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Recombinant translation initiation factor-1 of *Wolbachia* is an immunogenic excretory secretory protein that elicits Th2 mediated immune protection against *Brugia malayi*

Jeetendra Kumar Nag, Nidhi Shrivastava, Jyoti Gupta, Shailja Misra-Bhattacharya*

Division of Parasitology, CSIR-Central Drug Research Institute, BS 10/1, Sector 10, Jankipuram Extension, Sitapur Road, Lucknow (U.P.) 226021, Uttar Pradesh, India

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ABSTRACT

Wolbachia, the intracellular alpha-proteobacteria are required for the development, fertility and survival of filarial parasites. *Wolbachia* Translation initiation factor-1 (Wol TI IF-1) is one of the factors required for *Wolbachia* growth and viability. In the present study, we cloned, over expressed and purified Wol TI IF-1 that exhibited strong immuno-reactivity with various categories of bancroftian sera. Immunization with the recombinant protein resulted into significant reduction in microfilarial density (70–72%) and adult worm establishment (61–63%) in susceptible *Mastomys coucha*. Protection offered by Wol TI IF-1 was found associated with humoral immune arm as observed by an increased antibody level with preponderance of IgE, IgM, IgG1 and IgG2a isotypes. The anti-Wol TI IF-1 antibodies promoted profound adherence of peritoneal exudates cells to the surface of microfilariae and infective larvae causing cytotoxicity and their death. The present study indicates potential of recombinant Wol TI IF-1 as a promising vaccine candidate against human lymphatic filarial infection.

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1. Introduction

Lymphatic filariasis (LF) is a major mosquito borne human filarial disease with low mortality but very high morbidity. It causes severe social stigma and huge economic loss in developing nations. LF is mainly caused by *Wuchereria bancrofti* prevalent in the tropics, while *Brugia malayi* is confined to South-East Asia and *Brugia timori* to Timor and the Flores areas of Indonesia. Currently, more than 1.3 billion people in 81 countries are at risk and of these, approximately 65% live in the South-East Asia region, 30% in African region while remaining in other tropical areas. Latest statistics reveal that over

120 million people are infected, with about 40 million disfigured and incapacitated by the disease. India alone accounts for 40% of the global disease burden. Although diethylcarbamazine (DEC) and ivermectin in conjunction with albendazole are currently being used as antifilarial drugs but they possess limited macrofilaricidal efficacy. Emerging resistance to antifilarial drugs is an encumbrance for the ongoing available treatment programs and is of serious concern [1,2]. Discovery of an effective vaccine against filariasis is therefore desired that may ignore the natural host immune response and may direct a response against parasite proteins that are essential for the parasite survival and existence. Human filarial parasite in spite of being a eukaryote, intracellularly harbor an obligate alpha-proteobacterial endosymbiont, *Wolbachia* with which it maintains a mutualistic relationship. Depletion of *Wolbachia* produces profound defects in nematode development, fertility and viability. Recent report indicates that *Wolbachia* depletion leads to an extensive apoptosis in the

* Corresponding author. Tel.: +91 522 2612411–18x4221/4224;

fax: +91 522 2623405/2623938/2629504.

E-mail addresses: shailja.cdri@rediffmail.com, shailjacdri@yahoo.com (S. Misra-Bhattacharya).

adult germline, and in the somatic cells of the embryos, microfilariae (Mf) and fourth-stage larvae (L4) [3]. The use of tetracycline or rifampicin to deplete *Wolbachia* leads to an arrested development of larval and embryonic stages resulting in permanent sterilization of adult female worms [4–6]. The adult parasites die prematurely after 1–2 years following depletion of *Wolbachia*, compared to their typical lifespan of 10–14 years, delivering for the first time a safe and potent macrofilaricidal treatment for filariasis [7]. Thus *Wolbachia* could be one of the crucial targets for developing vaccine candidates. Presence of *Wolbachia* primarily in reproductive system of female worms but not males ensures its vertical transmission through cytoplasm of the egg and not through the sperm [8]. Recent detection of *Wolbachia* in the lumen of excretory-secretory canals of *B. malayi* embedded in the hypodermal lateral chords suggests the release of *Wolbachia* and/or their products from live adult worms into the vertebrate host tissues in addition to their release from dead or degenerating parasites [9]. In spite of an immense role of *Wolbachia* in filarial parasite biology and survival, very few proteins of *Wolbachia* have been characterized. These are, *Wolbachia* surface protein (WSP), NAD⁺-dependent DNA ligase (wBm-LigA), heat shock protein 60 (HSP60), independent phosphoglycerate mutase (iPGM) and the enzymes involved in heme biosynthetic pathway. Out of these, WSP possesses the antigenic role and generates an IgG response in cats infected with *Dirfilaria immitis* [10–13]. *Wolbachia* for their own existence depend on various essential factors such as Translation initiation factor-1 (Wol TI IF-1) which is present solely in prokaryotes and is essential for bacterial viability and growth [14]. It has been observed that anti-rickettsial antibiotic, tetracycline causes death of *Wolbachia* via blocking the translation process by preventing the binding of Wol TI IF-1 with A-subunit of 30S ribosome resulting in filarial parasite killing in the vertebrate host [15]. TI IF-1 recruits IF-2 and stimulates IF-2 dependent binding of fmet-tRNA to 30S ribosome several folds [16]. Its chaperon activity is responsible for melting of secondary structures formed in RNA [17,18]. These exceptionally excellent properties of TI IF-1 prompted us to clone this wolbachial factor and characterize it immunologically. In the current study, wolbachial gene *infA* (Wol *infA*) from *B. malayi* has been cloned, over expressed, purified and the immunological functions of the recombinant protein Wol TI IF-1 have been investigated in an experimental rodent model of *B. malayi*.

2. Materials and methods

2.1. Parasite

The sub periodic strain of *B. malayi* was cyclically maintained in the experimental rodent host, *Mastomys coucha* (GRA 'Giessen' strain) through laboratory bred mosquito vector *Aedes aegypti*. The infective larvae (L3) of *B. malayi* were recovered from the mosquitoes fed on donor *Mastomys* 9 ± 1 day earlier. L3 were isolated from gently crushed mosquitoes by Baermann technique, washed and counted in Ringer's solution. Adult parasites and Mf were obtained by lavaging the peritoneal cavity of jirds inoculated 3–6

months earlier with *B. malayi* L3 as described previously [19].

2.2. Amplification and cloning of Wol *infA* gene

Adult worms recovered from the peritoneal cavity of jirds were washed in PBS (phosphate buffer saline pH 7.2) and genomic DNA was isolated as per the established method [20]. The gene Wol *infA* (NCBI 3266784) encoding Wol-TI IF-1 was amplified with Taq DNA polymerase (Invitrogen, USA) lacking 3'–5' exonuclease activity, using gene specific forward primer (5' CAT ATG ATG GTA AAA GAT GAA AAA TCG AAA AC 3') having *Nde*I restriction site (underlined) and the reverse primer (5' CTC GAG TTT AGA AAT TGT TCT CTC ACT TGT ACC 3') having *Xho*I restriction site (underlined). The amplification of gene was performed by mixing 1 µM of each primer, 200 µM of each dNTP (Fermentas, USA), 2 units Taq DNA polymerase, 1× PCR buffer and 1.5 µM MgCl₂ under the conditions of initial denaturation at 95 °C/10 min, 29 cycles at 94 °C/45 s, 58.1 °C/1 min, 72 °C/1 min, and 1 cycle at 72 °C/10 min. The amplified gene (264bp) was cloned into pTZ57R/T (2.88 kb) vector (Fermentas, USA) as per manufacturer's instructions and transformed into competent DH5α cells. The transformants were screened for the presence of recombinant plasmids carrying Wol *infA* insert by gene-specific PCR under similar conditions and fidelity of the cloned product was verified by sequencing. pET28a(+) and Wol *infA* insert was cut from pTZ57R/T construct with *Nde*I and *Xho*I for directional ligation into pET28a(+) vector (Novagen, USA) to generate N and C terminal His-tag expressing plasmid (pET28a-His6-*infA*). The plasmid construct with pET28a(+) was confirmed by double digestion with *Nde*I and *Xho*I restriction endonucleases.

