CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Reagents and Chemicals

Chemicals of analytical grade were purchased from Sigma chemical company, St Louis, MO, USA and Merck GmbH, Germany. Restriction enzymes were procured from New England Biolabs, UK. Antibiotics Ampicillin was purchased from Ranbaxy, Delhi, India, chloramphenicol and Kanamycin were from Invitrogen, Sandiego, USA. Hibond nitrocellulose membranes, protein molecular weight markers, Q sepharose and IMAC chelating sepharose were procured from Amersham International, Birmingham, UK. DNA molecular weight markers and protein molecular weight markers were obtained from Fermentas (Fermentas, MD, USA). High binding 2HB plates (Thermo Scientific, MA, USA) for peptides and Maxisorb (Nunc, Maxisorp, Nalge Nunc International, Denmark) 96 wells were purchased for ELISA. The other molecular grade chemicals were procured from HiMedia, India. Primer synthesis and sequencing reactions were carried out at Microsynth, Balgach, Switzerland. The immunological reagents and secondary conjugates were also procured from Sigma, St.Louis, USA and Bangalore Genei, India. The capture ELISA kits for detection of the levels of cytokines like IL-4,IL-5 and IFN-γ, and antibody isotyping kit for detecting isotype antibody distribution in serum, were purchased from Pierce, Rockford, IL USA. For Splenocyte proliferation assay, 96-well flat bottom sterile tissue culture plates from NUNC, Roskilde, Denmark were used. RPMI
1640 and fetal calf bovine sera were obtained from Gibco BRL, USA. The radioactive [H3] Thymidine and scintillation fluid was purchased from Amersham International, Birmingham, UK. For micropore chamber protection study 3-mm ring, Millipore Filtration (MF) cement and durapore membranes were purchased from Millipore, Massachusetts, USA.

3.1.2 Culture Media

Luria Bertani (LB) broth was used for propagation of *E. coli* strains. The LB broth was prepared by dissolving 10 g of tryptone (HiMedia, Mumbai, India) and 5 g of sodium chloride in 1 L of distilled water and the pH adjusted to 7.2 with 1N NaOH. Media was supplemented with 100µg/ml of ampicillin or 50µg/ml of kanamycin or 35µg/ml of chloramphenicol wherever required. For preparing solid medium 1.5% agar was added to the liquid broth. BL21 (DE3) pLysS strains containing pLysS plasmids and recombinant plasmids were grown in the presence of double antibiotics chloramphenicol (35µg/ml) and ampicillin (100µg/ml) in LB. Luria Bertani broth without NaCl was used to propagate GJ1158. The chemicals were procured from Merck, Germany.

3.1.3 Bacterial Strains and Plasmids

*Escherichia coli* strains DH5α, BL21 (pLys S), BL21 (DE3), GJ1158 and Prokaryotic T7 expression vector pRSET B was purchased from Invitrogen, CA, USA. Genotype of the *E. coli* strains employed in this study is given in Appendix 1. Vector Map and restriction sites of the vector pRSET A, B and C are shown in Appendix 2.

3.1.4 Expression System Used in this Study

The recombinant clones were expressed in pRSET plasmid system based on T7 RNA polymerase (Studier and Moffat 1986). T7 promoter is
highly specific for T7 RNA Polymerase and the transcription by T7 polymerase is selective and 5 times faster than *E. coli* RNA polymerase thus leading to higher expression of genes cloned under T7 promoter. The metal-binding domain (six-tagged histidine moieties) at the N-terminal end forms a fusion peptide and has a high affinity for the divalent ions (nickel, copper and cobalt) and facilitates purification of the protein using immobilized metal affinity columns (IMAC) (Crowe et al 1995).

The pRSETB vector used for cloning in this study offers

- T7 promoter for high-level expression
- T7 gene-10 sequence to provide protein stability
- N-terminal 6-histidine tag for rapid purification with nickel resin and detection with an anti-histidine antibody
- N-terminal X-press epitope for protein detection with the Anti X-press antibody
- Enterokinase cleavage site for removal of fusion tag.

The T7 expression hosts used in this study are BL21 (DE3), BL21(pLysS) and GJ1158. BL21 strain contains a chromosomal copy of T7 RNA polymerase gene under the control of *lac* UV5 (DE3 lysogen) promoter which can be induced by Isopropyl-thio-galactoside (IPTG). T7 RNAP is expressed upon induction and transcribes the gene of interest, hence expression of genes under the control of T7 promoter in the plasmid can be induced with the gratuitous inducer IPTG (Calbiochem, Merck, Germany) at 1 mM final concentration. Further, BL21 (DE3) being a *lon* protease deficient strain protects the expressed heterologous proteins from proteolytic cleavage.

Another genetically engineered strain of BL21 (DE3) was developed called GJ1158 (Bhandari et al 1997). This strain (GJ1158) carries a
single chromosomally integrated copy of the gene for phage T7 RNA polymerase under transcriptional control of the cis-regulatory elements of the osmoreponsive proU operon. Plasmids that have been constructed to obtain overproduction of individual target gene products in strain BL21(DE3) (by addition of IPTG as an inducer) can directly be transformed into GJ1158. Induction of Pro-U by NaCl drives the transcription of the T7 RNA polymerase gene, which in turn switches on the expression of the genes under the control of T7 promoter in the recombinant plasmid.

The NaCl induction regimen was also shown to be associated with a decreased propensity for sequestration of overexpressed target proteins within insoluble inclusion bodies. The use of NaCl as an inexpensive inducer in large-scale expression cultures and increased stability makes GJ1158 a very suitable expression host. BL21(DE3) host was induced with 1mM IPTG for 3 hours, while GJ1158 host was induced with sterile NaCl to a final concentration of 0.3M.

In case of BL21(pLysS) strains, the native plasmid contains a chloramphenicol resistance marker and it produces small amounts of lysozyme which prevents leaky expression of genes under the control of T7 promoter in the uninduced condition and this is especially important in case of certain toxic proteins.

### 3.1.5 Recombinant Proteins Used in the Present Study

The clones *B. malayi* Thioredoxin (TRX-1) (Genbank Acc. No. AAM51563), Transglutaminase (TGA) (Genbank Acc. No. AAQ23042), Thioredoxin Peroxidase (TPX-1) (Genbank Acc. No. AAC23701) and Abundant Larval Transcript (ALT-2) (Genbank Acc. No. AAB41884) in pRSETB were available in the laboratory which were confirmed by DNA
sequencing and used for all the studies. The recombinant proteins were further purified and used for all the analysis.

