Vaccine 37 (2019) 571–580

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Immunogenicity and protective efficacy of Recombinase A from *Wolbachia* endosymbiont of filarial nematode *Brugia malayi* (wBmRecA)

Mamta Gangwar [a], Ruchi Jha [a], Manish Goyal [a,1,*], Mrigank Srivastava [a,b,*]

[a] *Parasitology Division, CSIR-Central Drug Research Institute, Sector 10, Jankipuram Extension, Sitapur Road, Lucknow 226031, UP, India*
[b] *Academy of Scientific and Innovative Research (AcSIR), New Delhi, India*

ARTICLE INFO

Article history:
Received 29 August 2018
Accepted 13 December 2018
Available online 27 December 2018

Keywords:
Filariasis
Brugia malayi
Wolbachia
Vaccine
wBmRecA

ABSTRACT

Lymphatic filariasis causes global morbidity. *Wolbachia*, an endo-symbiotic intracellular bacterium of the filarial nematode helps in their growth and development, regulates fecundity in female worms and contributes to the immunopathogenesis of the disease. However, genes and proteins of *Wolbachia* that may act as putative vaccine candidates are not known. In this study, we cloned recombinase-A protein of *Wolbachia* from *Brugia malayi* (wBmRecA) and carried out its detailed biochemical and immunological characterization. Bioinformatics analysis, circular dichroism and fluorescence spectral studies showed significant sequence and structural similarities between wBmRecA and RecA of other alpha-proteo- bacterial species. wBmRecA was ubiquitously expressed in all the three major life stages of *B. malayi*, including excretory-secretory products of the adult worm. *In silico* studies suggested immunogenic potential of wBmRecA, and mice immunized with wBmRecA exhibited elevated levels of immunoglobulins IgG1, IgG2a, IgG2b and IgG3 in their serum along with increased percentages of $CD4^+$, $CD8^+$ T cells and $CD19^+$ B cells in their spleens. Notably, splenocytes from immunized mice showed increased m-RNA expression of T-bet, elevated proinflammatory cytokines IFN-γ and IL-12, while peritoneal MΦs exhibited increased levels of iNOS, downregulated Arg-1 and secreted copious amounts of nitric oxide which contributed to severely impaired development of the infective larvae (Bm-L3). Interestingly, sera from immunized mice promoted significant cellular adherence and cytotoxicity against microfilariae and Bm-L3. Importantly, wBmRecA demonstrated strong immuno-reactivity with bancroftian sera from endemic normal individuals. These results suggest that wBmRecA is highly immunogenic, and should be explored further as a putative vaccine candidate against lymphatic filariasis.

1. Introduction

Human Lymphatic filariasis (LF) is a devastating vector borne neglected tropical disease that is caused by *Wuchereria bancrofti, Brugia malayi and Brugia timori*. Globally, 120 million people are infected, 40 million people are debilitated, and 856 million people are threatened by LF [1,2]. The disease damages the lymphatic system and causes severe and permanent disability and social stigma that leads to huge socioeconomic loses [2]. Presently Albendazole in combination with either Ivermectin or Diethylcarbamazine citrate (DEC) is the frontline chemotherapeutic option for preventing LF. However, since these drugs are mainly microfilaricidal, and exhibit only limited adulticidal efficacy, there is an urgent and unmet need for new and effective ways to treat LF [3–6].

Recently, Wolbachia, an obligate alpha-proteobacterial endosymbiont of the filarial nematodes has been suggested as a promising new target to control LF as it maintains a mutualistic relationship, regulates fecundity in female worms, and plays an indispensable role in worm development and survival [7–9]. Furthermore, *Wolbachia* contributes to pathological manifestations of LF, and its depletion eventually leads to death of the adult worms, making it an attractive target for anti-filarial drug therapy [7,10–13]. However, information about genes and proteins of *Wolbachia* that may act as putative vaccine candidates is relatively unknown. In this study, we focused our attention on Recombinase A (RecA) protein that is evolutionary conserved in many bacterial species and plays a crucial role in maintaining the integrity of the bacterial genome. RecA monomer consists of three domains;

* Corresponding authors at: Parasitology Division, CSIR-Central Drug Research Institute, Sector 10, Jankipuram Extension, Sitapur Road, Lucknow 226031, UP, India (M. Srivastava).

E-mail addresses: manish.goyal@mail.huji.ac.il (M. Goyal), Mrigank_Srivastava@cdri.res.in (M. Srivastava).

[1] Current Address: Department of Microbiology & Molecular Genetics, The Kuvin Center for the Study of Infectious and Tropical Diseases, IMRIC, The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel.

https://doi.org/10.1016/j.vaccine.2018.12.015
0264-410X/

the central domain is involved in DNA and ATP binding while amino and carboxy domains are important for the formation of RecA polymer and interfilamentous associations respectively. Besides being involved in homologous recombination and recombinational DNA repair, the two processes that maintain genome stability and genetic diversification, Rec A also acts as a co-protease for the LexA protein which is a repressor of genes encoding DNA repair proteins (SOS genes). Upon DNA damage, LexA catalyses its own digestion in the presence of ssDNA-RecA filament, thereby allowing synthesis of necessary SOS proteins. Thus, RecA plays a crucial role in SOS response that allows bacteria to survive sudden increase in DNA damage [14–18].

Here, we cloned, expressed and purified RecA ortholog from *Wolbachia* endosymbiont of *B. malayi* (wBmRecA) and evaluated its prophylactic potential.

2. Materials and methods

2.1. Animals and parasite

Six- to eight-week-old BALB/c mice were used for all experiments in accordance with our Institutional Animal Ethical Committee guidelines. *B. malayi* was maintained in *Mastomys coucha*, and the third infective larval stage of the parasite (Bm-L3, n = 50) recovered from infected *Aedes aegypti* was used to infect mice (i.p.). Control animals received sterile phosphate-buffered saline (i.p.).

2.2. Bioinformatics studies, cloning and biophysical studies of wBmRecA

Detailed methodology is given in the supplementary file.

2.3. Antibody generation and immunoblotting

Antibody against recombinant wBmRecA (r-wBmRecA) was generated, and its specificity was confirmed via immunoblotting as described earlier [19].

2.4. Presence of wBmRecA in different life stages of parasite

Total RNA from Adult worms (AW), Bm-L3 and Mf was isolated; reverse transcribed and amplified using wBmRecA gene specific primers. For immunoblotting, protein lysate from different life stages was used, resolved on to 12% SDS-PAGE and detected using anti-wBmRecA antibodies as described earlier [20,21]. To ascertain the presence of wBmRecA in the excretory-secretory (ES) product, female worms (n = 4/ml) were maintained *in vitro* in serum-free RPMI 1640 medium that was subsequently concentrated, and processed for SDS-PAGE analysis. Anti-wBmRecA antibody and goat anti-mouse IgG-HRP antibody was used to detect wBmRecA as described earlier [19].

2.5. Immunization of mice

BALB/c mice were immunized with r-wBmRecA as described previously [22]. Next, they were divided into four groups with 6 mice each. Animals in group 1 received PBS (non-immunized control group), animals in group 2 received Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) in PBS (Adjuvant group). Animals in the third group received r-wBmRecA, while animals in the fourth group received r-wBmRecA along with the adjuvant (r-wBmRecA+FCA). One week after the final booster, 3 mice from each group were randomly selected and euthanized to measure various immune parameters. Additionally, sera was collected and stored at −20 °C for subsequent experiments. Remaining mice were used for protection studies as described earlier [19].

2.6. Estimation of wBmRecA specific immunoglobulins

Antibody titers were measured in the sera of mice by indirect ELISA as described earlier [19] and subtyping was done using commercially available antibody isotyping kit (Sigma-Aldrich, USA).