2.3. Over-expression, purification and localization of recombinant Wol TI IF-1

The Wol *infA*-pET28a(+) construct was transformed into several *Escherichia coli* strains viz. BL21(DE3), BL21(DE3)pLysS, Origami(DE3), Origami(DE3) pLysS, Rosetta and C41 (all from Novagen), out of which, optimal protein expression was noticed in Rosetta. Thus subsequent experiments were carried out with Rosetta cells. Protein synthesis was induced with 0.5 mM IPTG (isopropyl β-D-thiogalactoside) (Sigma, USA) for 6 h at 25 °C with constant shaking at 220 rpm in cultures in logarithmic phase (OD₆₀₀ = 0.5–0.6). The cells were harvested at 5000 rpm for 10 min and the pellet was suspended in 50 mM NaH₂PO₄ buffer (pH 8.0) containing 250 mM NaCl, 1 µM PMSF and 10 mM imidazole. Cell suspension was disrupted by sonication and pelleted at 12,500 rpm for 30 min at 4 °C. The supernatant was run on nickel nitrilotriacetic acid (Ni-NTA) agarose affinity column pre-equilibrated with 50 mM NaH₂PO₄ buffer (pH 8.0) along with 250 mM NaCl and 10 mM imidazole. The column was subsequently washed with the same buffer containing 25 mM imidazole followed by 50 mM imidazole. The recombinant protein was finally eluted with 250 mM imidazole. The purity of eluted recombinant protein was analyzed on 15% SDS-polyacrylamide gel electrophoresis

(SDS-PAGE) and protein content was determined by the Bradford method. The protein was electrophoretically transferred to nitrocellulose (NC) membrane in a dry blot apparatus as per manufacturer's instructions (Invitrogen, USA). Membrane was blocked in 3% skimmed milk for 2 h and incubated at 37 °C with 1/5000 dilution of mouse anti-His monoclonal antibody (Novagen, USA), re-incubated with goat anti-mouse IgG-HRP (horseradish peroxidase) antibody (1/10,000; Sigma, USA) for 2 h at 37 °C. The blot was developed with the substrate, 3,3'-diaminobenzidine tetra hydrochloride (DAB) in the presence of 20 µl H₂O₂ (Sigma, USA).

2.4. *Wol* infA gene expression in various stages of *B. malayi*

Adult *B. malayi* and Mf were recovered from the peritoneal cavity of infected jirds as mentioned above. The worms were made free of host tissue under the dissecting microscope. Mf were pelleted after passing the peritoneal washing through the membrane filter having pore size of 5.0 µm. Mf get concentrated over the filter and are collected by immersing the filter disc in PBS at 37 °C and pelleting. L3 were recovered from experimentally infected laboratory bred mosquitoes after gentle crushing in Ringer's solution as described above and washed several times in sterile PBS. RNA was extracted from all the three life-stages using Trizol reagent (Invitrogen, USA) and quantified with a Gene-quant (Bio-Rad). Genomic DNA contamination was removed by treatment with DNase I and 2 µg of DNA-free RNA from each life-stage was used for first cDNA synthesis using a first-strand cDNA synthesis Kit (Fermentas, USA). The cDNA was amplified with *Wol* infA-specific primer pairs using the same incubation conditions as mentioned above.

2.5. *In silico* prediction of immunogenicity of *Wol* TI IF-1

The antigenicity of *Wol* TI IF-1 was determined by the semi-empirical method of Kolaskar and Tongaonkar, which predicts the antigenic determinants based on the physicochemical properties of amino acid residues and the frequencies of their occurrence in experimentally known segmental epitopes [21]. Prediction of immunodominant T cell antigenic sites from the primary sequence of *Wol* TI IF-1 was determined by the programme ProPred which predicts peptide binding to major histocompatibility complex (MHC), the key checkpoint on the antigen presentation pathway within the adaptive cellular immunity. ProPred implements robust statistical models for both Class I HLA alleles (A1, A2, B2702 and B2705) and Class II HLA alleles (DRB1.0301, DRB1.0306, DRB1.0307, DRB1.03011, DRB1.0701, DRB1.0703, DRB1.0801, DRB1.0802, DRB1.0804, DRB1.0806, DRB1.1107, DRB1.1301, DRB1.1327, and DRB1.1328) [22].

2.6. Immunoprophylactic studies with *Wol* TI IF-1

2.6.1. Animals

Purpose-bred, parasite naive, 6-week-old male *Mastomys* (*M. coucha*) were used for the experiments ($n=6$) in

replicates. Throughout the experimentation, animals were maintained in proper housing condition at the Animal House Facility of CSIR-CDRI, Lucknow, India. Animals were fed on standard pellet diet and water *ad libitum*. The animal handling procedures and experimental protocols were duly approved by the Animal Ethics Committee of the Institute.

2.7. Immunization studies

2.7.1. *Mastomys coucha*

The protective potential of the recombinant protein, *Wol* TI IF-1 was investigated in *Mastomys* against *B. malayi* L3 challenge since this rodent species is highly susceptible to most of the filarial species and is widely used for experimental manipulative research. The immunization protocol followed in the present study has been described earlier [23]. Briefly, animals were divided into three groups: (i) unimmunized control; (ii) adjuvant group; and (iii) *Wol* TI IF-1 immunized group. The animals were subcutaneously injected with 12.5 µg of the recombinant protein having endotoxin level of 1.72 U/ml (Lonza, Singapore) emulsified with Freund's complete adjuvant (FCA; Sigma, USA) on day 0, followed by two booster doses of equivalent quantity of protein in Freund's incomplete adjuvant (FIA) on days 14 and 21 post first immunizing dose. Age and sex matched adjuvant group received equivalent volume of FCA/FIA alone in PBS while the normal unimmunized control group received PBS only. One week following the final booster dose, all the groups of *Mastomys* were challenged with 100 L3 of *B. malayi* each through subcutaneous route in the back region. The animals were kept under observation till the completion of incubation period (90 days) and thereafter parasitemia was monitored to observe the immunization efficacy of the recombinant protein on Mf density and later on the establishment of adult parasites.

2.7.2. BALB/c mice

The immunological responses generated by *Wol* TI IF-1 were investigated in the 6-week old, male BALB/c mouse which is a well dissected model and majority of the immunological reagents are commercially available for murine host. The protocol for immunization was same as in case of *Mastomys* except that the BALB/c mice were not challenged with *B. malayi* as these do not support full development of *B. malayi* from L3 to adult stage, however, can support single life-stage of filarial parasite.

2.8. *Mastomys coucha*

2.8.1. Effect of immunization on microfilaraemia, adult worm recovery and female worm fecundity

Twelve week post-challenge (p.c.) with *B. malayi* L3, monitoring of microfilaraemia was initiated in the controls and immunized group of *Mastomys* and continued fortnightly up to 24th week. Briefly, 10 µl tail blood smears were prepared and stained with Leishman to assess Mf density microscopically. Percent change in mean Mf density at each time point was evaluated in comparison to that of control group. Animals were euthanized at the end of 24 weeks p.c. for the recovery of adult worms from the teased tissues of heart, lungs and testes in normal

saline. Adult worms were isolated, counted and live female worms were individually teased on a glass slide in a drop of saline to assess the effect of immunization on worm fecundity. The intrauterine contents were observed for any development arrest and the phenotype of the various developmental stages. Parasite recoveries and counting were performed without the knowledge of the experimental status of each animal. Arithmetic means were calculated for the total worm burden and the percentage protection was calculated as $[(u - v)/u \times 100]$, where u is the mean value for the control group and v is the mean value for the experimental group. Female worm sterilization was calculated as $[\text{number of sterile worms}/\text{total worm teased} \times 100]$.

2.8.2. Quantification of wolbachial load in female worms by real-time PCR

For quantification of the load of *Wolbachia* in female worms recovered from immunized and unimmunized *Mastomys*, genomic DNA was extracted from ten female worms each isolated from control, adjuvant and Wol TI IF-1 immunized animal groups using the genomic DNA isolation kit (Invitrogen). SYBR green I PCR amplification was performed using a LightCycler instrument (Roche, Mannheim, Germany). Amplification were carried out in a 20 μ l reaction mixture containing 1 μ g of genomic DNA from each group, 1 μ M concentration each of forward primer (5'-AAA GCA ACA AGA GAG GCA AGA G-3') and reverse primer (5'-AAG TTC CGC AAT ACG CAT ACG-3') specific against *ftsZ* gene (NCBI 3266907) of *Wolbachia* and 10 μ l (2 $\times$) of LightCycler-FastStart DNA Master SYBR green I (Takara, Japan). Amplification involved incubation at 95 °C for 10 min for initial denaturation, followed by 45 cycles consisting of (i) denaturation at 95 °C for 30 s, (ii) annealing at 60 °C for 40 s, (iii) extension at 72 °C for 40 s, and (iv) fluorescence acquisition (530 nm) at the end of extension. The temperature transition rate was 20 °C/s for each step. After real-time PCR, a melting curve analysis was performed by continuously measuring the fluorescence during heating from 60 to 95 °C at a transition rate of 0.1 °C/s. Threshold cycle (C_T) values were determined with the LightCycler software (version 1.5), using the second derivative method. Each sample was tested in three replicates. Fold change in *Wolbachia* load in the immunized group with respect to the control groups was evaluated in terms of ΔC_T as mentioned earlier [24].

