EVOLUTION OF PROPHYLACTIC ADEQUACY OF FILARIAL RECOMBINANT PROTEIN (rBmALT-2) WITH MONOPHOSPHORYL LIPID A (MPLA) ADJUVANT

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ABSTRACT

Brugia malayi Abundant larval transcript-2 (BmALT-2) is most potential prophylactic vaccine candidate. In order to increase the protection efficacy of BmALT-2, Monophosphory Lipid A (MPLA), as adjuvant, a detoxified derivative of lipopolysaccharide (LPS) known to promote Th-1 responses, was used in this study. Moreover to determine the percentage of protection obtained following a challenge infection with B. malayi L3 larvae in immunized Mastomys. This study used, 6-8 weeks aged healthy Mastomys (n=5-7/group), immunized intramuscularly with 50 μg of rBmALT-2 antigen either with MPLA or Alum as adjuvant and the control groups received MPLA or Alum only. The protective immunity elicited in the Mastomys was checked by in vitro antibody-dependent cellular cytotoxicity (ADCC) and in vivo microprobe chamber assay. The humoral and cellular immune responses were also analyzed. Our results demonstrate, high antibody response in all immunized group compared to control group. We observed increased levels of IgG2a and IgG2b antibodies in rBmALT-2+MPLA and rBmALT-2+Alum+MPLA immunized group, whereas, rBmALT-2+Alum immunized group generated IgG1, IgG2a and IgG3 antibodies. rBmALT-2+Alum group secreted high levels of IL-4 and IFN-γ cytokines and suggesting the balanced Th-1/Th-2 response. The increased level of IL-10 cytokine in rBmALT-2+MPLA or rBmALT-2+Alum+MPLA groups reflecting the inhibitory effect of Th-1 (IFN-γ) and Th-2 (IL-4) cytokines which do not demonstrate the classical role of MPLA. Furthermore, our, in vitro and in vivo assay results observed significantly high cytotoxicity in rBmALT-2+Alum group in vitro (80% p<0.001) and in vivo (79.79%; p<0.001) compared to rBmALT-2+MPLA and rBmALT-2+Alum+MPLA in vitro 61% and 67% and in vivo 59.84% and 65.96% cytotoxicity. This study illustrated immunomodulation, which includes an up-regulation of IL-10 cytokine and a decreased expression of TLR-4 (MPLA) by which filarial parasites have evolved to escape host immune mechanisms.

KEYWORDS

Lymphatic filariasis; Adjuvant; MPLA; BmALT-2; IL-10

1. INTRODUCTION

Lymphatic Filarialis (LF) is one of the neglected tropical disorders that impair the lymphatic system causing lymphoedema and hydrocele. LF caused by microscopical filarial parasites namely Wuchereria bancrofti Brugia malayi and B. timori. The infection is transmitted by wide range of mosquito species such as Culex, Anopheles, Mansonia and Aedes, depending on the geographic area. Globally, 893 million people in 49 countries remain threatened and necessitate preventive chemotherapy to stop the burden of this parasitic infection (WHO 2019). In 2000, WHO had launched the Global Programme to Eliminate Lymphatic Filariasis (GPELF) with the objectives to stop transmission of infection by mass drug administration (MDA) and to lessen suffering among affected population through morbidity management and disability prevention (MMDP). In several countries the incidence of LF has substantially reduced by this approach. WHO 2019, however, most endemic regions still face various difficulties in halting LF transmission and elimination suggesting that the mass drug administration alone as a prophylaxis against LF is not effective. A more effective approach, such as prophylactic vaccination, is required to avoid transmission and eradicate LF from the endemic areas (Jambulingam et al. 2016; Harris and Wiegand 2017). Our laboratory and many others have identified several possible vaccine antigens that demonstrated substantial protection in experimental animals against challenge infections (Thirunagaram et al., 2007; Veerapathran et al., 2009; Samkutty et al., 2010; Anand et al., 2011; Dakshinamoorthy et al., 2013; Arumugam et al., 2014; Immanuel et al., 2017). Among these, B. malayi Abundant Larval Transcript-2 (BmALT-2) is the most studied vaccine candidate and appeared to elicit high protective responses against B. malayi parasites (Gregory et al., 2000; Gnanasekar et al., 2004). BmALT-2 proteins are synthesized in the infective stage (L3) of the parasite and have no mammalian homologue. It is known to have immunomodulatory role in early host immune response (Pouthouse et al. 2006). BmALT-2, conferred high protection in Jirds and Mastomys when immunized alone with alum (Gnanasekar et al., 2004; Anand et al., 2008; Dakshinamoorthy et al., 2013; Nakhale et al., 2020). For parasite clearance both Th1 (IFN-γ stimulated toxic mediators) and Th2 responses (antibody-dependent cell mediated cytotoxicity) are essential.

