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 like 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). Maxisorp (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-\(\gamma\), 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) 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 100 µg/mL of ampicillin or 50 µg/mL of kanamycin or 35 µg/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 (35 µg/mL) and ampicillin (100µg/mL) in LB. Luria Bertani broth without NaCl was used to propagate GJ1158 strain. The chemicals were procured from Merck, Germany. For Pichia pastoris expression, YPG medium was used as follows: yeast extract 10- g L; peptone- 20 g; glycerol- 20 g in 1 L distilled water.

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. Pichia pastoris strain X-33 was used for expression of clones in pPICZα vector Invitrogen (San Diego, CA). The vector map of pPICZα is shown in Appendix 3.
3.1.4 Expression Systems Used in the study

E. coli T7 expression system

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 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 at 1 mM final concentration.
Further, BL21 (DE3) being a lon protease deficient strain protects the expressed heterologous proteins from proteolytic cleavage.

The GJ1158 strain, which is a genetically engineered strain of BL21 (DE3) (Bhandari et al 1997) 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. 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 its increased stability makes GJ1158 a very suitable expression host.

**P. pastoris AOX system**

In the production of recombinant proteins, yeasts have greater advantage over both bacterial and mammalian systems. The eukaryotic yeast offers the capacity to post-translationally modify the secreted protein and also glycosylate in par with mammalian expression system. *Pichia pastoris* has become an ideal host for the expression of recombinant proteins (Cereghino and Cregg 2000) due to the contribution of following factors: it can be easily manipulated at the molecular genetic level (e.g. gene targeting, high-frequency DNA transformation, cloning by functional complementation); it can express proteins at high levels due to its highly efficient and tightly regulated promoter from the alcohol oxidase I gene (AOX1); and it can perform many ‘higher eukaryotic’ protein modifications, such as glycosylation, disulfide-bond formation, and proteolytic processing.
P. pastoris, a methylotrophic yeast is one of approximately a dozen yeast species representing four different genera that are capable of metabolizing methanol as its sole carbon source. Recombinant P. pastoris strains are obtained by transforming the host strains with the constructed plasmid, which on electroporation gets integrated into the chromosome at a specific locus and generates a genetically stable transformants / clone. Methanol induces a specific methanol utilization pathway leading to expression of key enzymes under the control of tightly regulated promoters. One of these key enzymes, alcohol oxidase (AOX), catalyses the oxidation of methanol to formaldehyde and hydrogen peroxide. The AOX1 gene expression is controlled at the level of transcription and the presence of methanol is essential to induce high levels of transcription. About 5 % of RNA isolated from methanol grown cells is from the AOX1 gene, whereas AOX1 message is undetectable in cells grown on any other carbon source (Tschopp et al 1987). In a bioreactor, cultures with methanol feeding at growth limiting rates, AOX1 transcription levels can be as high as 30 % of total soluble protein.

For expression in Pichia pastoris system shake flasks containing 100 mL of the media were inoculated with 1 mL of Pichia culture, which were grown in 3 mL test tubes for 24 h. The shake flasks were kept incubated for approx. 36 – 48 h at 30 °C in a shaker and after the cultures attained stationary OD 600 (around 40), methanol was added to the flasks at a concentration of 0.5 % for every 24 h. Samples of the induced cultures were withdrawn for every 12 h intervals and were analyzed for the growth and recombinant expression profiles for upto 4 days post - induction. Samples were collected for further analysis like activity profile, amount of protein expressed after induction by SDS - PAGE and protein estimation by Bradford method.
3.1.5 Recombinant Proteins Used in the Study

The clone *W. bancrofti* Thioredoxin (TRX-1) (Genbank Protein Acc. No. AAN34968) without histidine tag was subcloned in pRSETB which were confirmed by DNA sequencing and used for all the studies. *W. bancrofti* Glutathione transferase (GST) (Acc No. AAO45827), Abundant Larval Transcript (ALT-2) (Acc No. AAB41884) and Venom Allergen Homologue (VAH) (Acc. No. AAK12274) cloned in pRSETB were available in the laboratory. *B. malayi* Glutathione Peroxidase (GP29) (Acc. No. CAA48881) was a kind gift from Prof. Murray Selkirk, Imperial College, London. The recombinant proteins were further purified and used for all the analysis.

