



Troponin 1 of human filarial parasite *Brugia malayi*: cDNA cloning, expression, purification, and its immunoprophylactic potential

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Abstract

In the search for immunoprophylactics for the control of human lymphatic filariasis, we recently identified troponin 1 (Tn1) in *Brugia malayi* adult worms. The present study reports the cloning and expression of the *B. malayi* Tn1 (Tn1bm), its immunoprophylactic efficacy against *B. malayi* infection, and the immunological responses of the host. The Tn1bm gene was cloned (Acc no. JF912447) and expressed, and the purified recombinant Tn1bm (rTn1bm) presented a single ~27 kDa band. Parasite load in rTn1bm-immunized BALB/c mice challenged with *B. malayi* infective larvae (L₃) was assessed. In rTn1bm-immunized animals, IgE, IgG, and IgG subclasses in the serum, cell proliferative response, Th1 and Th2 cytokine secretion (from splenocytes), and NO release (from peritoneal macrophages) were determined. Antibody-dependent cell-mediated cytotoxicity (ADCC) to L₃ was assayed using rTn1bm-immune serum. The innate immune response markers MHC class-I, MHC class-II, TLR2, TLR4, and TLR6 expression in peritoneal macrophages and CD3+, CD4+, CD8+, and CD19+ in the splenocyte population were determined in Tn1bm-exposed cells from naïve mice. rTn1bm-immunized L₃-challenged animals showed a 60% reduction in parasite burden. Immunization upregulated cellular proliferation, cytokine (IFN- γ , TNF- α , IL-1 β , IL-4, IL-6, and IL-10) secretion, NO release, and antigen-specific IgG, IgG1, and IgG2b antibody levels. rTn1bm-immune serum killed > 65% of L₃ in the ADCC assay. Increased MHC class-II, TLR2, and TLR6 expression and the relative CD4+ and CD19+ cell populations of naïve animal cells indicated the ability of rTn1bm to mobilize innate immune responses. This is the first report of the immunoprophylactic potential of rTn1bm against *B. malayi*.

Keywords Troponin 1 · *Brugia malayi* · Immunoprophylaxis · Th1 and Th2 responses · ADCC · MHC class-I · MHC class-II · TLRs · CD3+ · CD4+ · CD8+ · CD19+

Vikas Kushwaha and Prachi Tewari contributed equally to this work.

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Introduction

Lymphatic filariasis (LF), caused by *Wuchereria bancrofti*, *Brugia malayi*, and *B. timori*, is recognized as one of the world's most debilitating and disfiguring vector-borne neglected tropical diseases of developing countries and is a significant barrier to socioeconomic development. The disease is endemic in 73 countries with over 120 million people infected (WHO 2014), approximately 1.4 billion people at risk of infection (<http://www.who.int/wer>), and over 40 million seriously incapacitated or disfigured by the disease (Babu and Nutman 2012). In India, more than 553 million people are exposed to infection, 31 million have the infection, and 23 million have chronic manifestations of the infection (Sabesan et al. 2010).

The current strategy for the prevention/control of LF comprises the administration of antifilarial albendazole (ALB) diethylcarbamazine (DEC) and ivermectin (IVM) once a year in

the mass drug administration (MDA) program of the WHO Global Program to Eliminate Lymphatic Filariasis (GPELF) (Taylor et al. 2010; WHO 2017). Additionally, DEC is added to common table/cooking salt and supplied to the endemic population for a period of 1 year. By 2017, this MDA strategy greatly reduced the incidence of the infection (Molyneux et al. 2017) and even eliminated it in some countries (WHO 2018) (<https://www.who.int/westernpacific/news/detail/08-10-2018-three-more-countries-eliminate-lymphatic-filariasis>). Unfortunately, there are reports of the re-emergence of infection in some areas, especially in Sri Lanka (Rao et al. 2017). This outcome suggests alternative approaches, such as the development of filarial vaccines, are needed. To this end, several potential immunoprophylactic parasite molecules (Sahoo et al. 2009; Vanam et al. 2009; Joseph et al. 2012; Kushwaha et al. 2014; Verma et al. 2015, 2017) have been identified and even irradiated infective third-stage larvae (L₃) (Babayan et al. 2006) have been investigated. Some of the parasite proteins and formulations that have been investigated are myosin heavy chain, myosin-like proteins and members of the paramyosin family (Li et al. 1991, 1995), multivalent vaccine formulations of BmALT-2 and BmHSP (Joseph et al. 2012; Thirugnanam et al. 2007; Samykutty et al. 2010), chitinase, glutathione S-transferase (GST), tropomyosin, SXP-1 (Harrison et al. 1999; Li et al. 1999; Gupta et al. 2015), trivalent/multivalent fusion protein vaccine (Dakshinamoorthy et al. 2013, 2014), and tetravalent fusion protein vaccine (Chauhan et al. 2018; Khatri et al. 2018). However, a successful LF vaccine has yet to be found. We recently identified a disorganized muscle protein-1 (DIM-1) in *B. malayi* adult worm extract that showed almost complete lack of homology with the human counterpart and partially protected *Mastomys coucha* against *B. malayi* infection (Kushwaha et al. 2014). We previously identified another protein, troponin t protein 2, a member of the troponin family, in *B. malayi* adult worm extract (Sahoo et al. 2009), but our attempts to clone this protein were not successful. A few years later, Gomase et al. (2013) analyzed the troponin protein of *Wuchereria bancrofti* by a bioinformatic approach and reported that predicted epitopes of the protein are important determinants for protection against lymphatic filariasis (LF) and suggested that a small fragment of troponin protein might produce an immune response and may be used for designing subunit and synthetic peptide vaccines against LF. This finding revived our interest in troponin, and we selected a close member, troponin I, from GenBank; designed primers; and cloned the gene. We submitted the TnI sequence to GenBank, which named it troponin 1 of *B. malayi* (Tn1bm) (acc no. JF912447). Amino acid sequence alignment of Tn1bm (gene sequence submitted; JF912447.1) with that of Troponin I (*B. malayi*: XM_001901780.1) showed 81% similarity between the two, whereas essentially no similarity was found between TnI and troponin t (acc no. XP_001892507.1).

Tn1bm was localized by immunofluorescence (using mouse Tn1bm antiserum and Alexa Fluor 488-conjugated goat anti-mouse IgG) in the body wall of both male and female adult worms and in the uterine wall of females (unpublished data). The present study was undertaken to determine whether Tn1bm immunization has any prophylactic potential against filarial lymphatic infection. To this end, the objectives of the study were (i) to prepare recombinant Tn1bm (rTn1bm), (ii) to investigate the effect of immunization of BALB/c mice with rTn1bm on the worm burden of a subsequently introduced *B. malayi* infection in the animals, (iii) to study the immune mediators and immune responses [cellular proliferation, release of Th1, Th2 cytokines and nitric oxide (NO), and antibody levels of specific IgE, IgG and its subclasses] of rTn1bm-immunized animals, and antibody-dependent cell-mediated cytotoxicity (ADCC), and (iv) to study the influence of rTn1bm on the relative distribution of CD4+, CD8+, and CD19+ cells, and expression of the innate immune response markers MHC class I and II molecules and TLR-2, TLR-4 and TLR-6 in naïve animal cells.

Materials and methods

Animals

Thirty-two healthy laboratory-bred BALB/c mice (6 to 8 weeks old; 20–22 g body weight) procured from the National Laboratory Animal Center (NLAC), CSIR-CDRI, were used in the present study.

Isolation of parasites and preparation of parasite extract

Live L₃ from experimentally infected *Aedes aegypti* mosquitoes (fed on microfilaremic *M. coucha*) and adult *B. malayi* parasites from the peritoneal cavity (p.c.) of *B. malayi*-infected jirds, respectively, were used in the present study (parasites were a kind gift of the Head of the Parasitology Division, CSIR-CDRI, Lucknow). A soluble extract of *B. malayi* adult parasites (BmA) was prepared (Dixit et al. 2004), the protein content was estimated (Bradford 1976), and the extract was stored in 0.5 mL aliquots at −20 °C until use. L₃ were used for infecting the animals and in the ADCC assay.

Cloning, subcloning, and sequencing of *B. malayi* cDNA

The primers for the TnI gene containing the BamH1 and XhoI restriction enzyme sites were designed with the help of OLIGO software, according to the gene sequence available at NCBI (*B. malayi* draft genome database of TIGR, J. Craig Venter Institute). The Tn1bm gene was amplified by PCR. We

submitted the TnI sequence to GenBank, which named it troponin 1 of *B. malayi* (Tn1bm) (acc no. JF912447).