### 3.1.6 Monoclonal Antibodies

The monoclonal antibodies against *B. malayi* ALT-2 protein were developed by Dr. Anjali Karande, Department of Biochemistry, IISc, Bangalore. The mice were immunized with the recombinant protein and the spleen cells were fused with Sp2/0 mouse myeloma cells to establish hybridoma. The clones were screened with recombinant ALT-2 and native crude protein of L3 parasite by ELISA.

### 3.2 MAINTENANCE AND COLLECTION OF PARASITES

#### 3.2.1 Mosquito Colony

Colonies of the liver pool black eye strain mosquitoes, *Aedes aegypti* (SS Strain) obtained from Hindustan Ciba Geigy Research centre, Mumbai were maintained at temperature of 25-28°C and relative humidity of 70-80% and maintained at Mahatma Gandhi Institute of Medical Sciences animal house. Mosquito eggs collected on whatman No.1 filter paper were immersed in water sprinkled with a pinch of sodium chloride and ascorbic acid. Some eggs collected on filter paper were washed and stored in dessicator for future use. Within 24-48 hrs, larvae were obtained and maintained in water sprinkled with yeast extract and vitamin B complex powder. In around 7-10 days larvae would get metamorphosed in to pupae. The pupae were collected in small beaker, left inside nylon cages (about 750 pupae/cage) measuring 12"X12" and were allowed to emerge as adults. The adults mosquitoes were routinely fed on cotton pads soaked in 6% sucrose solutions. Six days old mosquitoes were given blood meal using healthy *Mastomys* (Suzuki and Seregeg 1979).
3.2.2 **Brugia malayi** Infection

*Brugia malayi*, the closely related filarial parasites to *W. bancrofti* was established and maintained in jirds and mastomys. *B. malayi* infected mastomys obtained from CDRI, Lucknow were used for infecting other animals. For maintaining the cycle of infection, four days old mosquitoes were used. Mosquitoes were fed on cotton pads soaked in 6% sucrose solution containing 0.05% Para Amino Benzoic Acid (PABA) (Rao et al 1984). Sugar pad was removed from the mosquito cages approximately 18-24 hours before they were infected and allowed to feed on infected *Mastomys* having 80-100 mf blood. The mosquitoes were then fed on cotton pads soaked with PABA solution. After 12-14 days, the mosquito were dissected and checked for L3 stage of larvae. For mass dissection the mosquito were collected using mosquito suction gun (Hausherr’s Machine words, NJ, USA) stunned by shaking placed in a Petri dish with 2-3 ml of insect saline (0.6% NaCl) and were gently crushed to release the L3 larvae. The contents were then transferred to a Bearmann’s apparatus and kept at 35-40 °C for 45-60 minutes. The 3rd stage larvae collected at the bottom were removed, counted with the help of dissecting microscope (Suzuki and Seregeg 1979) and used for infecting fresh animals. Male *Mastomys* of 6-8 weeks old infected by subcutaneous injection and male jirds of the same age were infected by intraperitoneal injection of approximately 100 L3 respectively (Sanger et al 1981).

3.2.3 Parasite Recovery

*B. malayi* adults, L3 larvae and mf were obtained from the filarial repository facility of Dr.Reddy, Department of Biochemistry, Mahatma Gandhi Institute of Medical Sciences (MGIMS), Wardha, Sevagram, Maharashtra. Microfilariae were obtained by lavage of the peritoneal cavity of
jirds with intraperitoneal filarial infection of 3 months or more duration. The mf were washed with RPMI 1640 medium, plated on sterile plastic petridishes and incubated at 37°C for 1hr to remove jird peritoneal cells. The mf were collected from petri dishes, washed with RPMI 1640 medium and used for in vitro maintenance.

Adult worms (both male and female) were recovered from the peritoneal cavity of infected jirds as described above using 18G needle. The adult worms were separated and washed in medium RPMI-1640. Adult worms were also recovered from the infected amicrofilaraemic Mastomys at necropsy. Worms were collected in sterile normal saline by dissecting heart, lungs, kidney, testes and seminiferous tubules. The recovered worms were transferred to medium RPMI-1640. L3 larvae were collected from infected mosquitoes as described above.

3.3 GENERAL MOLECULAR BIOLOGY TECHNIQUES

3.3.1 Polymerase Chain Reaction

The PCR cycling conditions and the template concentration was optimized and standardized for all the genes. The primers used for constructing ALT epitope protein gene is given in Table 3.1. Vector specific T7 primers were used for screening the positive colonies by lysate PCR. Each 50-μL PCR mixture contained 200 μM of each dNTP, 2.5 mM MgCl2, 0.5 mM of each primer, 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3) and two units of high fidelity vent polymerase (NEB, MA, USA). The PCR was carried out in MJ MiniCycler (Bio-Rad, CA, USA) and the cycling parameters are given below.
Table 3.1 List of primers used in PCR amplification of AEP construct

<table>
<thead>
<tr>
<th>S.No</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>AEP EDI  F</td>
<td>5' CGGGATCCGATGAATAAACTTTTAATAGCATTCCGG 3'</td>
</tr>
<tr>
<td>2.</td>
<td>AEP EDI  R</td>
<td>5’ GGAAGATCTTCCGGAGTCATCGAACTCTTCGTC 3'</td>
</tr>
<tr>
<td>3.</td>
<td>AEP EDII F</td>
<td>5' GGAAGATCTTCCGCATGGACTGACAGAGGCTGC 3'</td>
</tr>
<tr>
<td>4.</td>
<td>AEP EDII R</td>
<td>5'CCGGAATTCCGGCTATGCATTGCGGATGCAACCTGC3'</td>
</tr>
<tr>
<td>5.</td>
<td>T7 F</td>
<td>5'-ATTAACCCTCACTAAAGGA-3'</td>
</tr>
<tr>
<td>6.</td>
<td>T7 R</td>
<td>5'-TAATACGACTCCTATAGGG-3'</td>
</tr>
</tbody>
</table>

