariasis. These drugs are microfilaricidal, thereby interrupting the transmission cycle (Liu et al 1992), yet they are not macrofilaricidal (Weil et al 1988) leaving adult worms alive. They have the inherent disadvantage of requiring repeated and prolonged treatment for years leading to potential drug resistance (Schwab et al 2006, Schwab et al 2007). Chemotherapeutic agents can cause hypersensitivity reaction due to dead or dying adult worms, which could worsen the lymphatic pathology. Therefore, alternative strategies like vaccination could be beneficial along with chemotherapy and vector control in eradicating filariasis from endemic areas (Grieve et al 1995).

Endemic normal individuals, are constantly exposed to the infection without showing any symptoms of parasitemia (Helmy et al 2000), and are the most interesting group to study since they may carry circulating protective antibodies. If there are protective antibodies, it is important to identify unique antigens that induce their production. Strategies to identify candidate vaccine antigens against brugian or bancroftian filariasis have relied largely on screening of expression libraries with immune sera (Freedman et al 1989), differential screening of abundantly expressed mRNAs (Werner et al 1989, Gregory et al 2000) or by the EST sequencing approach (Blaxter et al 1996). Using these approaches several potential vaccine candidates have been identified and reported to have varying degrees of protection in animal models.

The presence of multiple stages in the life cycle of the parasite, lack of suitable animal model, paucity of parasite material and the complex immune mechanisms have severely hampered the development of efficient
vaccines for Filariasis. The non-availability of susceptible host animal models for \textit{W.bancrofti} is also responsible for delay in the immunological investigations. The only animals that can be infected with \textit{W.bancrofti} are the leaf monkeys \textit{Presbytis cristata}, (Palmieri et al 1982) \textit{P.melalophos} (Sucharit et al 1982) and Taiwan monkeys \textit{Macaca cyclopis} (Cross et al 1979). These animals are expensive and difficult to maintain in captivity. Semi permissive host such as Balb/c mice have been proposed as suitable models to asses immunity against developing larvae of \textit{B.malayi}. Alternatively maintainance of \textit{B.malayi} small animals like rodents has been succefful. The susceptibility of infection has been reported in different laboratory rodent hosts ranging from fully susceptible gerbils or desert rats (\textit{Meriones unguiculatus}-Mangolian Jirds and \textit{Mastomys coucha}) to semi-permissible mice. However, among the currently available animal models, gerbil or jird is a permissive host for \textit{B.malayi} and is a well established animal model for \textit{B.malayi} infection. Since \textit{B.malayi} genes like ALT, paramomysin, Tetraspanin have shown high sequence homology with \textit{W.bancrofti}, the \textit{Brugia malayi} in Mangolian Jirds (Sanger et al 1981, Lok & Abraham 1992) has been used, in the present study, for evaluation of immunoprophylactic responses against the parasite.

Experimental studies suggest that immunity is most likely to be operative in the early infection, and that the L3 and L4 larval stages are the targets of immune attack. When infective L3 stage of filarial parasite first encounter their definitive host they exhibit a specific pattern of gene expression. Potential vaccine candidates are likely to be among these genes. Abundant Larval Transcript (ALT) proteins are abundantly expressed in the infective L3 stage and neutralization of ALT function may be sufficient to protect the host from infection (Gomez-Escobar et al 2005). ALT-2 has been shown to be highly protective in experimental filariasis (Gregory et al 2000, Ramachandran et al 2004) and has immunomodulatory role (Hoerauf et al
ALT group of proteins share significant sequence homology with *Dirofilaria immitis* 20/22kDa developmentally regulated excretory/secretory protein, which is also a vaccine candidate. In this study Wb20/22 variant of ALT, which belongs to the chromadorea ALT family of protein, was cloned from Wb L3 cDNA library and immunological characterization and parasite challenge study was performed in Jirds.

Studies in our laboratory by Dr. Kaliraj’s group for the past 20 years have achieved identification and characterization of key target antigens like ALT-2, TRX, TPX, TGA and VAH (Gnanasekar et al 2004, Vanam et al 2009, Anugraha et al 2013, Prince et al 2013). Recombinant subunit vaccines have been promising and showed high protective responses. ALT-2 showed the highest protection of 69-75% in experimental models (Sharmila et al 2011). Multiple antigen combination of ALT-2 with TPX conferred 78% protection in mice (Anand et al 2008) and combination of TPX with TGA conferred 74% in Jirds (Vanam et al 2009).

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). Epitope-based vaccines containing well-characterized immunogenic regions have been extensively shown to be promising in various diseases capable of inducing protective immunity (Srinivasan et al 2004). Peptide vaccines first of its kind in filariasis, was studied in ALT-2, analyzing the epitope regions using bioinformatics tool like epipred and DNAstar. The regions 55–68 and 73–91 of ALT-2 induced very high levels of IL-10 secretion and hence could be involved in immunomodulation in the host (Madhumathi et al 2010c). Sequence similarity between *W. bancrofti* and *B. malayi* ALT was 98% suggesting they will show immunological cross-protection between these two species. The peptide regions of *Wb*ALT-2
showed similar pattern of reactivity, regions spanning (21-44) and (44-60) encompass the variable acidic domain which showed high reactivity and a protective isotype IgG1 and IgG2 in the host, that could be exploited in vaccine design (Madhumathi et al 2010a).

Unlike traditional vaccines, peptide vaccines are synthetic and do not carry the risk of reversion, or incomplete inactivation and in principle epitopes could be selected to avoid components that give rise to unwanted side effects. T cells recognize antigen or peptide by different MHC molecules. Endogenous antigens (e.g. Viral proteins) are processed by MHC class I pathway eliciting cytotoxic T cell responses (Zinkernagel & Doherty 1974). On the other hand exogenous antigens (e.g. Bacterial proteins) are processed by MHC class II pathway that predominantly elicits humoral response resulting in antibody formation or cell mediated response characterized by the activation of macrophages, expansion of the antigen reactive T cell pool and the production of cytokines. Though recombinant protein ALT offers significant protection in parasite challenge study, the immunodominant epitopes of ALT identified could be exploited as an alternative strategy by constructing the T and B epitopes as a MAP.

Various adjuvants have been used in order to enhance the immune response against specific antigens, when Ramon (1926) first demonstrated that it was possible to enhance artificially the diphteric and tetanic antitoxin level by the addition of substances such as agar, metallic salts, lecithin or saponin. A handful of adjuvants are currently licensed for human vaccination, and a number of new adjuvants have reached advanced development stages. These adjuvants have the potential to address previous development barriers by enhancing otherwise poorly immunogenic antigens or biasing the immune response toward the type of response needed to protect against specific pathogens. Aluminum salts, also known as alum, are the primary adjuvants
used in vaccines worldwide; however, alum is not optimally effective for vaccines against diseases where cell mediated immunity or mucosal immunity is likely required for protection (Schijns & Lavelle 2011).

Recombinant proteins or synthetic peptides are safer than crude inactivated micro-organism, but less immunogenic. Adjuvants are therefore required to assist new vaccines to induce potent and persistent immune responses, with the additional benefits that less antigen and fewer injections are needed (Vogel & Powell 1995). Hence, we made an attempt to increase the efficacy of recombinant *Bm*ALT antigen with an array of adjuvants including Alum, MPLA, MDP, Inulin, Chitosan, and QS21.

With the growing need of a prophylaxis and enhancement of the existing putative vaccine targets for protective response against filarial parasites we designed the study with the following objectives:

1. Immunological characterization of ALT homologue Wb20/22 and their evaluation as a putative vaccine candidate. The high similarity between *Bm*ALT and Wb20/22 probed us to characterize and analyze the immunoprophylactic efficacy in Jirds.

2. Construction of synthetic ALT MAP and encapsulation in microsphere. The immunodominant regions of *Bm*ALT were chemically synthesized, a step forward in the development of peptide vaccine for lymphatic filariasis.