The sequence of primers for Tn1bm is given in Table 1.

Amplification of the gene by PCR was carried out in 30 cycles with the initial denaturation at 95 °C for 5 min, final denaturation at 95 °C for 30 s, primer annealing at 58 °C for 1 min, and a final extension at 72 °C for 5 min. The amplified PCR product was ligated into the TA vector pTZ57R/T using a TA clone PCR kit (Fermentas, USA), transformed into *Escherichia coli* DH5- α competent cells, and plated on Luria Bertani (LB)-ampicillin plates treated with X-gal. The white colonies screened were positive, and the sequence was confirmed (Integrated DNA Technologies, USA). Plasmid DNA was isolated from TA clones using the Quick Plasmid miniprep Kit (Invitrogen, USA), subsequently digested with restriction enzymes, the insert ligated into expression vector pet43.1a and transformed into competent *E. coli* BL21-DE3 cells.

Expression of rTn1bm

Five milliliters of overnight incubated primary culture of transformed BL21-DE3 cells (containing pet43.1a-Tn1gene) was inoculated into 250 mL of LB medium (secondary cultures) containing ampicillin and incubated in a shaker incubator (200 rpm) at 37 °C until the O.D. was 0.6–0.8 (mid-log phase) at 600 nm. The secondary culture was induced with 1 mM isopropyl β -D thiogalactoside (250 μ l/250 mL culture, stock conc. 1 M; pH 7.2) and further incubated in a shaker incubator (200 rpm) at 22 °C for 4 h. The culture was centrifuged (5000 rpm) for 5 min at 4 °C and sonicated for 10 $\times$ 20 s with 10-s intervals in lysis buffer (50 mM Tris, 250 mM NaCl, pH 8.0) containing 10 mM imidazole, Triton-X, and supplemented with 1000 μ M phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, USA) and 1 mg/mL lysozyme (Sigma-Aldrich, USA) under chilled conditions (Kushwaha et al. 2014). The suspension was centrifuged at 2500 $\times$ g for

30 min at 4 °C, and 10 μ l of the suspension was checked by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of rTn1bm

The cell lysate was loaded in a Ni-NTA column and washed by graded concentrations of imidazole (10, 20 and 50 mM) prepared in 50 mM PBS (pH 7.8). The histidine-tagged rTn1bm was eluted using 100 mM imidazole, desalted by dialysis (cut-off: 10 kDa), concentrated to 1 mL using Amicon ultra filters (cut-off: 10 kDa; Millipore, USA), and checked by 10% SDS-PAGE. Purified rTn1bm was ~81 kDa.

Further purification of rTn1bm (~81 kDa) with recombinant enterokinase

Purified rTn1bm (~81 kDa) was treated overnight with recombinant enterokinase (22 °C), which removed Nus protein (size ~55–60 kDa). The digested protein was loaded in a Ni-NTA column and washed with 5 mM and 10 mM imidazole buffer to remove unbound proteins. The first His-tag (near the Nus-tag) had very low affinity to the column, while the second His-tag (at the C-terminus of the protein) had greater affinity to the column. The column was rewashed with low concentrations of imidazole (10, 20 and 50 mM) to remove the Nus protein (first His-tag), while rTn1bm with the second His-tag was purified using 100 mM imidazole. The final purified recombinant protein (~27 kDa) was eluted with 100 mM imidazole and confirmed by 10% SDS-PAGE.

The endotoxin level in the final purified rTn1bm was determined by a Limulus Amebocyte Lysate (LAL) test kit (E-TOXATE®; Sigma, USA) according to the manufacturer's instructions; the level of endotoxin in the purified protein was found to be negligible (< 1 EU/mg). The protein was then transferred to nitrocellulose paper (NCP) and localized with an anti-His antibody by western blot. A single band at ~

Table 1 Primer sequences used for PCR/qPCR

Gene	Primer	Sequence
Tn1bm (JF912447)	Forward	5'-GGATCCATGGAAAGAAAAATCGGACGACAGAAT-3'
	Reverse	5'-CTCGAGTTTCGTATTTTGCGCAATTTTCGACATCCAT-3'
TLR-2 (Gene ID: 31981332)	Forward	5'-CTGCACTGGTGTCTGGAGTC-3'
	Reverse	5'-GGGCACCTACGAGCAAGAT-3'
TLR-4 (Gene ID: 118130391)	Forward	5'-GGACTCTGATCATGGCACTG-3'
	Reverse	5'-CTGATCCATGCATTGGTAGGT-3'
TLR-6 (Gene ID: 58743328)	Forward	5'-GAGCCTGAGGCATCTAGACCT-3'
	Reverse	5'-TCAGGTTGCCAAATTCCTTAC-3'
GAPDH (Gene ID: 14433)	Forward	5'-TTCACCACCATGGAGAAGGC-3'
	Reverse	5'-GGCATGGACTGTGGTCATGA-3'

27 kDa represented the purified protein, and the protein content was quantified (Bradford 1976).

Grouping of BALB/c mice, immunization, and exposure to *B. malayi* infection

BALB/c mice were selected for the present study because (1) they fully support the molting of the L₃ to L₄ stage, which is taken as the short-term endpoint for the assessment of the effect of rTn1bm immunization on infection; (2) there is a large amount of information on the immune system and immune pathways of these mice, which would help in understanding the immune responses in the present study; and (3) all immunological reagents of mice are commercially available.

The grouping of the animals ($n = 26$) and the experimental schedule are given in Table 2. Groups of animals were immunized with three subcutaneous (s.c.) injections of rTn1bm/BmA in PBS or PBS only, mixed with Freund's complete adjuvant/Freund's incomplete adjuvant (FCA/FIA; Sigma-Aldrich, USA). The first injection of rTn1bm (7 µg/animal), BmA (10 µg/animal), or PBS was given mixed with FCA, whereas the second and third injections of rTn1bm (3.5 µg/animal), BmA (5 µg/animal), or PBS were given mixed with FIA. The second injection was given 2 weeks after the first injection, and the third injection was given 1 week after the second injection. On day 27/28 post-first immunization (p.f.i.), one set of rTn1bm-immunized and non-immunized (PBS + FCA/FIA; control) animals were euthanized, and various immunological parameters were investigated. Another set of rTn1bm-immunized and non-immunized animals was challenged with *B. malayi* by intraperitoneal injection of live L₃ (50 L₃/animal) and euthanized on day 27/28 post-first L₃ inoculation (p.i.) to assess parasite burden. Blood was collected from the animals by cardiac puncture at euthanization.

Assessment of parasite load

On day 27/28 post-L₃ inoculation (p.i.), parasites were recovered from the peritoneal cavity (p.c.) of animals (immunized and non-immunized), counted, and the viability assessed. The stage of the parasites recovered was determined, and the parasite dimensions were measured by micrometry (Joseph et al. 2011) under a microscope (Leica DMLB). The percentage reduction in parasite burden in immunized infected animals was calculated relative to parasites recovered from non-immunized infected animals.

Immune mediator responses in rTn1bm-immunized animals

For the lymphocyte transformation test (LTT) and cytokine release assays, spleens were collected from immunized and

Table 2 Grouping of BALB/c mice and experimental schedules

Group	Group abbreviation	No. of animals (total from 2 independent experiments)	Antigen ^a	Adjuvant	Infection ^b	Day of termination ^c of experiment; purpose
Non-immunized	Non-Im	5	- (PBS only)	FCA/FIA	-	27/28 p.f.i.; immunological study
rTn1bm-immunized	rTn1bm-Im	5	rTn1bm in PBS	FCA/FIA	-	27/28 p.f.i.; immunological study
Non-immunized+L ₃	Non-Im + L ₃	6	- (PBS only)	FCA/FIA	<i>B. malayi</i> L ₃	27/28 p.i.; parasitological study
rTn1bm-immunized+L ₃	rTn1bm-Im + L ₃	6	rTn1bm in PBS	FCA/FIA	<i>B. malayi</i> L ₃	27/28 p.i.; parasitological study
BmA ^e -immunized (positive control)	BmA-Im	4	BmA in PBS	FCA/FIA	-	27/28 p.f.i.; blood collection for serum isolation before termination
Naïve	Control	6	-	-	-	Blood collection for serum (for ADCC assay) before termination. Isolation of cells for ADCC assay and innate immune response studies

^a Three subcutaneous injections of antigen/vehicle (PBS) were given. First injection of rTn1bm (7 µg in PBS/animal)/BmA (10 µg in PBS/animal) or PBS only (0.1 ml/animal) was given mixed with FCA, and after 2 weeks, two injections each of ½ the amount of the antigen in 0.1 ml PBS/animal or PBS only mixed with FIA were given at weekly intervals

^b *B. malayi* L₃ (50/animal) were injected intraperitoneally on day 27/28 post first immunization injection (p.f.i.)