Adjuvants play a significant role in enhancing the antigen potency and promote Th1 or Th2 immune responses (Di Pasquale et al., 2015). Alum (aluminum salt precipitates) is the most popular vaccine adjuvant and is used in almost 80% of all vaccines (Gupta 1998; Brewer 2006; Marrack et al., 2009). Alum promotes predominantly Th2 bias immune response consisting of IgG4 and IgE isotypes (Hamaoka et al. 1973; Brewer 2006; Marrack et al., 2009), but does not activate cell mediated immune response (Borud 1980). Hence, it is essential to identify adjuvant that increased the protective efficiency of BmALT-2 and also capable for stimulating cell mediated immune
response (CMI). Among various adjuvants, the Monophosphoryl Lipid A (MPLA) is derived from the Lipopolysaccharide (LPS) fraction of the cell walls of gram-negative bacteria (Salmonella minnesota) and has been found to enhance adaptive immunity through Toll-like receptors (TLR-4). MPLA boosts IFN-γ production with antigen specific CD4+ T cells; thereby generating a Th1 biased immune response (Casella and Mitchell 2008). In this study, we attempted to evaluate the immune response of recombinant BmA-LT-2 antigen plus Aqueous Formulation of MPLA (MPLA-AF) with or without Alum in Mastomys and determine the percentage of protection obtained following a challenge infection with B. malayi L3 larvae.

2. MATERIALS AND METHODS

2.1 Experimental animals and B. malayi infective L3 larvae:
In the present study, Mastomys (Mastomys coucha) of 6-8 weeks aged were used, bred and kept in the central animal house amenity at Mahatma Gandhi Institute of Medical Sciences (MGIMS), Sevagram registered with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). This study was allowed by the Institutional Animal Ethics Committee, MGIMS, Sevagram (MGIMS/IAEC/July/8/2014), which follows the USA National Institutes of Health animal study guidelines (NIH, Office of Animal Care and Use. 2009; Office of Laboratory Animal Welfare Public Health Service Policy on humane Care and Use of Laboratory Animals 2015). The infective third-stage larvae (L3) of B. malayi parasites were obtained by using Baermann technique (Suzuki et al., 1979) and utilized for further study.

2.2 Antigen and adjuvant formulation:
The 3-O-desacylated Monophosphoryl Lipid A (MPLA) from Salmonella minnesota R595, was purchased from Avanti polar lipids inc. (Mumbai). Aqueous formulation of MPLA (1 mg/ml) was prepared by dissolving in 0.5% Triethanolamine (TEOA) (Sigma, Mumbai), subjected to sonication at 60°C until a homogenous clear suspension was obtained (Baldridge and Crane 1999). The Escherichia coli (E. coli) strain BL2I-DE3 (pLYsS) used for the expression of BmML-2 gene as described earlier (Veerapathran et al., 2009). His-tag recombinant BmA-LT-2 protein was purified by nickel (Ni2+) affinity chromatography column (Thermo Fisher Scientific, Mumbai) and confirmed by the toxin investigated by Limulus Amoeboocyte Lysate (LAL) assay kit (Thermo Fisher Scientific, Mumbai). The protein concentration was estimated using a Bicinchoninic Acid (BCA) protein assay kit (Thermo Fisher Scientific, Mumbai).

2.3 Immunization of Mastomys with recombinant BmA-LT-2 protein and MPLA adjuvant:
Mastomys were divided into six groups (n=7/group) and vaccinated intramuscularly (i.m.); two groups of Mastomys with 50 µg of tBmA-LT-2 antigen and 20 µg of one of the adjuvant formulation (MPLA or Alum), one group with 50 µg of tBmA-LT-2 plus 20 µg of MPLA plus Alum, and one group with 20 µg of MPLA plus Alum. Two groups of Mastomys immunized with 20 µg of MPLA or Alum only, served as controls. Each group were administered with three same doses at every four weeks of interval followed by one booster dose and sera were collected 10 days after the last dose of immunization. Sera were separated and used to check the total IgG antibody and isotypes of IgG antibodies.