PCR conditions used for amplification:

i. Gene-specific PCR cycling parameters

Step 1. Initial denaturation: 95°C, 5 min
Step 2. Denaturation : 95°C, 1 min
Step 3. Annealing : 50°C, 1 min
Step 4. Extension : 72°C, 1 min
Step 5. Cycling from step 2 to 4 for 30 more times.
Step 6. Final extension : 72°C, 5 min

Step 7. End

ii. T7-specific primers cycling parameters

Step 1. Initial denaturation : 95°C, 5 min
Step 2. Denaturation : 95°C, 1 min
Step 3. Annealing : 56°C, 1 min
Step 4. Extension : 72°C, 1 min
Step 5. Cycling from step 2 to 4 for 30 more times.
Step 6. Final extension : 72°C, 5 min

Step 7. End

3.3.2 Agarose Gel Electrophoresis

All the PCR amplicons were separated on horizontal submerged gels at 1 or 1.2% agarose (GE Healthcare, CA, USA) and resolved using TBE buffer of pH 8.3 (98 mM Tris, 89 mM boric acid and 2 mM EDTA) at 5–8 V/cm of the gel (Sambrook et al 1989). The gel loading buffer (Fermentas, MD, USA) was used for loading the amplicons and they were stained with 5 μg/mL of ethidium bromide and viewed under UV transilluminator (Bio-Rad, CA, USA). Either 100 bp ladder or 1000 bp ladder (Fermentas, MD, USA) was used as molecular weight markers. The relative amounts of PCR products in the gel were determined by scanning the gel in personal densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

3.3.3 Purification of DNA from Agarose Gel

Amplified gene products from various geographical locations were gel purified individually using Qiaquick gel extraction kit (Qiagen, Hilden, Germany) as described below:

Protocol

1. The expected amplified gene product was excised using a sterile scalpel blade from the agarose gel.

2. Binding buffer, thrice the weight of the excised gel piece, was added and incubated at 50°C until the gel melts completely.

3. Equal volume of isopropanol of the gel weight was added and mixed well.
4. The contents were then transferred to the column and centrifuged at 13,000 rpm for 1 min and the filtrate was discarded.

5. Column was washed with the wash buffer in the ratio 1:4 (wash buffer: alcohol) and centrifuged at 13,000 rpm for 1 min and the filtrate was discarded.

6. The empty column was centrifuged again at 13,000 rpm for 1 min to remove the excess alcohol.

7. The column was then placed in a new collecting tube and 30 µL of sterile water was added and incubated for 1 min and centrifuged at 13,000 rpm for 1 min.

8. The filtrate containing the purified PCR gene product was analyzed in 1.2% agarose gel and quantified.

3.3.4 **Restriction Digestion**

The restriction digestions were performed using enzymes from New England Biolabs, USA, and in the manufacturer-recommended buffers.

1. Restriction enzyme digestions were performed by adding the following:

   - DNA (2–3 µg) : 2 µL
   - 10X Buffer : 2 µL
   - Enzyme (2–3 units/µg of DNA) : 1 µL
   - BSA 10X : 2 µL

2. Total volume was made upto 20 µL with triple distilled water and incubated for 3–4 h at 37°C.
3. The completion of digestion was monitored by agarose gel (1%) electrophoresis.

4. When double digestions were performed, the most appropriate buffer as recommended by the manufacturer was used. Simultaneously the efficiency of each enzyme was verified separately in the selected buffer using control DNA. The domain EDI of ALT-2 was restricted using BamHI and Bgl II while the domain EDII was restricted with BglII and EcoRI. The recombinant clone B.malayi AEP gene was restricted with BamHI and EcoRI.

3.3.5 Ligation

Ligation of digested vector (pRSETB) and insert DNA (AEP) was performed as follows. The ligation mixture consisted of

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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<tbody>
<tr>
<td>10X ligation buffer</td>
<td>10µl</td>
</tr>
<tr>
<td>Vector (50 ng)</td>
<td>2µl</td>
</tr>
<tr>
<td>Insert (50 ng)</td>
<td>6µl</td>
</tr>
<tr>
<td>T4 DNA Ligase (NEB, USA)</td>
<td>1µl</td>
</tr>
</tbody>
</table>

The total reaction volume was made up to 20µl with distilled water and ligation was performed for 16 hours at 16°C and after completion stored at -20°C till use. The ligation mixture was transformed into E.coli host DH5α. The positive clones were further confirmed by restriction digestion and lysate PCR using gene-specific primers to check for the presence of insert. The recombinant construct containing the ALT Epitope Protein gene was named as pRSETB-AEP.
3.3.6 Screening the Clones by Lysate PCR

For screening the recombinant clones, a small portion of freshly grown transformant-positive colony was picked using a sterile toothpick and resuspended in 100 μL of 0.1X TE (1 mM Tris and 1 mM EDTA). The cells were lysed by boiling for 10 minutes, snap-chilled on ice, centrifuged at 12000g for 10 minutes and 1 μl of the supernatant was used as template for PCR (Sambrook et al 1989). Vector-specific T7forward and T7 reverse primer sites were used in lysate PCR. A direct analysis of the lysate PCR will reveal the possible presence of the gene insert. The clones were selected based upon the insert site and archived for further analysis.

3.3.7 Plasmid DNA Isolation

Freshly grown recombinant clones of *B. malayi* AEP were maintained in *E. coli* DH5α maintaining host, deficient for T7 RNA polymerase and hence does not express the foreign protein were used for plasmid DNA isolation. All the plasmids were isolated using plasmid mini isolation kit as per the manufacturer’s instructions (Qiagen, Hilden, Germany). The plasmids isolated were estimated and used for further studies.

3.3.8 Transformation of *E. Coli*

Recombinant plasmids were chemically transformed using CaCl₂ (Sambrook et al 1989) in *E. coli* GJ1158. Competent cells were prepared as stated below:

1. Pre-inoculum from a single colony of freshly revived *E. coli* culture was made in a 3-mL LB broth and grown o/n at 37°C and the control was grown with ampicillin (50 μg/mL).
2. 100 µl of overnight culture was inoculated into 50 mL LB medium in conical flask and allowed to grow at 37°C till OD600 reached 0.6.