2.8.3. Determination of Wol TI IF-1 specific serum IgG antibody pre- and post L3 challenge

Recombinant protein immunized, adjuvant immunized and unimmunized control *Mastomys* were bled for sera collection through retro-orbital plexus on days 0, 15, 30, 45, 60, 90, 120, 150 and 180 to monitor the Wol TI IF-1 specific serum IgG antibody level. Briefly, 96 well microtiter polystyrene plates (Nunc, USA) were coated with 1.0 μ g/ml concentration of Wol TI IF-1 in carbonate buffer, pH 9.6 (100 μ l/well) and kept overnight at 4 °C. At each step here after, plates were washed thrice with PBST (PBS containing 0.05% (v/v) Tween-20). Wells were blocked with 3% (w/v) skimmed milk (in PBS) at 37 °C for 2 h. Pooled serum from six experimental and naive healthy *Mastomys* were

initially tested at various two fold dilutions starting from 1:200 to 1:800 to assess the cut off dilution. All the test sera were finally used at 1:800 (cut off) dilution in PBST containing 0.3% skimmed milk in triplicate wells and the plate was incubated at 37 °C for 2 h. Bound primary antibodies were allowed to react with 1:10,000 dilution of HRP conjugated goat anti-mouse IgG (Sigma, USA). The antigen-antibody reaction was detected after adding substrate OPD (o-phenyl diamine, Sigma, USA) and H₂O₂. The reaction was stopped by adding 40 μ l of 2.5 M H₂SO₄. The OD was determined at 492 nm in a multiplate reader (Infinite Series M200, Tecan, Switzerland).

2.8.4. Determination of Wol TI IF-1 specific serum antibody isotypes

Antibody isotypes were determined by antibody isotype ELISA kit (Sigma, USA) as per the manufacturer's protocol. Briefly coating and blocking were done as described above except that the concentration of Wol TI IF-1 was 0.1 μ g/ml in the carbonate buffer. Post vaccinated and post challenged sera diluted to 1:800 taken from six experimental animals were added to the triplicate wells at 37 °C for 2 h. Secondary antibodies in the form of isotype-specific monoclonal antibodies (goat anti-mouse IgM, IgA, IgG1, IgG2a, IgG2b and IgG3) at 1:1000 dilution were added and plate was kept for 2 h at 37 °C. Detection of bound isotype specific antibodies was done with peroxidase labeled rabbit anti-goat IgG (1:10,000) and OPD substrate as above and OD at 492 nm was measured.

2.8.5. Reactivity of Wol TI IF-1 in immunoblot with IgE present in the immunized serum

To detect the presence of IgE in *Mastomys* sera after immunization with Wol TI IF-1, 20 μ g of purified Wol TI IF-1 protein along with prestained standard molecular weight marker was run on a 15% SDS-PAGE, transferred to NC membrane as mention above. The membrane was blocked for 2 h and incubated at 37 °C with 1:800 dilution of immunized and control *Mastomys* sera pool. Goat anti-mouse IgE-HRP (Southern Biotech) at 1:10,000 dilution was used as a secondary antibody and the membrane was developed with DAB.

2.8.6. Reactivity of human bancroftian antibodies with Wol TI IF-1

Reactivity of antibody present in the sera of human subjects staying in *W. bancrofti* endemic area with Wol TI IF-1 was observed in Western blot. Blood for isolation of serum samples was collected from the outskirts of Lucknow, and was categorized as, symptomatic microfilaremic, symptomatic amicrofilaremic and asymptomatic microfilaremic and ten samples were taken from each category. Sera from humans living in filaria free zones such as Jammu and Kashmir, India served as non-endemic control. Mf presence or absence was determined in 2-ml night blood by membrane filtration technique [25]. Endemic normals were Mf negative and free from any clinical filarial symptom when followed for five consecutive years. Purified recombinant protein (20 μ g) along with prestained molecular weight protein marker was run on a 15% SDS-PAGE transferred

to NC membrane as described above and processed for immune recognition with pooled human sera (1:200) as described above. Goat anti-human IgG-HRP (Sigma, USA) (1:10,000 dilutions each) were used as secondary antibody and DAB as substrate.

2.8.7. Determination of Wol TI IF-1 in excretory and secretory (ES) product of *B. malayi*

The male and female adult worms (10 worms/ml in triplicate) were separately maintained *in vitro* in serum-free RPMI 1640 (Sigma) supplemented with antibiotic-antimycotic (Invitrogen, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml of Amphotericin B) and 25 mM HEPES at 37 °C in 5% CO₂ in air. The spent media were collected and replaced with fresh medium every 24 h continuously up to 7 days during which the parasites remained highly motile and active. The collected media from the female worms were then centrifuged at 1000 × g for 5 min to remove the possibility of Mf and embryo existence and both the supernatants from male as well as female worm culture were passed through 0.2 µm membrane filters (Millipore, USA) and concentrated using Amicon Ultrafilters with 3 kDa cut-off membrane (Millipore). Concentrated ES products and Wol TI IF-1 were individually run on 15% SDS-PAGE and transferred to NC membrane as described above. Membrane was blocked with 3% skimmed milk for 1 h and incubated at 37 °C for 2 h with 1:800 dilution of Wol TI IF-1 immunized serum of *Mastomys* as described elsewhere [26]. The membrane was re-incubated with goat anti-mouse IgG-HRP antibody for 2 h at 37 °C with the dilution of 1:10,000 and the blot was developed with the substrate DAB.

2.8.8. *In vitro* antibody-dependant cellular adhesion and cytotoxicity (ADCC) to Mf and L3

Cell adhesion experiments were performed with Mf and L3 as described earlier [26]. Briefly, 100 live Mf or 50 live L3 were separately cultured with normal *Mastomys* peritoneal exudate cells (PECs) in triplicate in a 96-well plate containing RPMI 1640 supplemented with sera of normal and immunized *Mastomys*. Each well contained 37.5 µl PECs (1.0 × 10⁶ cells), 12.5 µl of serum of *Mastomys* generated against Wol TI IF-1 (1:16), 25 µl Guinea pig serum as a source of complement and 25 µl Mf (100 numbers) OR 25 µl L3 (50 numbers) in a final volume of 200 µl of RPMI 1640. The plates were kept at 37 °C in a CO₂ incubator (Binder, Germany). Cell-adherence on the surface of parasites impairing their motility was noted after 1, 3, 6 and 24 h of incubation. Percent Mf and L3 with cell adherence (≥5 cells adhered/Mf or ≥2 cells/L3) were assessed by microscopic examination of each well.

2.8.9. *In vitro* lymphocyte proliferation of splenocytes in the presence of mitogens and Wol TI IF-1

The lymphoproliferative responses of splenocytes isolated from different groups of *Mastomys* were determined post-vaccination as described earlier [27]. Briefly, the spleens from control and immunized group of *Mastomys* were aseptically removed, crushed gently with sterile glass

crusher in RPMI-1640 (Sigma, USA) and passed through the sterile nylon cell strainer (40 µm pore size; BD Falcon, USA) to prepare single cell suspension. Cell suspension (100 µl/well) from stock (5 × 10⁶ cells/ml) was plated in the 96 well Nunc culture plate in triplicate and the cells were stimulated with 100 µl of Wol TI IF-1 (2.5 µg/ml; optimized earlier) or 100 µl concanavalin A (2.5 µg/ml; Sigma, USA) for 48 h and pulsed with 1.0 µCi/well of [³H] thymidine (³H-Tdr, specific activity 18 Ci/m mole, BARC, India) for 18 h preceding cell harvest. The radioactive incorporation in the cells was measured by standard liquid scintillation β-counter (Beckman Instruments, Palo Alto, CA) in the form of counts per minute (cpm) and compared with that of controls.