3.2 CRYSTALLOGRAPHY AND STRUCTURE ANALYSIS

3.2.1 Crystallization of the Proteins

The purified recombinant proteins were evaluated for polydispersity and aggregates were removed by ultracentrifugation/ultrafiltration to yield homogenous monodispersed solutions. The proteins were immediately subjected to different stages of crystallization protocols.

3.2.2 Dynamic Light Scattering (DLS)

Dynamic Light Scattering (also known as Photon Correlation Spectroscopy (PCS)) detects the fluctuations of the scattering intensity due to the Brownian motion of molecules in solution. The back scattered light will have a frequency shift (Doppler shift) and the resulting intensity variation can be recorded by a highly sensitive detector. Thus DLS measures the velocity of the Brownian motion and relates it to the particle size by the Stokes-Einstein equation (Equation 3.1).
This equation can be used to convert the measured diffusion coefficient \( D \) into a hydrodynamic radius (diameter) \( d_H \) which is the size of a sphere that has the same diffusion behaviour. This equation also links diffusion velocity with temperature \( T \), Boltzmann constant \( k_B \), the viscosity \( \eta \) of the buffer. The statistics of the scattering signal can be further analysed with a correlator and the resulting correlation function can be inverted to find a size distribution for the biomolecules like proteins in solution (Pecora 1985, Berne et al 2000). This enables effective investigation of purified proteins in terms of homogeneity, integrity and stability.

The Dynamic Light Scattering was measured using Laser-Spectroscatter 201 (Molecular Dimensions, UK). Analysis of the autocorrelation function in terms of particle size distribution was done by program CONTIN (Provencher 1982). The result in terms of particle size distribution for monodispersed samples were obtained by fitting the decay of the correlation function.

### 3.2.3 Pre-Crystallization Test

The Pre-Crystallization Test (PCT) was used to determine the optimized protein concentrations for crystallization screening. Highly concentrated samples resulted in amorphous precipitate, while diluted samples had produced transparent drops. Amorphous precipitate and clear drop production was avoided by changing the protein concentration accordingly (PCT™, Hampton Research, USA).

The four reagents of PCT kit, used to evaluate protein concentration for crystallization screening, are:
1. Reagent A1: 0.1M Tris Hydrochloride pH 8.5, 2.0M Ammonium Sulfate
2. Reagent B1: 0.1M Tris Hydrochloride pH 8.5, 1.0M Ammonium Sulfate
3. Reagent A2: 0.1M Tris Hydrochloride pH 8.5, 0.2M Magnesium Chloride Hexahydrate, 30% w/v Polyethylene Glycol 4,000
4. Reagent B2: 0.1M Tris Hydrochloride pH 8.5, 0.2M Magnesium Chloride Hexahydrate, 15% w/v Polyethylene Glycol 4,000

3.2.4 Robotic Screening for Crystallization

Honeybee-961 sitting-drop crystallization robot (Genomic Solutions, Irvine, CA, USA) and Zinsser Pipetting Robot (Digilab Genomic Solution, Germany) were used for setting up the crystallisation trials. Crystal screening was carried out using Qiagen Crystallisation Suites (Jancarik and Kim 1991, The Classic Lite Suite, USA) in 96 well plates with a reservoir solution of 100 µL and 0.1 µL of protein solution equilibrated with equal volumes of reservoir solution. The plates were sealed with airtight transparent films and were incubated at 20°C. The plates were observed periodically for crystal growth by research stereo microscope (Olympus SZX12). The crystallization conditions of wells with visible signs of crystal growth were noted for further optimisation.

3.2.5 Optimization of Crystals

The positive crystallisation conditions obtained in the high throughput screening were further optimized in 24-well VDX plates (Hampton Research, USA) by hanging-drop vapour-diffusion method
(McPherson 1982). The drops were prepared on siliconized cover slips and
2μl of protein solution (7 to 15 mg ml\(^{-1}\) of Wb-GST in 50 mM TrisHCl pH 8.0,
15 mM GSH) was mixed with an equal volume of reservoir solution and
equilibrated against 1 mL of optimised reservoir solution (0.2 M magnesium
acetate, 0.1 M Sodium cacodylate, pH 6.5, 10% (w/v) PEG 8000) and
incubated at 20° C for ten days. The crystals of optimal sizes (0.2 x 0.2 x 0.3
mm) were selected for diffraction studies. Rigaku Rotating anode generator,
Rotaflex RU-200B series with a PSPC (position sensitive proportional
counter) type microdiffractometer with auxiliary cabinet, H.V. transformer,
water circulator and an IP scanner, was used for screening crystals based on
diffraction in 15% glycerol as cryoprotectant. The crystals that diffracted
well were later transported intact in cryo vials along with cryo-loop in liquid
nitrogen for diffraction data collection in synchrotron beamline.