3. Culture was chilled on ice for 5 min by gentle swirling and centrifuged at 3500 rpm for 10 min at 4°C.

4. The cell pellet was resuspended in 10 mL of 100 mM ice-cold MgCl2 and incubated on ice for 30 min.

5. Cells were pelleted as in step 3 and the pellet was resuspended in 2 mL of 100 mM ice-cold CaCl2 and incubated on ice for 1 h.

6. Approximately 10–20 ng of DNA was added to 100 µL of above cells and further incubated for 30 min on ice.

7. A heat shock at 42°C was given for 90 s and chilled again on ice for 10 min.

8. About 400 µL of LB medium was added to the tubes and allowed to grow in rotary shaker at 37°C for 45 min.

9. Around 100 µL was plated onto LB agar and the plates were supplemented with appropriate antibiotics.

10. A positive control plasmid (10 ng of pRSET B) was used in all the experiments to verify the transformation efficiency. Cells with no plasmid DNA served as negative controls.

For transformation in *E.coli* (GJ1158) LB medium without NaCl was used in all steps.
3.3.9 Induction of *E. coli* Culture

Recombinant protein expression was studied after inducing with specific inducers.

1. A single colony of fresh transformant was inoculated into 1.5 mL LB/ON and grown overnight (o/n) at 37°C.

2. About 50 µL of the o/n culture was inoculated into 50 mL LB and grown at 37°C with 150 rpm shaking, until OD$_{600}$ of the culture reached 0.6.

3. The culture was induced with 50-300 mM NaCl for GJ1158 strain or 1mM IPTG for BL21 strains and grown for 3 h at 37°C with 150 rpm shaking.

4. The culture was centrifuged at 10,000 rpm for 5 min. The supernatant was discarded and *E. coli* pellet containing the recombinant protein was suspended in 1X PBS and analyzed by SDS–PAGE for protein expression.

3.3.10 SDS–Polyacrylamide Gel Electrophoresis

Recombinant proteins extracted from the above clones were analyzed by SDS–PAGE (Laemmli 1970). The gel compositions of the SDS–PAGE are described below:

i) Monomer solution: 29.2% acrylamide and 0.8% N, N-methylene bis acrylamide in distilled water. The solution was filtered through Whatmann filter paper no.1 and stored in brown bottles at 4°C.

ii) Separating gel buffer: 1.5M Tris, pH 8.8.
iii) Stacking gel buffer: 1.5M Tris, pH 6.8.

iv) Electrophoresis buffer: 0.025M Tris–Cl, 0.192M glycine, 0.1% SDS, pH 8.3.

v) Ammonium persulphate (APS): 120 mg/mL (12%).

vi) SDS: 10% solution.

vii) TEMED

viii) Sample solubilizing buffer (SSB) (5X): 10% SDS, 10% (v/v) β-mercaptoethanol, 50% sucrose, 0.025% bromophenol blue in stacking gel buffer. 1X SSB was added to the cell pellet and resuspended with appropriate volume of 1X PBS and kept in boiling water bath for 10 min.

Depending on the proteins to be separated, 10–15% separating gel and 5% stacking gels were used. Stacking gel was approximately 1/5 of the separating gel. Protein estimations were performed (Bradford 1976) and equal amounts of total protein were loaded in each well. Electrophoresis was performed at room temperature with constant current of 20 mA for stacking gel and 30 mA for separating gel. Gels were stained with staining solution (0.25 g of Coomassie Brilliant Blue R-250 in 45% methanol, 10% acetic acid) overnight and destained with 45% methanol, 10% acetic acid solution until a clear background was obtained.

3.3.11 Western Blotting

After electrophoresis, the SDS–PAGE gel was transferred for Western blotting as described by Towbin et al (1979). The separating SDS–PAGE gel and nitrocellulose membrane (NC) (HyBond, Amersham Pharmacia, U.K) cut to the exact size of separating gel was incubated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS) for
10 min. The nylon mask was laid in the apparatus to block the extra area of transfer. Without trapping air bubbles, the NC was overlaid on the gel and sandwiched between filter papers and scotch brite pads. Electrophoretic transfer was carried out at 120 mA for 90 min using Hoefer TE 70 semi-dry electroblotting apparatus (Amersham Pharmacia Biotech, U.K). After transfer, the molecular weight marker lane was cut and stained with amido black (100 mg amido black in 45% methanol, 10% acetic acid). The rest of the NC was stained with Ponceau S (0.2% Ponceau S [Sigma, St Louis, USA] in 0.3% trichloroacetic acid and 0.3% sulfosalicylic acid) to ensure the transfer of the proteins. Membrane was washed in PBS and blocked overnight at 4°C with 5% non-fat milk powder in PBS. The NC was washed in wash buffer (PBS with 0.05% Tween-20) thrice for 5 min, followed by washing in 1X PBS thrice and then incubated with appropriately diluted primary antibody at room temperature for 1 h. The membrane was washed again as described above and was incubated in recommended dilution of secondary antibody conjugated with alkaline phosphatase for 1 h. After extensive washing, the blot was incubated in detection buffer (100 mM Tris–Cl, pH 9.5, 100 mM NaCl, 5 mM MgCl2) for 10 min. The colour development was achieved using 33 μL of 5-bromo-4-chloro-3-indolyl phosphate (50 mg/mL in dimethyl formamide; USB, Amersham Pharmacia) and 66 μL of nitroblue tetrazolium (50 mg/mL in 70% dimethyl formamide; USB, Amersham Pharmacia) in 10 mL of detection buffer. The reaction was stopped after 15 min by adding 10 mM EDTA. Primary antibodies, mouse monoclonal anti-His (Sigma, St Louis, USA), diluted at 1:20,000 in 1X PBS was used in detecting recombinant fusion protein expressed. Various human clinical sera such as microfilareamics (MF), chronic pathology (CP), endemic normals (EN) and non-endemic normals (NEN) was used at 1:100 dilution in human sera immunoblot analysis. Mouse anti-ALT (1:5000), mouse anti-TRX (1:1000), mouse anti-TGA (1:1000) and mouse anti-TPX-1 (1:5000) were used. The secondary antibodies anti-human (Sigma, St. Louis, USA) and anti-mouse (Sigma, St.Louis, USA) IgG-ALP conjugate were used at 1:1000 dilution.
3.3.12 Sample Preparation for Purification

The recombinant protein cultures were centrifuged and the cell pellet was resuspended at 2.5 g wet weight per mL of lysis buffer comprising 50 mM Na$_2$HPO$_4$–NaH$_2$PO$_4$, 50 mM Tris, 400 mM NaCl, 10 mM imidazole, pH 8, and 0.3 mg/mL lysozyme. The suspension was incubated at 4ºC for 1 h and subjected to sonication for five cycles with 30 seconds on and 30 seconds off. To the cell lysates, 1 mM PMSF and 1 mM β-mercaptoethanol were added and centrifuged at 12000rpm for 10 min prior to purification. The clear supernatant was loaded in the column for purification.