3. Enhancing the efficacy of *Bm*ALT using different adjuvants. An array of adjuvants was formulated with *Bm*ALT as a preliminary study to identify the best formulation which elicited protective immune response in mice model.
4.2 IMMUNOLOGICAL CHARACTERIZATION OF Wb20/22 A HOMOLOGUE OF ALT FAMILY AND THEIR EVALUATION AS A PUTATIVE VACCINE CANDIDATE

4.2.1 Wb20/22- a Homologue of ALT:

ALT a L3 stage specific protein is an important vaccine target due to (i) larval stage specificity in terms of immunogenicity (ii) high expression, offering an abundant target; and (iii) no known homologue in the mammalian host (Gregory et al 2000). ALT protein belongs to chromadorea ALT family, which is unique to filarial parasite, with distant similarities in C.elegans. ALT like genes are present in other filarial nematode species and are characterized by a signal peptide, a variable acidic domain, and a conserved cysteine-rich domain. The alt-2 genes of B.malayi and W.bancrofti share 79% and 73% identity at the amino acid level with the alt-1 gene (Ramachandran et al 2004). ALT-1 and ALT-2 genes differ in the highly acidic domain of the protein. The high protection elicited by ALT (Thirugnanam et al 2007, Sharmila et al 2011) probed us to study in depth, about Wb20/22 which also belongs to chromadorea ALT family, with 83% similarity against BmALT-2 and 54% similar to WbALT-2. Wb20/22 codes for 558base pair DNA sequence, and the translated protein constitutes 185 amino acids with a molecular weight of 21.7 KDa (Fig 4.1). This protein sequence was analysed by Scan Prosite tool, which identified glutamic acid rich region (Fig 4.2) that spanned a region of 25-104 amino acids (de Castro et al 2006). PROSITE is a database of protein families and domains. It is based on the observation that, while there is a huge number of different proteins, most of them can be grouped, on the basis of similarities in their sequences, into a limited number of families. Proteins or protein domains belonging to a particular family
generally share functional attributes and are derived from a common ancestor (Sigrist et al 2013).

(a)

<table>
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<tr>
<td>Definition</td>
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</tr>
<tr>
<td>Accession</td>
<td>AF285860</td>
</tr>
<tr>
<td>Source</td>
<td><em>Wuchereria</em> bancrofti</td>
</tr>
<tr>
<td>Coding sequences (CDS)</td>
<td>1-558</td>
</tr>
</tbody>
</table>

(b) ORIGIN: Coding sequences-AF285860

1 atgaacaaac ttttaatagt tttggtcta ataattcttt tgccacacc actttatgca
61 aaacaatcaa atgaagagga agaagagatg agtaacgaag aggaaaaaga gaaacgggaat
121 aaagaggaag agatgaaga gaactatagt gaagaggaag aggaggatgagaagaggaat
181 gaaagtgggg agaaagagga tgaagaagaa ggaagtagaa gtaaagagga agaggaggat
241 gaagcagg atgggtgtga agaggatgag atgaaaaag aaagaagatga tgattgtgaa
301 gagagagaag atatacggc taaaggagaa ttcgttaaaa ctgacggcaa gaagaaacaa
361 tgtgattctc acgtagcttg ctagatcaaatcgaaccacctgcctgtttcatattaaa
421 gagaatcgtgtgtctgtgcgtgaaagagataaagagagataattgttttaaatggcgtgccagagagaaacaa
481 atggaacgga agaatgggcgg taaattggacatacgtcctgaaattggcgtcctgc gaagaagatggmaatgggaaatggcgtgccagagagaaacaa
541 aagtgttcgt acagttga

(c) Translation: Protein id-AAG31481.1

MNKLLIVFGLIILFATPLYAKQSNSEEEEEMSNEEEEKENSKKEEEEDEEDY SEEEEDEEKNEESGEKEDEEEREESRSKEEEEDEEDGDEDEDEKENDDD CEEREETYAKGEFVKTGDKGKKQCDSHVCYDQREPOQWCILKENQSWTSDKGFCDEKRHLCVMERKNGKLEYAYCAPAKDWKCSYD

Figure 4.1: Characterisation of Wb20/22.

(a) Gen bank accession number, source and CDS of Wb20/22
(b)nucleotide sequence of Wb20/22 (c) translated protein sequence of Wb20/22
(d) Characteristics of Wb20/22:

Number of amino acids : 185
Molecular weight : 21.703 kDa
Theoretical pI : 4.18
Instability index : 85.92

Negatively charged residues (Asp + Glu) : 67
Positively charged residues (Arg + Lys) : 25

Figure 4.1 contd: Characterisation of Wb20/22
(d) Biochemical characteristics of Wb20/22

Figure 4.2: Domain analysis of Wb20/22 by SCAN PROSITE: It reveals the presence of glutamic acid rich region

Gomez-Escobar et al, (2005) constructed truncated mutant of ALT-2 (lacking acidic domain) and transfected in L.mexicana promastigote that showed abolition of ALT phenotype, indicating the critical role of this acidic domain. Multiple sequence alignment using sequences of B.malayi ALT-1, ALT-2, W.bancrofti ALT-2 and Wb20/22 was performed by clustalW (Larkin et al 2007)which showed the glutamic acid rich region of Wb20/22 was not aligned with other sequence (Fig 4.3). Since Wb20/22 carried glutamic acid rich region we intended to analyze the role of this domain in immune response elicited: Th1 or Th2 or Th1/Th2 pattern. Using in silico bioinformatics tools we analysed the epitopic regions of Wb20/22, which predicted the glutamic acid rich region as an immunodominant B epitope region, while the N terminal signal sequence predicted as T epitope region.
With a vision to characterize Wb20/22 a variant of ALT was cloned: Wb20/22 and its mutants.

![Multiple sequence alignment of Bm ALT-2, Wb ALT-2, BmALT-1 and Wb20/22](image)

The highlighted box indicates the acidic region of Wb20/22 which is absent in other ALT sequences.

In this study Wb20/22 was cloned from *W. bancrofti* L3 cDNA library and made mutant constructs of the same based on epitope prediction, and previous findings of Sharmila et al (2011). It was reported that the signal peptide sequence alone, administered as a gene vaccine or synthetic peptide, can induce protective immunity against a microbial pathogen (Jiang et al 2002). Gomez-Escobar et al, (2005) reported that the acidic domain deleted region of ALT showed abolition of ALT phenotype, indicating the essentiality of the acidic domain. Recombinant proteins Wb20/22, Wb20/22 without signal sequence (WOSS) and Wb20/22 without acidic domain (WOAD) were analysed in human samples and Jird model to identify the potential domains contributing to their protective efficacy.
4.2.2 Cloning of Wb20/22 and construction of mutants

The DNA sequence of Wb20/22 was PCR amplified from *W. bancrofti* L3 cDNA library using gene specific primers (Fig 4.4). The PCR amplified product was cloned in pRSETA vector using *BamHI* and *HindIII* sites. The clone was confirmed by PCR, restriction analysis and sequencing. Construction of Wb20/22 mutants: WOSS and WOAD was performed by PCR amplification. WOSS carried regions from 63-558bp excluding the signal region of Wb20 (1-62bp). Gene specific primers were used to amplify WOSS from pRAWb20/22 clone. The amplified product was cloned in pRSETB vector in *BamHI* and *HindIII* sites. WOAD excluded the glutamic acid region, carrying two regions: 1-62bp and 103-558bp. Primers were designed for both regions and ligated using *XhoI* restriction site in between them. The ligated product was further amplified using forward primer of region 1-62bp and reverse primer of 103-558bp. Both mutant constructs were confirmed by PCR, restriction analysis and sequencing (Fig 4.5).

![PCR amplification of Wb20/22](image)

**Figure 4.4:** PCR amplification of Wb20/22
Amplification of Wb20/22 using gene specific primers (1) 100 bp DNA ladder, (2) Wb20/22 gene of 558bp amplified from *Wuchereria bancrofti* L3 cDNA library.
Figure 4.5: Confirmation of Wb20/22, WOSS and WOAD clones

Restriction analysis using BamHI and HindIII double digestion. (a) (1) 100bp DNA ladder (2) pRSETA-Wb20/22 clone showing the insert of 558 bp and vector backbone (3) pRSETA vector. (b) (1) 100bp DNA ladder (2) pRSETB-WOSS clone showing the insert of 495 bp and vector backbone (3) pRSETB vector. (c) (1) 100bp DNA ladder (2) pRSETA-WOAD clone showing the insert of 330 bp and vector backbone (3) pRSETA vector.

4.2.3 Expression and Purification of Wb20/22, WOSS and WOAD

The Wb20/22 protein was expressed in E. coli BL21-CodonPlus (DE3)-RIL (Stratagene) induced with 1mM IPTG at 0.8 OD$_{600nm}$ for 3hrs at 28°C. The 25 kDa rWb20/22 protein was purified by Immobilized Metal Affinity Chromatography (IMAC). The purified rWb20/22 was further confirmed by western blotting with anti-His antibody (Fig 4.6) against vector control which did not show the protein band.
Figure 4.6:  Expression and purification of pRSETA Wb20/22
(a) Expression of 25KDa recombinant Wb20/22 protein was analysed on 12% SDS-PAGE Lane 1: Molecular weight marker, Lane 2: Vector control, Lane 3: Uninduced culture of Wb20/22, Lane 3: E. coli culture induced with 1mM IPTG for 3 hours showing expression of Wb20/22. (b) Reactivity of purified rWb20/22 with mouse anti-His confirmed by immunoblotting. Lane 1: Protein molecular marker, Lane 2: Purified Wb20/22

WOSS was expressed in E. coli BL21-CodonPlus (DE3)-RIL induced with 1mM IPTG at 0.8 OD_{600nm} for 3hrs at 28°C. The protein was purified by IMAC and confirmed by western blotting using anti-His antibody (Fig 4.7).