^c Animals were killed humanely under anesthesia

^d p.i.: post first L₃ inoculation

^e BmA: soluble extract of *B. malayi* adult parasites

non-immunized animals, and a splenocyte suspension was prepared in complete RPMI-1640 fortified with antibiotics (penicillin: 100 U/mL; streptomycin: 100 µg/mL; Sigma-Aldrich, USA) + 10% fetal bovine serum (FBS; Sigma-Aldrich, USA) as described elsewhere (Dixit et al. 2006). The viability of the cells was checked by the 0.1% trypan blue dye exclusion method, and the final concentration of the cells was adjusted to 2×10^6 cells/mL.

NO determination

NO was determined as described by Sahoo et al. (2009) with some modifications. Briefly, 2 ml of RPMI 1640 medium containing EDTA (0.1%) and antibiotics was injected aseptically into the peritoneal cavity of the immunized and non-immunized animals, and peritoneal lavage was collected. Peritoneal cells thus collected were washed thoroughly with the medium, suspended in medium containing 10% fetal bovine serum, and dispensed (1×10^6 cells/500 µL/well) into sterile 48-well tissue culture plates (Nunc, Roskilde, Denmark). After overnight incubation at 37 °C in a 5% CO₂ atmosphere, non-adherent cells were removed, and the wells were replenished with fresh medium. rTn1bm (1 µg/mL) or LPS (1 µg/mL) was added to each well and incubated under the same conditions. The presence of nitrite in 48 h post-stimulation (PS) culture supernatants was quantified as described by Thomas et al. (1997). Assays were carried out in triplicate for each sample.

Cellular proliferation

LTT was performed as described by Dixit et al. (2006). A splenocyte suspension (1×10^6 /mL) prepared from the spleens of immunized and non-immunized animals was dispensed (200 µL/well) into a sterile 96-well plate (Nunc, Roskilde, Denmark) and exposed to rTn1bm (1 µg/mL) or Con A (10 µg/mL) and incubated as above. After 72 h of incubation, ³H-thymidine (BRIT, Mumbai) was added (1 µCi/well), and incubation was continued for 16–18 h. The cells were then harvested, suspended in scintillation fluid, and ³H-thymidine incorporation was quantified in a scintillation counter (LS Analyzer, Beckman Inc.). The results were expressed as counts per minute (CPM).

Cytokine secretion assay

Splenocyte suspensions (2×10^6 /mL) prepared from the spleens of immunized and non-immunized animals were plated in sterile 24-well plates (Nunc, Roskilde, Denmark) and exposed to rTn1bm or LPS (1 µg/mL) for 48 h at 37 °C in a 5% CO₂ atmosphere. Secretion of IFN-γ, IL-1β (Pierce Endogen), TNF-α, IL-4, IL-6, IL-10, TGF-β (BD Biosciences, USA), and IL-13 (R & D, USA) into culture

supernatants was determined by sandwich ELISA using paired antibodies following the manufacturer's instructions with some modification (Murthy et al. 2000). Triplicate wells were run for each sample. The color reaction was read after incubation with O-phenylenediamine dihydrochloride (OPD; Sigma-Aldrich, USA) prepared in citrate buffer+H₂O₂ at 490 nm in a spectrophotometer (Bio-Rad, Model-680, Microplate Reader, CA, USA). The concentration of all cytokines, except TGF-β, was calculated from OD readings (490 nm) of the respective standards obtained from the same manufacturers. The results of TGF-β were expressed in OD.

Determination of IgE, IgG, and IgG subclasses

The specific IgE, IgG, and four subclasses of IgG in both immunized and non-immunized animals were determined by indirect ELISA (Sahoo et al. 2009; Joseph et al. 2011) using the optimum concentration of rTn1bm (0.5 µg/ml) and optimum dilutions of rTn1bm antiserum (IgE 1:4; IgG 1:100; IgG-subclasses 1:50), HRP-conjugated goat anti-mouse IgE antibody (1:1000; Bethyl Laboratories Inc., USA), HRP-conjugated rabbit anti-mouse-IgG (1:50,000; MP, Biomedicals, Ohio, USA), and goat anti-mouse-IgG1 (dilution 1:1500), IgG2a, IgG2b, or IgG3 (dilution 1:2000; AbD Serotec, USA). After washing, OPD prepared in citrate buffer+H₂O₂ was added to the wells, and the absorbance was read in a plate reader (Bio-Rad, Model-680, Microplate Reader, CA, USA) at 490 nm.

ADCC assay

The assay was carried out to determine the anti-rTn1bm antibody-dependent cell-mediated cytotoxic effect on L₃ in vitro. The assay was performed as described by Chandrashekar et al. (1990) with some modifications. Briefly, viable peritoneal exudate cells (PECs) of healthy naïve mice ($n = 6$; Table 2) were prepared in RPMI 1640 medium containing 100 U/mL penicillin and 100 µg/mL streptomycin. Triplicate wells of a 48-well sterile culture plate (Nunc, Roskilde, Denmark) received, per well, 4×10^6 PEC, 40 L₃ and 50 µL of serum from naïve (negative control), non-immunized, Tn1bm-immunized, and BmA-immunized (positive control) animals (final volume of mix/well = 400 µl). After 24 h of incubation at 37 °C in a 5% CO₂ atmosphere, the viability of the L₃ was assessed under a microscope. Dead/dying L₃ showing adherent cells on the surface were counted, and the results were expressed as the percentage cytotoxicity to L₃. The experiment was repeated twice.

Determination of innate immune response markers

Peritoneal macrophages of the naïve BALB/c mice ($n = 6$) were collected, the viability of the cells was checked, and a

cell suspension was prepared (Roy et al. 2014; Tewari et al. 2017). The cells were dispensed into a sterile 12-well flat-bottom plate (Genetix Biotech Asia Pvt. Ltd., India) at a density of 2×10^6 cells/mL. After 3 h of incubation (37 °C with 5% CO₂), non-adherent cells were removed, and the adherent cells were exposed to LPS (10 µg/mL; Sigma–Aldrich, USA) or rTn1bm (2 µg/mL) for 24 h and used to determine the expression of TLRs, MHC class-I, and MHC class-II.

Unexposed cells were used as a control, and LPS/Con A-exposed cells were used as a positive control.

Expression of MHC class-I and MHC class-II in rTn1bm-exposed peritoneal macrophages of naïve BALB/c mice

MHC class-I and MHC class-II expression on the surface of the macrophages were determined as described by Roy et al. (2011, 2014). Briefly, the cells were suspended in staining buffer (PBS containing 0.2% FBS and 1% sodium azide) and stained with PE-conjugated anti-MHC class-I and FITC-conjugated anti-MHC class-II antibodies for 20 min at 4 °C. The stained cells were washed with PBS and suspended in 500 µl of PBS and analyzed by flow cytometry by gating the MHC class-I and MHC class-II population individually in a dot plot showing the live cells only.

Expression of TLR2, 4, and 6 in rTn1bm-exposed macrophages of naïve BALB/c mice

The expression of TLR2, 4, and 6 genes in macrophages was analyzed by real-time PCR gene-specific primers (Table 1). Total RNA from macrophages was isolated using RNazol®RT (Molecular Research Center, Inc. Cincinnati, OH). cDNA from different groups was prepared by using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California 94404, USA) following the manufacturer's instructions. Quantitative RT-PCR was carried out using a SYBR Green PCR master mix (Thermo Scientific, MA, USA) according to the manufacturer's instructions. Amplification of the housekeeping gene GAPDH was used as an endogenous control. Normalized values were used to plot the bar graphs.