3.2.6 Data Collection

3.2.6.1 Diffraction Data

A single crystal of Wb-GST was mounted on a nylon loop and
flash-cooled in cold nitrogen-gas stream at 100 K. The X-ray diffraction data
was collected by exposing the crystal at the synchrotron Consortium-
Beamline X13 DESY (0.8123 Å), Hamburg. All intensity data were indexed,
integrated and scaled with the HKL-2000 package (Otwinowski et al 1997)
and Mosflm of CCP4i suite (Potterton et al 2003). Molecular Replacement
(MR) strategy was used for generating the phase information of Wb-GST.
The program Molrep of CCP4i suite (Potterton et al 2003) was used in
calculating the phases and the pi-class GST from human river blindness
parasite Onchocerca volvulus (Protein Data Bank entry code 1TU7) was used
as the search model.
3.2.6.2  Model Building and Refinement

The preliminary three dimensional structure model of Wb-GST after solving the phase was built in an iterative fashion using the macromolecular model building software Crystallographic Object-Oriented Toolkit (COOT) (Emsley et al 2004) and was refined in a tandem manner with the program Refmac5 (Murshudov et al 1999, Vagin et al 2004). Systematic model building of Wb-GST involved idealization of amino acids residues in line with electron density maps, real space refinement, manual rotation/translation, rigid-body fitting, ligand search, solvation, mutation, rotamers validations, Ramachandran plots, skeletonization and non-crystallographic symmetry validation with COOT. The atomic model of Wb-GST was refined by adjusting the model parameters (coordinates, B-factors, TLS etc.) in order to obtain the model which best explains the experimental data (i.e. maximizes the likelihood).

The progress of model building was monitored and brought to completion by reduction in the likelihood scores of the R-factor and Free R-factor (Brunger et al 1993). Solvent molecules were automatically added at the end of the refinement process using the program COOT add water features and checked based on chemically reasonable positions where difference density exceeded 3 sigma. The ‘Check/Delete Waters’ routine and peaks in the mFo–DFc difference electron-density maps were used to detect additional problem areas in the refinement results. COOT was used to evaluate anomalies detected by WHAT_CHECK in the context of the experimental data. The ‘Check/Delete Waters’ routine and peaks in the mFo–DFc difference electron-density maps were used to detect additional problem areas in the refinement results. The quality of the final model was verified using the program PROCHECK (Laskowski et al 1993).
Overall Methodology adapted for Determination of \textit{Wb-GST} Structure

1. Cloning/Expression/Purification and Concentration of the recombinant protein – Purification of proteins in reproducible quantities preferably by affinity chromatography methods

2. DLS (Dynamic Light Scattering) & PCT (Precrystallisation Test) studies – suitability of proteins in terms of homogeneity and concentration for crystallization

3. High throughput screening (HTS) for crystallization with various conditions (Honeybee 96 well plates/Robotic handling)

4. Positive hits of HTS reproduced and amplified – Hanging drop vapor diffusion method 24 well plates

5. Crystal screening by preliminary X-ray diffraction studies – Bench top rotating anode with cryo-protectants

6. Enhanced X-ray diffraction data collection using synchrotron radiation at beam line with cryo-protectants

7. Processing of diffraction data – Indexing, integration, refinement of data using Mosflm/Denzo

8. Macromolecular structure elucidation – Phasing by Molecular replacement (Molrep), Model building (Coot), refinement (Refmac) and statistical analysis using CCP4i suite, PDB online Addit tool

Figure 3.1 Methodology for Structure Studies
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 (Sambrook et al 1989). The primers used for TRX 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 TRX