2.7. In vitro antibody dependent cell mediated cytotoxicity (ADCC)

Peritoneal exudate cells (PECs; 1×10^6) from naive mice were cultured in the presence of immunized or non-immunized mouse serum along with Live Mf (∼100) or Bm-L3 (∼20) and cellular adherence and cytotoxicity to parasite stages was examined microscopically as described earlier [23]. Percentage killing of Bm-L3 or Mf was calculated using the formula (Number of dead Bm-L3 or Mf/Total number of Bm-L3 or Mf) × 100.

2.8. Isolation of peritoneal exudate cells and splenocytes

PECs and splenocytes were isolated following previously published protocols [24,25]. Erythrocytes in the splenocytes fraction were lyzed and cells were suspended in ice cold MACS buffer [1 × PBS pH 7.2, 0.5% bovine serum albumin (BSA), 2 mM EDTA] for further use.

2.9. Flow cytometry

Splenocytes (1×10^6 cells) were incubated with CD4-FITC, CD8-PE and CD19-FITC monoclonal antibodies (BD Biosciences, USA) and subjected to flow-cytometric analysis as described earlier [26]. Acquisition was done on FACS Calibur using Cell quest software (BD Biosciences, USA) and analysis was done using FACS-DiVA software.

2.10. Real time RT-PCR

RNA from splenocytes and peritoneal MΦs was isolated, quantified, reverse transcribed and processed for real time RT-PCR using the SYBR green master mix as described previously [25,27]. Primer sequences listed in Supplementary Table 1 were either taken from previously published reports [28,29] or were designed using the Primer3 input software (http://bioinfo.ut.ee/primer3-0.4.0/).

2.11. ELISA

Splenocytes (∼2×10^6) were incubated with either LPS (1 μg/mL) or r-wBmRecA (2.5 μg/mL) for 48 h at 37 °C in a humidified CO_2 incubator and concentrations of Th1 (IFN-γ, IL-12) and Th2 cytokines (IL-4 and IL-10) were measured in the culture supernatant using ELISA kit (R&D, USA) [30].

2.12. Estimation of nitric oxide (NO)

PECs were plated for 2 h in a humidified CO_2 incubator at 37 °C. Thereafter, supernatant was removed and adherent cells (mostly MΦs; 1×10^6 cells/well) were cultured for an additional 48 h with either r-wBmRecA (2.5 μg/mL) or LPS (1 μg/mL). NO generation was quantified by measuring the accumulation of nitrite in culture supernatant using Griess reagent [29].

2.13. In vitro lymphocyte proliferation

Splenocytes ($\sim 0.5 \times 10^6$) were stimulated with either r-wBmRecA (2.5 μg/ml), concanavalin A (2.5 μg/ml) or LPS (1 μg/mL) for 48 h in a humidified CO_2 incubator at 37 °C. Thereafter, mitochondrial activity as a measure of T-cell proliferation was measured by XTT assay as described earlier [31].

2.14. Sero-reactivity of wBmRecA

Sero-reactivity of r-wBmRecA was ascertained using sera from *W. bancrofti* infected individuals viz. endemic normals (EN), asymptomatic microfilariaemics (MF+), or symptomatic microfilaraemic carriers (SYMPT) based on the clinical manifestations and presence or absence of microfilariae (Mf) in the blood as described earlier [19].

2.15. Statistical analysis

Data shown are mean ± standard deviation (SD) of at least two-three different sets of independent experiments containing 3–5 animals/group. Human sera sample size was 10 individuals/group. Results were analyzed by Student's *t* test or one-way ANOVA followed by Dunnett's post-test using Graph Pad Prism software (version 5.0). P value of ≤0.05, ≤0.01, and ≤0.001 between different groups were considered significant, highly significant, and very highly significant respectively.

3. Results

3.1. Sequence analysis and phylogenetic studies of wBmRecA

Multiple sequence alignment revealed that wBmRecA was 92% identical with RecA of *Wolbachia* endosymbiont of *Armadillidium vulgare*, *Culex quinquefasciatus*, and *Drosophila simulans*, while >60% similar to RecA from other bacterial species (Fig. 1A) Phylogenetic tree showed that RecA formed a distinct cluster that was further subdivided into three branches containing RecA proteins from alpha-proteo bacteria (group I), beta and gamma proteobacteria (group II) and gram positive bacteria (group III). Detailed analysis showed that wBmRecA was most closely associated with group I (Fig. 1B).

3.2. Cloning, expression and purification of recombinant wBmRecA

wBmRecA gene was PCR amplified and an amplicon of 1068 bp was obtained (Fig. 2A, lane1). Next, it was cloned into pTZ57R/T (T/A) cloning vector (Supplementary Fig. 1) and sub-cloned into pET28a vector for over-expression into the bacterial system. *wBmRecA*-pET28a clone was confirmed through DNA sequencing