2.8.10. Activation of antigen presenting cells (APCs) by Wol TI IF-1

Intracellular reactive oxygen species (ROS) in PECs were determined through a fluorometric assay using 2',7'-dichlorofluorescein diacetate (DCF-DA) as described earlier [28]. Briefly, freshly harvested PECs from control, adjuvant and Wol TI IF-1 immunized animals were washed with PBS 3 × and distributed in FACS tubes (1 × 10⁶ cells/tube). For probe loading, cells were incubated with DCF-DA at a final concentration of 1 µM for 15 min at 37 °C and washed twice in PBS. ROS levels in individual living cells were determined by sequentially measuring their fluorescence intensity (FI) on FACS Calibur (Becton Dickinson, USA). Data were analyzed by CellQuest Software (Becton Dickinson, USA) and mean ROS values were evaluated for cell population.

2.9. BALB/c mice

2.9.1. Staining of cell surface markers of T and B lymphocytes

For immuno-phenotyping of T and B cells, 1 × 10⁶ splenocytes were incubated with fluorochrome conjugated mouse monoclonal antibodies (BD) directed against receptors to CD4+ (FITC), CD8+ (phycoerythrin or PE) and CD19+ (PE) antigens following manufacturer's protocol. After 15 min of incubation in dark at room temperature, cells were washed and finally suspended in 500 µl PBS and analyzed by FACS using CellQuest analysis software (Becton-Dickinson, San Diego, CA) after gating the forward- and side-scatter settings to exclude debris. For each determination 10,000 cells were analyzed and the results are reported as percentage of each cell population.

2.9.2. Determination of intracellular Th1/Th2 cytokines

The measurement of intracellular cytokines in the spleen was performed as per manufacturer's instructions using antibodies and reagents from BD. Briefly, splenocytes (4 × 10⁶/ml) were incubated with Brefeldin A (10 µg/ml) (AbD Serotec, UK) in dark for 6 h in CO₂ incubator at 37 °C. After washing, cells were incubated with mouse seroblock FcR at RT for 10 min, re-incubated in dark at 4 °C in the presence of FITC labeled CD4 antibody and washed with PBS. The cells were fixed with Leucoperm A, permeabilized in the presence of Leucoperm B for 15 min and dispensed

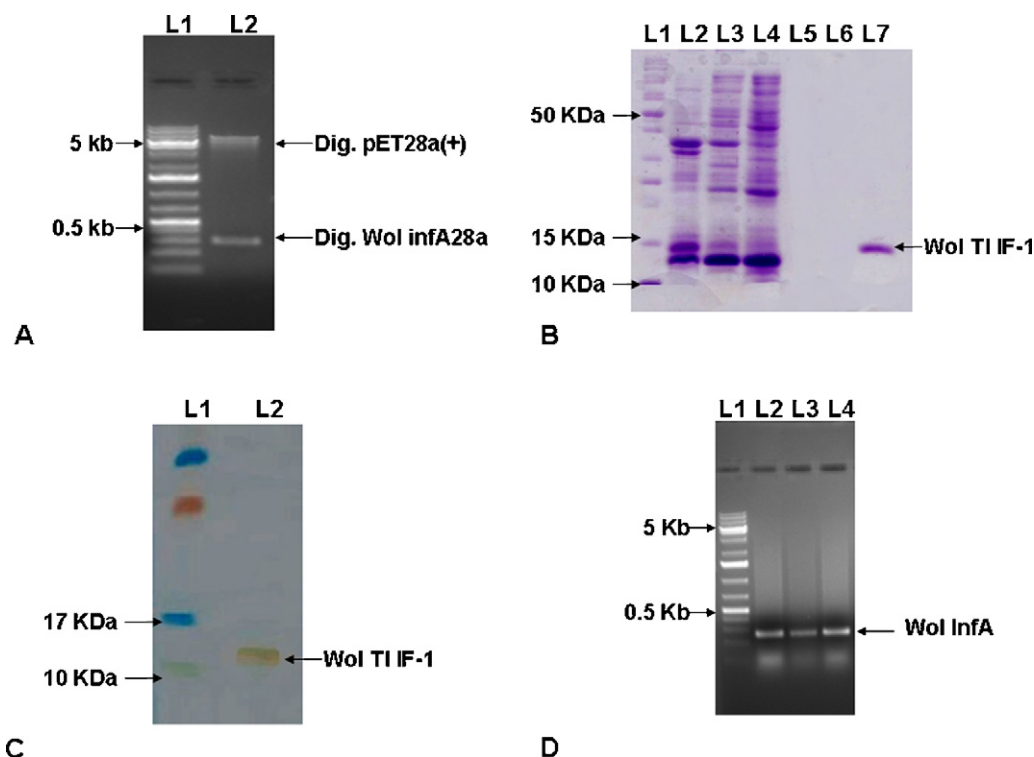


Fig. 1. Cloning, purification, Western blot and stage specific expression of recombinant Wol TI IF-1. (A) Restriction digestion of Wol infA-pET28a(+) construct. Lane 1, 1 kb plus DNA ladder; lane 2, double digestion of Wol infA-pET28a(+) plasmid construct using restriction enzymes *Nde I* and *Xho I*. (B) Purification of Wol TI IF-1. Recombinant Wol TI IF-1 was purified by affinity chromatography using Ni-NTA beads. Lane 1, standard protein molecular weight marker; lane 2, soluble *E. coli* proteins following induction with 0.5 mM IPTG at 25 °C for 6 h; lane 3, flow through from the affinity column; lanes 4–6, washed fractions from column prior to elution; lane 7, elution of His-tagged purified recombinant Wol TI IF-1 at 250 mM imidazole concentration. (C) Western blot analysis of recombinant Wol TI IF-1. Lane 1, standard prestained protein molecular weight marker; lane 2, localization of ~13 kDa His-tagged recombinant Wol TI IF-1 with monoclonal anti-His antibody. (D) Stage-specific expression of Wol infA gene. Lane 1, 1 kb plus DNA ladder; lane 2, amplification of Wol infA gene with Wol infA-specific primer pairs using cDNA template of microfilariae; lane 3, amplification of Wol infA gene with Wol infA-specific primer pairs using cDNA template of infective larvae; lane 4, amplification of Wol infA gene with Wol infA-specific primer pairs using cDNA template of adult worms (both sexes).

in four tubes each containing 1×10^6 cells/100 μ l. Rat-PE antimouse monoclonal antibodies to cytokines TNF, TGF- β , IFN- γ , IL-2, IL-4, IL-5 and IL-10 were added to matching tubes and incubated for another 30 min. Cells were washed and finally suspended in 250 μ l of 0.5% Para formaldehyde for FACS reading and data were analyzed by CellQuest software.

2.10. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) as appropriate. Individual comparisons following ANOVA were made using Newman–Keul's multiple comparison method with the help of statistical software PRISM 3.0. Results of worm recovery, Mf density, immunophenotyping, cytokines levels and absorbance values have been presented as mean $\pm$ S.D. The criterion for statistical significance between the results of immunized and control group were as follows: $p < 0.05$ was considered significant, $p < 0.01$ as highly significant, $p < 0.001$ as very highly significant. Unless otherwise indicated, all values were derived from the mean of replicates data and the standard deviation is presented.

3. Results

3.1. Wol infA was successfully amplified, cloned and subcloned

The Wol infA gene was amplified from the genomic DNA isolated from adult *B. malayi* worms. The amplicon of Wol infA encoding recombinant TI IF-1 was successfully cloned in pTZ57R/T vector for maintaining the integrity of the construct. The sequencing of the cloned gene showed no mutation in the amplified gene product. The gene was subsequently sub-cloned in pET28a(+) and confirmed by restriction digestion of the recombinant construct (Fig. 1A).

3.2. Wol TI IF-1 recombinant protein was successfully over-expressed and confirmed in immunoblot

Wol infA gene was expressed in Rosetta strain of *E. coli* as ~13 kDa protein (Wol TI IF-1) with 6-His-tag fused at both N and C terminus. SDS-PAGE analysis revealed release of ~70% of the target protein into the soluble fraction with a natively folded conformation (data not shown). The soluble recombinant protein was purified by affinity chromatography on a Ni-NTA agarose column and the tight binding

Table 1

Prediction of antigenic determinants for the Wol TI IF-1 using Kolaskar–Tongaonkar semi-empirical method.