Determination of CD3+, CD4+, CD8+, and CD19+ immunophenotypes in rTn1bm-exposed splenocytes of naïve BALB/c mice

The CD3+, CD4+, CD8+, and CD19+ immunophenotypes were determined in the splenic cell population from naïve animals according to the method

of Yadav et al. (2012, 2013). Briefly, splenocytes (2×10^5 cells/100 µl) were seeded into sterile 12-well plates (Genetix Biotech Asia Pvt. Ltd., India) and exposed to LPS (10 µg/mL; Sigma–Aldrich, USA) for B lymphocytes, Con A (5 µg/mL; Sigma–Aldrich, USA) for T lymphocytes, and rTn1bm (2 µg/mL) for 72 h at 37 °C in a 5% CO₂ atmosphere. The cells were harvested and suspended in staining buffer (0.2% FBS, 1% sodium azide in PBS) and stained with APC-Cy-7-conjugated CD3+, FITC-conjugated CD4+, PerCP-conjugated CD8+, and Alexa Fluor 700-conjugated CD19+ (BD Biosciences, San Diego, CA) antibodies for 20 min at 4 °C. The stained cells were washed in wash buffer and suspended in PBS as above. The samples were analyzed by gating on CD3+ cells, and the CD3+ population was further used to gate CD4+ and CD8+ populations in a dot plot showing the live cells only.

Statistical analysis

Data were analyzed using GraphPad Prism 6.0 software. The results were expressed as the mean $\pm$ SD/median $\pm$ quartile of two experiments and compared by the 't' test/Mann-Whitney U test. One-way ANOVA was performed, and the significance of the difference between means was determined by Newman-Keuls/Tukey's/Bonferroni's multiple comparisons test depending on the datasets. Differences with $p < 0.05$ were considered significant.

Results

Cloning, expression, and purification of rTn1bm

The Tn1bm gene amplified from *B. malayi* cDNA yielded a 696 bp product (Fig. 1a). The protein could be successfully cloned (Fig. 1b) and expressed (Fig. 1c) as a single band of ~81 kDa, which represented the Nus-rTn1bm fusion protein (Fig. 1d). Treatment with recombinant enterokinase and affinity purification provided a protein eluted by 100 mM imidazole, which resolved as a single band of ~27 kDa (Fig. 1e). The level of the endotoxin in the purified rTn1bm protein was found to be < 1 EU/mg.

Immunization with rTn1bm reduced the parasite load

Immunization with rTn1bm reduced the parasite burden of subsequently infected mice by 60% (Fig. 2).

The size/dimensions (mean $\pm$ SD) of L₃ before injection into the animals were as follows: length 1.18 ± 0.24 mm and width 21.90 ± 2.88 µm. Live parasites recovered from rTn1bm-immunized+L₃ infected animals and non-immunized+L₃-infected animals were motile and were in the

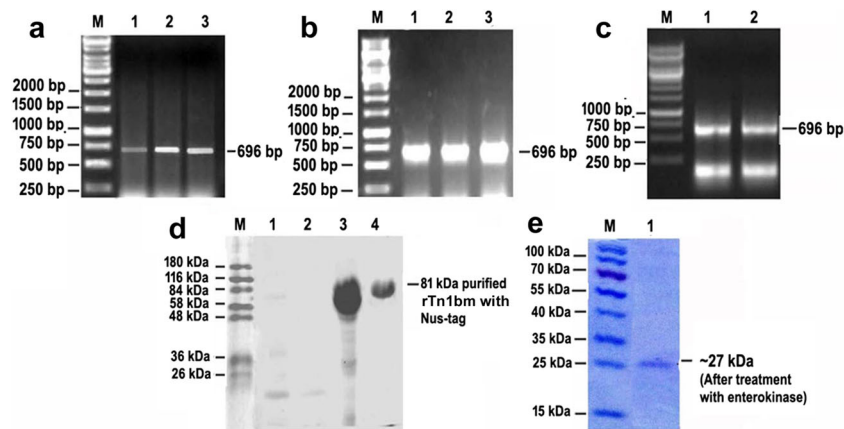


Fig. 1 Amplification of Tn1bm gene by PCR at optimal temperature (58 °C) using Tn1bm gene-specific primers. **a** M: 1 kb DNA ladder (Fermentas, USA). Lanes 1–3: PCR product (696 bp) of Tn1bm gene. **b** Colony PCR of transformed white colonies containing Tn1bm gene in pTZ57R/T (TA cloning vector) using Tn1bm gene specific primers. M: 1 kb DNA ladder. Lanes 1–3: PCR product of gene from different colonies, 696 bp PCR product shows successfully cloned Tn1bm gene in TA vector. **c** Colony PCR of Tn1bm gene cloned in pet43.1a expression vector using Tn1bm gene-specific primers. M: 1 kb DNA Ladder. Lanes 1–2: PCR product of Tn1bm gene from different colonies,

696 bp PCR product represents Tn1bm gene insert. **d** Purified rTn1bm (with Nus-tag) by Ni-NTA column checked by 10% SDS-PAGE. M: Molecular weight marker (Sigma–Aldrich, USA). Eluted rTn1bm at 10, 20, 50 and 100 mM imidazole in lanes 1, 2, 3, and 4, respectively. A single band corresponding to ~81 kDa represents the recombinant fusion protein (with Nus-tag) in western blot. **e** Purified rTn1bm by affinity chromatography after treatment with recombinant enterokinase and checked by 10% SDS PAGE. M: Molecular weight marker (Fermentas, USA). Lane 1: Eluted rTn1bm at 100 mM imidazole. A single band corresponding to ~27 kDa represents purified protein

L₄ stage with characteristic bulbous anterior extremity. There was no difference in the size of the L₄ recovered from rTn1bm-immunized+L₃-infected animals (length 4.90 ± 0.44 mm and width 47.00 ± 5.44 μ m) and non-immunized+L₃-infected animals (length 5.79 ± 1.59 mm and width 45.24 ± 4.93 μ m).

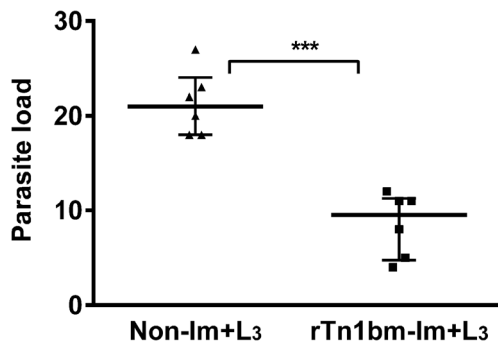


Fig. 2 Parasitological responses of BALB/c mice immunized with rTn1bm and subsequently inoculated with *B. malayi* infective third stage larvae (L₃) and assessment of morphological features of the recovered parasites. All the animals receiving L₃ on day 27/28 post first immunization (p.f.i.) were euthanized on day 27/28 post larval (L₃) inoculation (p.l.i.). The morphological features of the parasites recovered from peritoneal cavity (p.c.) of the animals were examined, identified, and measured the length and width under a microscope (Leica DMLB) by micrometry. Non-Im + L₃: non-immunized+L₃ infected; rTn1bm-Im + L₃: rTn1bm-immunized+L₃ infected. Values are mean $\pm$ SD of data from six animals. Scattered plot showing median and interquartile region and compared by 't' test/Mann-Whitney U test. *** $p < 0.001$

Immunization upregulated cellular proliferation and secretion of the cytokines IFN- γ , TNF- α , IL-1 β , IL-4, IL-6, and IL-10 and NO release

Splenocytes from immunized mice proliferated significantly when exposed to rTn1bm or Con A ($p < 0.001$; Fig. 3a) compared to cells from non-immunized animals; however, Con A-induced cell proliferation was >6 times greater than that in cells of immunized animals exposed to rTn1bm.

NO release from peritoneal macrophages of rTn1bm-immunized animals was greater ($p < 0.001$; Fig. 3b) than from cells of non-immunized controls.

Splenocytes from rTn1bm-immunized animals showed increased release of IFN- γ ($p < 0.01$; Fig. 3c), TNF- α ($p < 0.001$; Fig. 3d), IL-1 β ($p < 0.001$; Fig. 3e), IL-4 ($p < 0.001$; Fig. 3f), IL-6 ($p < 0.001$; Fig. 3g), and IL-10 ($p < 0.05$; Fig. 3h) in response to rTn1bm challenge in vitro compared to the non-immunized counterpart. However, immunization failed to alter the levels of IL-13 (Fig. 3i) and TGF- β (Fig. 3j) release in response to in vitro rTn1bm challenge.