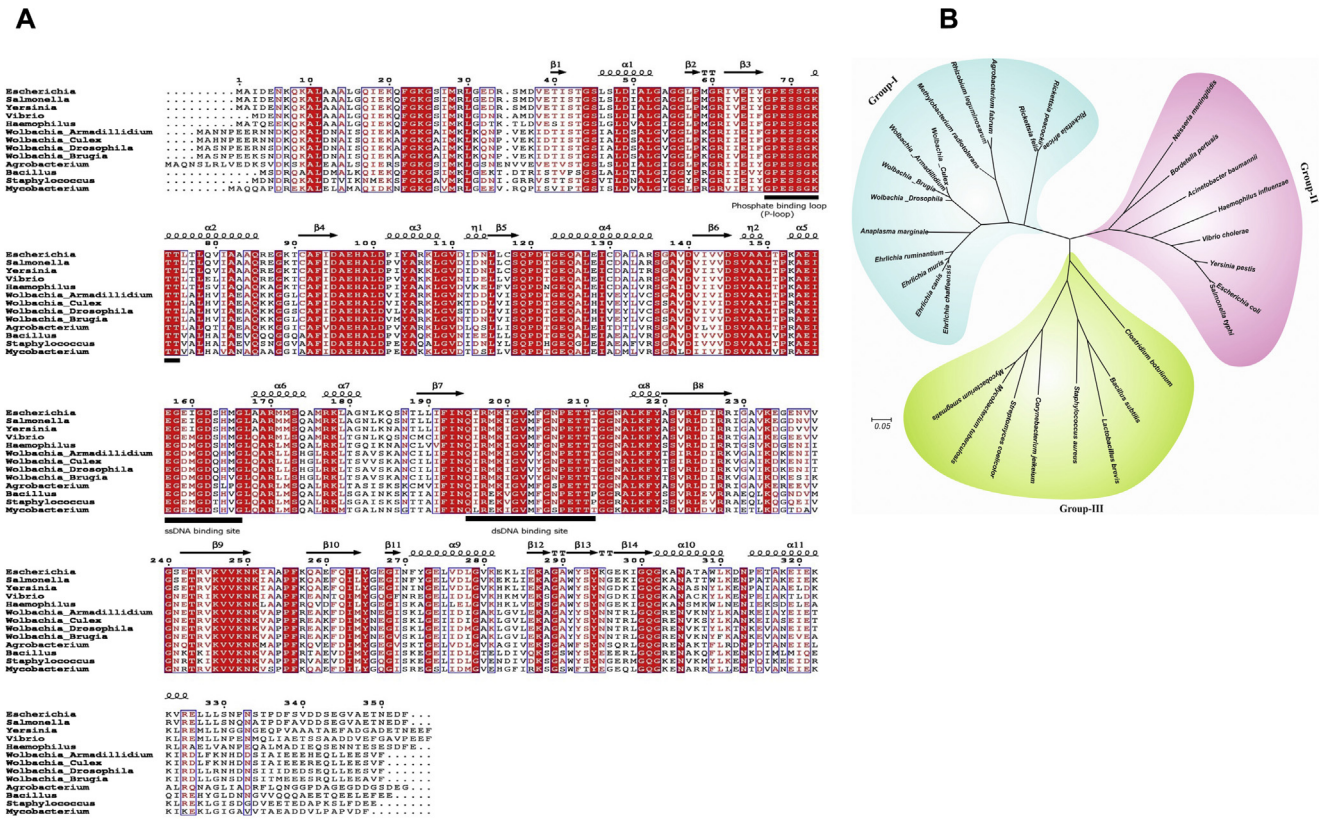

Fig. 1. Sequence analysis and evolutionary relationship of wBmRecA. (A) Multiple sequence alignment of wBmRecA amino acid sequence with other RecA sequences was performed using CLUSTALW software. Identical and similar residues are denoted by red color background and boxed, respectively. The secondary structure is displayed onto the alignment. The amino acid residues involved in ATP binding (phosphate binding P-loop), single and double strand DNA binding are represented as bold underline. Complete species name and their respective gene bank accession number/swissprot are as follows: *Escherichia coli* (NP_417179.1), *Salmonella typhi* (NP_457222.1), *Yersinia pestis* (YP_002348203.1), *Vibrio cholerae* (EET24901.1), *Haemophilus influenzae* (NP_438757.1), *Wolbachia* endosymbiont of *Armadillidium vulgare* (AIT39401.1), *Wolbachia* endosymbiont of *Culex quinquefasciatus* (WP_007302323.1), *Wolbachia* endosymbiont of *Drosophila simulans* (WP_010963008.1), *Wolbachia* endosymbiont of *Brugia malayi* (WP_011256625.1) *Agrobacterium tumefaciens* (WP_003495306.1) *Bacillus subtilis* (NP_389576.2), *Staphylococcus aureus* (WP_000368166.1/AAK15276.1), and *Mycobacterium tuberculosis* (NP_217253.1). (B) Evolutionary relationship of wBmRecA was studied by constructing phylogenetic tree using a total set of 31 RecA sequences from different bacterial species. Multiple sequence alignments of these sequences were obtained from CLUSTALW and bootstrapped neighbor-joining tree was obtained using MEGA version 6.0. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

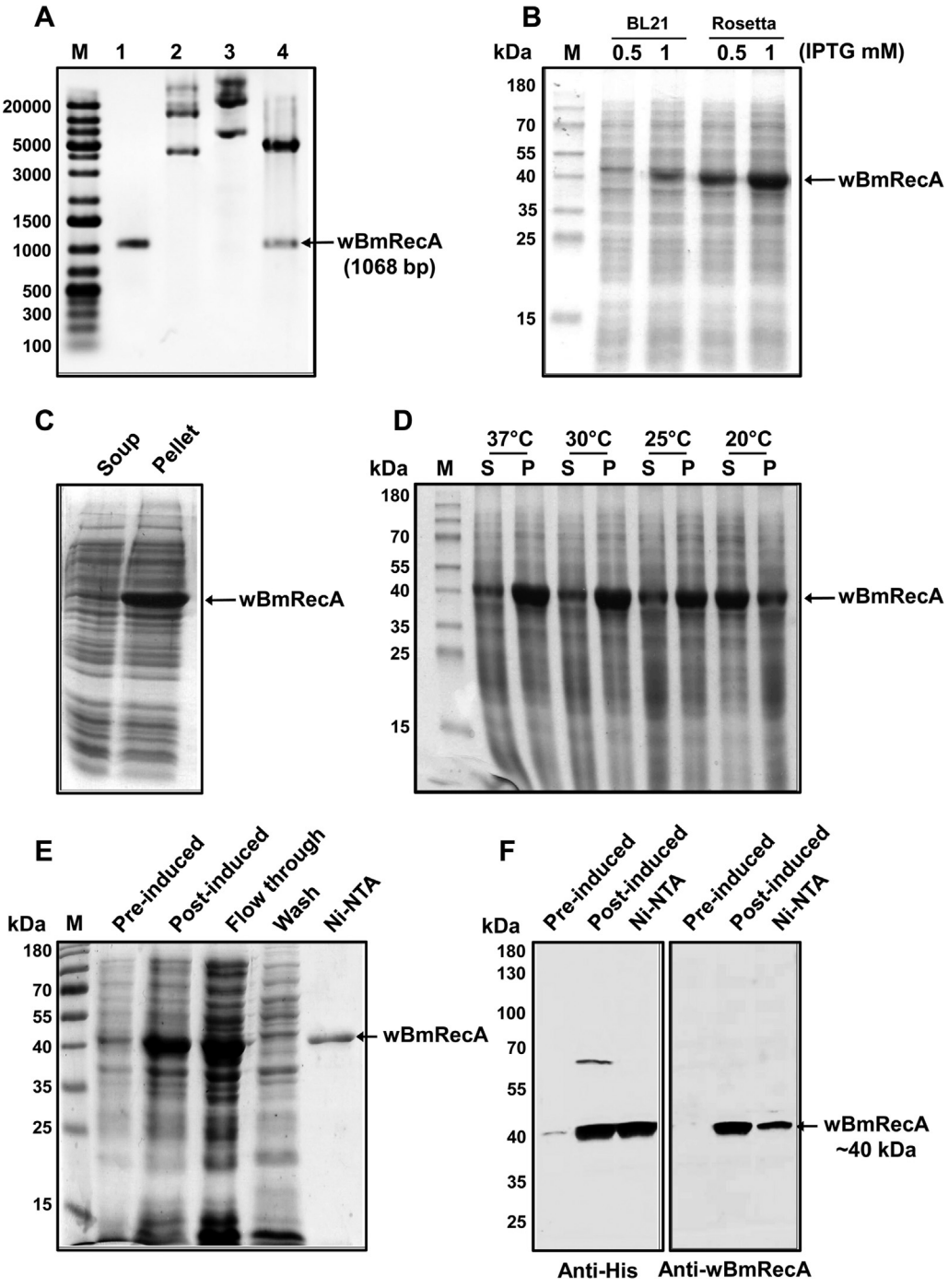

Fig. 2. Cloning, overexpression, purification and western blotting of wBmRecA. (A) *wBmRecA* gene was PCR amplified using gene specific primers from genomic DNA and cloned in expression vector pET28a as described in materials and methods. Lane M - DNA ladder, lane 1 - amplified *wBmRecA* gene, lane 2 - undigested pET28a vector, lane 3 - undigested construct *wBmRecA*-pET28a, lane 4 - *NcoI* and *XhoI* digested *wBmRecA*-pET28a. (B) Recombinant *wBmRecA*-pET28a was transformed into *E. coli* strain BL21 (DE3, lane 2 and 3) and BL21 (DE3) Rossetta strain (lane 4 and 5) and induced with IPTG (0.5–1.0 mM) at 37 °C. (C) Soup and pellet fractions from induced whole cell lysates were separated on 12% SDS–PAGE. (D) Recombinant construct *wBmRecA*-pET28a was transformed and overexpressed in *E. coli*, BL21 (DE3) Rossetta strain at different temperatures (0.5 mM IPTG). Following IPTG induction soup and pellet fractions were separated and analyzed on 12% SDS–PAGE. (E) Purification of recombinant wBmRecA from soluble fraction was carried out using Ni-NTA affinity chromatography. (F) Specificity of wBmRecA was analyzed by western blotting using anti-His and anti-wBmRecA antibodies.

and restriction digestion (Fig. 2A, lane 2–4) and showed retarded mobility in comparison to pET28a vector (Fig. 2A, lane 2 and 3). Restriction digestion of *wBmRecA*-pET28a liberated a fragment of 1068 bp equal to that of wBmRecA (Fig. 2A, lane 4).