S. no.	Antigenic determinant	Position
1	TLFEVEGAVTALLPAAEFRV	9
2	HEIICHVSG	34
3	IGDRVLVEM	53

could not be disrupted until eluted with 250 mM concentration of imidazole (Fig. 1B). The recombinant protein was localized with monoclonal anti-His antibody in Western blot (Fig. 1C).

3.3. Wol *infA* gene is expressed in all the major life-stages of *B. malayi*

To find out the presence of Wol TI IF-1 in major life-stages of *B. malayi*, cDNA synthesis was carried out with the RNA isolated from Mf, L3 and adult *B. malayi*. PCR amplification of the target gene with *infA* specific primers was confirmed in each cDNA sample demonstrating the abundance of this protein in *B. malayi* throughout its development (Fig. 1D).

3.4. Wol TI IF-1 reveals MHC-II restricted high antigenicity in silico

Kolaskar–Tongaonkar semi-empirical method predicted three antigenic determinants in protein i.e. TLFEVEGAVTALLPAAEFRV, HEIICHVSG, and IGDRVLVEM. The average antigenic propensity of the protein was found to be 1.0234 revealing its highly immunogenic nature (Table 1). ProPred analysis for HLA-DR alleles showed three peptide sequences (MVKDEKSKT, VRRSKIRII, and IIR-RLKGTS) to be the highest scoring promiscuous binders that could bind to almost all HLA-DR alleles (Table 2). In ProPred analysis for HLA-A/B alleles, the identified peptide sequences viz. KDEKSKTLF had affinity to HLA-A1, HLA-B2702 while VKDEKSKT had affinity to HLA-A2. However, these two peptide sequences possessed inefficient affinity for HLA-B2705 thus failed to prove as promiscuous binder (Table 3).

Table 2

ProPred analysis of HLA-DR binding Wol TI IF-1 peptides.

S. no.	HLA-DR allele	Peptide sequences	Position	Peptide score	Threshold score
1	DRB1_0301	MVKDEKSKT	0	5.27	2.96
2	DRB1_0306	MVKDEKSKT	0	4.3	2.08
3	DRB1_0307	MVKDEKSKT	0	4.3	2.08
4	DRB1_0311	MVKDEKSKT	0	4.3	2.08
5	DRB1_0701	VRRSKIRII	43	7.0	4.1
6	DRB1_0703	VRRSKIRII	43	7.0	4.0
7	DRB1_0801	IIRRLKGTS	72	4.6	1.8
8	DRB1_0802	IIRRLKGTS	72	4.6	1.0
9	DRB1_0804	IIRRLKGTS	72	5.6	1.6
10	DRB1_0806	IIRRLKGTS	72	5.6	2.4
11	DRB1_1107	MVKDEKSKT	0	4.57	2.1
12	DRB1_1301	VRRSKIRII	43	4.8	2.6
13	DRB1_1327	VRRSKIRII	43	4.8	2.6
14	DRB1_1328	VRRSKIRII	43	4.8	2.6

3.4.1. Wol TI IF-1 provides considerable protection against *B. malayi* L3 challenge in Mastomys

Blood Mf density remained significantly low in Wol TI IF-1 immunized Mastomys throughout the observation period post L3 challenge. On the day of autopsy, Wol TI IF-1 immunized Mastomys had 58.4 ± 17.3 Mf in $10 \mu\text{l}$ of tail blood as opposed to 199 ± 25.24 and 210.4 ± 36.91 in PBS or adjuvant control groups respectively thereby demonstrating around 70–72% reduced Mf density in the recombinant protein immunized group ($p < 0.001$) (data not shown). In addition to microfilaremia, vaccination with Wol TI IF-1 also significantly ($p < 0.001$) reduced (61–63%) the adult parasite establishment (Table 4). Not only this, the adult female worms recovered from the vaccinated group also exhibited higher embryostatic effect ($63.33 \pm 3.51\%$; $p < 0.001$) (Table 4).

3.4.2. Wol TI IF-1 treated worms demonstrate reduced Wolbachia load

Results of real-time PCR (qRT-PCR) for wolbachial load are expressed in terms of the threshold cycle (C_T). The difference between the mean C_T values of immunized and control female worms are indicated as ΔC_T ($\Delta C_T = C_T$ immunized – C_T control). ΔC_T is proportional with the exponential relationship to the difference in wolbachial content. ΔC_T value of PBS group and adjuvant group are 8 and 7 respectively that suggests 2^8 -fold (i.e. 256-fold) and 2^7 -fold (i.e. 128-fold) less wolbachial load in the immunized group as compared to PBS and adjuvant control groups (Table 5).

3.4.3. Wol TI IF-1 triggers the production of IgG antibody in immunized Mastomys

Vaccination with Wol TI IF-1 resulted in the production of specific IgG in the pooled sera which showed a gradual increase in immunized group, the antibody level increased tremendously after L3 challenge and maintained itself till the end of observation period, that is, the day of autopsy (24 weeks). Pooled sera from adjuvant or control group of Mastomys did not react with Wol TI IF-1 demonstrating the antibody specificity. Each point in the figure represents a value obtained with the pooled sera of six animals (replicates) and the data revealed a highly significant increase

Table 3

ProPred analysis of HLA-A/B binding Wol TI IF-1 peptides.

S. no.	HLA-A/B allele	Peptide sequences	Position	Peptide score	Threshold score
1	HLA-A1	KDEKSKTLF	2	0.025	−0.693
2	HLA-A2	VKDEKSKTL	1	6.667	1.553
3	HLA-B2702	KDEKSKTLF	2	0.100	0.000
4	HLA-B2705	KDEKSKTLF	2	1.5	2.996
		VKDEKSKTL	1	0.030	2.996

Table 4

Adult worm recovery and female worm fecundity.

Animal groups	No. of animals/surviving animals	Adult parasite counts/animal	Adult worms (mean ± S.D.)	Total adult worm recovery (mean ± S.D.)	Percentage reduction in worm burden	Percentage female parasite sterilization (mean ± S.D.)
PBS alone	6/6	♀ 16, 17, 9, 15, 14, 16 ♂ 10, 7, 6, 6, 5, 8	14.5 ± 2.88 7 ± 1.78	21.50 ± 4.03	0	21.67 ± 2.08
Adjuvant alone	6/6	♀ 13, 15, 18, 8, 14, 15 ♂ 6, 8, 8, 6, 5, 7	13.83 ± 3.3 6.6 ± 1.2	20.50 ± 4.135	0	20.33 ± 2.082
Wol TI IF-1 with adjuvant	6/6	♀ 3, 4, 9, 5, 6, 5 ♂ 1, 3, 2, 4, 2, 3	5.33 ± 2.06*** 2.5 ± 1.04***	7.83 ± 2.31***	61.80 with respect to adjuvant	63.33 ± 3.5***

*** $p < 0.001$, values significantly different from control group.

($p < 0.001$) in IgG in the immunized group over those of two control groups (Fig. 2A).

3.4.4. Wol TI IF-1 predominantly triggers the production of specific IgM and IgG1

Immunization with Wol TI IF-1 caused a significant increase in the specific IgG1 and IgM antibody isotype whose levels further got elevated after L3 challenge ($p < 0.001$). IgG2a ($p < 0.01$) was also found to be increased when compared with the adjuvant and PBS control groups. IgA, IgG2b and IgG3 antibody isotype generation was however minimal ($p > 0.05$) (Fig. 2B).

3.4.5. Wol TI IF-1 also triggers considerable production of IgE

SDS-PAGE and subsequent transfer of the recombinant protein on NC membrane revealed characteristic band of Wol TI IF-1 after reaction with the vaccinated *Mastomys* sera developed with antimouse IgE as secondary antibody.

Table 5Quantification of wolbachial load in *Brugia malayi* female worms by real-time PCR.

Animal groups	C_T	Mean	ΔC_T^a
PBS alone	23.27 23.34 23.37	23.33	8.11
Adjuvant alone	24.12 24.74 24.39	24.42	7.02
Wol TI IF-1 with adjuvant	31.39 31.63 31.29	31.44	–

^a ΔC_T is the difference between the mean C_T values of immunized and control female worms.

Control sera on the other hand did not show any reaction (Fig. 2C).

3.4.6. Wol TI IF-1 cross-reacts with the bancroftian antibodies present in the sera of putatively protective endemic normal subjects

The strong reactivity of purified Wol TI IF-1 with pooled bancroftian human sera of various categories from *W. bancrofti* endemic area indicates sharing of Wol TI IF-1 amongst the two human lymphatic filarial species. The reactivity of serum collected from endemic normal population substantiates the immunogenic nature of this wolbachial protein. There was no reaction with the sera from non-endemic normal subjects staying in filaria-free zone demonstrating filarial specificity (Fig. 2D).