2.4 Determination of titer of antigen-specific immunoglobulin G (IgG) antibody in sera of Mastomys:
An indirect enzyme linked immunosorbent (ELISA) assay was performed to analyse the titer of antigen-specific IgG antibody as described previously (Dakshinamoorthy et al., 2013, Chauhan et al., 2017). Serum samples were diluted (1:100, 1:500, 1:1000 and 1:10000) and detected for IgG antibodies titer using HRP conjugated goat anti-mouse secondary antibody (1:10,000 diluted Thermo Fisher Scientific, Mumbai) and color development using TMB substrate (Thermo Fisher Scientific, Mumbai). Reaction was stopped by adding of 2M H2SO4 and absorbance was taken at 450 nm using a spectrophotometer (Biotek, New Delhi).

2.5 Estimation of antigen-specific IgG isotypes in the sera of Mastomys:
The levels of antigen-specific IgG1, IgG2a, IgG2b & IgG3 antibodies against tBmA-LT-2 were determined in the sera of immunized Mastomys using isotype-specific HRP labelled anti mouse IgG1 (1:1000), IgG3 (1:5000); IgG2a and IgG2b (1:15000) antibodies.

2.6 Analysis of splenocytes proliferation:
Spleens were collected two weeks after last immunization and single cells suspension (0.2x10^7 cells/well) were prepared in 100µl of complete RPMI media and incubated and stimulated with 1 µg/well of Concanavalin A. As a positive control (Con A; Sigma-Aldrich, Mumbai) or tBmA-LT-2 antigen, un-stimulated cells were served as negative control and incubated for 48 h (at 37°C with 5 % CO2). The cell proliferation was calculated after 48 h incubation using an MTS assay kit (Promega, New Delhi). Proliferation of cells expressed as stimulation index (SI) has been determined as described previously (Nakhaile et al., 2020).

2.7 Estimation of secreted levels of cytokines in culture supernatant:
For cytokine estimation, single cell suspension of splenocytes (2x10^6 cells/well) were prepared and placed in 24 wells tissue culture plates; stimulated with 2 µg/well of Con A (positive control) or 10µg/well of antigen (tBmA-LT-2) and incubated for 72 h (at 37°C in 5% CO2). The cell supernatants were collected and centrifuged at 5000 rpm for 15 min. The cytokine levels assayed (IL-4, IL-10 and IFN-γ) using ELISA kits (Invitrogen Bioservices, Mumbai) as per the manufacturer’s instructions.

2.8 Analysis of antigen-specific protective antibodies:
For the determination of the cytokote effect antibody dependent cellular cytotoxicity (ADCC) assay was performed as described previously (Veerapathran et al., 2009; Dakshinamoorthy et al., 2013). In brief, around 10-12 infective stage larvae (L3) of B. malayi were incubated with peritoneal exudates cells (PECs) collected from normal Mastomys at 2x10^7 cells/well plus 50 µl of sera samples for 48 h at 37°C with 5 % CO2 environment. The larval (L3) viability of B. malayi was determined microscopically after 48 h of incubation. Percentage cytotoxicity was expressed as the ratio of number of immobile or dead larvae (L3) to the total number of larvae used for ADCC multiplied by 100.

2.9 Study of vaccine-induced protection in Mastomys (Microscope chamber technique):
To study the vaccine-induced protection plexi-glass microcype chamber with a hole; Millipore India technique was used as described previously (Abraham et al., 1993; Anand et al., 2008). Ten days after the last dose of immunization microcpe chamber containing approximately 20 B. malayi infective L3 were implanted into the peritoneal cavity of Mastomys. After 48 h of implantation, microcpe chambers were recovered from the peritoneum of challenged Mastomys and the contents of each chamber were observed microscopically for the cell adherence and cytotoxicity. The percentage cytotoxicity was calculated as a proportion of the total number of dead larvae out of the total number of larvae collected during the microcpe chamber experiment.

2.10 Statistical analysis:
Data are presented as the Mean ± Standard Deviation (S.D.). The statistical analyses of the data by one-way ANOVA with Bonferroni correction test were performed using SPSS software (21.0 IBM, India). The significance level was defined as probability (P) value ≤ 0.05.