Next, *wBmRecA*-pET28a clone was transformed in BL21 (DE3) and Rossetta strain of *E.coli* and induced with IPTG. Rossetta strain showed enhanced expression of wBmRecA with 1 mM IPTG as compared to BL21(DE3) strain (Fig. 2B). Most wBmRecA (~80%) aggregated and accumulated as inclusion bodies in the pellet, and only ~15–20% was observed in the soup (Fig. 2C), hence, protein expression conditions were optimized and best results were seen with 0.5 mM IPTG, at 20 °C temperature for 16 h (Fig. 2D). Next, r-wBmRecA was purified to ~90% purity using Ni-NTA column chromatography and a single band of 40 kDa was obtained (Fig. 2E), this was further verified by MALDI-TOF analysis and found to be 39574.23 Da (Supplementary Fig. 2A). Specificity of

r-*w*BmRecA was further confirmed by immunoblotting using commercially available anti-His and generated anti-wBmRecA antibodies (Fig. 2F).

3.3. wBmRecA is present in all the major life-cycle stages of B. malayi

RT-PCR (Fig. 3A) and immunoblotting (Fig. 3B) showed presence of wBmRecA (~38 kDa) in all the life stages of *B. malayi* with highest expression seen in adult worms followed by Bm-L3 and Mf. Notably, anti-wBmRecA antibodies showed cross reactivity with ES product from female worms highlighting the secretory nature of wBmRecA (Fig. 3C).

3.4. wBmRecA is a potent immunogen and anti-wBmRecA antibodies provide protection to the host

In silico studies revealed highly immunogenic nature of wBmRecA (Supplementary Tables 3 and 4). Also, mice immunized with r-wBmRecA showed significantly elevated levels of antigen specific IgG antibody (Fig. 4A) as well as concentrations of IgG1, IgG2a, IgG2b and IgG3 but not that of IgA and IgM when compared to other groups (Fig. 4B). Also, PECs cultured in the presence of serum from r-wBmRecA immunized mice showed considerable cellular adherence to the surface of Bm-L3 and Mf as compared to sera from non-immunized mice (Fig. 4C and E) resulting in death of Bm-L3 (53.87%) and Mf (64.03%) within 48 h of incubation (Fig. 4D and F). Furthermore, larval development in immunized mice was severely impaired with 53.01% and 64.50% reduction observed in the recovery of Bm-L3 in r-wBmRecA and r-wBmRecA+adjuvant group respectively as compared to control and/or adjuvant administered mice (Fig. 4G). Interestingly, wBmRecA exhibited strong immuno-reactivity with human bancroftian sera from EN individuals, followed by MF+ and SYMPT individuals, but no cross-reactivity was observed with sera from NEN individuals (Fig. 4H). Taken together these results showed that anti-wBmRecA antibodies provided protection to the host.

3.5. Immunization with wBmRecA recruits lymphocytes in the spleens of mice

Multi-colour immunophenotyping showed significant expansion of $CD4^+$ T helper cells, $CD8^+$ T cytotoxic cells and $CD19^+$ B cells in the spleens of r-wBmRecA immunized mice (Fig. 5A–D). While

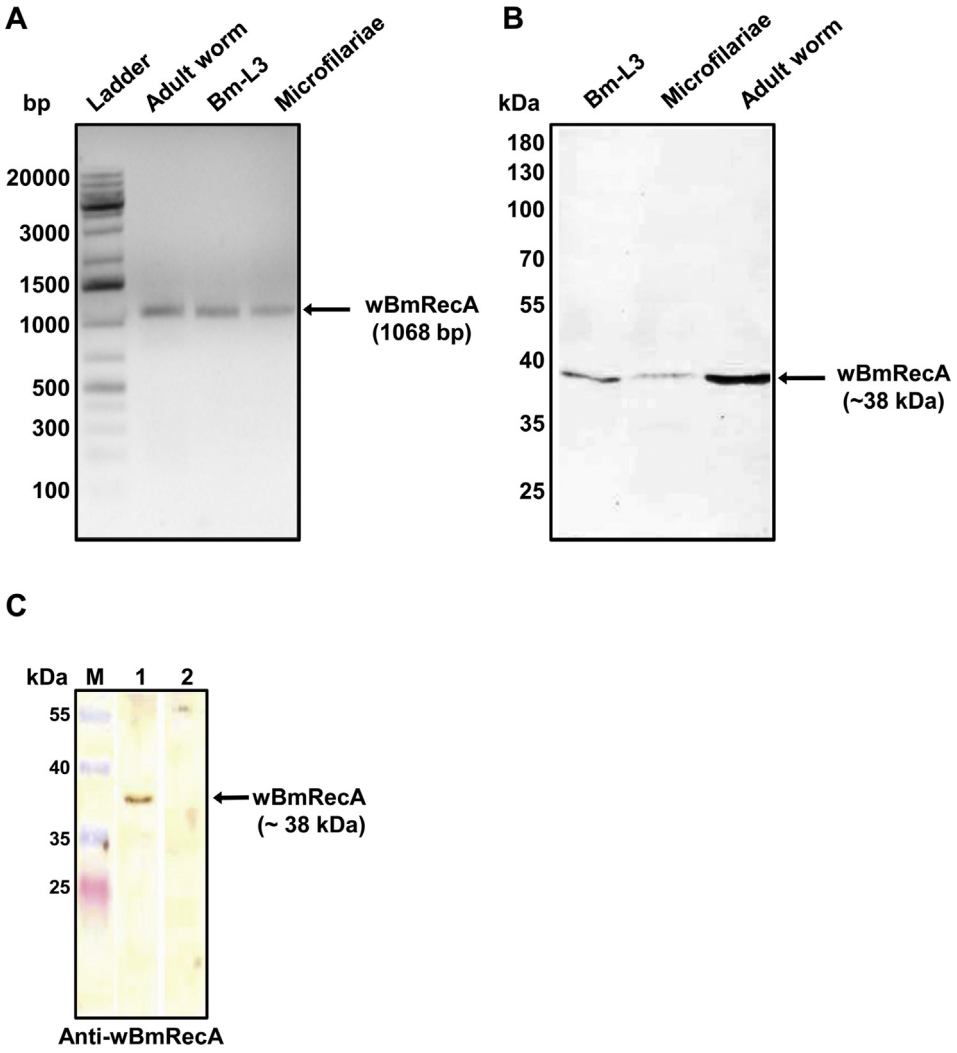

Fig. 3. Expression of wBmRecA in different life stages of *B. malayi* (A) RT-PCR analysis using cDNA from different stages of *B. malayi*. Lane M - DNA ladder; lane 1 - adult worms (AW); lane 2 - third stage infective larva (Bm-L3); lane – 3 microfilariae (Mf). (B) Protein lysate from Bm-L3, Mf and AW was resolved onto 12% SDS-PAGE and subjected to western blotting using anti-wBmRecA antibody. (C) Cross reactivity of anti- wBmRecA antibody with ES product from female worms. No reactivity was observed in case of control sera. Lane M: Protein molecular weight marker; Lane 1: ES product treated with anti-wBmRecA antibodies; Lane 2: ES product treated with control sera.