3.4.7. Wol TI IF-1 is secreted-excreted out by the live adult *B. malayi* in vitro

Polyclonal antibody developed in the sera of *Mastomys* post immunization reacted with the ES products of male and female adult *B. malayi* collected by *in vitro* culture of each sex of worms separately demonstrating the release of TI IF-1 by the live male and female adult parasites. It may therefore be inferred that Wol TI IF-1 is one of the wolbachial proteins which comes in contact with the host and elicits strong immune response (Fig. 2E).

3.4.8. Anti-Wol TI IF-1 antibodies promote cellular adherence to the Mf and L3 causing cytotoxicity

Markedly increased antibody mediated cellular adherence was noticed on the surface of Mf and L3 of *B. malayi* in the presence of Wol TI IF-1 immunized *Mastomys* serum. 67.25% of the Mf and 71.6% of the L3 had profound adherence of PECs while sera from control groups showed negligible cell adhesion (5.75–6.8%) within 24 h of co-culture (Table 6). The cellular adherence also brought

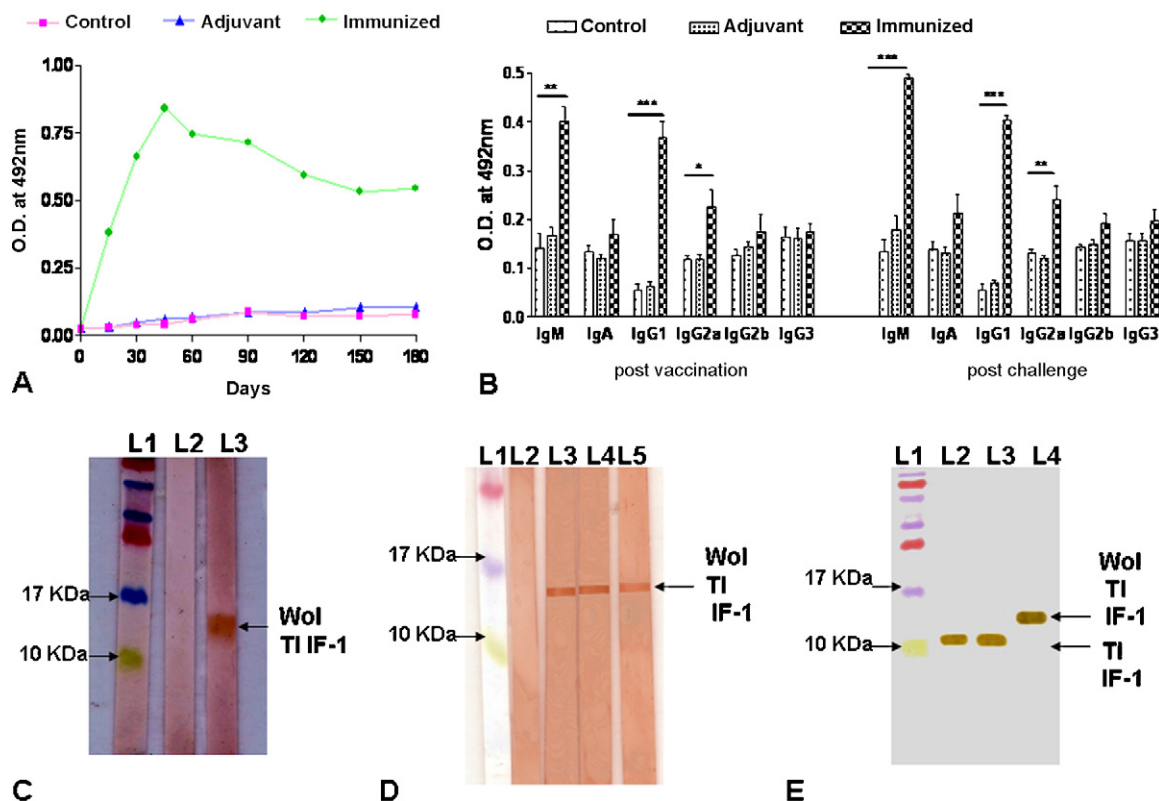


Fig. 2. Immunoprophylactic efficacy of recombinant Wol TI IF-1 in *Mastomys coucha*. (A) Kinetics of Wol TI IF-1 specific IgG antibody titer in *Mastomys* sera. Groups of *Mastomys* were immunized with Wol TI IF-1 (immunized), FCA/FIA (adjuvant) and PBS alone (control), and Wol TI IF-1 specific antibody levels were detected by ELISA at different time intervals. Gradual increase in IgG level occurred in immunized group, that reached maximum after L3 challenge and remained high even up to the day of autopsy. Control groups did not develop Wol TI IF-1 specific IgG. Each point represents a value obtained with pooled sera of six experimental animals. (B) Anti-Wol TI IF-1 isotypes level in sera of different groups of *Mastomys* at post vaccination and post *B. malayi* L3 challenges. IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3 antibodies were checked by ELISA for specific antibody against Wol TI IF-1. Each point represents a value obtained with sera of six experimental animals. (C) Reactivity of Wol TI IF-1 with IgE present in the immunized sera was carried out by Western blot. Immune sera of *Mastomys* showed reactivity with purified recombinant protein. However no reactivity was observed with control sera. Lane 1, standard prestained protein molecular weight marker; lane 2, 20 µg of purified Wol TI IF-1 incubated with 1:800 dilution of control *Mastomys* sera; lane 3, 20 µg of purified Wol TI IF-1 incubated with 1:800 dilution of immunized *Mastomys* sera. (D) Cross-reactivity of Wol TI IF-1 with pooled bancroftian human sera. Lane 1, standard prestained protein molecular weight marker; lane 2, reactivity of 20 µg of purified Wol TI IF-1 with nonendemic normal sera at 1:200 dilution; lane 3, reactivity of 20 µg of purified Wol TI IF-1 with asymptomatic MF positive sera at 1:200 dilution; lane 4, reactivity of 20 µg of purified Wol TI IF-1 with symptomatic MF negative sera at 1:200 dilution; lane 5, reactivity of 20 µg of purified Wol TI IF-1 with symptomatic MF positive sera at 1:200 dilution. (E) Presence of Wol TI IF-1 in ES product of adult *B. malayi* worms. Lane 1, standard prestained protein molecular weight marker; lane 2, concentrated ES products of male adult worms; lane 3, concentrated ES products of female adult worms; lane 4, recombinant purified Wol TI IF-1. Statistical significance of the differences between mean values of immunized and control groups is depicted as * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

about significant ($p < 0.001$) cytotoxicity and mortality to majority of the parasites *in vitro* (Fig. 3).

3.4.9. Wol TI IF-1 immunized *Mastomys* demonstrated profound *in vitro* lymphoproliferation in the presence of recombinant protein and mitogen

The splenocytes taken from the immunized group proliferated significantly on exposure to the recombinant protein ($p < 0.01$). The cells also proliferated in the presence

of Con A ($p < 0.05$). The control animals on the other hand demonstrated very high proliferation ($p < 0.001$) in the presence of Con A but not so when the recombinant protein was added to culture (Fig. 4A).

3.4.10. Wol TI IF-1 activates macrophages in the immunized *Mastomys* to produce profound ROS

A real time monitoring of ROS generated from PECs of immunized, adjuvant and control groups of *Mastomys*

Table 6

Antibody-dependant cellular cytotoxicity to L3 and Mf induced by sera of *Mastomys* immunized with Wol TI IF-1.