Figure 4.7:  Expression and purification of pRSETB WOSS
(a) Expression of 21 KDa recombinant WOSS protein was analysed on 12% SDS-PAGE Lane 1: Molecular weight marker, Lane 2: Vector control, Lane 3: Uninduced culture of WOSS, Lane 4: E. coli culture
induced with 1mM IPTG for 3 hours showing expression of WOSS. (b) Reactivity of purified rWOSS with mouse anti-His confirmed by immunoblotting. Lane 1: Protein molecular marker, Lane 2: Purified WOSS

WOAD, as an 18KDa protein, was expressed in in E. coli salt inducible strain (GJ1158) induced with 100mM NaCl and 1% glycerol at 0.6 O.D for 4 hrs at 37°C. The protein was purified by gel elution and confirmed by western blotting using anti-His antibody (Fig 4.8). Since WOAD was present in the insoluble fraction, the probability that it forms an aggregate is higher. Hence the same was observed in Figure-4.8b which showed a ~36KDa band that can be attributed as a dimer. This can be further confirmed by performing mass spectrometry of the sample.

Figure 4.8: Expression and purification of pRSETA WOAD
(a) Expression of 18 KDa recombinant WOAD protein was analysed on 12% SDS-PAGE. Lane 1: Molecular weight marker, Lane 2: Vector control, Lane 3: Uninduced culture of WOAD, Lane 4: E. coli culture induced with 100 mM NaCl for 4 hours showing expression of WOAD. (b) Reactivity of purified rWOAD with mouse anti-His confirmed by immunoblotting. Lane 1: Protein molecular marker, Lane 2: Purified WOAD

The immunoblot analysis of purified recombinant Wb20/22 against the pooled clinical sera of EN, MF, CP and NEN populations revealed the
presence of anti Wb20/22 antibodies in the EN population (Fig 4.9). Earlier reports have shown that filarial antigens like ALT-2, VAH, TPX, GST, and TRX show significantly high recognition of the natural immune sera (EN) than the MF and CP sera and also show high protective efficacy (Allen et al 2000, Hoerauf et al 2005, Veerapathran et al 2009).

Figure 4.9: Immunoblot of purified Wb20/22 with clinical sera samples
Lane 1: Protein molecular marker, Lane 2: EN sera, Lane 3: Mf sera, Lane 4: CP sera, Lane 5: NEN sera

4.2.4 Humoral Response

4.2.4.1 Reactivity with human clinical sera

Residents of areas endemic for lymphatic filariasis are continually exposed to infection with mosquito-transmitted infective larvae (L3), some of which survive to become adult worms and subsequently produce mf transmission stages. The question of whether naturally acquired resistance occurs in adult residents of endemic areas has recently become of interest as the development of molecular vaccines against filarial parasites is being
considered (Day 1991). The reactivity pattern of recombinant proteins were analyzed with different clinical sera. The ELISA of Wb20/22, WOSS, WOAD with clinical sera showed significantly high reactivity with endemic normal individuals (p<0.0001). However endemic normal sera reactivity against WOAD showed mean absorbance (>1.84 O.D) significantly higher than Wb20/22 and WOSS groups, suggesting the signal region of Wb20/22 could be more immunodominant compared to glutamic acid rich region (Fig 4.10).

Figure 4.10: Reactivity of Recombinant proteins with human clinical sera samples.
ELISA of the recombinant proteins was carried out with Endemic Normal (EN), Microfilaraemic (MF) and Chronic Pathology (CP) sera (n=20) from endemic region of South India. Data is represented as aligned scatter plot where each symbol represents absorbance of individual sera and the horizontal line in each group represents the mean O.D. The mean absorbance ± 3 SD of the negative control Non Endemic Normal (NEN) sera was taken as cut off value which is represented as the horizontal line parallel to X axis.
4.2.4.2 Antibody titer and isotype ELISA

The total IgG raised against all recombinant proteins in Jirds were measured by ELISA and was represented as antibody titer. The peak titer of Wb20/22 was found to be 15,000 on day 21 post immunization, WOSS elicited a titer of 27,000 on day 35 post immunization while WOAD groups showed antibody titer of 34,000 (Fig 4.11). This difference in antibody response elicited by WOAD group compared to Wb20/22 and WOSS suggest that the N-terminal signal sequence of Wb20/22 could also be an immunodominant region as present in BmALT-2 (Sharmila et al 2011). The low profile immune response elicited by Wb20/22 could be attributed to the longest linear B epitope present in glutamic acid rich region of Wb20/22 which might be an immunomodulatory region as already reported in ALT (Gomez-Escobar et al 2005).

The antibody isotype elicited by WOAD showed high levels of IgM, followed by IgG1. Both IgG1 (Thirugnanam et al 2007) and IgM (Rajan et al 2005, Anthony et al 2007) have been reported to be involved in vaccine induced protection in experimental filariasis. The isotype profile elicited by WOSS, WOAD primed Jirds indicates high IgG1 followed by IgG2b probably indicating a Th1/Th2 balance (Fig 4.12). Similar response was observed with Merozoite surface protein (MSP-2) immunized mice which skewed the immune response to induce high levels of IgG2b antibodies in C57BL/6 mice (Tongren et al 2005). Antibodies which bind Fc receptors and mediate antibody-dependent cytotoxicity, growth inhibition, or phagocytosis (so-called “cytophilic” antibodies) have repeatedly been shown to be highly effective at clearing blood stage malaria parasites, both in humans (Sabchareon et al 1991) and in mice (von der Weid et al 1996). Mouse IgG2b are minor components of normal serum, that bind with high affinity to Fc receptor, fix complement, and preferentially induced during Th1 immune
responses, especially effective at mediating immunity to blood stage malaria infection. Since lymphatic filariasis is associated with Th2 biased response (King et al 1993) high levels of IgG1, IgG2b induced by WOSS, WOAD is a positive aspect in inducing both Th1 and Th2 response.

Figure 4.11: Antibody Titer of Wb20, WOSS, WOAD in Jirds
Peak antibody titers elicited by Jirds immunized with Wb20/22, WOSS and WOAD. Comparison of peak titers shows that the WOAD and WOSS were highly immunogenic.
4.2.5 Cellular Response

The splenocytes of Jirds immunized with the recombinant proteins were stimulated \textit{in vitro} with respective antigens or Wb20/22, WOSS, WOAD to assess the T cell proliferative response in the permissive models (Fig 4.13). Each of the antigens showed highest proliferation (P<0.01) in the respective antigen immunized Jirds. A remarkable feature of filariasis is T cell hypo-responsiveness characterized by the production of IL-10, an anti-inflammatory cytokine, that down regulates the production of IFN-$\gamma$ thereby creating a conducive environment for the parasites to establish in the host (Yazdanbakhsh 1999). The dominant T epitopes mapped in \textit{B. malayi} ALT-2 protein showed peptide regions P1 (2–21), P3 (44–60), P4 (55–68), P7 (90–110) and P8 (112–128) that induced high T cell proliferation in Balb/c mice (Madhumathi et al 2010c). The Wb20/22, WOSS, and WOAD immunized
Jirds showed high proliferation to respective antigens with comparable proliferation to other antigens also. This could be attributed to the cross reactivity since the mutants are derived from single protein Wb20/22.

The high levels of IFN-γ (267.8±1.83) (Table 4.1) in Wb20/22 stimulated cells compared to WOSS and WOAD shows an elevated Th1 signature cytokine. Though IFN-γ was high, the levels of regulatory cytokine IL-10 was also significantly high, which could be a possible reason for the low antibody titer elicited by this Wb20/22 group. The peak antibody titer elicited by Wb20/22 was high on the 21st day compared to 35th and 42nd day where fall in titer was observed that could be attributed to the low levels of Th2 cytokines IL-4 and IL-5. WOSS elicited low levels of IL-10 compared to Wb20/22 yet the production of IFN-γ was comparatively high. The other Th2 cytokines IL-4, IL-5 was also comparatively low. WOAD elicited high levels of IL-4, IL-5. BmALT-2 protein vaccination with alum elicited a Th2 biased response evidenced by the antibody response and cytokine levels and thus conferred protection against parasites (Thirugnanam et al 2007). Many other studies also suggest that Th2-biased responses are associated with protective immune responses against various nematode parasites (Babu et al 2000, Le Goff et al 2000, Martin et al 2000, Volkmann et al 2003). The regions P1 and P7 of BmALT-2 have shown high levels of IFN-γ in murine model (Madhumathi et al 2010c).
Figure 4.13: Splenocyte proliferation assay in Jirds
Splenocyte proliferation of Wb20/22, WOSS and WOAD immunized Jirds stimulated with the respective proteins, or ConA compared to that of the alum control group. The data is represented as mean stimulation index (S.I) of six animals±SEM. The asterix on top of the bars indicate a significantly high (Two-way ANOVA P<0.01) S.I value compared to corresponding stimulation in the control cells.