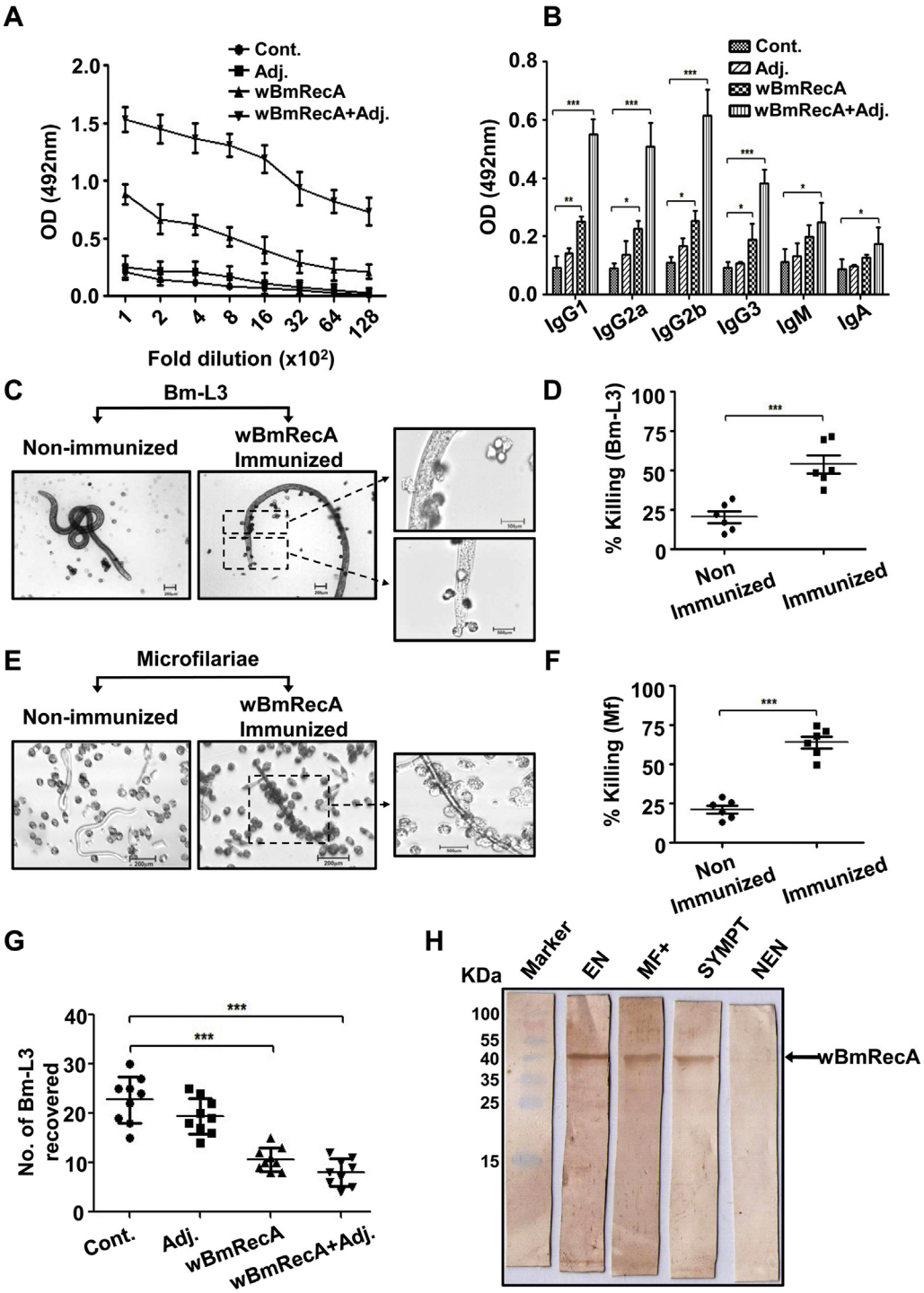

Fig. 4. Host protective nature of wBmRecA. (A) IgG titers, (B) r-wBmRecA specific Immunoglobulins in pooled sera of mice. (C–F) Peritoneal exudate cells from naive mice were separately cultured with (C) Bm-L3 and (E) Mf in the presence or absence of sera from r-wBmRecA immunized animals and (D) Bm-L3 and (F) Mf killing was observed microscopically. Representative images (C and E) were taken by phase contrast microscope (Carl-Zeiss, Germany) at 10× (Bm-L3) and 20× magnification (Mf). Boxed region of the respective image was further photographed at 40× magnification. Images are representative from one of the three independent experiments. (G) Recovery of Bm-L3 in mice from different treatment groups. (H) Sero-reactivity of wBmRecA with human bancroftian serum samples. Lane 1- Protein molecular weight marker; lane 2- serum from endemic normal (EN); lane 3- serum from asymptomatic microfilariaemic carriers (MF+); lane 3- serum from symptomatic microfilariaemic (SYMPT) and lane 4- serum from non-endemic normal (NEN) controls. Data shown are mean ± SD values from three independent experiments having 3–5 mice/group. Statistical significance between experimental groups was calculated by one-way ANOVA with Dunnett's post-test (A-B, G) and Student's *t* test (D, F) using Graph Pad Prism version 5.0 and indicated as $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$.

both $CD4^+$ and $CD8^+$ T cells almost doubled (15% $CD4^+$ Th cells in control and 31.8% $CD4^+$ Th cells in immunized mice; 8.8% $CD8^+$ Tc cells in control and 16.2% $CD8^+$ Tc cells in immunized mice), that of $CD19^+$ B cells increased by 1.3-fold in immunized mice (50.6% $CD19^+$ B cells in control and 64.7% $CD19^+$ B cells in immunized mice) (Fig. 5A–D). These results showed that T and B lymphocytes helped in boosting the host immune response thereby attenuating parasite growth and development.

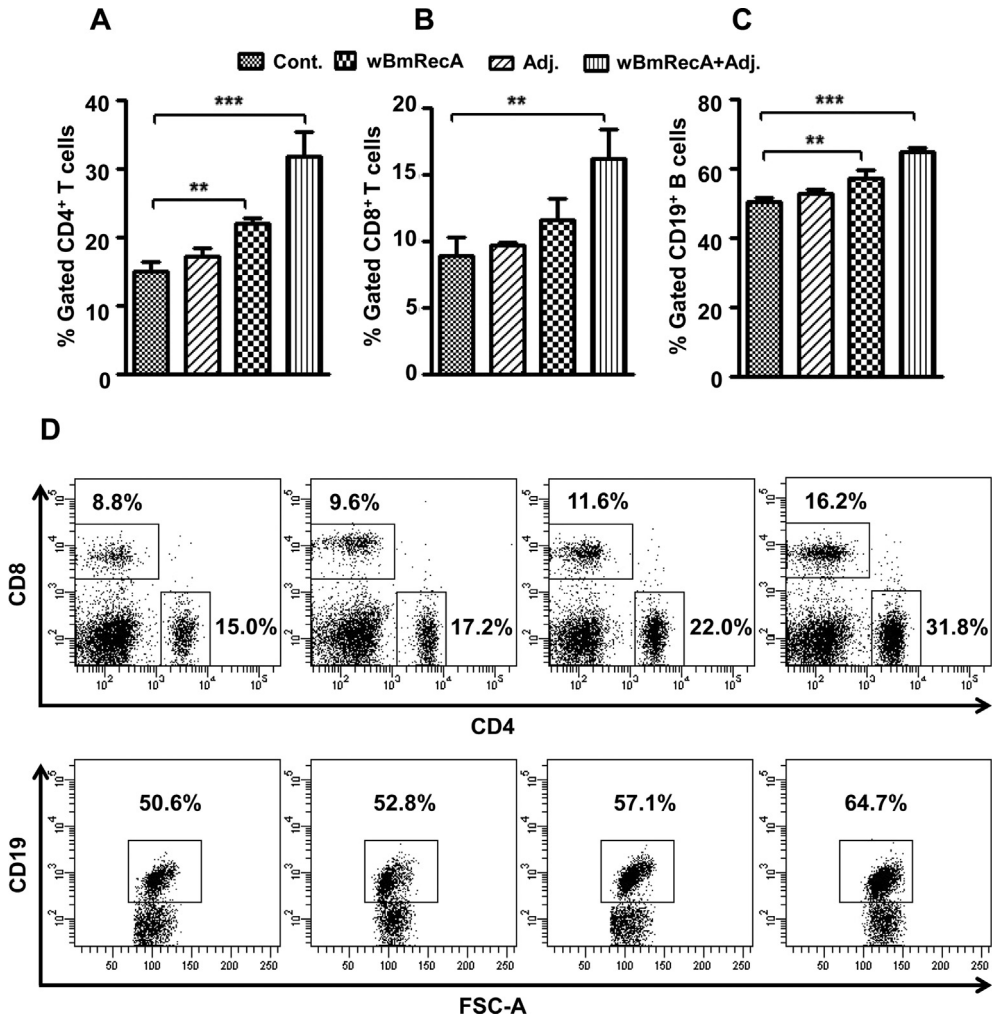

Fig. 5. Immunophenotyping of T and B lymphocytes in the spleens of mice. (A–C) Percentages and (D) representative flow cytometry dot plots of (A) $CD4^+$ T helper cells, (B) $CD8^+$ T cytotoxic cells and (C) $CD19^+$ B cells present in the spleens of different groups of mice as enumerated by multicolor flow cytometry using fluorochrome conjugated monoclonal antibodies. Data shown are mean ± SD values from three independent experiments having 3–5 mice/group. Statistical significance between experimental groups was calculated by one-way ANOVA with Dunnett's post-test using Graph Pad Prism (version 5.0) and indicated as $^{**}p < 0.01$ and $^{***}p < 0.001$.