3. RESULTS

3.1 Mastomys immunized with rBmA-LT-2+Alum or rBmA-LT-2+Alum+MPLA developed a high titer of antigen-specific IgG antibodies:
Antigen-specific IgG antibodies titer in the sera of immunized Mastomys was determined by using indirect ELISA. Results have shown that a high titer of antigen-specific IgG antibodies have been produced in all vaccinated Mastomys compared to control groups. rBmA-LT-2 plus Alum or rBmA-LT-2+Alum+MPLA immunized group of Mastomys gave higher IgG titer compared to Mastomys vaccinated with either rBmA-LT-2+MPLA or MPLA+Alum (Fig. 1).

3.2 Analysis of antigen-specific protective antibodies:
For the determination of the cytokote effect antibody dependent cellular cytotoxicity (ADCC) assay was performed as described previously (Veerapathran et al., 2009; Dakshinamoorthy et al., 2013). In brief, around 10-12 infective stage larvae (L3) of B. malayi were incubated with peritoneal exudates cells (PECs) collected from normal Mastomys at 2x10^7 cells/well plus 50 µl of sera samples for 48 h at 37°C with 5 % CO2 environment. The larval (L3) viability of B. malayi was determined microscopically after 48 h of incubation. Percentage cytotoxicity was expressed as the ratio of number of immobile or dead larvae (L3) to the total number of larvae used for ADCC multiplied by 100.
3.2 Antigen-specific IgG isotypes were produced in the sera of immunized Mastomys:
The levels of antigen-specific IgG1, IgG2a, IgG2b and IgG3 antibodies in the sera of Mastomys immunized with *rBmALT-2* alone or in combination with MPLA adjuvant formulation were analysed using indirect ELISA. Our results showed that, Mastomys immunized *rBmALT-2* plus Alum gave significantly high level of IgG1 and IgG3 

\[(p<0.001)\] antibodies compared to other immunized and control groups (Fig. 2). Also, *rBnALT-2* immunized Mastomys showed higher level of IgG2a 

\[(p<0.001)\] compared to *rBmALT-2* plus MPLA immunized Mastomys group and control groups. Antigen specific IgG2a and IgG2b antibodies predominantly observed in Mastomys immunized with *rBnALT-2*+MPLA or *rBmALT-2*+Alum+MPLA followed with increased level of IgG1 and IgG3 antibodies compared to control groups (Fig. 2).

3.3 Sera of immunized Mastomys showed significant cytotoxicity by ADCC assay:
Several preceding studies have shown that antigen-specific antibodies can kill filarial parasites through an ADCC-mediated mechanism (Veerapathan et al., 2009; Veerapathan et al., 2013; Immanuel et al., 2017) Our ADCC study results showed that all immunized Mastomys sera showed significant cytotoxicity 

\[(p<0.05)\] compared to control groups. *rBmALT-2* plasmid immunized Mastomys induced significant death of L3 larvae (80.90%; \(p<0.05\)) compared to *rBmALT-2* plus MPLA immunized Mastomys sera (60.98%; \(p<0.05\)). Whereas, *rBALT-2*+Alum+MPLA immunized Mastomys sera showed (66.81%; \(p<0.05\)) compared to control groups (Table 1).

3.4 In *vivo* micropore chamber assay showed significant protection against filarial parasites in immunized Mastomys:
In *in vivo* challenged study, the microscopic observation of micropore chamber implanted in the peritoneum of Mastomys showed migration of host macrophages and polymorph nuclear cells into the chambers. In immunized Mastomys cells adhered to infective L3 leading to killing/inactivation of larvae. Almost all dead L3 had cells adhered to their surfaces. In contrast, cells in micropore chambers of control group did not adhere to larvae (Fig 3 a & b). The study showed significant protection 

\[(p<0.05)\] against challenged infection in immunized Mastomys compared to control groups. Mastomys immunized with *rBmALT-2* plus Alum challenged with *B. malayi* parasites gave highest protection (79.79%; \(p<0.05\)) compared to *rBmALT-2*+Alum+MPLA or *rBALT-2*+Alum+MPLA (Table 1)