3.3.13 Purification by Immobilized Metal Affinity Chromatography (IMAC)

The recombinant protein BmTGA was expressed with 6 histidine residues as an N-terminal fusion peptide. The metal binding domain in the fusion peptide allows simple one step purification of recombinant protein by IMAC (Crowe et al 1995). Briefly the following protocol was adopted for purification:

i) The column was equilibrated with 3 column volumes binding buffer (pH 8.0). Samples were applied to the NiCl$_2$ charged NiNTA column (Amersham Pharmacia Biotech, Hong kong) at the rate of 5mg of recombinant protein per ml of column matrix and allowed to bind.

ii) Column was washed with elution buffer (0.1M phosphate buffer pH 6.5), to remove all contaminating proteins, followed by elution with increasing concentrations of imidazole (25mM - 250 mM in elution buffer)
iii) The protein was eluted at 150mM and 200mM imidazole concentration. The purity of the protein fractions were checked on SDS-PAGE and the presence of histidine-tagged recombinant protein was confirmed by immunoblotting with anti-histidine tag antibodies (anti-HisG, Invitrogen, Sandiego, USA).

iv) The protein fractions were dialyzed against 0.1x PBS overnight at 4°C, in snake-skin dialysis bags (Pierce, Rockford, USA) and then concentrated by vacuum concentrator. The concentration of the protein was determined by Bradford method (Bradford 1976) using Bradford reagent (BioRad, California) and stored in aliquots at -80°C in aliquots till further use.

3.3.14 Purification by Gel Elution

After resolving the proteins in SDS-PAGE, the gels were stained using 1M KCl for 5-10 min’s. The protein band of interest was located and excised. The gel pieces were rinsed in 1M NaCl for 10-20 minutes till the gel becomes clear. The proteins were then electroeluted (Hager and Burgess 1980) in a dialysis bag filled with 500 µl Tris-Glycine buffer (0.025M Tris–Cl, 0.192M glycine) for 3 hrs. It was dialysed against Millipore water with three changes overnight to remove the salts.

3.4 PEPTIDES

3.4.1 Epitope Prediction and Synthesis of Candidate Peptides

The bioinformatics tools BcePred (Saha and Raghava 2004) and IEDB (Peters et al 2005) were used for B epitope prediction which utilizes the physiochemical properties of protein like hydrophilicity, flexibility and
surface probability. For prediction of HLA binding peptides, SVMHC based on support vector machines (Donnes and Elofsson 2002) and Propred (Singh and Raghava 2001) were used to identify putative T epitopes. T-cell epitopes for H-2\textsuperscript{d} class II alleles (I-E\textsuperscript{d} and I-A\textsuperscript{d}) were predicted using the PRED\textsuperscript{BALB/c} (Zhang et al 2005).

The epitopes of the filarial vaccine targets TRX, TGA and TPX in the regions non-homologous to host proteins were analyzed. Based on the protein sequence homology analysis and \textit{in-silico} epitope prediction, peptides carrying B and T epitopes in the host non-homologous regions were screened. Thus five such peptides were identified and named as - TRX\textsubscript{P1}, TRX\textsubscript{P2}, TGA\textsubscript{P1}, TGA\textsubscript{P2}, and TPX\textsubscript{P1}.

TRX\textsubscript{P1} : NH\textsubscript{2}- ADLLANINLKADGTVKKGSDLANK-COOH
TRX\textsubscript{P2} : NH\textsubscript{2}- SEIEKLKNKYEVAGIP-COOH
TGA\textsubscript{P1} : NH\textsubscript{2}- KEFLLHETNGLVGIRTSENRYQFD-COOH
TGA\textsubscript{P2} : NH\textsubscript{2}- PKNRKDKPEPYSGGREVDDFIKYIAKHATEE-COOH
TPX\textsubscript{P1} : NH\textsubscript{2}- GIRPLGPKNKAPDFS-COOH

The B and T epitopes of ALT-2 protein were predicted and eight peptide regions carrying either putative B or T or both epitopes were identified named in order from P1 to P8 as given below.

**ALT Peptides**

P1 : NH\textsubscript{2}-NKLIAFGLVILLVTLPCASESDEEFDDG-COOH
P2 : NH\textsubscript{2}-SEDEEFDDSADDTDDSEAGGGS-COOH
P3 : NH\textsubscript{2}-SEGGDEYVTKGEVVETD-COOH
P4 : NH\textsubscript{2}-EVVETDGKKKECSS-COOH
P5 : NH₂-HEACYDQREPQAWCRP-COOH
P6 : NH₂-YDQREPQAWCRPNENQSWT-COOH
P7 : NH₂-WTDKGCFCEDKLHSCVIERKNNGKLE-COOH
P8 : NH₂-GKLEYSYCAPEAGWQCA-COOH

3.4.2 Peptide Synthesis and Entrapment

The peptide sequences were synthesized by solid phase technique, using f-moc chemistry and assembled on wang resin. The peptides were purified and the purity was analyzed by high performance liquid chromatography. Micro particles with entrapped peptide were prepared using poly (poly-DL-lactide-co-glycolide)/ PLG (50:50, mol wt. 84 kDa) microparticle by water-in-oil-in water (w/o/o) solvent evaporation technique (Tripathi et al 2006). The peptides were synthesized and encapsulated by Dr. D.N. Rao, Department of Biochemistry, AIIMS, New Delhi.

3.4.3 Synthesis of Chimeric Peptide Conjugates

The chosen epitope peptides were synthesized as a single conjugate with two Glycine residues as spacers in between the two peptide sequence by F-moc solid phase technique and encapsulated in microparticles by water-in-oil-in water (w/o/o) solvent evaporation technique. The peptide conjugates were named as PC1, PC2 and PC3 and their constituent peptides with sequences are given below.