<table>
<thead>
<tr>
<th>S.No</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>TRX F</td>
<td>5’-CATATGGATGATTTACTTGCTATTATCG-3’</td>
</tr>
<tr>
<td>2.</td>
<td>TRX R</td>
<td>5’-AAGCTTCTACGCTGCTGCTAACCAGCTT-3’</td>
</tr>
<tr>
<td>3.</td>
<td>T7 F</td>
<td>5’-ATTAACCCTCACTAAAGGGA-3’</td>
</tr>
<tr>
<td>4.</td>
<td>T7 R</td>
<td>5’-TAATACGACTCACTATAGGG-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 : 58°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 were gel purified individually using Qiaquick gel extraction kit (Qiagen, Hilden, Germany). The expected amplified gene product was excised using a sterile scalpel blade from the agarose gel. Binding buffer was added at thrice the weight of the excised gel piece and incubated at 50°C until the gel melted completely. Equal volume of isopropanol of the gel weight was added and mixed well. The contents were then transferred to the column and centrifuged at 13,000 rpm for 1 min and
the filtrate was discarded. The Column was washed with wash buffer in the ratio 1:4 (wash buffer: alcohol) and centrifuged at 13,000 rpm for 1 min and the filtrate was discarded. The empty column was centrifuged again at 13,000 rpm for 1 min to remove the excess alcohol. 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. 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.

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

The total volume was made upto 20 μL with triple distilled water and incubated for 3–4 h at 37°C. The completion of digestion was monitored by agarose gel (1%) electrophoresis. 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 *W. bancrofti* TRX gene was restricted using *Nde I* and *Hind III*.

3.3.5 Ligation

Ligation of digested vector (pRSETB) and insert DNA (TRX) was performed as follows. The ligation mixture consisted of
10X ligation buffer : 10µL  
Vector (50 ng) : 2µL  
Insert (50 ng) : 6µL  
T4 DNA Ligase (NEB, USA) : 1µL

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.

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. 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 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 cold CaCl$_2$ (Sambrook et al 1989) in E. coli. 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). 100 μL of overnight culture was inoculated into 50 mL LB medium in conical flask and allowed to grow at 37°C till OD$_{600}$ reached 0.6. Culture was chilled on ice for 5 min by gentle swirling and centrifuged at 3500 rpm for 10 min at 4°C. The cell pellet was resuspended in 10 mL of 100 mM ice-cold MgCl$_2$ and incubated on ice for 30 min. Cells were pelleted as above and the pellet was resuspended in 2 mL of 100 mM ice-cold CaCl$_2$ and incubated on ice for 1 h. Approximately 10–20 ng of DNA was added to 100 μL of above cells and further incubated for 30 min on ice. A heat shock at 42°C was given for 90 s and snap chilled again on ice for 10 min. About 400 μL of LB medium was added to the tubes and allowed to grow in rotary shaker at 37°C for 45 min. Around 100 μL was plated onto LB agar and the plates were supplemented with appropriate antibiotics. 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. A single colony of fresh transformant was inoculated into 1.5 mL LB/ON and grown overnight (o/n) at 37°C. 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. The culture was induced with 100 mM NaCl for GJ1158 strain and grown for 3 h at 37°C with 150 rpm shaking. 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 membrane 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 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 was developed using 33μL of 5-bromo-4-chloro-3-indolyl phosphate (50 mg/mL in dimethyl formamide; USB, Amersham Pharmacia) and 66μL of nitroblue tetrazolium (50 mg/mL in 70% dimethyl formamide; USB, Amersham Pharmacia) in 10 mL of detection buffer. Primary antibodies, mouse monoclonal anti-His (Sigma, St Louis, USA), diluted at 1:20,000 in 1X PBS was used in detecting recombinant fusion proteins. Various human clinical sera were used at 1:100 dilution in human sera immunoblot analysis. Mouse anti-ALT (1:5000), anti-TRX (1:1000) and anti-TGA (1:1000) were used for confirmation of proteins. 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 Na2HPO4–NaH2PO4, 50 mM Tris, 400 mM NaCl, 10 mM imidazole, pH 8, and 0.3 mg/mL lysozyme. The suspension was incubated at 4°C for 1 h and subjected to sonication for 5 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 **Wb-GST Purification Using Glutathione Affinity Chromatography**