Table 4.1: Cytokine levels (pg ml⁻¹) in Jirds

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<th>Antigens</th>
<th>IFN-γ pg/ml</th>
<th>IL-2 pg/ml</th>
<th>IL-4 pg/ml</th>
<th>IL-5 pg/ml</th>
<th>IL-10 pg/ml</th>
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<td>Wb20</td>
<td>267.8±1.83</td>
<td>43.78±0.7</td>
<td>66.72±9.2</td>
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<td>WOSS</td>
<td>194.56±4.6</td>
<td>21.24±0.46</td>
<td>88.21±14.09</td>
<td>42.75±2.94</td>
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<td>WOAD</td>
<td>210.75±2.27</td>
<td>89.11±0.13</td>
<td>126.02±9.94</td>
<td>83.33±11.29</td>
<td>55.12±4.78</td>
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The cytokine levels were measured in the culture supernatants of spleen cells from Jirds immunized with the Wb20/22, WOSS, and WOAD proteins and stimulated in vitro with respective proteins. Experiments were performed thrice and data represented as mean concentration ± SEM.
4.2.6 Protection Study

Many experimental models for filariasis have demonstrated that protection can be induced in animals by irradiated third stage infective larvae (L3) (Babayan et al 2006). The protection efficacy of the recombinant antigens, Wb20/22, WOAD, WOSS and ALT were assessed by micropore chamber method involving the implantation of L3 larvae in Jirds. In order to check the immunoprophylactic efficacy of Wb20/22 which is a L3 stage specific protein, we have also taken ALT a well known L3 stage specific candidate for comparison. Examination of the micropore chamber contents at 48 h after implantation showed that 6.83±1.17, 4.83±0.98, 3.0±0.89, 2.17±0.75, worms were alive in Wb20/22, WOSS, WOAD and ALT immunized groups respectively compared to 8.50±1.05 live worms in alum control group respectively (Table 4.2). The results showed that WOAD had reduced worm burden followed by Wb20/22, WOSS when compared to the control group. However ALT showed significantly reduced worm count compared to Wb20/22 (P<0.0001) (Fig 4.14). The acidic domain of ALT appears to have a major role in the clearance of parasite, which is well exhibited in this study, since WOAD which lacked the glutamic acid rich region the variable acidic domain demonstrated a comparable worm reduction. Our results were in consensus with Sharmila et al (2011) who has demonstrated that the lack of signal sequence in ALT elicited only 49% immunoprotection, while the whole protein without any adjuvant elicited 71% protection against parasites. In addition, our study indicates the immunomodulatory role of glutamic acid rich region, which when removed (WOAD) elicited highest protection in Jirds.
Figure 4.14: Protection study of Wb20/22, WOSS, WOAD and ALT-2 in Jirds
Percentage protection conferred by recombinant proteins (Wb20/22, WOSS, WOAD and ALT-2) compared to alum control in Jirds against L3 larvae. Data is represented as mean percentage of six animals ± SEM.

Table 4.2: Percentage reduction of *B. malayi* L3 in Wb20/22, WOAD and WOSS immunized Jirds

<table>
<thead>
<tr>
<th>Number of live parasites</th>
<th>Alum</th>
<th>Wb20/22</th>
<th>WOSS</th>
<th>WOAD</th>
<th>ALT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean± SEM</td>
<td>8.50±1.05</td>
<td>6.83±1.17</td>
<td>4.83±0.98</td>
<td>3.0±0.89</td>
<td>2.17±0.75</td>
</tr>
<tr>
<td>Percentage Reduction in worm burden</td>
<td>49.82%</td>
<td>54.78%</td>
<td>62.26%</td>
<td></td>
<td>78.18%</td>
</tr>
</tbody>
</table>

The data represented as mean ± SEM of six animals. The percentage is expressed as the average number of worms recovered from the control animals minus average number of worms recovered from the vaccinated animal’s ÷ average number of worms recovered from the control animals x 100. #, *, $, represents a 2Tail T-test P<0.05, <0.001, <0.0001 respectively compared to Wb20/22.
The protection study in Jirds exhibited interesting data, where Wb20/22 showed 49.82% protection which was significantly low compared to ALT. The removal of signal sequence (WOSS) elicited 54.78% immunoprotection. WOAD showed 62.26% protection which was significantly high compared to Wb20/22, WOSS (Table 4.2) and parasite clearance can be attributed to immune mediated Antibody Dependant Cellular Cytotoxicity (ADCC) (Lawrence 2001). Classically, the immunological mechanism used by the host to kill a large multicellular helminth is thought to involve ADCC and the same has been attributed for parasite clearance in earlier studies including synthetic di-peptide conjugate PC1 (Madhumathi et al 2010a). Our study also shows that the N and C terminal of the Wb20 carries the protective epitopes, which showed this significant difference in protection. The elimination of the variable acidic region, glutamic acid rich region of Wb20/22 in WOAD mutant has thrown light in determining the immunomodulatory role of this region, in spite of it being identified as a B epitope region by different epitope prediction software tools.

Identification and characterization of ALT homologue Wb20/22 led to the findings on the variable acidic region of ALT proteins. Elimination of the immunomodulatory role of glutamic acid rich region gave rise to 62.26% protection which was significantly higher than Wb20/22, yet not on par with ALT a putative vaccine candidate. Hence, further enhancement of ALT vaccine was attempted by constructing MAP using immunodominant regions of ALT.
4.3 CONSTRUCTION OF SYNTHETIC ALT MAP AND ENCAPSULATION IN MICROSPHERE

Traditional vaccines have been prepared by attenuating the organism or inactivating entire organism or part of it (toxin) which does have limitations like chances of reversion to virulence, reactogenecity, need for cold chain storage. An alternative approach would be to identify the epitopes that would induce favorable protective response, which can be synthesized chemically. Since these preparations are synthetic they avoid the risk of reversion or incomplete inactivation. The previous attempts to identify the epitope regions of ALT manifested into a construction of ALT MAP. Madhumathi et al (2010a) identified immunodominant B and T epitopes of ALT-2, the sequence of these peptides were extrapolated to construct ALT MAP. It is essential that the putative vaccine candidate should elicit a humoral response and cell mediated response, since lymphatic filariasis is characterized by hyporesponsiveness (Dimock et al 1996). Studies in ALT-2 peptides have revealed the immunomodulatory region P4 that was found to react well against microfilaeremic sera than with other clinical sera, which could be used for diagnosis of active infection (Madhumathi et al 2010c). Regions of peptide P1 (2-21), P2 (21-44), P3 (44-60), P7 (90-110) and P8 (112-128) (Fig 4.15) were constructed on a Fmoc-lysine-(ivDde) core exploiting the solid phase peptide synthesis technique.

---

| P1 : NH₂-NKLLIAFGLVILLVTLCASESDEEFDDG-COOH |
| P2 : NH₂-SESDEEFDDSAADDTDDSEAGGS-COOH |
| P3 : NH₂-SEGGDBYVTGKGEVVETD-COOH |
| P7 : NH₂-WTDKGCFCEDKHKNCVIERKNNGKLE-COOH |
| P8 : NH₂-GKLEYSYCAPEAGWQCA-COOH |

Figure 4.15: Sequences details of the selected peptide regions of BmALT-2 for construction of ALT MAP
4.3.1 Synthesis and Characterization of ALT MAP

The ALT MAP was constructed by solid phase peptide synthesis using Fmoc chemistry (Fig 4.16). It was then cleaved from resin and purified by gel permeation chromatography. Since peptides are weak immunogens it was encapsulated in PLGA microparticles. PLGA is a polymeric delivery system which has been able to elicit both humoral and cellular immune responses (Men et al 1995). PLGA microparticles are biocompatible as the degraded products lactide and glycolide are eliminated by Krebs cycle as CO₂ and in urine (Bazile et al 1992). ALT MAP was encapsulated in PLGA microparticles since optimal immune responses require their efficient delivery and presentation to the immune system which is achieved by the encapsulation of antigens into polymeric microspheres (Hickey et al 2002). The ALT MAP construct was synthesized and encapsulated at Department of Biochemistry, AIIMS, New Delhi. The peptides were analyzed by 12.5% Tricine SDS-PAGE gel (Fig 4.17). Microsphere diameter plays an important role in the interactions with phagocytic cells. Particles smaller than 10 µm are phagocytosed faster by macrophages, which are recruited to the site of administration after subcutaneous injection, and particles larger than 10 µm act as a depot releasing the antigens in a second step (Eldridge et al 1993). In this study, rALT-2 was also encapsulated in microsphere to observe if the controlled delivery system enhanced the immune response elicited.
4.3.2 Humoral Response of ALTMAP Against Clinical Sera

Human lymphatic filariasis is often associated with a variety of clinical manifestation which reflect the nature and intensity of the host immune response to the parasite (King & Nutman 1991). Between the hyper
reactive diseased state (CP) and immune suppressed parasitized individuals (MF) there lies the third group of individuals who despite having lived their entire lives (or prolonged periods) in areas endemic for lymphatic filariasis, and continually exposed to infected vectors, show no parasitological or clinical signs of infection. These individuals are described as putative immune or endemic normals (Hoerauf et al 2005) and are frequently cited as providing evidence for the existence of protective immunity against filarial infections.