PC1: TRX<sub>P1</sub>-TRX<sub>P2</sub>

NH₂-ADLLANINLKKADGTVKKGSDALANK-GG-SEIEKLKNKYEVAGIP-COOH
**PC2: TRX\textsubscript{P1} -TGA\textsubscript{P1}**

\[\text{NH}_2\text{-ADLLANINLKKADGTVKKGSDALANK-GG-KEFLLHETNGLVGIRTSENRYQFD-COOH}\]

**PC3**: TRX\textsubscript{P2} -TGA\textsubscript{P1}

\[\text{NH}_2\text{-SEIEKLKNKYEVAGIP-GG-KEFLLHETNGLVGIRTSENRYQFD-COOH}\]

### 3.4.4 Estimation of Peptide Concentration

The peptides were estimated using BCA method (Smith et al 1985) which is a more sensitive assay for estimation. The peptides were by adding suitable solvents. 10 \(\mu\)l of peptide sample was added to 190 \(\mu\)l of BCA (Bicinchoninic acid) working reagent (Pierce, Rockford, USA) and incubated at 37\(^\circ\)C, dark, for 30 min. The Reaction mixture was incubated in dark for 30 minutes and the absorbance was read at 540 – 590 nm.

### 3.5 Immunological Studies

#### 3.5.1 Human Sera Samples

All serum samples used in this study were obtained from volunteers at Chennai, Vellore and Chengalpet, India. Informed consent was obtained from all patients in accordance with U.S. Department of Health and Human Services Human Experimentation Guidelines and Department of Public Health, Chennai, Tamil Nadu, India. All the procedures followed were in accordance with the guidelines issued by Department of Public Health, Government of Tamil Nadu, India, for dealing with human subjects. The Institutional review board at the Center for Biotechnology, Anna University, India also approved the protocols. Sera were classified into MF, CP or EN based on the detection of circulating parasites, parasite antigens or by
evaluating clinical symptoms of lymphatic filariasis. Circulating microfilariae were detected in the blood of subjects as described previously (Rao et al 2000). The presence of circulating antigen was detected using an Og4C3 kit (Lalitha et al 1998) and a WbSXP-based enzyme-linked immunosorbent assay (ELISA) (Rao et al 2000). Subjects with no circulating antigen or microfilariae were classified as EN, whereas subjects with circulating microfilariae and/or circulating antigen, as detected by ELISA, were considered as MF. Subjects showing lymphedema and other visible clinical symptoms of filariasis were grouped into CP. (Control non-endemic normal (NEN) sera were kindly provided by Professor Murray Selkirk, Imperial College London, London).

3.5.2 Immunoreactivity with Clinical Sera

The optimum dilutions for assay reagents were determined by titration, and the blocking/assay conditions were determined by a series of comparative trials. BmTRX, TGA, TPX, ALT and AEP antigens were (100 ng/well) diluted in coating buffer (0.1M carbonate/bicarbonate, pH 9.6). The antigens were then coated in 96-well plates (Nunc Maxisorp, Nalge Nunc International, Denmark) and incubated o/n at 4°C. For ELISA of peptides, wells of polystyrene microtitre plates (Immulon II HB, Dynatech, ThermoFisher) were coated with 500 ng of peptides in carbonate buffer and incubated at 4°C o/n.

After washing three times with PBS-T, the plates were blocked with 5% skimmed milk powder at 37°C for 1 h. Human clinical sera of various stages, viz., MF, CP, EN and NEN, were diluted in PBS-T (1:100), added to the wells (100 μL/well) and incubated at 37°C for 1 h. After washing with PBS-T, human anti-IgG alkaline phosphatase conjugate (Sigma, St Louis, USA), (1:1000 dilution in PBS-T) was added (100 μL/well) and incubated for 1 h at 37°C. Plates were washed three times with PBS-T and the
substrate pNPP (p-nitrophenyl phosphate, disodium salt) was added to the wells (Sigma, St Louis, USA) at 1mg/ml in substrate buffer (NaHCO$_3$ -0.84 g/L; Na$_2$CO$_3$ - 1.25 g/L; MgCl$_2$ -0.2 g/L). The absorbance was measured 405 nm after 30 min using a micro plate ELISA reader (BioTek Instruments, Inc., USA).

### 3.5.3 Human Isotype ELISA

Human antibody isotypes were determined to measure the efficacy of the antigens in eliciting the immune responses. The pattern of IgG subclass elicited by different antigens was measured. The plates were coated with the antigens, and the human clinical samples were used as primary antibody as described above. After washing, mouse anti-human IgG isotypes (IgGl [1:500], IgG2 [1:2000], IgG3 [1:5000] and IgG4 [1:5000]) were added as secondary antibodies (Sigma, St Louis, USA) and incubated at 37°C for 1 h. After washing the plates with PBS-T followed by PBS, mouse anti-human IgG-ALP (Sigma, St Louis, USA) conjugate was added (1:1000 dilution) as secondary antibody and incubated for 1 h at 37°C. Plates were washed three times with PBS-T and pNPP (p-nitrophenyl phosphate, disodium salt) substrate was added (Sigma, St Louis, USA). The optical density was read at 405 nm after 30 min.

### 3.5.4 Human PBMC Proliferation Assay

Peripheral blood mononuclear cells (PBMC’s) were isolated from heparinized venous blood collected from endemic normal individuals (n=10) by gradient centrifugation over lymphocyte medium (Lymphoprep; Nycomed Pharma AS, Oslo, Norway). All the procedures were performed in aseptic conditions under a laminar hood. The cells were washed and the viability was determined by trypan blue dye exclusion method. The cells were then cultured in round-bottomed microtiter plates at a concentration of $0.2\times10^6$
cells/well in RPMI 1640 supplemented with gentamicin (80 μg/ml), 25 mM HEPES, 2 mM glutamine and 10% FCS. The cells were stimulated with protein (1 to 20 μg/ml), peptides (5-10 μg/ml), soluble crude extract of BmL3 (10 μg/ml) and positive control PHA (10 μg/ml). An unrelated peptide sequence (TRX 72-90) was used as a negative control and Wells with medium alone were used as unstimulated controls. Cultures incubated for 72 h. at 5% CO2 and 37°C. The cultures were pulsed with [H3] thymidine (USB, Amersham Pharmacia, UK) at 0.5 μCi per well. The cells were incubated for 18 h and harvested onto glass fiber disks (Millipore, Massachusetts, USA) placed on 96 well opaque opti plates (Perkin Elmer, MA, USA). The filter disks were allowed to dry completely and 50μl of MicroScint 20 scintillation fluid (Perkin Elmer, MA, USA) was added to the wells. The amount of thymidine incorporation was measured by a TopCount liquid scintillation counter (Packard, CT, USA). Proliferative responses were expressed as stimulation index (SI) calculated as counts per minute (cpm) of cells stimulated with antigen divided by cpm of unstimulated cells (Ausiello et al 1986). All cultures were taken in triplicates and the results expressed as mean S.I ± SEM.