3.6. Immunization with wBmRecA generates Th1 biased pro-inflammatory response

r-wBmRecA alone or in combination with adjuvant significantly upregulated Th1 transcript T-bet in splenocytes, while almost no change was reported for Th2 transcript GATA-3 (Fig. 6A). Notably, upregulation of T-bet was marked by 6-fold and 4-fold increased transcript levels of pro-inflammatory cytokines IFN-γ and IL-12 in immunized mice as compared to adjuvant administered mice (Fig. 6B). Interestingly, moderate increase in the transcripts of IL-4 and IL-10 was also observed, but this was significantly lower when compared to the overall transcript levels of Th1 cytokines (Fig. 6B). Peritoneal MΦs from immunized mice also showed increased transcript levels of iNOS along with concomitantly reduced expression of Arg-1 which showed that immunization with r-wBmRecA generated a biased Th1 pro-inflammatory response that classically activated peritoneal MΦs and arrested their alternative activation (Fig. 6C).

Assessment of wBmRecA specific recall response showed that concentration of IFN-γ and IL-12 increased significantly, while that of IL-4 and IL-10 increased moderately in immunized mice which corroborated with our real time RT-PCR data (Fig. 6D–G). Similarly, peritoneal MΦs from r-wBmRecA immunized mice produced significantly higher nitric oxide (NO) as compared to non-immunized animals. (Fig. 6H). Similarly, r-wBmRecA induced proliferation of lymphocytes from immunized animals which proved its ability to generate a strong memory T helper cell response (Fig. 6I).

4. Discussion

Lymphatic filariasis is a major health problem due to lack of a safe adulticidal drug and absence of any effective vaccine that can control the disease [1,2,32–34]. In recent years, new advances in the understanding of the disease etiology have offered hope by targeting the filarial endosymbiont *Wolbachia* because of its mutualistic relationship with the parasite and its unambiguous role in regulating the fecundity in female worms [12].

Past research has shown that the removal of *Wolbachia* using tetracycline class of antibiotics results in sterilization of the female worm [10,35–38]. However, duration and dose of treatment coupled with the risk of introducing resistance against antibiotics means that such strategies cannot be adopted on a mass scale [7,10,11,13,35]. Previously we showed that recombinant *Wolbachia* surface protein (r-Wsp) induced expansion of

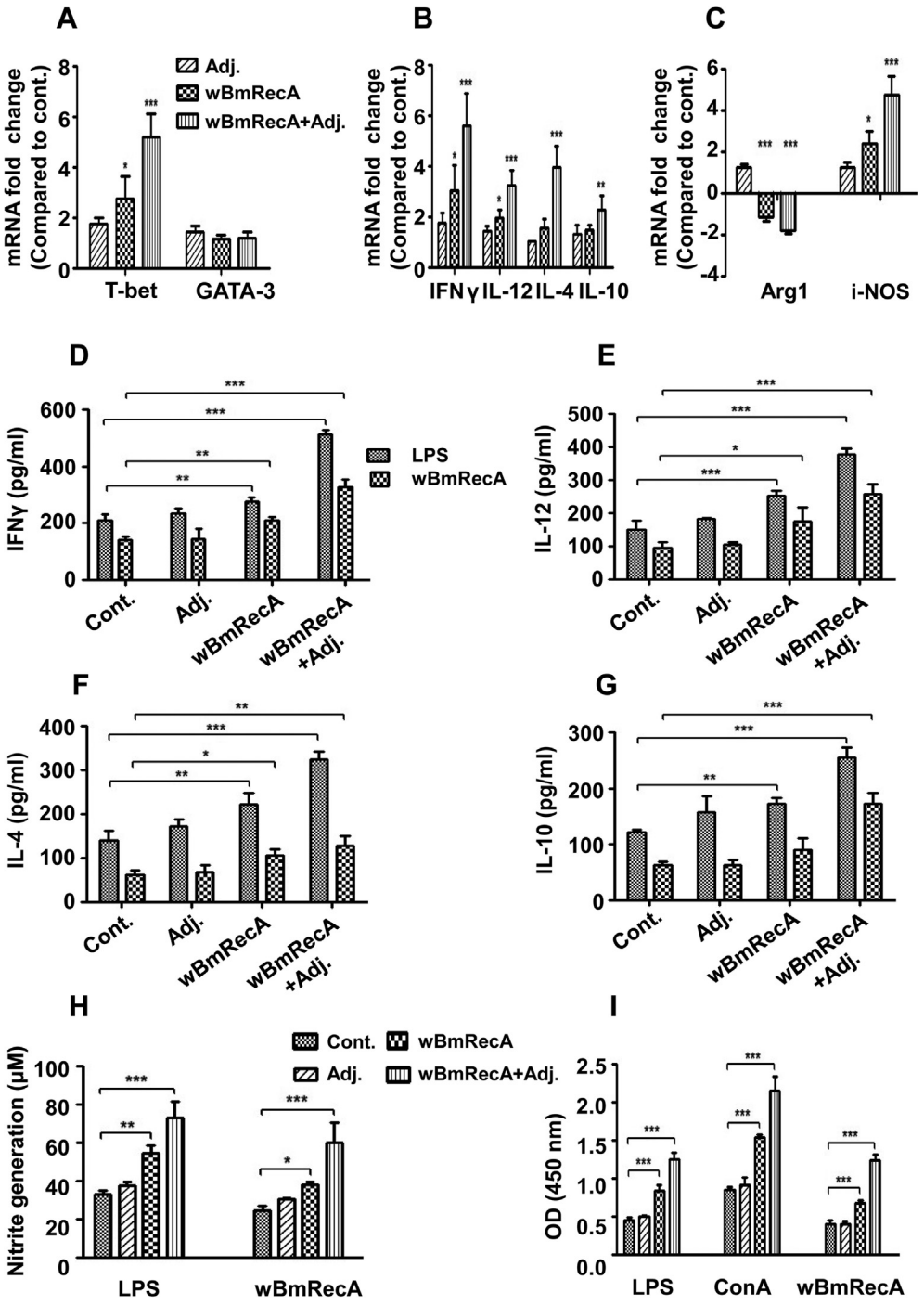

Fig. 6. Host immune response to wBmRecA. Transcript levels of (A) T-bet and GATA-3, (B) IFN-γ, IL-12, IL-4 and IL-10, and (C) Arg1 and iNOS were ascertained in either splenocytes or peritoneal MΦs from mice of different experimental groups using SYBR green chemistry and fold changes were calculated using 2^-DDCT method as described in material and methods. β-actin served as internal reference control. Splenocytes were stimulated by either LPS or r-wBmRecA and concentrations of (D) IFN-γ, (E) IL-12, (F) IL-4 and (G) IL-10 were measured in the culture supernatant by ELISA. (H) Nitric oxide (NO) levels present in the culture supernatant of peritoneal MΦs was estimated by Griess assay. (I) Lymphocyte proliferation was assessed by XTT-dye based assay by stimulating splenocytes with either LPS, concanavalin A (Con A) or recombinant wBmRecA as described in materials and methods section. Data shown are mean ± SD values from three independent experiments having 3–5 mice/group. Statistical significance between experimental groups was calculated by one-way ANOVA with Dunnett's post-test using Graph Pad Prism (version 5.0) and indicated as $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$.