Immunization of BALB/c mice with rTn1bm-augmented specific IgG and its subclasses IgG1 and IgG2b but not IgE (Fig. 4)

In the present study, the levels of rTn1bm-specific IgE, IgG2a, and IgG3 antibodies remained unaltered in immunized-mice compared to non-immunized animals. However, antigen-

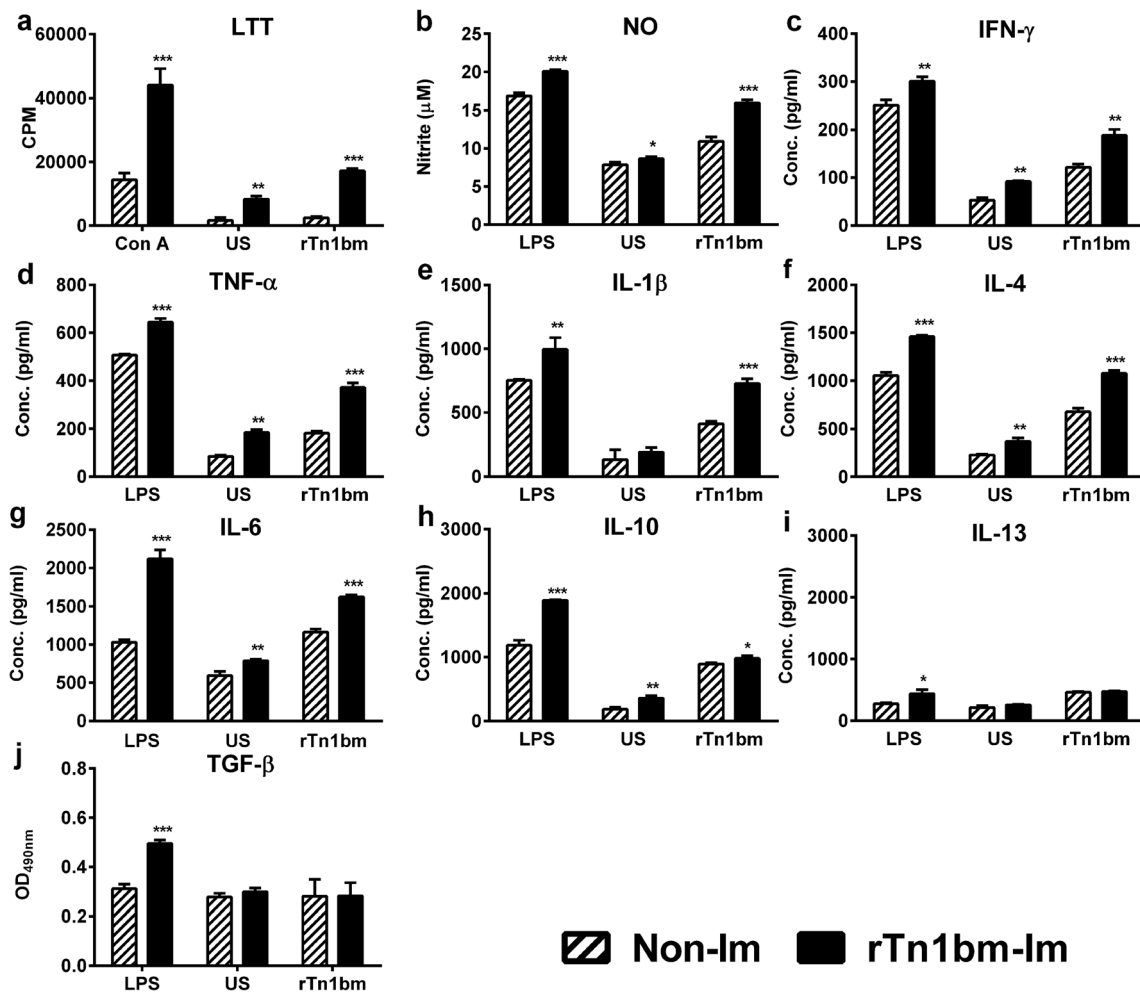


Fig. 3 Immunological responses of BALB/c mice immunized with rTn1bm. Splenocytes and peritoneal lavages were collected from non-immunized and rTn1bm-immunized animals and their suspensions were prepared. Splenocytes ($1-2 \times 10^6$ /mL) and peritoneal macrophages (2×10^6 /mL) were stimulated with Con A (10 μ g/mL)/rTn1bm (1 μ g/mL)/LPS (1 μ g/mL) for 48–72 h at 37 °C in 5% CO₂ atmosphere. Splenocytes were used for LTT and cytokine secretion assays using 0.2×10^6 cells/well in 96-well tissue culture plate and 2×10^6 /mL/well in 24-well tissue culture plates (Nunc-Roskilde, Denmark), respectively. In all the assays, triplicate wells were run for each sample. **a** In LTT assay, after 72 h incubation, ³H-thymidine (BRIT, Mumbai) was added (1 μ Ci/well) followed by 16–18 h incubation at the same atmosphere. The cells were harvested and ³H-thymidine incorporation was quantified by scintillation counter (LS Analyzer, Beckman Inc.). Results were expressed as counts per minute (CPM), **b** NO production was determined in 48 h cultured supernatants of peritoneal macrophages (1×10^6 cells/500 μ L/well in 48-

well tissue culture plate) stimulated with rTn1bm/LPS using Griess reagent and nitrite was quantified against the standard. Secreted cytokines: **c** IFN- γ , **d** TNF- α , **e** IL-1 β , **f** IL-4, **g** IL-6, **h** IL-10, **i** IL-13, **j** TGF- β in 48 h rTn1bm/LPS-exposed splenocytes (2×10^6 /mL) culture supernatants were determined by sandwich ELISA using paired antibodies following the manufacturer's instructions. Color reaction was read, after incubation with O-phenylenediamine dihydrochloride (OPD; Sigma-Aldrich, USA) prepared in citrate buffer+H₂O₂ at 490 nm in a spectrophotometer (Bio-Rad, Model-680, Microplate Reader, CA, USA). The concentration of all cytokines, except TGF- β , was calculated from OD readings (490 nm) of the respective standards obtained from the same manufacturers of the cytokines. The results of TGF- β were expressed as OD. US: Unstimulated, Non-Im: Non-immunized; rTn1bm-Im: rTn1bm-immunized. Values are mean $\pm$ SD of data from 5 animals. Statistics: Student's *t* tests. **p* < 0.05; ***p* < 0.01; ****p* < 0.001

specific IgG (*p* < 0.001), IgG1 (*p* < 0.01), and IgG2b (*p* < 0.01) antibody levels were elevated compared to non-immunized animals; the level of IgG2b was more than two-fold higher than that of IgG1. In summary, immunization resulted in upregulated rTn1bm-specific IgG, IgG1, and IgG2b; the level of IgG2b was highest.

Elimination of parasites by ADCC (Fig. 5)

In the ADCC assay, serum from rTn1bm-immunized animals killed ~67% of L₃ compared to serum from non-immunized animals, which killed 11% of L₃; BmA-immunized animal serum killed ~17% of L₃.

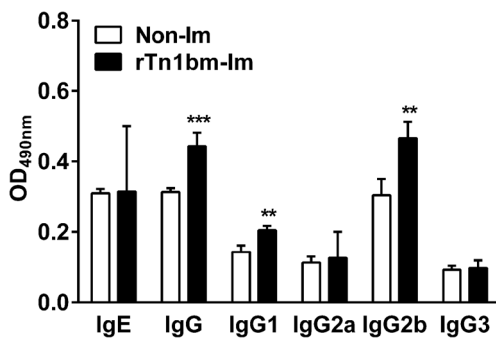


Fig. 4 Specific IgE, IgG, and IgG-subclass responses of BALB/c mice immunized with rTn1bm. The levels of IgE, IgG, and four subclasses of IgG in sera of non-immunized and rTn1bm-immunized animals were determined by indirect ELISA. The optimum concs. of rTn1bm protein (0.5 µg/mL) and optimum dilutions of rTn1bm serum (IgE: 1:4; IgG: 1:100; IgG subclasses: 1:50), HRP-conjugated goat anti-mouse-IgE (1:1000; Bethyl Laboratories Inc., USA), HRP-conjugated rabbit anti-mouse-IgG (1:50,000; MP Biomedicals, Ohio, USA), and goat anti-mouse-IgG1 (1:1500), IgG2a, IgG2b, or IgG3 (1:2000; AbD Serotec, USA). OPD prepared in citrate buffer+H₂O₂ was added to the wells. The color reaction was measured as OD at 490 nm in a plate reader (Bio-Rad, Model-680, Microplate Reader, CA, USA). Non-Im: non-immunized, rTn1bm-Im: rTn1bm-immunized. The values are mean ± SD of data from 5 animals. Statistics: Student's *t* tests. **p* < 0.05; ***p* < 0.01; ****p* < 0.001

rTn1bm exposure upregulated MHC class-II, TLR2 and TLR6 expression, and the relative distribution of the CD4+ and CD19+ cell populations in naïve animals

rTn1bm exposure downregulated the expression of MHC class-I in peritoneal macrophages of naïve animals compared