3.5.5 Animal, Immunization, Sera Collection

Six to Eight weeks old female BALB/c (H-2d) mice were procured from TamilNadu Veterinary and Animal Sciences University, Chennai. All experiments were performed in accordance with ‘Indian Animal Ethics Committee’ regulations. A group of 6 mice were injected via intraperitoneal route with: 30 μg of protein in PBS and mixed with alum at 1:1 ratio to a total of 100 μl. Peptides were immunized via intramuscular route with 50 μg-peptide equivalent in microspheres suspended in 100 μl of PBS. The control group of mice received alum alone in 100 μl PBS. For all proteins same dose of booster was given on days 7,14 and 21 whereas for peptides same dose of booster was given on days 14 and 28. Blood was collected on days 0, 14, 28
and 42. For peptide immunized mice sera was collected on days 0, 28, 42, 60, 74 and 88 since microsphere encapsulation provides long-lasting immunity due to sustained release of antigen. About 200 ~ 300 µl of blood was collected by tail bleeding in mice. The blood was allowed to clot and centrifuged at 2500 rpm for 10 min. The sera were separated and stored at −20°C until further use.

3.5.6 Measurement of Total IgG

Peptide/Protein specific IgG levels in the mice sera were determined by ELISA as described above. 96-well microtiter plates were coated with 100 µl of peptide (500 ng/ well) or protein (100 ng/well). After washing and blocking with 5% skimmed milk powder, a serial two-fold dilution (1:500-1,28,000) of antisera was used. Antibody titers were assessed as the highest serum dilution giving an absorbance (0.15) higher than that of preimmune sera. The color was developed using p-nitrophenyl phosphate substrate (1 mg/ml) in substrate buffer and absorbance was read at 405nm.

3.5.7 Mouse IgG Isotypes

For estimating IgG isotypes, mice sera (dilution 1:100) from different immunization groups were incubated for 1 h at 37°C, with respective peptides/protein coated on ELISA plates. The IgG isotype binding was detected using secondary rabbit anti-mouse IgG specific for each subclass (Pierce Isotyping kit, Pierce, USA,) as per the manufacturer instructions. The absorbance was read at 405 nm.

3.5.8 Direct Binding Assay

The plates were coated with peptide (500 ng/well) in high-binding 96-well titre plates (Thermo Scientific, MA, USA) and incubated o/n at 4°C. The plates were washed with PBS-T followed by PBS and blocked in 5%
skimmed milk powder as described above. The anti-sera raised against corresponding proteins in mice were diluted in 1% skimmed milk powder (1:1000) and incubated at 37°C for 2 h. After washing as described above, anti-mouse IgG-ALP (1:1000) (Sigma, St Louis, USA) was added and kept at 37°C for 1 h, washed and reacted with pNPP (p-nitrophenyl phosphate, disodium salt) substrate system (Sigma, St Louis, USA). The optical density of the reaction product was read at 405 nm after 30 min (Tripathi et al., 2006).

3.5.9 Competitive Inhibition ELISA

The inhibition assay was done for peptides to determine the specific binding affinity of peptides with antibodies raised against corresponding protein (Tripathi et al 2006). ELISA plates were precoated with recombinant proteins TRX, TGA or TPX (100ng/well) and blocked with 5% skimmed milk powder. Different amounts of peptides derived from the respective protein (0.5- 50 μg) were incubated with anti-TRX, anti-TGA or anti-TPX sera (1:1000) for 1 hr at 37°C. TRX, TGA and TPX antigens were also incubated at different amounts (1-10 μg) with respective antisera. The solution was transferred onto the precoated plates and incubated for 1 hr at 37°C. The plates were then developed after washing as described above using IgG-ALP secondary conjugate and pNPP substrate. The peptides with high affinity for the antibodies act as the competitor binding strongly during incubation and show a fall in the O.D_{405nm} due to inhibition of normal antigen-antibody interaction. The peptides showing weak binding are displaced quickly and thus the antibodies bind the respective protein coated in the plate instead. The results were expressed as the percentage of antibody binding in presence of competitor peptide as compared to the control value in the absence of competitor. The IC50 values were determined from graphs plotted for percent binding versus concentration of competitor.
3.5.10 Dot Blot Analysis

To analyze the reactivity of the antisera with native parasitic antigens, dot blot assay was performed (Ermens et al 1997). The NC membrane was cut in appropriate size and dipped in transfer buffer. The membrane was placed above a filter paper strip in the dot blot apparatus and fixed firmly. 500 ng of recombinant protein or 500 ng of peptide conjugate or 1 µg of crude protein extract from *B. malayi* mf, L3 or adult parasites were added onto the separate slots of the dot blot apparatus. Vacuum was applied to the apparatus for 30 seconds and the blot was blocked with 5% skimmed milk powder. After washing the blot, antisera at appropriate dilutions were incubated for 1 hr 37°C. TRX antiserum was also used as positive control. After washing, bound antibodies were detected with alkaline phosphatase conjugated goat anti-mouse IgG and NBT-BCIP substrate (Sigma).