In this study ALT MAP, ALT was checked for their reactivity against 20 human sera samples including EN, MF, CP and NEN by performing ELISA. The clinical sera reactivity showed significantly (P<0.0001) higher reactivity with endemic normal individuals in both ALT and ALT MAP groups (Fig 4.18) compared to MF, CP, and NEN indicating that the epitope portion of ALT used in ALT MAP construction was immunodominant. The ALT MAP showed higher reactivity with all EN sera (mean= 0.95±0.08) which was higher than ALT (mean= 0.74±0.07). The preferential recognition of ALT MAP by putatively immune sera shows that this region may be involved in inducing protective immunity in endemic normals and could be exploited in vaccine design. The presence of ALT MAP specific antibodies in putative immune individuals may be protective to host.

To be used as a vaccine, the peptides must induce antibodies which on the one hand is able to recognize the cognate, native antigen (i.e., cross-reactive immunogenicity) and also neutralizes the infectivity of the pathogen harboring the antigen (i.e., cross protective immunogenicity) (Van Regenmortel 2006). Also, epitope-based vaccines are more effective if multiple epitopes covering a region of immunological hot spots are simultaneously delivered as a single chimeric construct for induction of a broad spectrum of immune responses (Srinivasan et al 2004). In this study, we
have constructed the ALT MAP based on the immunological hot spots of ALT-2 which is the first of its kind in the peptide vaccine development for lymphatic filariasis. Reactivity pattern of EN sera showed higher reactivity of ALT MAP compared to ALT, which supports the idea of MAP construction, which comprises of the immunodominant B and T epitopes of ALT.

![Reactivity of ALT, ALT MAP with human clinical sera samples.](image)

**Figure 4.18: Reactivity of ALT, ALT MAP with human clinical sera samples.**

ELISA of the peptides was carried out with Endemic Normal (EN), Microfilaraemic (MF) and Chronic Pathology (CP) sera (n=21) from endemic region of South India. Data is represented as aligned scatter plot where each symbol represents absorbance of individual sera and the horizontal line in each group represents the mean O.D. The mean absorbance ± 3 SD of the negative control Non Endemic Normal (NEN) sera was taken as cut off value which is represented as the horizontal line parallel to X axis.
4.3.3 Antibody Titer in Jirds

The total IgG levels in Jirds immunized with ALT MAP microsphere (MS), ALT MS was analysed by ELISA. The peak titer of ALT MAP MS and ALT MS were found to be 32,000 on day 21 in the former and day 28 in the latter group (Fig 4.19). The earlier antibody titer peak indicates the antigenicity and the efficient delivery of the ALT MAP by the microspheres. The similar titer elicited by both groups reveals that ALT MAP construct containing immunodominant epitopes were able to produce immune response on par with ALT. This immune response could be attributed to the microsphere delivery system used in the study which enhances both humoral and cellular response in host.

Use of biodegradable polymer microparticles as vaccine delivery systems has many potential advantages including optimized immune response via selective targeting of antigens, elimination of need for booster doses by control release of antigens over a longer period and their proven safety profile (Rosas et al 2001, Jiang et al 2005). The release mechanism generally involves diffusion of the polypeptide through a complex pore structure in the polymer matrix. The factors influencing the rate of release include protein particle size, loading, protein solubility and molecular weight, polymer composition and the dimensions and shape of the matrix (Hsu & Langer 1985, Ogawa et al 1988, Saltzman & Langer 1989). The first food and drug administration (FDA) approved peptide delivery system, Lupron Depot®, was an injectable polylactide–coglycolide microsphere formulation of leuprolide acetate used for the treatment of prostrate cancer and capable of delivering a sustained therapeutic level for one month (Langer 1990).
4.3.4 Antibody Isotype

The ALT MAP and ALT exhibited high antibody titer in Jirds which indicate the presence of immunodominant B epitopes. The antibody isotype elicited by ALT MAP MS, ALT MS showed similar patterns of antibody isotype profile. High levels of IgG2b and IgG2a was seen in ALT MAP MS groups. However ALT MS groups elicited comparable levels of IgG2b and IgM which paralleled with ALT MAP MS (Fig 4.20). The high levels of IgM antibodies indicate host defense mechanism. Studies conducted by Rajan, (2005) highlighted that IgM might be involved in adherence of peritoneal cells to the larvae to initiate granuloma formation. Although IgM is the first isotype elicited in response to various infectious agents, class switching and affinity maturation result in the production of high titers of other isotypes in later infection. It has been established that the infective
larval (L3) and adult stages of the parasite primarily induce type 2 responses which are known to be protective against helminth infections (Lawrence 2001, Anthony et al 2007). IgM and IgG1 have been implicated in protection in experimental filariasis (Rajan et al 2005, Thirugnanam et al 2007).

![Graph showing antibody isotype induced by ALT MS and ALT MAP MS in Jirds.](image)

**Figure 4.20: Antibody isotype induced by ALT MS and ALT MAP MS in Jirds.**

Isotype ELISA was carried out to measure the levels of antibody subtypes elicited by the MAP and recombinant protein in Jirds using class or subclass specific antibodies. Data is represented as mean absorbance of MAP and recombinant protein with the respective antisera. Control represents Jirds immunised with only microspheres (MS).

### 4.3.5 Cellular response

Balanced humoral and cellular arm of the immune system are essential to combat infection and thus any vaccine antigen should be capable of eliciting both antibody and T cell responses in humans, especially in filariasis, since there is a hallmark T cell suppression immediately after infection (Lawrence, 2001). Filarial infections induce severe downregulation of T cell responses and consequently reduced cellular immunity. For ideal
humoral and cellular immune response, peptide vaccine should contain both B epitope and Th epitope which ‘help’ the B cells along with other effector mechanisms. Also it was observed that, the B cell and T cell determinants should be derived from the same protein antigen to maintain consistent peak antibody levels and memory response (Sabhnani et al 2003). ALT MAP immunized Jirds showed a slightly higher Splenocyte proliferation to both ALT and ALT MAP antigens (10.42±0.57 and 9.15±0.89 respectively) when compared to the proliferation in ALT immunized Jirds (9.41±1.33 and 7.50±1.04 respectively) (Fig4.21). Hence, ALT MAP construct carries both B/T epitope regions of ALT evidenced by the proliferation of T cells to both antigens. Maximum proliferation, almost to identical levels, was observed for ALT in both the groups, revealing the ability of ALT MAP to produce recall response.

Figure 4.21: Splenocyte proliferation assay in Jirds
Splenocyte proliferation of ALT MS, ALT MAP MS and MS immunized Jirds stimulated with ALT, ALT MAP or ConA. The data is represented as mean stimulation index (S.I) of six Jirds ± SEM.
4.3.6 PBMC Proliferation Assay

To analyze the ALT MAP specific T cell response human PBMCs were separated from endemic normal individuals and stimulated *in vitro* with ALT and ALT MAP. The PBMCs showed significantly high proliferation (P<0.007) when stimulated with ALT MAP compared to ALT (Fig 4.22). The elevated levels of ALT MAP specific proliferation can be attributed to the dominant T cell epitopes used in the ALT MAP construct. The immunomodulatory regions in ALT play a crucial role in the diminishing of the ALT specific proliferation. The human filarial infection is characterized by a profound bias in the Th cell response of infected individuals, with a dominating Th2 response and impaired parasite specific Th1 response (Ottesen et al 1977, King et al 1993, Maizels et al 1995). For a prophylactic vaccine to be effective, a balanced Th1/Th2 response is required. Multiple epitopes covering regions of immunological hot spots when delivered simultaneously as a single chimeric construct are known for induction of a broad spectrum of immune responses (Srinivasan et al 2004).