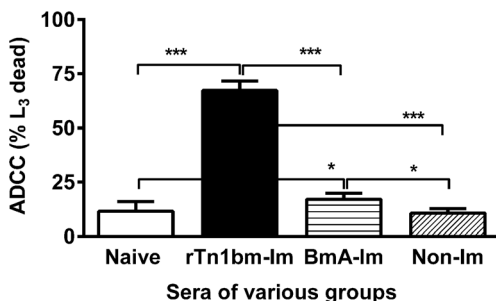


Fig. 5 Antibody-dependent cell-mediated cytotoxicity against L₃ of *B. malayi*. The viable peritoneal exudate cells (PECs) of healthy naïve mice (*n* = 6) were prepared in RPMI 1640 containing 100 U/mL penicillin and 100 µg/mL streptomycin. Triplicate wells of a 48-well culture plate (Nunc, Roskilde, Denmark) received, per well, 4 × 10⁶ PEC, 40 L₃ and 50 µL serum each from naïve (negative control), non-immunized, rTn1bm-immunized, and soluble extract of *B. malayi* adult parasites (BmA)-immunized (positive control) animals. The final volume of mix/well was 400 µL. After 24 h incubation at 37 °C in a 5% CO₂ atmosphere, the viability of L₃ was assessed under a microscope. Dead/dying L₃ showing adherent cells on the surface were counted, and the results were expressed as the percentage cytotoxicity to L₃. The experiment was repeated twice. Values are mean ± SD of data from two experiments. Non-Im: non-immunized, rTn1bm-Im: rTn1bm-immunized, BmA-Im: BmA-immunized, Naïve animals, Statistics: Newman-Keuls multiple comparisons test. **p* < 0.05; ****p* < 0.001

to control or LPS-exposed (*p* < 0.01; Fig. 6a) cells. MHC class-II, on the other hand, was significantly (*p* < 0.01) upregulated by rTn1bm exposure compared to the control treatment, but the increase was lower than that of LPS (*p* < 0.01; Fig. 6b).

We investigated TLR2, 4, and 6 expression in macrophages. rTn1bm upregulated TLR2 expression (*p* < 0.01) compared to the control treatment, but LPS-induced expression was more than twofold than that of rTn1bm-induced expression (*p* < 0.01; Fig. 7a). TLR4 expression was low in rTn1bm-exposed cells compared to unexposed cells. However, in LPS-induced cells, TLR4 expression was the highest (Fig. 7b). rTn1bm-exposed cells showed the highest TLR6 expression compared to LPS-exposed or control cells (*p* < 0.01; Fig. 7c).

Splenocytes of naïve animals exposed to rTn1bm did not show any change in the relative distribution of CD3+ cells. The CD3+ population was therefore used for gating CD4+ and CD8+ cells. rTn1bm exposure resulted in an increased CD4+ population (*p* < 0.001; Fig. 8a) but decreased CD8+ population compared to Con A (*p* < 0.001; Fig. 8b) or control treatments (*p* < 0.05; Fig. 8b). CD19+ cells were selectively expanded in rTn1bm-treated splenocyte cultures compared to unexposed (*p* < 0.001) or LPS-stimulated cultures (*p* < 0.01) (Fig. 8c). These changes in innate immune response markers suggest that rTn1bm is capable of mobilizing innate immune responses in the host.

Discussion

The final purified rTn1bm prepared from adult *B. malayi* worm extract and used in the present study was a ~27 kDa protein, and our preliminary fluorescence microscopy investigation revealed that the protein was localized in the body wall of both male and female adult worms and in the uterine wall of females (unpublished data). Immunization with rTn1bm reduced the parasite burden of L₃-infected mice by 60%, and all of the remaining live worms recovered were in the L₄ stage. This finding clearly suggests that although Tn1bm was isolated from adult worms, it is also evidently present in L₃. Further proof for this was provided by (1) an ADCC assay wherein incubation with serum from rTn1bm-immunized animals killed the L₃ and (2) sera of endemic normal (EN) subjects from *W. bancrofti*-endemic regions, who are considered to be putatively immune to L₃, reacted with rTn1bm (unpublished observation).

Protective immunity against filarial parasites is a complex process involving type 1 and type 2 immune responses via a variety of mediators and effector molecules (Verma et al. 2015, 2017; Dixit et al. 2004). Cell-mediated immunity (CMI) of the Th1 type plays a significant role in protection against filarial infection (Maizels et al. 2004), and this is evident from elevated levels of Th1 cytokines in putatively

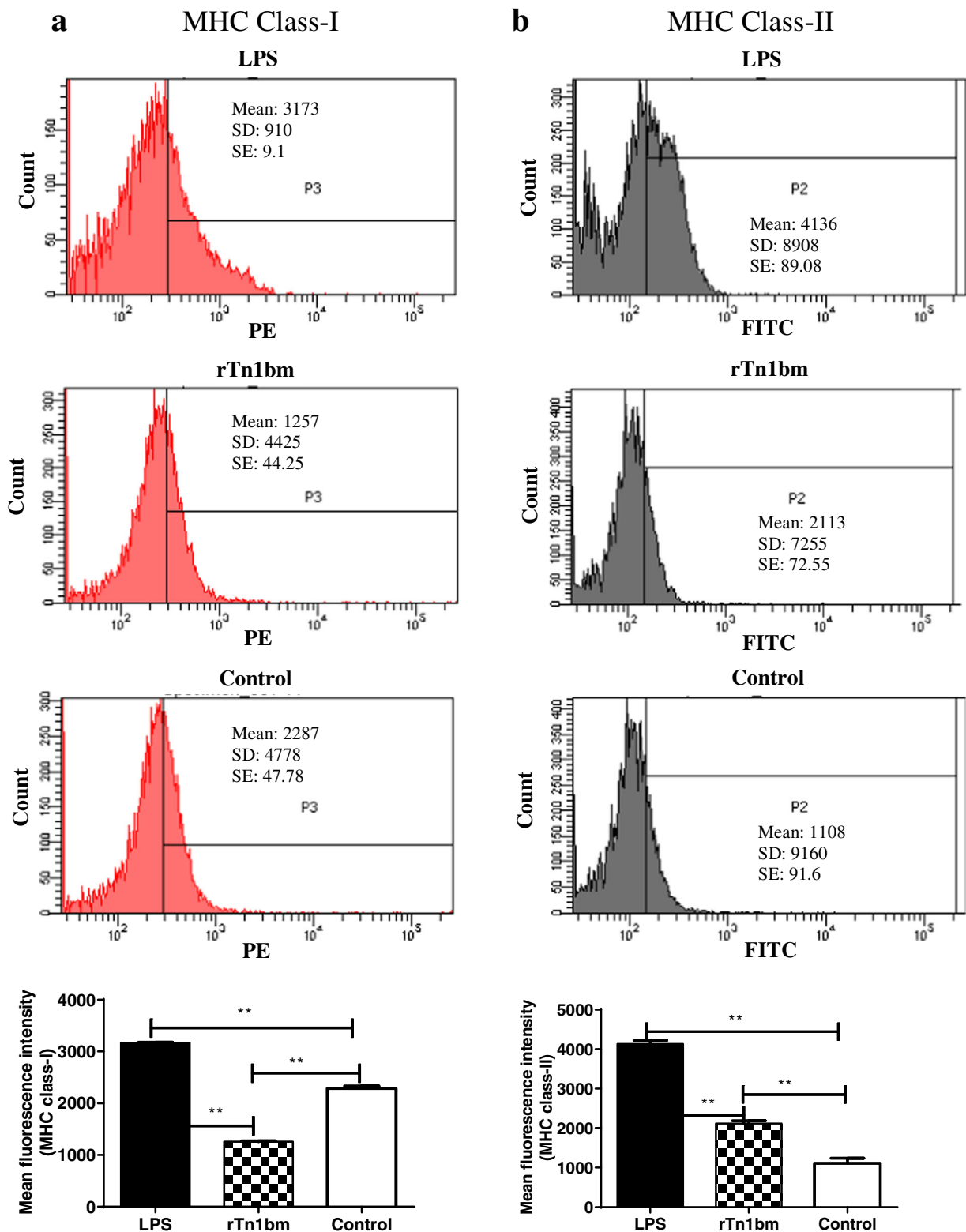


Fig. 6 Expression of MHC class-I and MHC class-II in rTn1bm-exposed peritoneal macrophages of naïve BALB/c mice. Stimulants used were: rTn1bm (2 µg/mL) and LPS (10 µg/mL). Unexposed cells served as control. **a** MHC class-I. **b** MHC class-II markers on macrophages. Data

are expressed as mean $\pm$ SE of six values. rTn1bm (recombinant troponin 1 of *B. malayi*). Statistics: Bonferroni-multiple comparisons test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

