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Immunological evaluation of an rsmD-like rRNA methyltransferase from *Wolbachia* endosymbiont of *Brugia malayi*

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ABSTRACT

Wolbachia is a wonderful anti-filarial target with many of its enzymes and surface proteins (WSPs) representing potential drug targets and vaccine candidates. Here we report on the immunologic response of a drug target, rsmD-like rRNA methyltransferase from *Wolbachia* endosymbiont of *Brugia malayi*. The recombinant protein generated both humoral and cell-mediated response in BALB/c mice but compromised its immunity. The humoral response was transient and endured barely for six months in mice with or without *B. Malayi* challenge. In splenocytes of mice, the key humoral immunity mediating cytokine IL4 was lowered (IL4↓) while IFNγ, the major cytokine mediating cellular immunity was decreased along with upregulation of IL10 cytokine (IFNγ↓, IL10↑). The finding here indicates that the enzyme has low immunogenicity and triggers lowering of cytokine level in BALB/c mice. Interestingly the overall immune profile can be summed up with equivalent response generated by WSP or whole *Wolbachia*.

1. Introduction

Lymphatic filariasis (LF), the second most common cause of long-term disability in tropical and subtropical countries, is caused by lymphatic-dwelling nematodes *Wuchereria bancrofti*, *Brugia malayi* and *B. timori* transmitted through a mosquito vector [1–3]. The disease globally affects ~130 million people with 1.3 billion at risk [1,4] resulting into heavy socio-economic loss to the developing nations [5] and India itself accounts for about 40% of the total cases. The infection due to filarial parasite result into a wide range of clinical and pathological manifestations represented by asymptomatic microfilarial carriers (MF), chronic symptomatic pathology of filariasis (SYMPT), endemic normals (EN) and tropical pulmonary eosinophilic syndrome [6]. MF people (carriers) generally have microfilaria (mf or L1, the early stage parasites [7]) in their bloodstream but do not show phenotypic signs of filarial disease [8]. These carriers harbour fecund adults in the lymphatics, susceptible to infection and have immunological responses known as modified 'T helper 2 (Th2) responses' [9,10]. Th2 type response in humans involves IgG4 isotype dominance with relatively very low IgE antibody titre. These individuals often have clinically silent infections and are the main reservoir for onward transmission. On the other hand, chronic cases are characterized by uncontrolled inflammatory responses (Th1 type) which leads to alteration in the walls of affected lymphatic vessels, with both dilation and obstruction [11], leading to lymphoedema or hydrocele, etc. [12]. In such SYMPT patients, low levels of IgG4 but moderate IgE levels are evident which ultimately cause failure of lymphatic drainage and opportunistic secondary infection leading to elephantiasis. A minor population mounts some degree of immunity and prevents maturation of infective L3 and called 'endemic normals' or EN [13], in contrast to non-endemic normal (NEN) population who are free from any filarial infection.

All the filarial worms harbours an endosymbiotic gram-negative bacteria which belongs to α-class of proteobacteria called *Wolbachia* and are highly evolved and successful manipulators of host reproductive system [14]. The distribution and phylogenetic patterns of *Wolbachia* in filarial nematodes indicate that the association is stable and specific, and suggests a long co-evolutionary history [15]. These endosymbionts in filarial parasites appear to play an important role in the fertility, development and survival of these filariids [16]. Though *Wolbachia* has been reported to have a very small role in generating host immune response against worm infection [17], however the most dominant *Wolbachia* surface proteins (WSP) have been shown to involve and induce a significant T cell mediated immune response as well as

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immunosuppression during filarial infections [18,19]. The WSP or other *Wolbachia* contents are released through either secretion of the worm or the death of either worm or sometimes a larger biomass of circulating microfilaria, releasing the *Wolbachia*. WSP activate toll-like receptors (TLR2/TLR6) on the surface of resident innate immune cells in the tissues such as corneal fibroblasts in ocular onchocerciasis, or resident macrophages and endothelial cells in the lymphatic and perilymphatic tissues in lymphatic filariasis [20]. TLR2/TLR6 signalling stimulates production of proinflammatory and chemotactic cytokines that leads to the release of vasoactive molecules leading to capillary leakiness, recruitment of neutrophils, macrophages, and other effector cells to the affected tissue, and the propagation of the inflammatory response. Continued and repeated inflammation in the perilymphatic tissues may induce changes in lymphatic vessel architecture and eventual lymphatic scarring and lymphedema.