![Figure 4.22: PBMC proliferation assay in human endemic normal samples](image)

Proliferation of human PBMC’s stimulated with ALT, ALT MAP. ConA was used as positive control. The data is represented as mean Stimulation Index (S.I) of ten endemic
normal individual’s ± SEM. The line drawn parallel to the X axis represents the cut off value (Mean ± SEM of negative control). The asterix on top of the bars indicate a significantly high S.I value compared to that of cells stimulated with recombinant protein ALT (P<0.007).

4.3.7 Protection Study

Immunoprophylactic efficacy of the ALT MS, ALT MAP MS was tested by protection studies using micropore chamber method with L3 larvae in Jirds (Lok & Abraham 1992). Infective larvae (L3) of *B. malayi* were collected following the Baermann’s technique. In contrast to the classical parasite challenge studies, where the duration of animal housing is more than 3 months to recover adult worms (Sharmila et al 2011), we adopted the micropore chamber method since the duration of this study was comparatively less and the cost of animal maintenance was lower. The micropore chamber method has been successfully used for similar studies previously from our lab (Madhumathi et al 2010b). Examination of the micropore chamber contents at 48h after implantation showed that 1.67±0.52 and 2.17±0.75 worms were alive in ALT MAP MS and ALT MS immunized groups respectively compared to 8.50±1.05 live worms in the alum control groups respectively (Table 4.3). ALT MAP MS showed a significantly reduced worm count compared to alum control. The analysis of percentage reduction in worm burden compared with ALT MS showed that ALT MAP MS construct conferred 78.18% (Table 4.3) worm reduction which was significantly higher (P<0.0001) compared to the alum control (3.7%). Gregory et al, (2000) reported 76% reduction in parasite survival following immunization with recombinant *B. malayi* ALT-1. The level of reduction in parasite survival on immunization with the larval stage antigen of the ALT family is the highest level of protection so far for an individual antigen. The protective role of Thioredoxin (TRX) antioxidant enzyme of *B. malayi* is established. The multi epitope vaccine designed with the 2 immunodominant epitopes of TRX
(TRXp1 and TRX p2) named as PC1 conferred 75.14% protection in Jirds (Madhumathi et al 2010b). Our results also confer significantly high parasite reduction of 78.18% by ALT MAP MS. The ALT MAP construct was able to elicit high titers in Jirds, with high levels of IgM, IgG that have been implicated in protection in experimental filariasis (Rajan et al 2005). ALT MAP yielded comparable and higher protection percentage than ALT, which can be used as multi antigenic peptide vaccine which is first of its kind for filariasis.

Table 4.3: Percentage reduction of *B. malayi* L3 in the ALT MS, ALT MAP MS immunized Jirds

<table>
<thead>
<tr>
<th>Number of Live Parasites recovered</th>
<th>Alum</th>
<th>ALT MS</th>
<th>ALT MAP MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean± SEM</td>
<td>8.50±1.05</td>
<td>2.17±0.75</td>
<td>1.67±0.52</td>
</tr>
<tr>
<td>Percentage reduction in worm burden</td>
<td></td>
<td>74.17%</td>
<td>78.18%</td>
</tr>
</tbody>
</table>

The data is represented as mean±SEM of six animals. The percentage is expressed as the average number of worms recovered from the control animals minus average number of worms recovered from the vaccinated animal’s ÷ average number of worms recovered from the control animals x 100.

4.4 ENHANCING THE EFFICACY OF ALT USING DIFFERENT ADJUVANTS

Adjuvants are compounds that can increase and (or) modulate the intrinsic immunogenicity of an antigen. Adjuvants are required to assist new vaccines to induce potent and persistent immune responses, with the additional benefits that less antigen and fewer injections are needed. New vaccine targets often require the induction of strong cellular responses, including T helper (Th) cells and sometimes cytotoxic T lymphocytes (CTLs),
in addition to antibodies. Since ALT has already been established as a putative vaccine candidate that has given the highest protection of 74% as a single antigen (Gregory et al 2000) we decided to enhance and study the immune response by administering them with different adjuvant formulations.

FDA approves adjuvants only in combination with vaccine and not alone. Alum was the first adjuvant to be approved by FDA for human use. Recently MF59, AS04 adjuvants have been approved by FDA for flu vaccine and viral vaccine. Yet there are many vaccine adjuvants that have undergone clinical testing which includes calcium phosphate, MPLA, Montanide ISA-51, CpG and many others. Lymphatic filariasis is characterized by hyporesponsiveness, which indicates the need for strong cellular response along with humoral response for a successful adjuvant formulation.

As already mentioned previously ALT-2 is a putative vaccine candidate with 74% protection efficacy we focused on enhancing the protective immune response elicited by this antigen with different adjuvants in mice model. The adjuvants included in this study were alum, QS21, MPLA, MDP, Inulin and Chitosan. In this study, the pattern of immune response elicited by different adjuvant formulations was analysed as a step forward towards the development of a prophylactic vaccine formulation for lymphatic filariasis at a preliminary level.

4.4.1 Humoral Responses to ALT Adjuvant Formulations

4.4.1.1 ALT+Alum

Mineral based adjuvant alum, oldest and predominantly administered adjuvant known to stimulate the humoral response was used in this study. Mice immunized with ALT+Alum elicited a peak titer of 32,000 on day 28 (Fig 4.23a). The IgG1 isotype was significantly high followed by
IgG2b compared to alum control (Fig4.23b). This result was in consensus with the early reports that aluminium adjuvants are very effective in promoting the expansion of humoral immune responses including IgE production (Hamaoka et al 1973). Antigen needs to be adsorbed to the adjuvant surface thereby transforming soluble antigen to a particulate antigen to favor APC uptake (Rimaniol et al 2004, Rimaniol et al 2007). Macrophages are activated by alum to present antigen which induced in vivo priming and expansion of antigen specific B cells (Jordan et al 2004).

![Figure 4.23: Humoral response in mice immunized with ALT+Alum, Alum](image)

(a) The total IgG induced by ALT+Alum, at different intervals post immunization in mice models (n=6 per group) was assessed by ELISA. Mice were immunized with 30µg of protein in alum intraperitoneally and blood was collected at different intervals. Serum from the mice immunized with alum alone was taken as negative control. (b) Isotype ELISA was carried out on 28th day to measure the levels of antibody subtypes elicited by the ALT+alum and alum in mice using class or subclass specific antibodies.
4.4.1.2 ALT+QS21

Saponin based adjuvant QS21, formulated with ALT yielded a peak titer of 1,28,000 on 35th day (Fig 4.24a). The antibody isotype pattern elicited by ALT+QS21 showed an overall high level of IgG subclasses, with predominantly elevated levels of IgG2b and IgG1 (Fig 4.24b). Studies highlight the ability of QS21 to induce Th1 cytokines and antibodies of IgG2a isotype in mice along with a Th1-bias (Kensil et al 1991, Kensil 1996). The overall high level of IgG reflects the balance of Th1/Th2 responses. QS21 has shown to stimulate humoral immune responses in mice, including antigen-specific IgG1, IgG2b and IgG2a titers. Most QS-21 formulations in mice have been administered by the subcutaneous or intramuscular route, but intranasal and oral administration have also been shown to be effective. It induced production of IgG responses to ganglioside antigen in melanoma vaccine in human Phase I clinical trials (Helling et al 1995). QS21 elicited protective immune response to recombinant malaria vaccine in human Phase I clinical trials (Stoute et al 1997).

![Figure 4.24: Humoral response in mice immunized with ALT+QS21, QS21](image)

(a) The total IgG induced by ALT+QS21, at different intervals post immunization in mice models (n=6 per group) was assessed by ELISA. Mice were immunized with 30µg of protein with 10µg of QS21 intraperitoneally and blood was collected at
different intervals. Serum from the mice immunized with QS21 alone was taken as negative control. (b) Isotype ELISA was carried out on 35th day to measure the levels of antibody subtypes elicited by the peptides and proteins in mice using class or subclass specific antibodies.

### 4.4.1.3 ALT+MPLA, ALT+MDP

ALT+MPLA immunized mice elicited a titer of 2,56,000 on day 35(Fig 4.25a), while ALT+MDP group mice elicited a low peak titer of 64,000 on 35th day (Fig 4.26a). It was shown that MPLA elicited a 16 fold increase in ELISA titers against ovalbumin (Ribi et al 1985). N-acetylmuramyl- L-alanyl-D-isoglutamine when administered subcutaneously in saline with antigen increased the humoral response in mice (Audibert et al 1976). The antibody isotype pattern of both the groups showed significantly high levels of IgG2b with other IgG subclass at low levels pointing a Th1 biased response (Fig 4.25b & 4.26b).