Th-17 cells, which was further enhanced by neutralization of regulatory T cells leading to reduced parasite burden in Bm-L3 infected mice [22,28].

Since very limited information is available regarding the genes and proteins of *Wolbachia* that can be targeted, we set out to explore genes and proteins of *Wolbachia* that may act as possible vaccine candidates. We came across RecA protein which plays an important role in the maintenance of bacterial genome integrity under basal and stress (SOS response) conditions [18], thus making it a vital factor for further characterization in *Wolbachia* [14,15]. *In-silico* analysis showed that very high sequence similarity existed between wBmRecA and RecA of other species, while phylogenetic

studies showed wBmRecA to be most closely associated with alpha-proteo bacteria.

Cloning, characterization, CD and fluorescence spectroscopic studies showed that r-wBmRecA was a ~40 kDa protein with mixed α/β topology. Further investigations revealed ubiquitous nature of wBmRecA as it was expressed during all the major life stages of *B. malayi* and was also found in the ES products of the female worm. Interestingly, r-wBmRecA immunized mice responded with significantly high host protective IgG antibodies and robust expansion of antigen specific IgG1, IgG2a IgG2b, and IgG3 immunoglobulins along with moderate increase in the levels of IgM and IgA which highlighted generation of a mixed Th1 and Th2 response. Of note, Th1 cells drive a dominant IgG2a response and to a lesser, but significant extent, isotype switching to IgG1 and IgG2b, whereas Th2 cells induce Ig isotype switching to IgG1 which confirms the established notion that immunoglobulins play a major role in mediating protection to filarial infections and that protective immunity to filarial infection requires co-ordination of both Th1 and Th2 responses. Similarly, pentameric IgM antibodies have been shown to play major role in parasite killing *in vitro* and *in vivo*, while the expansion of secretory IgA antibodies is important for mucosal associated parasitic infections [39]. In fact, studies have shown that immunoglobulins are required for eliciting an optimal immune response against filarial infection which resonates with the findings of the present study [40,41].

Mice immunized with r-wBmRecA and challenged with Bm-L3 showed significant reduction in the recovery of pre-adult parasites (Bm-L4 stage). These results were further supported by *in vitro* ADCC assay where prominent cell adhesion and killing of Bm-L3 and Mf was observed when these parasite stages were incubated with PECs in the presence of immunized mouse serum. In sum, these results showed that increased titers of different immunoglobulins provided significant protection against Bm-L3 which corroborated with previously published reports [42].

r-wBmRecA immunized mice also showed significant expansion of $CD4^+$ T helper cells, $CD8^+$ T cytotoxic cells and $CD19^+$ B cells which was noteworthy as filarial infections are associated with impaired parasite specific proliferative responses, as well as down regulation of $CD4^+$ T cell responses [43]. It is worth mentioning that while T cells are absolutely critical for elimination of filarial infection, both T and B cells are required for protective immunity. Though we did not look specifically into the role of plasma cells in the present study, but elevated levels of serum immunoglobulins might signify expansion of plasma cells.

r-wBmRecA immunized mice also responded with a mixed T-helper response albeit with a skewed Th1 bias that was characterized by elevated levels of Th1 transcription factor T-bet, and cytokines IFN-γ and IL-12 in their spleens. Notably, splenocytes from immunized mice also induced proliferation of lymphocytes, while peritoneal MΦs showed increased transcript level of iNOS and high secretion of nitric oxide along with concomitantly down-regulated expression of Arg-1. These results highlighted the classical activation of MΦs (CAMs) and signified that immunization with r-wBmRecA successfully prevented the polarization of splenic MΦs from M1 (classical activation) to M2 phenotype (alternative activation) which was noteworthy as infection with *B. malayi* has been reported to hijack the classical activation pathway in host MΦs, while promoting an alternatively activated phenotype (AAMs) that help in parasite establishment and survival within the host.

Interestingly, wBmRecA showed highest cross reactivity with human bancroftian sera collected from endemic normal individuals which suggested that humans infected with *W. bancrofti* contained anti-wBmRecA antibodies thereby underscoring the prophylactic potential of wBmRecA. In summary, we demonstrate that immunization with r-wBmRecA elicits a robust host protective immune response that contributes to reduced parasite burden in infected animals. Further studies in a permissive rodent model will be needed to fully explore the immunoprophylactic potential of wBmRecA, and evaluate its potential as a putative vaccine candidate against lymphatic filariasis.

Author contribution

MG cloned, characterized and performed immunological studies with wBmRecA and drafted the initial manuscript. RJ carried out ADCC and Flow cytometry experiments with wBmRecA. M. Goyal carried out bioinformatics and biophysical studies, helped MG in the experiments and participated in drafting the initial manuscript. MS conceptualized and designed the experiments, analysed the data and finalized the manuscript for important intellectual content.

Acknowledgements

Authors thankfully acknowledge excellent technical support provided by Shikha Mishra and O. P Yadav for maintaining *B. malayi* infection in the laboratory. Authors also acknowledge the technical support of Mr. Achchhe Lal Vishwakarma for FACS experiments. MG and RJ thankfully acknowledge fellowship support from Council of Scientific and Industrial Research (CSIR), and Indian Council of Medical Research (ICMR), New Delhi respectively.

Declaration of conflict of interest

None.

Funding

This work was supported by DST-SERB, India, Grant No. EMR/2016/001880 and grants provided under CSIR, India - Network projects "New approaches toward understanding of Disease dynamics and to accelerate drug discovery (UNDO)" and "Emerging and re-emerging challenges in infectious diseases: Systems based drug design for infectious diseases (SPLenDID)" to MS. The funders had no role in study design, data collection, analysis, decision to publish, or preparation of the manuscript. This is communication number 9785 from CSIR-CDRI.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2018.12.015.

References

[1] Cano J, Rebollo MP, Golding N, et al. The global distribution and transmission limits of lymphatic filariasis: past and present. Parasit Vectors 2014;7:466.
[2] WHO. Global Programme to Eliminate Lymphatic Filariasis: progress report on mass drug administration. WHO 2010, Global Programme to Eliminate Lymphatic Filariasis: progress report on mass drug administration.
[3] Pink R, Hudson A, Mouries MA, Bendig M. Opportunities and challenges in antiparasitic drug discovery. Nat Rev Drug Discov 2005;4(9):727–40.
[4] Hotez PJ, Strych U, Lustigman S, Bottazzi ME. Human anthelminthic vaccines: rationale and challenges. Vaccine 2016;34(30):3549–55.
[5] Lustigman S, Prichard RK, Gazzinelli A, et al. A research agenda for helminth diseases of humans: the problem of helminthiases. PLoS Negl Trop Dis 2012;6(4):e1582.
[6] Srivastava M, Misra-Bhattacharya S. Overcoming drug resistance for macro parasites. Future Microbiol 2015;10(11):1783–9.
[7] Taylor MJ. Wolbachia endosymbiotic bacteria of filarial nematodes. A new insight into disease pathogenesis and control. Arch Med Res 2002;33(4):422–4.
[8] Landmann F, Foster JM, Slatko B, Sullivan W. Asymmetric Wolbachia segregation during early Brugia malayi embryogenesis determines its distribution in adult host tissues. PLoS Negl Trop Dis 2010;4(7):e758.