Experimental group	% cytotoxicity of Mf (mean $\pm$ S.D.)	% cytotoxicity of L3 (mean $\pm$ S.D.)
Control (n=6)	5.75 $\pm$ 1.70	6.8 $\pm$ 1.09
Immunized (n=6)	67.25 $\pm$ 1.25***	71.60 $\pm$ 3.8***

*** $p < 0.001$, values significantly different from control group.

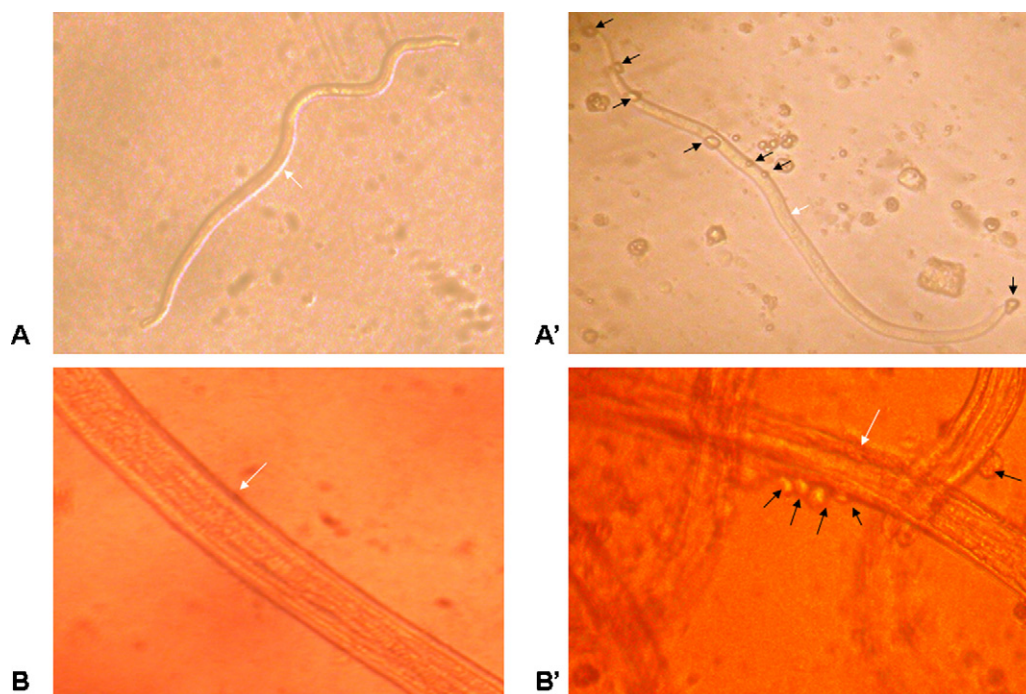


Fig. 3. Antibody dependent cellular adhesion of peritoneal exudate cells (PECs) on the body surface of microfilariae (MF) and infective larvae (L3) were observed within 24 h led to their death within 48 h. Percent Mf and L3 with cell adherence (≥ 5 cells adhered/Mf or ≥ 2 cells/L3) were assessed by phase contrast fluorescent microscope (Nikon, Japan). White arrow indicates Mf, L3 and black arrow indicate adhered PECs. (A) 40 $\times$ magnified image of Mf incubated with control sera at 1:16 dilution. (A') 40 $\times$ magnified image of Mf incubated with Mastomys sera raised against Wol TI IF-1 at 1:16 dilution. Similarly (B) 40 $\times$ magnified image of L3 incubated with control sera at 1:16 dilution. (B') 40 $\times$ magnified image of L3 incubated with Mastomys sera raised against Wol TI IF-1 at 1:16 dilution.

was done post-vaccination in order to determine *in vivo* milieu of macrophage activation. FACS data indicate that immunization with Wol TI IF-1 activates the APCs and leads to considerable oxidative burst ($p < 0.001$) in peritoneal macrophages over that of adjuvant and control animals (Fig. 4B).

3.4.11. Wol TI IF-1 provokes expansion of CD4 $^{+}$ T and CD19 $^{+}$ B lymphocytes in BALB/c mice

Immunophenotyping of splenic lymphocytes isolated from Wol TI IF-1 immunized mice revealed a significant increase in the population of CD4 $^{+}$ T cells ($p < 0.05$) and CD19 $^{+}$ B cells ($p < 0.01$). The CD8 $^{+}$ cytotoxic T cell population though showed a marginal increase, it was statistically not significant ($p > 0.05$) (Fig. 4C).

3.4.12. Wol TI IF-1 preferentially up-regulates the Th2 cytokines, IL-4 and IL-5

Intracellular content of various Th1 and Th2 cytokines by staining of CD4 $^{+}$ cells in FACS revealed significantly high IL-4 ($p < 0.01$) and IL-5 ($p < 0.001$) content. TGF- β content is low and remained unchanged with IL-10 post immunization. TNF and IFN- γ levels were rather down-regulated when compared with those of adjuvant group ($p < 0.001$) and PBS control group ($p < 0.05$). Thus Wol TI IF-1 predominantly promotes the production of Th2 cytokines, IL-4 and IL-5 (Fig. 4D).

4. Discussion

In absence of an effective adulticidal antifilarial drug, vaccination approach provides a logical alternative. In recent years, research on *Wolbachia*, the major contributor of inflammatory disease has gained considerable momentum. *Wolbachia* killing by some of the antirickettsial antibiotics adversely affected the morphogenesis, development, survival and fecundity of filarial parasites [29]. The complete genomic information on *Wolbachia* (TRS) of *B. malayi* has now facilitated investigations on the intriguing relationship of *Wolbachia* with nematode hosts. As mentioned in the beginning few proteins/enzyme pathways of *B. malayi Wolbachia* have been explored however, no attempt has ever been made to assess the immunoprophylactic efficacy of *Wolbachia* protein although WSP elicited antibody response has been well characterized in humans against lymphatic filariasis [30]. In the present investigation, a vital factor of *Wolbachia*, Wol TI IF-1 has been cloned, over-expressed and further investigated for immunoprophylactic efficacy against *B. malayi* larval challenge in susceptible rodent host, Mastomys. This factor does not exist in the eukaryotes therefore its epitopes showed greater structural disparity with the mammalian host. Wol TI IF-1 exhibited reactivity with the sera of humans residing in filaria-endemic regions but not with the filaria-free individuals demonstrating filarial-specificity of the protein. It was also released by both sexes of live adult *B. malayi* parasites in the form of ES products, thus