3.5.11 Splenocyte Proliferation Assay

All the procedures were performed in aseptic conditions under a laminar hood. The peptide and protein immunized animals were sacrificed on day 60 and the spleens were removed aseptically (Anand et al 2008). Splenocytes were separated and washed twice with fresh culture medium (RPMI 1640). Lysis buffer (0.1% ammonium chloride) was added to the pellet to remove the RBC’s and the cells were counted. The single cell suspension was cultured in triplicate in 96well plates at 2 x 10^5 cells/ml in RPMI 1640 medium (100 µl/well) (Sigma, St. Louis, USA) supplemented with gentamycin (80 µg/ml) (Ranbaxy Laboratories, India), 25 mM HEPES (USB, Amersham Pharmacia, UK), 2 mM glutamine (USB, Amersham Pharmacia, UK) and 10% fetal bovine serum. The cells were then stimulated *in vitro* with protein (0.1, 1, 5 and 10 µg/well), peptides (1, 5, 10, 25 µg /well) or positive control Con A (1µg/well). Wells with medium alone were used as unstimulated controls. The cultures were pulsed with [H3] thymidine (USB,
Amersham Pharmacia, UK) at 0.5 μCi per well. The cells were incubated for 18 h and harvested onto glass fiber disks and thymidine incorporation was measured by a liquid scintillation counter. Proliferative responses were expressed as stimulation index (SI) calculated as counts per minute (cpm) of cells stimulated with antigen divided by cpm of unstimulated cells. All cultures were taken in triplicates and the results expressed as mean S.I ± SEM.

3.5.12 Cytokine Assays

Separate cultures were set up for cytokine assay. Spleen cells were washed and plated in 24-well flat-bottomed tissue culture plates (Costar) at 4×10⁶ cells/ml in a volume of 1 ml of RPMI 1640 supplemented with 10% fetal calf serum and 80μg/ml gentamycin. The cells were stimulated as described above and incubated for 72 hrs. Supernatants were harvested and stored at −80°C. Capture ELISAs (Pierce Biotechnology, Rockford, IL) were performed to detect IFN-Gamma, IL-2, IL-4, IL-5 and IL-10 from culture supernatants according to the manufacturer’s protocol. ELISA plates were coated with optimal concentration of capture antibody (mouse anti-mouse IL-2, IL-4, IL-5, IL-10 or IFN-γ) in PBS and incubated overnight at 4°C. The wells were washed thrice using wash buffer (PBS with 0.05% of Tween-20) and then incubated with blocking buffer (1% BSA and 0.05% sodium azide in PBS) for 2 h at room temp. The wells were washed and incubated with 100 μl volume of culture supernatants collected from 72 h old culture (for IL-2, IL-4, IL-5 and IL-10 estimation) and 96 h old culture (for IFN-γ estimation). Followed by another wash, the wells were incubated, 100 μl of optimal concentration of biotin labeled detection antibody for 2 h at room temp.

The wells were washed and further incubated with optimally diluted (1:200) streptavidin-HRP conjugate for 1 h. After final washing, the wells were incubated with 100μl of the substrate Tetramethyl benzidine
The reaction was stopped after 20 min by adding stop solution (50 µl/well of 1N H₂SO₄) and the absorbance was measured at 450 nm wavelength. All concentration values were derived from standard curves run simultaneously and data expressed in pg/ml after deducting the values of unstimulated cultures.

3.6 IMMUNOPROPHYLACTIC STUDIES

3.6.1 Animals for Protection Study, Immunization and Parasites

Four to Six week old, male *Mastomys coucha* were used in this study (Lok and Abraham 1992). Animals were obtained from Mahatma Gandhi Institute of Medical Sciences, India. Mastomys were handled in accordance with the institutional guidelines, and was approved by an Institutional animal care committee. Six animals were used per group. The animals were immunized with 50 µg of recombinant protein in alum or 100 µg of peptide conjugates in microspheres suspended in PBS. Four doses at weekly intervals were administered intraperitoneally for proteins or intramuscularly for peptide conjugates. The control group received PBS alone in alum. Sera collected periodically after immunization was used to check the antibody titre by ELISA. For *Brugia malayi* infection, the mosquito colony and parasites were maintained as described previously (Thirugnanam et al 2007). Twelve days after infection, *B. malayi* L3 larvae were obtained by crushing the insects (Suzuki and Seregeg 1979) and carefully removing the L3s. Larvae were counted under a microscope and used for protection study.

3.6.2 Preparation of Micropore Chambers

Micropore chambers were assembled using 14X2 mm plexi glass rings and 3.0µm nucleopore polycarbonate membranes. Membranes were attached to the plexi glass rings with mf cement (Millipore, MA, USA). Chambers were sterilized at 80°C in oven for 10 hrs. The chambers were loaded, under a dissecting microscope, with ten L3 larvae in media via an
aperture (1mm diameter) at the side of plexi glass ring, which was subsequently sealed by MF Cement (Millipore).

3.6.3 Protection Study in *Matomys coucha*

Protection study was done by micropore chamber method as described previously (Abraham et al 1993 and Chenthamarakshshan et al 1995; Weiss and Tanner 1979). After the final booster immunization, animals were challenged by intraperitoneal surgical implantation of a micropore chamber containing 10 live *B. malayi* L3 larvae in RPMI 1640 media (Gnanasekar et al 2004). Mice were anaesthetised with sodium pentobarbital, and the chambers with live L3 were inserted through a small incision of 3 mm in the peritoneum. After placing the micropore chamber the peritoneum was stitched with surgical suturing needle. Strict aseptic conditions were followed for surgical procedures. Betadine and neosporin were applied to the peritoneum of each animal. Housing, handling and treatment of mice were performed in accordance with animal ethical committee guidelines.

![Figure 3.1](image_url)

(a) *Mastomys coucha*  
(b) Micropore Chamber experiment

**Figure 3.1** Protection Study for Evaluating the Immunoprophylactic Efficacy of Filarial Vaccines. (a) The Animal Model for Filarial Infection, *Mastomys coucha* and (b) the in *Situ* Micropore Chamber Challenge Experiment.
After 48 h the micropore chamber was harvested and the contents were removed onto a glass slide and examined microscopically for cytotoxicity. The number of live and dead L3 was enumerated by observing movement of L3 under a Nikon microscope. The percentage protection was expressed as the average number of worms recovered from the control animals minus average number of worms recovered from the vaccinated animals ÷ average number of worms recovered from the control animals x 100.

3.7 STATISTICAL ANALYSIS

All statistical analyses were done using Graphpad prism software version 5.0. The difference in two means was compared using non-parametrical analysis of Student’s t-test. For multiple comparisons, non-parametric Kruskal-Wallis test was used along with the Bonferroni’s post test. For T cell proliferation studies Two way ANOVA was used. A probability (p) value ≤ 0.05 was considered statistically significant.