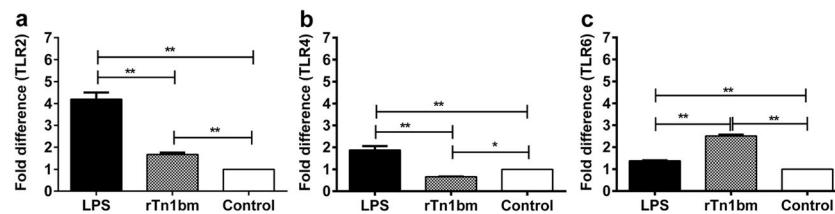


Fig. 7 Expression of TLR2, 4 and 6 in rTn1bm-exposed macrophages of naïve BALB/c mice. Stimulants used were rTn1bm (2 µg/mL) and LPS (10 µg/mL). Unexposed cells served as control. mRNA expression of **a** TLR2, **b** TLR4, and **c** TLR6 determined by real-time PCR. Real-time

data represents mean $\pm$ SE of 6 values. rTn1bm (recombinant troponin I of *B. malayi*). Statistics: Tukey's multiple comparison test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

immune endemic normals (Elson et al. 1995; Dimock et al. 1996). Th2 responses are also known to be associated with control of filarial infections, and therefore, protective immunity to filarial infections requires co-ordination of both Th1 and Th2 responses (Sahoo et al. 2009; Dakshinamoorthy et al. 2013; Babu and Nutman 2014). In the present study, we found that an enhanced rTn1bm-specific cellular proliferative response was associated with parasite elimination. In the host, exposure to antigen molecules leads to the differentiation of T-helper cells into Th1 and Th2 subsets, which perform distinct functions (Chen et al. 2000). Th1 cells generate IL-2, IFN- γ , and lymphotoxin- β , which arbitrate proinflammatory functions important for the development of cell-mediated immune responses, whereas Th2 cells produce IL-4, IL-6, and IL-10, which augment humoral immunity. In the present study, immunization of animals with rTn1bm enhanced both Th1 (IFN- γ , TNF- γ , IL-1 β) and Th2 cytokine (IL-4, IL-6, and IL-10) responses without altering IL-13 and TGF- β release, which mirrors the enhanced IgG, IgG1, and IgG2b levels in these animals. In our exploratory studies, we found elevated levels of rTn1bm-specific IgG in the sera of bancroftian EN subjects (unpublished data). We previously reported that immunization with F6 (a proinflammatory fraction of *B. malayi* adult worm) intensely upregulated IgG1, IgG2a, and IgG2b in *M. coucha* (Sahoo et al. 2009). ADCC is a well-known part of the adaptive immune mechanism by which filarial parasites are killed in vitro and in vivo (Chandrashekar et al. 1990; Veerapathran et al. 2009). In the present study, serum of rTn1bm-immunized animals killed a significant number of L₃. This finding suggests the generation of protective antibodies in the sera of rTn1bm-immunized animals and indicates that the Th2 immune response to rTn1bm is also involved in reducing the parasite load by 60%. The lack of IgE response in rTn1bm-immunized hosts clearly rules out the possibility of the generation of undesirable type I hypersensitivity reactions by rTn1bm.

Toll-like receptors (TLRs) are thought to play an important role in inflammation and in host defense immune responses. TLR2 and TLR6 present on the surface of macrophages are involved in host defense against helminth/filarial infections, whereas TLR4 is involved in defense against bacterial infection. In the present study, rTn1bm-exposed cells showed the

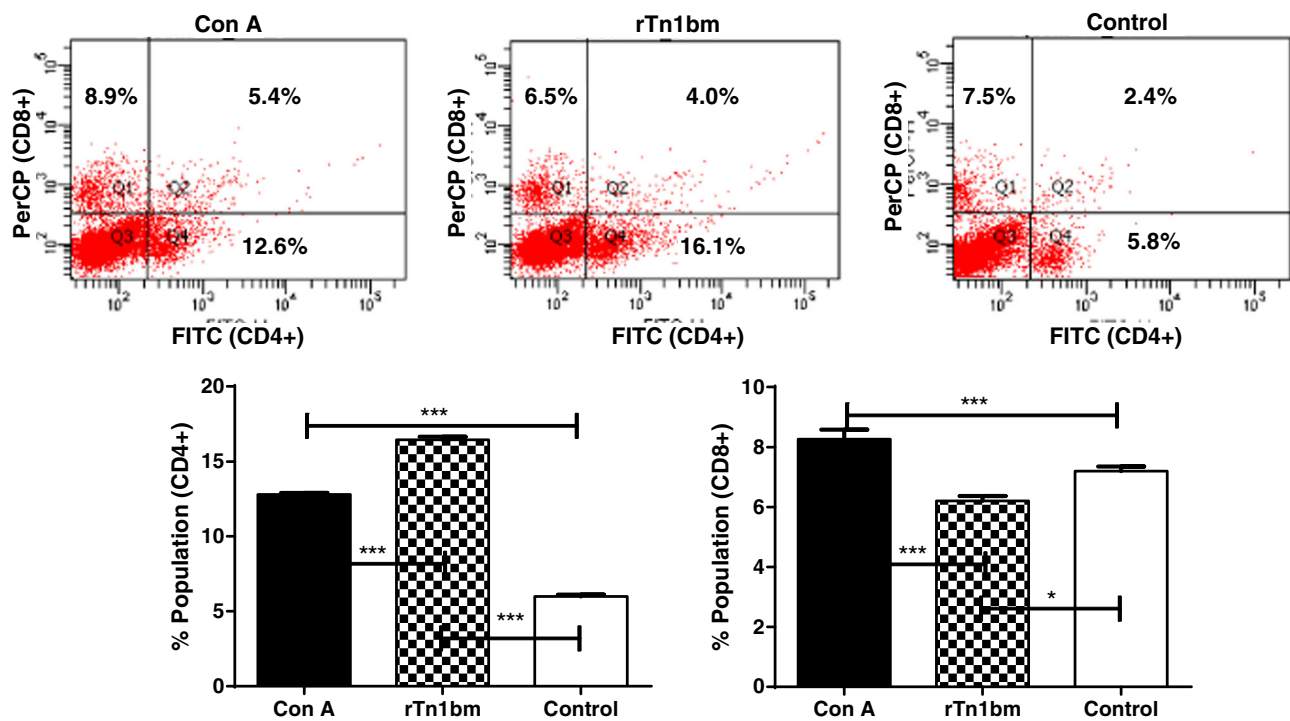
highest TLR6 expression compared to LPS-exposed or control cells; rTn1bm did not induce TLR4, but as expected, LPS elicited enhanced TLR4 expression. TLR4 has been shown to be important for the induction of Th2-dependent immune responses in mice, and TLR2/TLR6 activation has been reported to elicit tolerogenic IL-10 secretion, indicative of a balance between immunosuppressive and proinflammatory responses (Netea et al. 2007).

Employing changes in the relative population of CD4+, CD8+, and CD19+ cells in splenocytes from naïve animals as markers of innate immune responses, we investigated the direct effect of rTn1bm on these cells. The change in these splenocyte populations is considered a useful predictor of the potential of rTn1bm to mobilize innate immune responses in the whole animal. Splenocytes of naïve mice exposed in vitro to rTn1bm showed an enhanced relative distribution of CD4+ and CD19+ cells, suggesting that rTn1bm could induce an antibody-mediated response via expansion of T-cells and B-cells, respectively. Concomitantly, rTn1bm also upregulated the expression of MHC class-II molecules in macrophages, which are involved in the antigenic activation of helper T cells, a vital prerequisite for efficient vaccination.