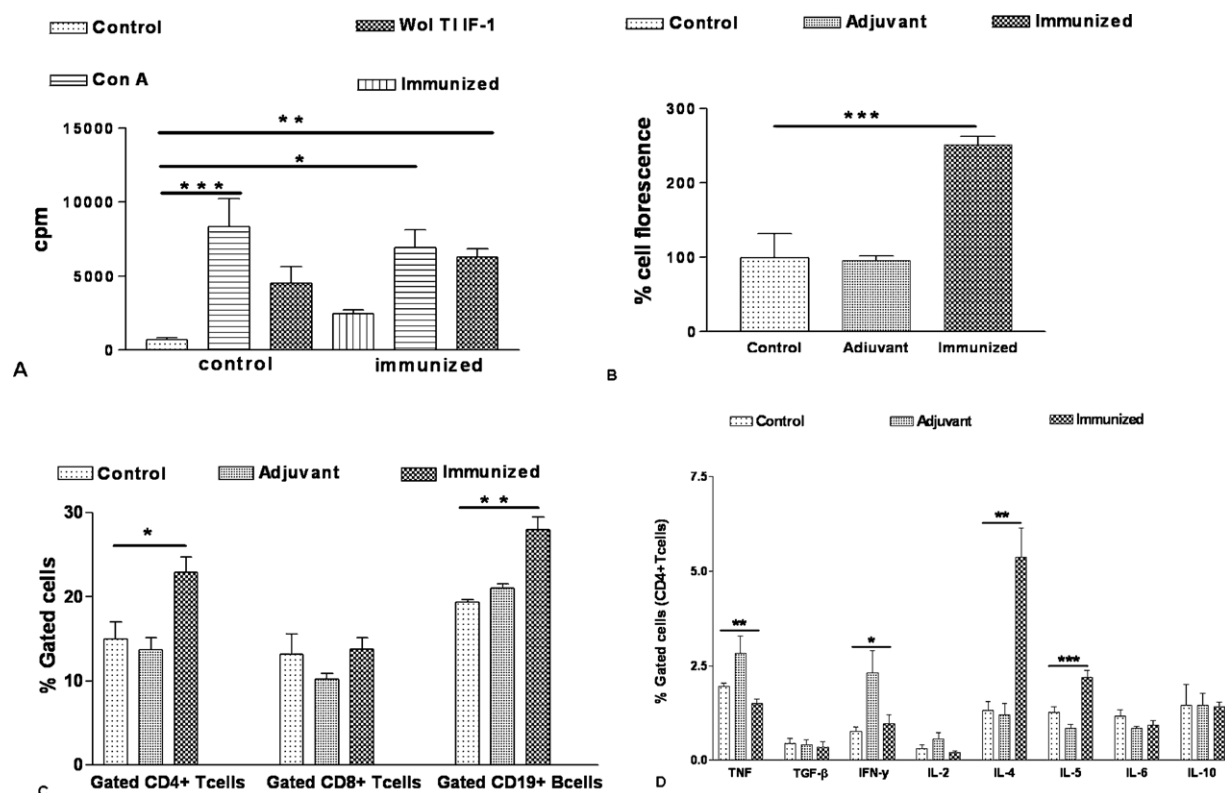


Fig. 4. Cellular immune responses of Wol TI IF-1. (A) *In vitro* lymphocyte proliferation was assessed in the splenocytes isolated from immunized, adjuvant and control groups by radioactive incorporation of [^{3} H]-thymidine after stimulation with either Concanavalin A (2.5 μ g/ml) or Wol TI IF-1 (2.5 μ g/ml). Radioactive incorporation in the cells was measured and results are expressed as mean counts per minute (cpm). (B) Total reactive oxidative burst was measured in the PECs harvested from control, adjuvant and immunized group of Mastomys at day 180 post L3 challenge. Significantly high oxidative burst was observed in PECs of Wol TI IF-1 vaccinated group. (C) Flow cytometric analysis of CD4, CD8 and CD19 cell surface markers of T and B cells. Level of CD4 and CD19 was significantly increased as compared to control groups. (D) Flow cytometric detection of intracellular cytokines TNF, TGF- β , IFN- γ , IL-2, IL-4, IL-5, and IL-10 production in CD4+ T cells. CD4+ T cells were fixed, permeabilized and stained with PE conjugated antibodies directed against particular cytokines and acquired on FACS. Level of IL-4 and IL-5 in immunized group was significantly increased as compared to the control groups. Statistical significance of the differences between mean values of immunized and control groups in all figures is depicted as * p < 0.05; ** p < 0.01 and *** p < 0.001.

likely to be immunogenic and capable of triggering the specific immune response in bancroftian patients. This fact is strengthened by the recent report which indicates that the vertebrate host gets exposed to *Wolbachia* or their products that are released through the lumen of the secretory-excretory canals of *B. malayi* [9]. These characteristics make Wol TI IF-1 extremely attractive target for the development of novel vaccine. In addition, the abundance of Wol infa gene among all the major life-stages of *B. malayi* reinforces its worth as vaccine candidate that can act against various life-forms. We used *M. coucha*, a susceptible host to several filarial parasite species including *B. malayi* which has earlier been used for vaccination studies [26]. The purified recombinant Wol TI IF-1 elicited considerable protection against establishment and transformation of L3 into adult parasites and the resulting microfilaremia was also found to be profoundly reduced. Majority of the established female parasites had defective embryogenesis too which could be correlated with the several fold reduction in the wolbachial load in female worms recovered from vaccinated animals as compared to those recovered from unimmunized Mastomys. Tetracycline is known to impair the development of L3–L4 by blocking the binding of TI

IF-1 of *Wolbachia* endosymbiont with A-subunit of 30S ribosome [31]. The high antibody level generated against the recombinant protein might possibly have inhibited the ribosomal binding of this factor resulting in impaired development of introduced L3. The recombinant protein triggered a dominant Th2 immune response as evidenced by the production of high levels of serum IgM and IgG1 antibodies that increased further upon L3 challenge. Antibodies are considered to play a protective role in helminth infections including infections with filariids [32]. Considerable increase in the levels of specific IgG1, IgM and IgG2a was evident and these antibodies are ascribed to play an important role in killing of helminth parasite via classical pathway [33]. IgM is essential for the elimination of *Brugia* worms while IgG1 and IgG2a antibodies are reported to offer adequate protection against challenged filarial larvae post irradiated L3 vaccination [34,35]. The recombinant factor in the current investigation provoked CD19+ B cell and MHC class II restricted CD4+ T helper cell expansion. These findings are correlated with the *in silico* antigenic prediction data that demonstrated the highly antigenic nature of protein. The peptide sequences, TLFEVEGAVTALL-PAAEFRV, HEIICHVSG and IGDRLVEM demonstrated more

than 1.0 antigen propensity that could be attributed to up-regulated B cell proliferation [36]. On ProPed analysis, the residues MVKDEKSKT at position zero, VRRSKIRII at position 43 and IIRRLKGTS at position 72 displayed high binding affinity with almost all the MHC-II restricted HLA-DR allele used in analysis. Two other peptide sequences KDEKSKTLF and VKDEKSKT which proved to be nonpromiscuous binders interacted with only a few MHC-I restricted alleles but failed to bind to majority of the HLA-A/B alleles. Thus it appears that Wol TI IF-1 selectively acts as a MHC-II restricted antigen, activates CD4+ T helper cells and triggers antibody production. CD4+ T cells and B cells are considered to be indispensable for producing a helminth vaccine [37,38]. The recombinant larval protein Na-ASP-2 from the human helminth parasite *Necator americanus* is a lead candidate for a human hookworm vaccine which also induced a strong cellular and antibody mediated immune response characterized by high specific IgG1, IgG2a, IgM, and IgG titers with latter being maintained up to three months post vaccination. In addition, the specific cellular proliferation was also noticed. The immune response thus generated by hookworm vaccine matched the immune response generated by Wol TI IF-1 in Mastomys [39]. Antibody-dependent cell-mediated cytotoxicity is believed to be one of the principal immunological mechanisms responsible for killing of helminth larvae including filariids. Antibodies to Wol TI IF-1 induced prominent adherence of PECs to the surface of Mf and L3 resulting into high percentage of cytotoxicity and their death. Basis for adhesion of PECs to L3 and Mf is that Wol TI IF-1 generated high level of IgM, essential for the recognition and cell adhesion to the surface of the larvae. In addition, sera taken from sec IgM^{-/-} mice that lack this isotype is incapable of showing adhesion of PECs to the larvae [34]. Among the PECs, macrophages are the principal cells involved in the killing of invading parasites by oxidative burst mechanism [40]. PECs also release complement proteins that kill parasites and correlate with elevated IgG1, IgG2a subclass and IgM antibodies [41]. In addition, the recombinant wolbachial factor also triggered the production of IgE and the two major Th2 cytokines IL-4 and IL-5. These two cytokines have been associated with resistance of the host to filarial infection as these prevent the development of adult worms as well as development of microfilaremia [42–45]. In the *Litomosoides sigmodontis* model, IL-4 and IL-5 deficient mice illustrated a massive elevation of the worm burden and higher level of microfilaremia [45,46]. In other murine models, IL-5 played an important role during expression of protective immunity to *Onchocerca lienalis* infective larvae in mice [47]. Thus IL-4 and IL-5 were the dominant cytokine response coinciding with parasite clearance. IL-5 appears to perform its function by recruiting eosinophils, which degranulate to kill the parasite [48]. While MHC-II restricted CD4+ T helper cells produce IL-4, which activates B cells to ooze anti-parasitic antibody IgE that plays a pivotal role in protective anti-helminth immunity in animal experimentations and human sero-epidemiological studies [49]. In Brazil, adolescents resistant to *S. mansoni* possess six to eight fold higher concentration of specific IgE than those with low resistance [50]. Recent rodents studies have demonstrated that helminth vaccines predominantly induce the type 2

immune response with IL-4 and IL-5 cytokines induction that can be protective in conjunction with antigen-specific IgE that are desired for the creation of effective vaccine against filarial parasites [44,51,52]. The possible mechanism of immunogenic TI IF-1 of *B. malayi* endosymbiont *Wolbachia* could be the stimulation of APCs that may have activated the CD4+ helper cells which in turn release Th2 cytokines IL-4 and IL-5 that elicit an effector immune response including activity of other 'front-line' cell types such as eosinophils, basophils, mast cells, macrophages and isotype-switch to B-cell responses. The activated B cells ooze IgE, IgM, IgG1 and IgG2a that play an important role in killing of brugian parasite via classical pathway. The antibodies produced against Wol TI IF-1 induced profound adherence of PECs to L3 and Mf surfaces leading to the parasite immobility and subsequent cytotoxicity.

Though a few filarial recombinant proteins have been used to immunize experimental animals with varying success, this is the first ever report on the immunoprophylactic evaluation and protective efficacy of a *Wolbachia* excretory secretory protein that comes out of *B. malayi* worms in the medium when parasites are cultured *in vitro* demonstrating the role of *Wolbachia* in generating protective immune response via Th2 cytokine milieu. Thus, Wol TI IF-1 has a scope for developing into a candidate vaccine antigen not only against lymphatic filariasis but also against all the major human filarial parasites.

Conflict of interest

None.

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