### Table 1: The number of larvae recovered from different sets of experiments

<table>
<thead>
<tr>
<th>Vaccination groups</th>
<th>% cytotoxicity (Mean ± SD)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>In vitro ADCC assay (%)</td>
</tr>
<tr>
<td>Alum-control</td>
<td>11.11±0.00</td>
</tr>
<tr>
<td>MPLA-control</td>
<td>25.63±5.14</td>
</tr>
<tr>
<td>MPLA+Alum</td>
<td>31.66±2.35a</td>
</tr>
<tr>
<td><em>rBmALT-2</em>+Alum+MPLA</td>
<td>66.81±4.49ab,c</td>
</tr>
<tr>
<td><em>rBmALT-2</em>+MPLA</td>
<td>60.98±3.74ab,c</td>
</tr>
<tr>
<td><em>rBmALT-2</em>+Alum</td>
<td>80.90±1.28ab,c</td>
</tr>
</tbody>
</table>

\[\text{In *vivo* cytotoxicity assay against *B. malayi* L3 larvae in *vivo*}

\[\text{using indirect ELISA. Our results showed that, Mastomys immunized}

\[\text{with *rBmALT-2*+Alum, *rBmALT-2*+MPLA, *rBmALT-2*+Alum+MPLA,}

\[\text{MPLA+Alum and control (Alum and MPLA). Each data point}

\[\text{was analysed by Kruskal–Wallis test.}

\[\text{Statistically significant (p<0.001) compared to control (Alum and}

\[\text{MPLA) and MPLA+Alum group}

\[\text{Statistically significant (p<0.001) compared to *rBmALT-2*+MPLA}

\[\text{and *rBmALT-2*+Alum+MPLA group}

\[\text{Data were analysed by Kruskal–Wallis test followed by Bonferroni}

\[\text{correction test.}

\[\text{Figure 3: Photomicrograph of *B.malayi* infective L3 larvae}

\[\text{recovered after 48 h from micropore chamber assay (a) no cells}

\[\text{adhered to the larvae and the larvae were active collected from}

\[\text{control group of Mastomys (b) Several cells were found attached to}

\[\text{the dead *B.malayi* L3 larvae in the vaccinated Mastomys (c) high}

\[\text{resolution of figure (b) showing cells attached to the dead larvae.}

\[\text{The clusters of cells adhered throughout the surface of the larva}

\[\text{causing death of the larva.}

\[\text{3.5 Spleen cells from vaccinated animals showed an antigen-}

\[\text{specific recall response:}

\[\text{Spleen cells from vaccinated animals were expressed as proliferative}

\[\text{stimulation index (SI) significantly (p<0.05) in response to the antigen}

\[\text{stimulation compared to the Alum control groups. Spleen cells from}

\[\text{Mastomys vaccinated with *rBmALT-2*+Alum or MPLA+Alum group}

\[\text{stimulated with Con A (mitogen) showed significant stimulation}

\[\text{index (SI) significantly (p<0.05) compared to the Alum control groups. Spleen cells from}

\[\text{vaccinated animals were expressed as proliferative stimulation index (SI) significantly (p<0.05) in response to the antigen stimulation compared to the Alum control groups. Spleen cells from Mastomys vaccinated with *rBmALT-2*+Alum or MPLA+Alum group stimulated with Con A (mitogen) showed significant stimulation indices (S.I =3.70±0.62; 3.99 ±1.32) confirming the proliferation of T cells. When, *rBmALT-2*+Alum+MPLA groups were pulsed with *rBmALT-2* antigen, showed high proliferation (S.I =3.36±0.44; 5.99±2.02). Whereas, Mastomys immunized with *rBALT-2*+Alum+MPLA or *rBALT-2*+MPLA groups showed decrease in proliferative response.}

\[\text{International Journal of Scientific Research
3.6 Splenocytes of vaccinated Mastomys predominantly secreted both Th1 and Th2 cytokines:

The secreted levels of cytokines in culture supernatants of rBmALT-2 plus Alum vaccinated Mastomys secreted significantly high level of IFN-γ and IL-4 (p<0.05) with much decreased level of IL-10 compared to other vaccinated and control groups (Table 2). Whereas, rBmALT-2+MPLA or rBmALT-2+Alum+mMPLA immunized Mastomys secreted very high level of IL-10 cytokine (p<0.05) compared to rBmALT-2+Alum and control groups (Table 2). The IL-10 levels of MPLA control group were higher than rBmALT-2+Alum immunized group. While IFN-γ and IL-4 levels were found to be higher in MPLA+Alum immunized group compared to control groups.