![Figure 4.25: Humoral response in mice immunized with ALT+MPLA, MPLA](image)

(a)The total IgG induced by ALT+MPLA, at different intervals post immunization in mice models (n=6 per group) was assessed by ELISA. Mice were immunized with 30µg of protein with 5µg of MPLA via intramuscular route and blood was collected at different intervals. Serum from the mice immunized with MPLA alone was taken as negative control. (b) Isotype
ELISA was carried out on 35th day to measure the levels of antibody subtypes elicited by the peptides and proteins in mice using class or subclass specific antibodies.

Figure 4.26: Humoral response in mice immunized with ALT+MDP, MDP
(a) The total IgG induced by ALT+MDP, at different intervals post immunization in mice models (n=6 per group) was assessed by ELISA. Mice were immunized with 30µg of protein with 5µg of MDP via intramuscular route and blood was collected at different intervals. Serum from the mice immunized with MDP alone was taken as negative control. (b) Isotype ELISA was carried out on 35th day to measure the levels of antibody subtypes elicited by the peptides and proteins in mice using class or subclass specific antibodies.

4.4.1.4 ALT+Inulin, ALT+Chitosan

An ideal adjuvant should promote both cellular and humoral response. Carbohydrates consist of repetitive units enabling them to follow a T cell independent immune response (Mond et al 1995, Snapper & Mond 1996). The persistent antibody-mediated immunity elicited by vaccination of pneumococcal capsular polysaccharide has been established previously (Heidelberger et al 1950). The ALT+Inulin formulation elicited a peak titer of 1,28,000 on day 28 which sustained even till 42nd day (Fig 4.27a). Another carbohydrate adjuvant formulation ALT+ Chitosan elicited a peak titer of 1,28,000 on 35th day which eventually declined to a titer of 64,000 on 42nd
day (Fig 4.28a). The high antibody titers elicited by carbohydrate adjuvant formulations are in consensus with the earlier reports of persistent antibody mediated immunity. Both the adjuvant formulations elicited high levels of IgG2b, IgG1 indicating a Th1/Th2 balance (Fig 4.27b & 4.28b). However, ALT+Inulin elicited overall high levels of IgG2b, IgM and IgG1 (Fig 4.27b).

Figure 4.27: Humoral response in mice immunized with ALT+Inulin, Inulin
(a) The total IgG induced by ALT+Inulin, at different intervals post immunization in mice models (n=6 per group) was assessed by ELISA. Mice were immunized with 30µg of protein with 50µg of Inulin via intraperitoneal route and blood was collected at different intervals. Serum from the mice immunized with Inulin alone was taken as negative control. (b) Isotype ELISA was carried out on 28th day to measure the levels of antibody subtypes elicited by the peptides and proteins in mice using class or subclass specific antibodies.
Figure 4.28: Humoral response in mice immunized with ALT+Chitosan, Chitosan

(a) The total IgG induced by ALT+Chitosan, at different intervals post immunization in mice models (n=6 per group) was assessed by ELISA. Mice were immunized with 30µg of protein with 4% chitosan solution via intra peritoneal route and blood was collected at different intervals. Serum from the mice immunized with Chitosan alone was taken as negative control.

(b) Isotype ELISA was carried out on 35th day to measure the levels of antibody subtypes elicited by the peptides and proteins in mice using class or subclass specific antibodies.

Thus to sum up, a mixed Th1/Th2 response, pertaining to the antibody repertoire generated, was obtained in the two carbohydrate adjuvants (Inulin and Chitosan) and the saponin adjuvant QS21 (Table 4.4). The highest antibody titer was also obtained in them only. The sustainance of the high antibody titer even on day 42 post immunization was observed only in Inulin (Fig 4.27a) indicating it to be a potentional adjuvant for ALT with respect to the humoral response.
Table 4.4: Comparative analyses of the Humoral immune response pattern elicited by different adjuvant formulations with ALT.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Adjuvant formulation</th>
<th>Peak Titer</th>
<th>Isotype</th>
<th>Immune response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Alum+ALT</td>
<td>1:32000</td>
<td>IgG1</td>
<td>Th2</td>
</tr>
<tr>
<td>2.</td>
<td>QS21+ALT</td>
<td>1:1,28,000</td>
<td>IgG2b, IgG1, IgG3</td>
<td>Mixed Th1/Th2</td>
</tr>
<tr>
<td>3.</td>
<td>MPLA+ALT</td>
<td>1:2,56,000</td>
<td>IgG2b</td>
<td>Th1</td>
</tr>
<tr>
<td>4.</td>
<td>MDP+ALT</td>
<td>1:64,000</td>
<td>IgG2b</td>
<td>Th1</td>
</tr>
<tr>
<td>5.</td>
<td>Inulin+ALT</td>
<td>1:1,28,000</td>
<td>IgG2b, IgG1, IgG3</td>
<td>Mixed Th1/Th2</td>
</tr>
<tr>
<td>6.</td>
<td>Chitosan+ALT</td>
<td>1:1,28,000</td>
<td>IgG2b, IgG1, IgG3</td>
<td>Mixed Th1/Th2</td>
</tr>
</tbody>
</table>

### 4.4.2 Cellular Responses to ALT Adjuvant Formulations

#### 4.4.2.1 ALT+Alum

The cellular responses elicited by mice immunized with alum, ALT+ alum were studied by stimulation of spleen lymphocytes of immunized mice with ConA or ALT which leads to proliferation of T cells. The mitogen ConA showed high proliferation in both alum (SI=7.99±0.55) and ALT+alum (SI=10±0.50) groups, while ALT showed a moderate proliferation in ALT+alum groups (SI=3.29±0.44) (Fig 4.29). The significant problem faced by alum is the restricted range of immune response induced, failing to induce Th1 response, such as IFN-γ production and B cell IgG2a secretion (Grun & Maurer 1989, Brewer & Alexander 1997). This adjuvant cannot be used against the three diseases causing most global mortality, HIV, tuberculosis and malaria, which are entirely or partially dependent on the generation of Th1 type immunity. Since filariasis is characterized by hyporesponsiveness, it is essential that the adjuvant formulation aids in restoring cellular response.
4.4.2.2 ALT+QS21

There was difference in the proliferation of T cells on stimulation with ALT in control group as well as ALT+QS21 immunized groups (Fig 4.30). However, with mitogen both the groups showed high proliferation. The splenocyte proliferation induced by ConA was similar in both ALT+Alum (SI=10±1.40; Fig 4.29) and ALT+QS21 (SI=9.5±0.50) immunized groups, however the stimulation by ALT was higher in ALT+QS21 (SI=7.78±0.77; Fig 4.30) group than the control QS21(SI=2.61±0.61) or the ALT+Alum (mean SI=4.95; Fig 4.29) immunised group. Thus, it suggests QS21 to be a more potent adjuvant than alum in this model. QS21 has shown potent adjuvancity enhancing antigen presentation to APCs and predominantly inducing CTL production, eliciting both Th1 and Th2 cytokine secretion in animal models (Kim et al 2006). The overall high levels of IgG produced by ALT+QS21 immunized group along with higher proliferation rates allows it to be further tested for parasite challenge studies to be used as successful adjuvant formulation with the putative vaccine candidate ALT.
Figure 4.30: Cellular response in mice immunized with QS21, ALT+QS21
Splenocyte proliferation of QS21, ALT+QS21 immunized mice stimulated with ALT or ConA. The data is represented as mean stimulation index (S.I) of six mice ± SEM.

4.4.2.3 ALT+MPLA, ALT+MDP

ConA mitogen pulsed cells, of both ALT+MPLA immunised and control (MPLA immunised) groups showed overwhelming stimulation indices (S.I=30.83±3.29 and 42.44±4.83 respectively) confirming the proliferation of T cells (Fig 4.31). When both groups were pulsed with ALT antigen, control groups showed high proliferation (S.I=32.28±3.27) whereas ALT+MPLA group showed almost 6 fold decrease in proliferation (P<0.0001).
Figure 4.31: Cellular response in mice immunized with MPLA, ALT+MPLA

Splenocyte proliferation of MPLA, ALT+MPLA immunized mice stimulated with ALT or ConA. The data is represented as mean stimulation index (S.I) of six mice ± SEM.