Here we report on the immunologic response generated by a wonderful drug target enzyme, rsmD-like rRNA methyltransferase/MTase [21] in BALB/c mice which unlike WSP, is neither a surface protein nor dominantly expressed but exhibits a comparative and similar profile to WSP. Although rsmD-like rRNA-MTase has been targeted, where filarial worms have been shown to be destroyed by various inhibitors directed specifically against its DPPY motif, it may have other alternative paths/targets to kill the worms as discussed in the previous article which requires further investigation on its inhibitors. Since *Wolbachia* is known to play a very little role in mounting immune response in the host animal, this another wolbachial drug target protein enzyme not present on the surface but inside the bacterial cell was evaluated for its immune properties if any in mice model BALB/c. The epitopes of the enzyme sequence to be presented by B and T cells were predicted with online software tool [22] to compare and analyze the experimental data (http://tools.immuneepitope.org/bcell). The rsmD-like rRNA-MTase was cloned and overexpressed as a recombinant protein in *Escherichia coli* and purified to homogeneity. BALB/c mice were administered with the recombinant protein along with an adjuvant through subcutaneous (s.c.) or intravenous (i.v.) route and then the humoral and cellular immune responses evidenced by changing concentration of cytokines of mice were monitored for a scheduled time. The recombinant protein was investigated for assessing serum antibodies including their isotypes, oxidative burst in peritoneal macrophages, B and T populations and cytokines (IL2, IL4, IL10 and IFNγ) expression level on T helper (Th) cells. Concurrently expression profile of major immune regulating cytokines in CD4+ cells (Th) were measured to assess the response of rsmD-like rRNA-MTase and compare it to the response generated by WSP or whole *Wolbachia*.

2. Materials and methods

2.1. Isolation of adult B. malayi worms and preparation of genomic DNA

Adult worms of *B. malayi* were harvested from euthanized jirds by washing their peritoneal cavity. The worms were washed repeatedly in phosphate buffer saline (PBS) and subjected for genomic DNA isolation as per the standard protocol of Sambrook et al. [23] with slight modification of using lysozyme in the extraction buffer. The genomic DNA isolated from *B. malayi* also contains the genomic DNA of its endosymbiont, *Wolbachia*.

2.2. Cloning of rsmD-like rRNA-MTase gene of Wolbachia

The ~546 bp rsmD-like rRNA-MTase gene of *Wolbachia* (ANwbm0791) was amplified from the *B. malayi* genomic DNA using forward sense primer – 5′ GGATCCTTACGTATTATTGCAGGAAAGTATCGT 3′ and reverse antisense primer – 5′ CTCGAGAGTTGATAGAGAAAGAAAAATTATTCG 3′ containing flanking restriction sites *BamHI* and *XhoI* (underlined) respectively. Amplification of gene was carried out by mixing 1 μM of each primer, 200 μM each dNTPs, 0.5 unit Taq DNA polymerase, 1× PCR buffer and 1.5 μM $MgCl_2$ under the conditions of initial denaturation at 95 °C/4 min, 29 cycles at 95 °C/45 s, 55 °C/1.30 min, 72 °C/1 min, and 1 cycle at 72 °C/20 min. The amplified product was subcloned into cloning/T vector pTZ57R (MBI Fermentas) and subjected to automated sequencing.

2.3. Protein expression and purification

The clone from pTZ57R/T vector was transferred into pET28a(+) (Novagen, USA) expression vector. The expression vector containing clone was done in *E. coli* Rosetta (p-LysS, DE3) cells. The protein expression was checked on SDS-PAGE using protein molecular weight marker followed by western blotting. The bacterial culture was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h, centrifuged, and pellet was resuspended in PBS (pH 7.4). It was subjected to sonication at 30/10 s on/off cycle at amplitude of 20 at 4 °C for 30 min. The lysate was and centrifuged at 12,500 × *g* for 30 min to separate pellet from supernatant. The presence of protein was checked in both pellet as well as supernatant. The pellet was run on a 10% SDS-PAGE preparatory gel to separate the protein band. After the complete run, it was negatively stained with 4 M sodium acetate solution on a rocker to visualize protein band as highly pronounced unstained row in the gel [24]. The band was excised from the gel, minced and bagged in 12 kDa cut-off dialysis tubing preequilibriated with elution buffer. The protein was electro-eluted at 50 V for 4 h at 4 °C, and then concentrated after passing through 10 kDa cut-off Amicon ultra centrifugal filters (Millipore, USA). The protein concentration was determined by Bradford assay [25] and was used for immunological study in BALB/c mice.

2.4. Ethics statement

BALB/c mice were approved by Animal Ethics Committee of CDRI (Institutional Animal Ethics Committee, IAEC-CDRI) duly constituted under the provision of CPCSEA (The committee for the purpose of Control and Supervision of Animals) rules and guidelines 1998, Govt. of India. The study bears the approval No. 86/9/PARA/IAEC/RENEW01 (44/10) dated 22.01.2010 for BALB/c mice. The study was carried out in strict accordance and recommendations in the guidelines for the care and use of laboratory animals. The animals were housed at the National Laboratory Animal Centre (NLAC) at CDRI, Lucknow, India under controlled conditions of temperature (23 ± 1 °C), humidity (RH: 55 ± 10%) and photoperiod (12:12 h light/dark cycle). They were fed on standard rodent pellet diet and drinking water ad libitum.

2.5. Mice and immunization

The BALB/c mice were administered recombinant rsmD-like rRNA-MTase emulsified in Freunds complete adjuvant (CFA) for priming while incomplete Freunds adjuvant (IFA) was used in booster doses. CFA was administered cautiously to avoid any inflammations that are often caused if not administered carefully. At all the times of immunization we maintained sterility and aseptically prepared the injection site. Inbred BALB/c male mice were divided into three groups viz. PBS, adjuvant and recombinant protein + adjuvant of 5 animals each (Table 1). In a parallel experiment, three separate groups of BALB/c mice as above were observed for humoral response for a period of 6 months. Mice receiving only