Table 2: Cytokine levels in culture supernatants of spleen cells from immunized and control group of Mastomys

<table>
<thead>
<tr>
<th>Groups</th>
<th>IFN-γ (pg/ml)*</th>
<th>IL-4 (pg/ml)*</th>
<th>IL-10 (pg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>rBmALT-2+Alum</td>
<td>1512.07±469.56</td>
<td>1507.16±65.56</td>
<td>508.57±101.01</td>
</tr>
<tr>
<td>rBmALT-2+MPLA</td>
<td>736.97±184.87</td>
<td>955.64±36.73</td>
<td>2076.19±170.01</td>
</tr>
<tr>
<td>rBmALT-2+Alum+mMPLA</td>
<td>672.90±214.88</td>
<td>979.35±42.83</td>
<td>1932.52±35.51</td>
</tr>
<tr>
<td>MPLA+Alum</td>
<td>661.36±146.16</td>
<td>964.01±62.58</td>
<td>555.65±110.22</td>
</tr>
<tr>
<td>MPLA-control</td>
<td>182.97±144.52</td>
<td>535.02±168.25</td>
<td>657.37±194.77</td>
</tr>
<tr>
<td>Alum-control</td>
<td>276.56±47.52</td>
<td>555.52±73.92</td>
<td>599.73±57.84</td>
</tr>
</tbody>
</table>

*Each data represents the Mean ± SD value of cytokine levels (pg/ml) in culture supernatants of spleen cells, from immunized and control group of Mastomys

1a-statistically significant (p<0.05) compared to MPLA+Alum and control (MPLA or Alum) groups

1c-statistically significant (p<0.001) compared to rBmALT-2+MPLA and rBmALT-2+Alum+mMPLA groups.

*ns-not significant. Data were analyzed by Kruskal–Wallis test followed by Bonferroni correction test.

4. DISCUSSION

Lymphatic filariasis is a worldwide leading cause of perpetual and long-term disability. In the year 2000, WHO has started programme for the elimination of LF using MDA approach. Though the prevalence of infection has reduced using MDA, re-emergence has been highlighted in several countries (Nujum et al., 2012; Krentel et al., 2013; Sunish et al., 2014; Bhattacharjee 2016; Dyson et al., 2017). Several factors hampered the effectiveness of the MDA-based elimination strategy: 1) non-compliance of human subject (Nujum et al., 2012; Krentel et al., 2013) ii) Diethylcarbamazine (DEC) and albendazole have little impact on the adult parasites living within the lymphatic vessels that persist to cause lymphatic damage and pathology (Crittchley et al., 2005; Bennuru and Nutman, 2009; Babayan et al., 2012). Hence, additional safe solutions such as an effective prophylactic vaccine are required to complement the MDA approach. A prophylactic vaccination may protect from the infection and can improve immunity through natural exposure to endemic areas. Several research have been working to developed a potential vaccine against LF (Gnanasekar et al., 2004; Thirugnanam et al., 2007; Veerapathran et al., 2009; Samykutty et al., 2010; Kalyansundaram and Balunmi, 2011; Dakshinamoorthy & Kalyansundaram 2013, Immennel et al., 2017). Breggia malayi ALT-2, a novel antigen in Fjirs has been proved to clear filarial parasites (Gnanasekar et al., 2004). Monophosphory Lipid A (MPLA), an lipopolysaccharide derivative that is known to induce cellular response, was used in this study as an adjuvant in the improvement of the prophylactic efficacy of this antigen.