MDP another bacterial derived adjuvant showed an unusual pattern of splenocyte proliferation. The control group immunized mice elicited high splenocyte proliferation rates against ConA and ALT (S.I=15.2±1.56, 12.2±1.31) (Fig 4.32). While the ALT+MDP immunized group elicited significantly low mitogen and much lower antigen specific T cell proliferation (P<0.0001) (fig 4.32). Though MPLA and MDP are good immunopotentiators, adjuvant formulation failed to induce proliferation and recall-response to the ALT antigen. This could be due to (1) low expression of co stimulatory signals on APC (2) low production of pro-inflammatory cytokines (3) insufficient production of memory cells, which result in impaired T cell proliferation, rendering T cells tolerant (Harry et al 2010).
4.4.2.4 ALT+Inulin, ALT+Chitosan

The lymphoproliferative response of inulin, and ALT+Inulin immunized groups to ConA was found to be high (SI=6.73±0.73, 7.32±0.84) (Fig 4.33). ALT pulsed spleen cells showed significantly high stimulation indices (SI=6.85±0.2) in ALT+inulin immunized mice compared to control groups. The adjuvant properties of γ-inulin studied in animal models demonstrated the capacity to enhance both cellular and humoral response against a wide variety of antigens, including KLH, T. ovis surface antigen, diphtheria and tetanus toxoids, influenza virus, HBsAg and malaria surface antigens (Silva et al 2004). Our study also has provided evidences for good cellular response (Fig 4.33) and humoral response as evidenced by sustained high antibody titer even after the 42nd day of immunization (Fig 4.27a). This suggests the potential efficacious property of ALT+inulin formulation.
Figure 4.33: Cellular response in mice immunized with Inulin, ALT+Inulin

Splenocyte proliferation of Inulin, ALT+Inulin immunized mice stimulated with ALT or ConA. The data is represented as mean stimulation index (S.I) of six mice ±SEM.

The pattern of T cell response elicited by chitosan, chitosan+ALT was slightly different from that of inulin formulation group. The control group chitosan showed low splenocyte proliferation to ConA and ALT antigens (Fig 4.34) while the chitosan+ALT immunized mice showed high proliferation against both these antigens (SI=19±1.40; 11.09±1.86 respectively) (Fig 4.34).

It has been demonstrated that chitosan solution improved humoral and cell-mediated immune responses to a subcutaneous vaccination with a model protein antigen in the absence of additional adjuvants (Zaharoff et al 2007). The present study's data was obtained by intra peritonial immunization for comparison with other adjuvants. Thus, a study by subcutaneous immunization may be essential to say the last word on the efficacy of chitosan in combination with ALT.
Figure 4.34: Cellular response in mice immunized with Chitosan, ALT+Chitosan
Splenocyte proliferation of Chitosan, ALT+Chitosan immunized mice stimulated with ALT and ConA. The data is represented as mean stimulation index (S.I) of six mice ± SEM.

4.4.3 Cytokine ELISA

An array of cytokines were analysed for different adjuvant formulations with ALT in mice model (Table 4.5). Alum and QS21 formulations elicited high levels of IFN-γ, with significantly low levels of IL-10. Studies have reported that there is a overwhelmed Th2 response in both humans (Maizels & Lawrence 1991) and mice (Osborne et al 1996). This state of Th1 tolerance/impairment is been reversed by QS21, which is evident from the values seen in Table 4.5. QS21 has shown potent adjuvant abilities enhancing antigen presentation to APCs and predominantly inducing CTL production, eliciting both Th1 and Th2 cytokine secretion in animal models (Kim et al 2006). The cytokine pattern elicited by bacterial derived adjuvants was skewed to IL-10, which in general gives raise to predominant Th1 cytokine IFN-γ and IL-2 (Tomai & Johnson 1989). Hence the increase of IL-10 in ALT+MPLA and ALT+MDP may be due to immunomodulatory effect of ALT-2 (Kurniawan et al 1993, Ravichandran et al 1997). Filarial immunity
is characterized by T cell hyporesponsiveness in asymptomatic state (Ottesen et al 1977), whereas people residing in endemic area, still resistant to infection, show a balanced Th1/Th2 response (Maizels & Yazdanbakhsh 2003). Vaccine adjuvant formulation that mimics the immunity of endemic normal will aid in preventing this dreadful disease. However, this ALT+MPLA formulation has elicited antibody titer, which may be a protective immune response, but the impaired/ hypo-responsive state of T cells and the milieu of cytokines produced establish the role of IL-10 a regulatory cytokine which has dampened the T cell mediated response (Ramanathan & Perumal 2014).

The carbohydrate adjuvants inulin and chitosan in formulation with ALT produced high levels of IFN-γ. ALT+chitosan also induced high levels of IL-10 a Th2 cytokine. Both the adjuvant formulations elicited high levels of IgG2b, IgG1 indicating a Th1/Th2 balance (Table 4.4). Diego et al, 2004 studied γ inulin (γ-IN) in combination with merozoite surface protein (MSP) an antigen from *Plasmodium falciparum* in mice. The cellular immune response to MSP 5 antigens elicited in vitro by Splenocyte re-stimulation showed that γ-IN was as effective as FCA in inducing Th1 (IFN-γ) and Th2 (IL-10) cytokines. The ALT+inulin and ALT+Chitosan formulation elicited a high antibody titer, balanced Th1/Th2 cytokines and a high cellular proliferation (Table 4.6) indicating the prospects of being a successful formulation for future studies in Jirds.
Table 4.5: Cytokine levels (pg ml\(^{-1}\)) in mice

<table>
<thead>
<tr>
<th>Antigen</th>
<th>IL-2</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-10</th>
<th>IFN-(\gamma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT+Alum</td>
<td>108.57 ± 0.66</td>
<td>230.7±7.36</td>
<td>145.71±1.42</td>
<td>115.6±2.58</td>
<td>202.5±18.39</td>
</tr>
<tr>
<td>ALT+QS21</td>
<td>125.54±7.94</td>
<td>378.56±3.13</td>
<td>112.65±7.15</td>
<td>56.87±1.62</td>
<td>895.45±13.24</td>
</tr>
<tr>
<td>ALT+MPLA</td>
<td>132.73±8.17</td>
<td>329.75±6.37</td>
<td>92.44±4.36</td>
<td>1243.11±41.77</td>
<td>662.46±53.49</td>
</tr>
<tr>
<td>ALT+MDP</td>
<td>112.4±7.79</td>
<td>312.43±6.1</td>
<td>87.43±5.4</td>
<td>1354.21±49.11</td>
<td>546.7±17.51</td>
</tr>
<tr>
<td>ALT+Inulin</td>
<td>143.56±6.69</td>
<td>267.78±10.44</td>
<td>279.56±1.94</td>
<td>176.12±5.15</td>
<td>2789.9±70.67</td>
</tr>
<tr>
<td>ALT+Chitosan</td>
<td>118.5±17.24</td>
<td>200.5±4.98</td>
<td>256.83±3</td>
<td>180.75±19.32</td>
<td>1420.65±51.45</td>
</tr>
</tbody>
</table>

The cytokine levels were measured in the culture supernatants of spleen cells from mice immunized with the ALT protein and different adjuvant formulation and stimulated in vitro with ALT protein. Experiments were done thrice and data represented as mean concentration ± SEM.

Table 4.6: Comparative analysis of the Cellular immune response pattern elicited by different adjuvant formulations with ALT.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Adjuvant formulation</th>
<th>Cell proliferation</th>
<th>Cytokine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Alum+ALT</td>
<td>Moderate proliferation</td>
<td>IL-4</td>
</tr>
<tr>
<td>2.</td>
<td>QS21+ALT</td>
<td>Moderate proliferation</td>
<td>IL-4, IFN-(\gamma)</td>
</tr>
<tr>
<td>3.</td>
<td>MPLA+ALT</td>
<td>Low proliferation</td>
<td>IL-10</td>
</tr>
<tr>
<td>4.</td>
<td>MDP+ALT</td>
<td>Low proliferation</td>
<td>IL-10</td>
</tr>
<tr>
<td>5.</td>
<td>Inulin+ALT</td>
<td>High proliferation</td>
<td>IFN-(\gamma)</td>
</tr>
<tr>
<td>6.</td>
<td>Chitosan+ALT</td>
<td>High proliferation</td>
<td>IFN-(\gamma), IL-5</td>
</tr>
</tbody>
</table>
Hence with the above analysis of humoral and cellular response of different adjuvant combinations with ALT (Table 4.4 & 4.6), the formulation which elicited Th1/Th2 response can be further investigated in Jirds. However, enhanced Th1 response elicited formulations can also be investigated since filariasis is related with impaired antigen specific cellular immunity. ALT+QS21, ALT+Inulin, ALT+Chitosan can be studied further to identify the successful formulation which elicits protective immunity.