[9] Taylor MJ, Hoerauf A. Wolbachia bacteria of filarial nematodes. Parasitol Today 1999;15(11):437–42.
[10] Hoerauf A, Nissen-Pahle K, Schmetz C, et al. Tetracycline therapy targets intracellular bacteria in the filarial nematode Litomosoides sigmodontis and results in filarial infertility. J Clin Invest 1999;103(1):11–8.
[11] Slatko BE, Taylor MJ, Foster JM. The Wolbachia endosymbiont as an anti-filarial nematode target. Symbiosis 2010;51(1):55–65.
[12] Taylor MJ, Hoerauf A, Townson S, Slatko BE, Ward SA. Anti-Wolbachia drug discovery and development: safe macrofilaricides for onchocerciasis and lymphatic filariasis. Parasitology 2014;141(1):119–27.
[13] Wu B, Novelli J, Foster J, et al. The heme biosynthetic pathway of the obligate Wolbachia endosymbiont of Brugia malayi as a potential anti-filarial drug target. PLoS Negl Trop Dis 2009;3(7):e475.
[14] Chen Z, Yang H, Pavletich NP. Mechanism of homologous recombination from the RecA-ssDNA/dsDNA structures. Nature 2008;453(7194). 489–484.
[15] Horii T, Ogawa T, Ogawa H. Organization of the recA gene of Escherichia coli. Proc Natl Acad Sci U S A 1980;77(1):313–7.
[16] Lin Z, Kong H, Nei M, Ma H. Origins and evolution of the recA/RAD51 gene family: evidence for ancient gene duplication and endosymbiotic gene transfer. Proc Natl Acad Sci U S A 2006;103(27):10328–33.
[17] Karlin S, Brocchieri L. Evolutionary conservation of RecA genes in relation to protein structure and function. J Bacteriol 1996;178(7):1881–94.
[18] Horii T, Ogawa T, Nakatani T, Hase T, Matsubara H, Ogawa H. Regulation of SOS functions: purification of E. coli LexA protein and determination of its specific site cleaved by the RecA protein. Cell 1981;27(3 Pt 2):515–22.
[19] Kushwaha S, Singh PK, Gupta J, Soni VK, Misra-Bhattacharya S. Recombinant trehalose-6-phosphate phosphatase of Brugia malayi cross-reacts with human Wuchereria bancrofti immune sera and engenders a robust protective outcome in mice. Microbes Infect 2012;14(14):1330–9.
[20] Rana AK, Chandra S, Siddiqi MI, Misra-Bhattacharya S. Molecular characterization of an rsmD-like rRNA methyltransferase from the Wolbachia endosymbiont of Brugia malayi and antifilarial activity of specific inhibitors of the enzyme. Antimicrob Agents Chemother 2013;57(8):3843–56.
[21] Shahab M, Verma M, Pathak M, Mitra K, Misra-Bhattacharya S. Cloning, expression and characterization of UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) from Wolbachia endosymbiont of human lymphatic filarial parasite Brugia malayi. PLoS One 2014;9(6):e99884.
[22] Pathak M, Verma M, Srivastava M, Misra-Bhattacharya S. Wolbachia endosymbiont of Brugia malayi elicits a T helper type 17-mediated pro-inflammatory immune response through Wolbachia surface protein. Immunology 2015;144(2):231–44.
[23] Nag JK, Shrivastava N, Gupta J, Misra-Bhattacharya S. Recombinant translation initiation factor-1 of Wolbachia is an immunogenic excretory secretory protein that elicits Th2 mediated immune protection against Brugia malayi. Comp Immunol Microbiol Infect Dis 2013;36(1):25–38.
[24] Gupta J, Pathak M, Misra S, Misra-Bhattacharya S. Immunogenicity and protective efficacy of Brugia malayi heavy chain myosin as homologous DNA, protein and heterologous DNA/protein prime boost vaccine in rodent model. PLoS One 2015;10(11):e0142548.
[25] Sharma A, Sharma P, Vishwakarma AL, Srivastava M. Functional impairment of murine dendritic cell subsets following infection with infective larval stage 3 of Brugia malayi. Infect Immun 2017;85(1).
[26] Sharma P, Sharma A, Srivastava M. In vivo neutralization of alpha4 and beta7 integrins inhibits eosinophil trafficking and prevents lung injury during tropical pulmonary eosinophilia in mice. Eur J Immunol 2017;47(9):1501–12.
[27] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25(4):402–8.
[28] Pathak M, Sharma P, Sharma A, Verma M, Srivastava M, Misra-Bhattacharya S. Regulatory T-cell neutralization in mice during filariasis helps in parasite clearance by enhancing T helper type 17-mediated pro-inflammatory response. Immunology 2016;147(2):190–203.
[29] Sharma P, Sharma A, Vishwakarma AL, Agnihotri PK, Sharma S, Srivastava M. Host lung immunity is severely compromised during tropical pulmonary eosinophilia: role of lung eosinophils and macrophages. J Leukoc Biol 2016;99(4):619–28.
[30] Sharma A, Sharma P, Ganga L, et al. Infective larvae of Brugia malayi induce polarization of host macrophages that helps in immune evasion. Front Immunol 2018;9:194.
[31] Bhardwaj J, Siddiqui AJ, Goyal M, et al. Host immune response is severely compromised during lethal Plasmodium vinckei infection. Parasitol Res 2015;114(9):3445–57.
[32] Geary TG, Mackenzie CD. Progress and challenges in the discovery of macrofilaricidal drugs. Expert Rev Anti Infect Ther 2011;9(8):681–95.
[33] Hoerauf A, Pfarr K, Mand S, Debrah AY, Specht S. Filariasis in Africa–treatment challenges and prospects. Clin Microbiol Infect 2011;17(7):977–85.
[34] Michael E, Bundy DA, Grenfell BT. Re-assessing the global prevalence and distribution of lymphatic filariasis. Parasitology 1996;112(Pt 4):409–28.
[35] Townson S, Hutton D, Siemienska J, et al. Antibiotics and Wolbachia in filarial nematodes: antifilarial activity of rifampicin, oxytetracycline and chloramphenicol against Onchocerca gutturosa, Onchocerca lienalis and Brugia pahangi. Ann Trop Med Parasitol 2000;94(8):801–16.
[36] Albers A, Esum ME, Tendongfor N, et al. Retarded Onchocerca volvulus L1 to L3 larval development in the Simulium damnosum vector after anti-wolbachial treatment of the human host. Parasit Vectors 2012;5:12.
[37] Langworthy NG, Renz A, Mackenstedt U, et al. Macrofilaricidal activity of tetracycline against the filarial nematode Onchocerca ochengi: elimination of Wolbachia precedes worm death and suggests a dependent relationship. Proc Biol Sci 2000;267(1448):1063–9.
[38] Landmann F, Voronin D, Sullivan W, Taylor MJ. Anti-filarial activity of antibiotic therapy is due to extensive apoptosis after Wolbachia depletion from filarial nematodes. PLoS Pathog 2011;7(11):e1002351.
[39] Rajan B, Ramalingam T, Rajan TV. Critical role for IgM in host protection in experimental filarial infection. J Immunol 2005;175(3):1827–33.
[40] Dash Y, Ramesh M, Greiner D, Shultz LD, Klei TR, Rajan TV. Determinants of memory in experimental filarial infections in mice. Parasite Immunol 2007;29(11):567–74.
[41] Folkard SG, Bianco AE. Roles for both CD4+ and CD8+ T cells in protective immunity against Onchocerca lienalis microfilariae in the mouse. Parasite Immunol 1995;17(10):541–53.
[42] Chandrashekar R, Rao UR, Parab PB, Subrahmanyam D. Brugia malayi: serum dependent cell-mediated reactions to microfilariae. Southeast Asian J Trop Med Public Health 1985;16(1):15–21.
[43] King CL, Nutman TB. Regulation of the immune response in lymphatic filariasis and onchocerciasis. Immunol Today 1991;12(3):A54–8.