In this study we used aqueous MPLA formulations with or without Alum in order to determine their ability to boost vaccine-induces safety in addition to improving the prophylactic efficacy of vaccine antigen tBmALT-2. Alum is the most popular vaccine adjuvant and is used in almost 80% of all vaccines (Gupta 1998; Brewer 2006; Marrack et al., 2009). When combined with other adjuvants, alum has a synergistic effect and can enhance the adjuvant properties of liposomes, QS21, MPLA and Cpg (Gupta et al., 1995). MPLA as adjuvant which is known to stimulate mostly Th1 type of immune response (Casella and Mitchell 2008) was used either with Alum or alone. Our results demonstrated that, when tBmALT-2 antigen has been used with MPLA as an adjuvant with or without Alum, generate high antibody response. Mastomys vaccinated with rBmALT-2+Alum+MPLA generated antigen-specific IgG1, IgG2a and IgG2b antibodies suggesting Th1 and Th2 responses our results correlates with previous studies (Sharmila et al. 2011). Whereas, tBmALT-2+MPLA and rBmALT-2+Alum+mMPLA vaccinated Mastomys predominantly generated IgG2a and IgG2b antibodies, this suggests Th1 immune response. The antigen-specific antibodies findings were further confirmed by the study of cytokine responses of spleen cell proliferation assays. The splenocyte proliferation assays demonstrated reduced stimulation index in tBmALT-2+MPLA and tBmALT-2+Alum+MPLA vaccinated group when compared to tBmALT-2+Alum and control groups, which could be associated to various in vivo factors our results in harmony with Apampa and Kaliraj 2014. The spleen cells from Mastomys vaccinated with tBmALT-2+Alum secreted elevated levels of IL-4 and IFN-γ cytokines suggesting the balanced Th1/Th2 response and correlate with our humoral immune response study. Interestingly, higher level of IL-10 in tBmALT-2+MPLA and tBmALT-2+Alum+mMPLA immunized group, compared to tBmALT-2+Alum and control group propose that BmALT-2 may be responsible for the down regulation of IFN-γ, possibly due to the immunomodulatory effect of BmALT-2 (Kurniawan et al. 1992; Raviachandran et al., 1993; Aparnaa and Kaliraj 2007). Several studies reported that the up-regulation of IL-10 resulted possibly due to the immunomodulatory effect of BmALT-2+Alum (Poncini et al., 2008; Silva et al., 1992; Murphy et al., 2001; Bhattacharyya et al., 2001; Murray et al., 2003). IL-10 also impairs the activation of conventional dendritic cells (DCs), impeding the up-regulation of co-stimulatory molecules (Murray et al., 2003; Perona-Wright et al., 2009; Poncini et al., 2008; Ronet et al., 2010; Owens et al., 2012). IFN-γ is the signature cytokine of Th-1 cells and IL-4 cytokine is of Th-2 cells, were measured in the MPLA+Alum vaccinated group, since MPLA know to promote Th-1 responses (Casella and Mitchell 2008) was used either with Alum or alone. Our results in harmony with Aparnaa and Kaliraj 2007). Several studies have shown that the in vitro antibody-dependent cell mediated cytotoxicity (ADCC) is one of the important immunological mechanisms that functions of parasite killing in tBmALT-2 vaccinated animals (Gnanasekar et al., 2004; Thirugnanam et al., 2007; Veerapathran et al., 2007; Zveirapathran et al., 2007). Thus, both cells and antibodies are necessary for the killing of infective L3 larvae. Our results showed that, the sera of Mastomys immunized with 50µg of tBmALT-2+Alum showed significantly high cytotoxicity (80% p<0.001) compared to other immunized group. This is maybe the only recombinant filarial antigen that can provide this high protection rate (Gregory et al., 2000). The mechanism of this high protection is unknown, but these proteins may play an important role in host immunomodulation (Maizels et al., 2013).

Ethical Approval:

Further, we analyzed in vitro ADCC assay result by using in vivo micropore chamber technique. Similar to the in vitro ADCC assay, present study using in vivo micropore chamber approach confirming our previous observations that tBmA LT-2+MPLA immunized Masts provides highest protection (79.79%; p<0.001) compared to tBmA LT-2+MPLA and tBmA LT-2+Alum+MPLA showed 59.84% and 65.90% cytotoxicity, respectively. In vivo technique provides a confined physiological environment, can be used to study the growth and survival of parasites and also to assess the host effector mechanism. (Kalyanasundaram and alumn 2011; Dakshinamoorthy et al., 2013; Nakhale et al., 2020). In rodent model, in vivo protective responses after vaccination restricted the migration of L3 into subcutaneous spaces where they were trapped and killed by eosinophil-rich granulomas (Sim et al., 1982; Babu et al., 2005).

Conflict of Interest: There is no conflict of interest.

Ethical Approval: All authors hereby declare that “Principles of laboratory animal care” (NHP publication No. 15-8013, revised 2015) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the Institutional Animal Ethics Committee (IAEC).

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