Wb-GST has G-site for glutathione binding which was utilized in this purification using ligand affinity chromatography first described by Simons and Vander Jagt (1981). The induced cell pellet was resuspended in binding buffer (PBS-pH-7.3) and sonicated for 10 cycles with 5s on and 5s pause by keeping the sample on ice for heat sink. Through the empty column double deionized water was passed and 2 mL glutathione sepharose fast flow matrix was packed. The column was incubated with 12 mL PBS for 10 min. Additional 12 mL was allowed to pass through the column. After sonication, sample was spun at 12000rpm and the supernatant was used for chromatography on GSH sepharose column (2 mL matrix) pre-equilibrated with PBS (pH-7.3). The column was equilibrated with 12 mL of sample by passing through the column once followed by 5 min incubation. This step was followed thrice. Flow through (2 mL) was collected. The unbound proteins were washed through the column with 24 mL (with eight-bed volume) of the binding buffer. A 2 mL binding buffer eluted fraction was also taken. GST (bound protein) was eluted from the column with 14 mL of elution buffer (50mM Tris pH-8.0) containing 10 mM GSH. Fractions (2 mL each) were collected. The fractions were run on 12% SDS-PAGE and band corresponding to 25kDa of Wb-GST was observed.

3.3.14 **Purification of Wb-TRX by Ion Exchange Chromatography**

The Wb-TRX which is an acidic protein was purified using ion exchange chromatography as described by Amersham Biosciences (2002). The column packed with the matrix Q-Sepharose fast flow (GE healthcare), was washed thoroughly with water and equilibrated by passing 10 column volumes of binding buffer (20mM Tris, 5mM EDTA, pH 8.0). The cell pellet containing the recombinant protein was solubilized by sonication in binding
buffer. The sample was centrifuged at 7,000g for 10 minutes and the supernatant was used for purification of the recombinant protein. The matrix was equilibrated with binding buffer till the absorbance of the flow through stabilized at 280 nm. The sample was loaded on to the column till the matrix was saturated. Then the matrix was washed with elution buffer (20mM Tris, 5mM EDTA, pH 8.0) till the flow through reached an absorbance value of 0.01 at 280 nm. The contaminating proteins bound to the matrix were washed with 15 column volumes elution buffer containing 5050mM NaCl. The proteins bound to the matrix were eluted with elution buffer containing 100mM, 150mM and 200mM of NaCl. The purity of the protein was checked on SDS-PAGE. The NaCl fraction containing the purified protein was dialyzed overnight against 0.1 X PBS at 4°C. The concentration of the purified protein was determined (Bradford 1979) and aliquots were made and stored in -20°C until further use.

3.3.15 Estimation of Protein

The proteins were estimated using BCA method (Smith et al 1985) which is a more sensitive assay for estimation. 10 μL of protein sample was added to 190 μL of BCA (Bicinchoninic acid) working reagent (Pierce, Rockford, USA) and incubated at 37°C, dark, for 30 min. The Reaction mixture was incubated in dark for 30 minutes and the absorbance was read at 540 – 590 nm.

3.3.16 CD Spectra Analysis

CD measurements in the far-UV region from 190-250 nm were performed with a Jasco-600 spectropolarimeter to analyze the secondary structural features of protein (Greenfield 2006). Samples containing proteins of 0.3-0.5 mg/mL concentration were scanned at a rate of 10 nm/min. The temperature of the sample compartment was maintained at 20.0°C with a circulating-water bath. The cuvette used had a light path of 1mm. Each
spectrum was corrected by subtracting the corresponding blank. The data was obtained in milli degrees which were converted to molar ellipticity to normalize protein concentration and molecular weight.

3.4 ENZYMATIC ASSAYS

3.4.1 Preparation of Wb-GST Protein for Enzyme Kinetic Studies

The purified Wb-GST proteins were dialyzed to remove excess glutathione against PBS overnight at 4°C. The dialysed rWbGST protein was concentrated by amicon ultra filters (Millipore, USA) with 3 kDa cut off. The ultra filters were centrifuged at 400g for 20 minutes at 25°C. The concentrated protein was estimated by Bradford method using BSA as standard.

3.4.2 Kinetic Assay Using CDNB as Substrate

For determining the specific activity of Wb-GST for GST-pi substrate CDNB, the assay was carried out with different concentrations (0.5 & 1mM) of the substrate against a constant concentration of co-substrate (GSH) (1 mM). The assay was performed as described by (Mannervik et al 1985). The assay was carried out in 96 well plates containing 5 μg of GST protein in 20 μL, with 10 μL CDNB and 20 μL GSH in a 200 μL of total reaction mixture. The formation of CDNB-GSH produces a dinitrophenyl thioether was detected spectrophotometrically at 340 nm. The activity of GST is defined as – one unit of enzyme will conjugate 1μmole of CDNB with reduced GSH per minute at 25°C (Equation 3.2).