NO-mediated macrophage effector mechanisms are involved in the control of filarial parasite survival in vitro (Taylor et al. 1996) and in vivo (Verma et al. 2015, 2017), and filarial parasites can also induce and release NO from endothelial cells, which may directly affect their own survival in lymphatics (Furchgott 1999). In the present study, we found elevated levels of NO release from peritoneal macrophages of rTn1bm-immunized animals, and this finding is consistent with our earlier observations that IFN- γ (Th1) and NO are involved in the protection of the host against *B. malayi* in *M. coucha* (Verma et al. 2015).

In the present study, splenocytes from rTn1bm-immunized mice showed an enhanced release of TNF- α . TNF- α is required at the early stage of infection to synergize with the Th2 effector mechanism that determines parasite elimination or persistence (Artis et al. 1999). We have previously shown that BmAFII, a proinflammatory fraction of *B. malayi*, has potent TNF- α stimulating molecules and that immunization with BmAFII eliminated intraperitoneally transplanted adult worms in *M. coucha* (Dixit et al. 2004).

a Relative distribution of CD4+ and CD8+ lymphocytes



b Relative distribution of CD19+ lymphocytes

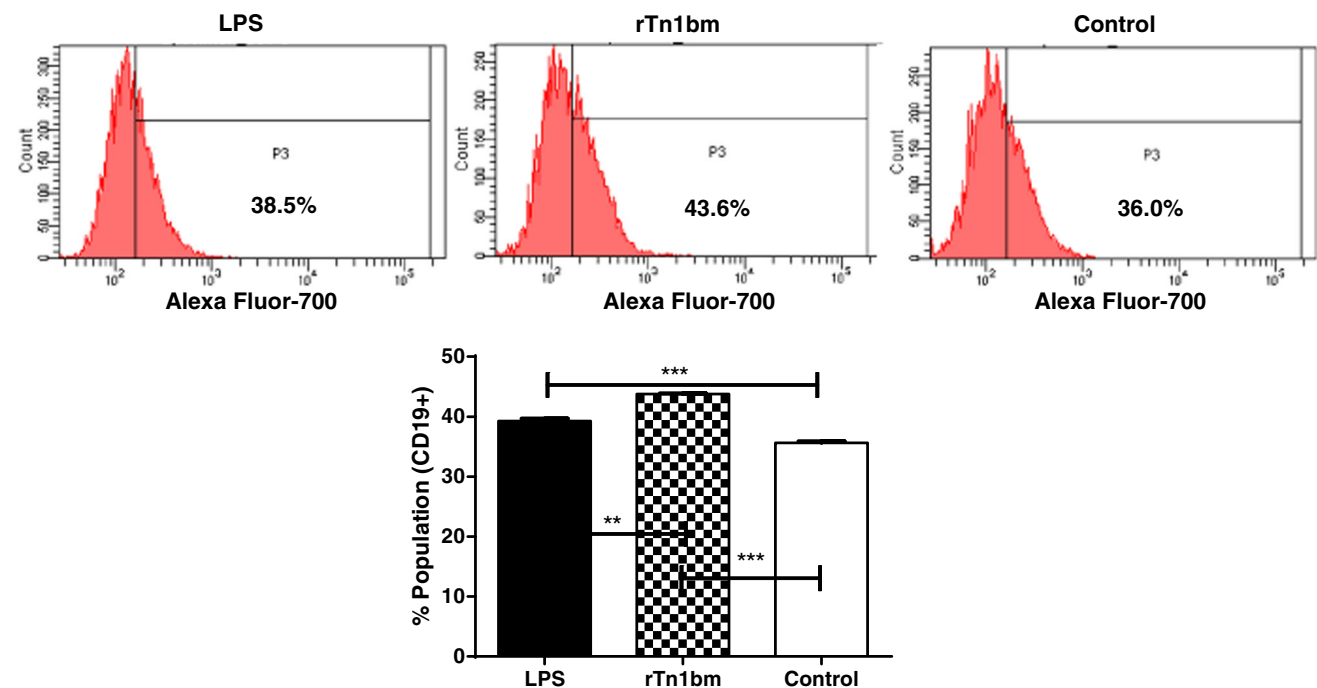


Fig. 8 Relative distribution of CD4+, CD8+, and CD19+ immunophenotypes in rTn1bm-exposed splenocytes of naïve BALB/c mice. Stimulants used were rTn1bm (2 µg/mL) and Con A (5 µg/mL). Unexposed cells served as control. **a** CD4+ and CD8+. **b** CD19+

population. Population of these cells were presented in percentage. Data are expressed as mean ± SE of six values. rTn1bm (recombinant troponin 1 of *B. malayi*). Statistics: Tukey's multiple comparison test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

IL-4 (a signature Th2 cytokine) is the key determinant of susceptibility of the host against filariasis (Volkman et al. 2003). IL-4 is known to stimulate IgG1 production (Snapper and Finkelman 1999), and therefore, the increased IgG1 level found in the present study is related to increased IL-4 levels. Thus, increased IgG1 and IgG2b (Th2 type) and unchanged IgG2a (Th1 type) fortified the Th2 milieu. However, during high cell proliferation responses, lower levels of antibodies are produced and thus the antibody responses during the Th1 (IFN- γ , TNF- α , and IL-1 β) response synergize the cellular components for efficient removal of the pathogen (Kanai et al. 2007). Thus, in the present study, we observed increased IgG1 and IgG2b, which may have helped in parasite elimination via the ADCC mechanism.

In this study, rTn1bm-immunized animals showed elevated levels of IL-6, which may have contributed to a reduction in parasite load. This finding is consistent with the finding of Muhsin (2013), who reported increased *Litomosoides sigmodontis* worm recovery in IL-6-deficient mice. IL-6-mediated immune activation to eliminate parasites was also evident from the increased serum levels of the proinflammatory cytokines IL-1 β and TNF- α , which are associated with progressive immune activation. It has been suggested that in the chronic stages of infection, IL-6-mediated immune activation is due to increased bacterial and fungal loads in the damaged lymphatics that form a source of immune activation in filarial lymphedema (Babu and Nutman 2014).

The anti-inflammatory cytokine (IL-10 and TGF- β) network plays an important role in modulating the host's effector mechanisms against longstanding parasite survival in filarial infection (Doetze et al. 2000). TGF- β activates several functions of monocytes/macrophages but deactivates the release of oxidative mediators (Dixit et al. 2006). In our present study, we observed an enhanced release of IL-10 but not TGF- β in immunized animals, which could be the reason for the incomplete removal of the infection.

In summary, we successfully cloned and expressed the *B. malayi* Tn1 gene and produced rTn1bm; the purified rTn1bm protein presented a single ~27 kDa band. Immunization with rTn1bm protected the host to an extent of 60%, and the protection was correlated with increased cellular proliferation, secretion of IFN- γ , TNF- α , IL-1 β , IL-4, IL-6, IL-10, the release of NO, and levels of specific IgG, IgG1, and IgG2b antibodies. rTn1bm-immune serum killed L₃ in the ADCC assay. The lack of an IgE response suggests that rTn1bm may not generate an undesirable type I hypersensitivity reaction. Increased MHC class-II expression, CD4+ and CD19+ cell populations, and the predominant expression of TLR6 (all used as markers of innate immune response) in cells from naïve animals exposed to rTn1bm indicate that the protein has the ability to mobilize innate immune responses.

This is the first report of the cloning of the Tn1bm gene and the immunoprophylactic potential of the rTn1bm protein against *B. malayi*. rTn1bm protein may hold promise as an immunoprophylactic agent.

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Compliance with ethical standards

Ethical approval The animal study protocol was approved by the National Laboratory Animal Centre (NLAC), CSIR-Central Drug Research Institute (CDRI), Lucknow, India, following the rules and guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environments and Forests, Govt. of India, New Delhi, for use and handling of animals. The animals used in the study were housed in climatically controlled pathogen-free animal quarters (Temp.: 23 $\pm$ 2 °C; RH: 60%; and photoperiod: 12 h light–dark cycles) at NALC, CSIR-CDRI, Lucknow, India, and they were fed standard rodent maintenance diet (prepared in-house) and water ad libitum. The animal study protocol was approved (approval number: IAEC/2011/145 renewed dated 03/07/2014).

Conflict of interest The authors declare that they have no conflict of interest.

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