The activity was calculated as follows:

\[
\frac{\Delta A_{340}(\text{Time 2}) - \Delta A_{340}(\text{Time 1})}{\text{Time 2(min)} - \text{Time 1(min)}}
\] (3.2)
GST activity (µmol/min/mL):

\[
\frac{\Delta A_{340} \text{ min} \times V}{0.0096 \mu\text{mol}^{-1} \text{ cm}^{-1} \times 1000 \text{ mL} \times 0.524 \text{ cm} \times \text{A}} \times \text{dil}
\]

(or)

\[
\Delta A_{340} \times 1.988
\]

(3.3)

for a sample volume of 20 µL in a 200 µL reaction volume when the sample dilution (dil) is 1.

where \( \Delta A_{340} = \) Change in absorbance / min

V = Total Reaction Volume

Dil = Sample dilution factor

A = Sample volume in ml

0.0096µmol^{-1} cm^{-1} = Extinction coefficient for CDNB (\( \varepsilon \))

0.524 cm = Path length for a well in 96 well plate in 0.2 mL reaction

The specific activity was calculated as Activity/concentration in mg/mL and expressed as µmol/min/mg or U/mg protein (Equation 3.3).

### 3.4.3 Insulin Reduction Assay

Thioredoxin-mediated catalysis of insulin reduction was measured spectrophotometrically at 650 nm, 25°C as an increase in turbidity resulting from precipitation of the free insulin chains (Holmgren 1979). The assay mixture contained 100 mM potassium phosphate, 2 mM EDTA (pH 7.0), 0.13 mM insulin (0.75 mg/ml), and various concentrations (25, 50, 100 and 300 ng per reaction) of Wb-TRX-1, or recombinant human TRX (Sigma, St Louis, USA). The reaction was initiated by the addition of 0.33 mM dithiothreitol.
(DTT). Anti-TRX-1, anti-PC-1 and control sera were used for inhibition assay and were incubated with protein at a dilution of 1:500. The increase in turbidity was measured at 650 nm for 60 minutes and absorbance was plotted against reaction time. One unit of enzyme will cause a $\Delta A_{650\text{nm}}$ of one in one minute at 25°C in insulin assay (Equation 3.4). The specific activity expressed as units per mg was calculated as follows:

$$\frac{\Delta A_{650\text{nm}} \text{(test)} - \Delta A_{650\text{nm}} \text{(blank)}}{C} \times V \times \text{dil}$$

(3.4)

where $\Delta A_{650\text{nm}}$ = Change in O.D per minute at 650 nm
V = Total reaction volume in ml
dil = dilution factor
C = Amount of enzyme in mg

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 Wb-SXP-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 Prof. Murray Selkirk, Imperial College, 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. BmGP29 and VAH antigens (100 ng/well) were 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. 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 1 mg/mL in substrate buffer (NaHCO₃ -0.84 g/L; Na₂CO₃- 1.25 g/L; MgCl₂ -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 (IgG1 [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 tryphan blue dye exclusion method. The cells were then cultured in round-bottomed microtiter plates at a concentration of 0.2×10^6 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 Animals, Immunization and Sera Collection

Six to Eight weeks old female BALB/c (H-2d) mice were procured from Tamil Nadu 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. 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. Blood was collected on days 0, 14, 28 and 42. 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

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 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 alum control sera. The colour 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 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 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.

3.5.9 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, USA) at 4×10^6 cells/mL in a volume of 1 mL of RPMI 1640 supplemented with 10% fetal calf serum and 80 μg/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 100 µL of the substrate Tetramethyl benzidine (TMB/H₂O₂). 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 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" X 12" 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).

3.6.2 Brugia malayi Parasite Maintenance

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.

3.6.3 Immunization and Parasite Collection

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.

3.6.4 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).

3.6.5 Protection Study in Matomys coucha

Protection study was done by micropore chamber method as described previously (Weiss and Tanner 1979, Abraham et al 1993 and Chenthamarakshshan et al 1995). 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 3.2 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.
Figure 3.3 Experiments for Filarial Vaccination Studies

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 (Man-Witney 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.