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

Biochemistry

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ABSTRACT

Brugia malayi Abundant larval transcript-2 (*Bm*ALT-2) is most potential prophylactic vaccine candidate. In order to increase the protection efficacy of *Bm*ALT-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 r*Bm*ALT-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* micropore 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 r*Bm*ALT-2+MPLA and r*Bm*ALT-2+Alum+MPLA immunized group, whereas, r*Bm*ALT-2+Alum immunized group generated IgG1, IgG2a and IgG3 antibodies. r*Bm*ALT-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 r*Bm*ALT-2+MPLA or r*Bm*ALT-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 r*Bm*ALT-2+Alum group *in vitro* (80%; $p<0.001$) and *in vivo* (79.79%; $p<0.001$) compared to r*Bm*ALT-2+MPLA and r*Bm*ALT-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 Filariasis (LF) is one of the neglected tropical disorders that impair the lymphatic system causing lymphoedema and hydrocele. LF caused by microscopic 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 has acknowledged sixteen countries and territory (Cambodia, The Cook Islands, Egypt, Kiribati, Maldives, Marshall Islands, Niue, Palau, Sri Lanka, Thailand, Togo, Tonga, Vanuatu, Viet Nam, Wallis and Futuna, and Yemen) for eliminating of LF as a public health problem (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 (Thirugnanam *et al*., 2007; Veerapathran *et al*., 2009; Samykutty *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 (*Bm*ALT-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 (Porthouse *et al*. 2006). BmALT-2, conferred high protection in Jirds and Mastomys when immunized along 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 (aluminium 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 (Bomford 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 *Bm*ALT-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 homogeneous clear suspension was obtained (Baldridge and Crane 1999). The *Escherichia coli* (*E. coli*) strain BL21-DE3 (pLysS) used for the expression of *Bm*alt-2 gene as described earlier (Veerapathran *et al*., 2009). His-tag recombinant *Bm*ALT-2 protein was purified by nickel (Ni^{2+}) affinity chromatography column (Thermo Fisher Scientific, Mumbai) and levels of endotoxin investigated by Limulus Amebocyte 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 *Bm*ALT-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 r*Bm*ALT-2 antigen and 20 µg of one of the adjuvant formulation (MPLA or Alum), one group with 50 µg of r*Bm*ALT-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 colour developed using TMB substrate (Thermo Fisher Scientific, Mumbai). Reaction was stopped by adding of 2M H_2SO_4 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 r*Bm*ALT-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 (0.2x10^6 cells/well) were prepared in 100µl of complete RPMI media and stimulated with 1 µg/well of Concanavalin A as positive control (Con A; Sigma-Aldrich, Mumbai) or r*Bm*ALT-2 antigen, un-stimulated cells were served as negative control and incubated for 48 h (at 37°C with 5 % CO_2). 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 (Nakhale *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/ml/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 (r*Bm*ALT-2) and incubated for 72 h (at 37°C in 5% CO_2). The culture 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 cytotoxic 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 2x10^5 cells/well plus 50 µl of sera samples for 48 h at 37°C with 5 % CO_2 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 (Micropore chamber technique):

To study the vaccine-induced protection plexi-glass micropore chamber (diffusion 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 micropore chamber containing approximately 20 *B. malayi* infective L3 were implanted into the peritoneal cavity of Mastomys. After 48 h of implantation, micropore 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 micropore 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 $\leq$ 0.05**.**

3. RESULTS

3.1 Mastomys immunized with rBmALT-2+Alum or rBmALT-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. r*Bm*ALT-2 plus Alum or r*Bm*ALT-2+Alum+MPLA immunized group of Mastomys gave higher IgG titer compared to Mastomys vaccinated with either r*Bm*ALT-2+MPLA or MPLA+Alum (Fig. 1).

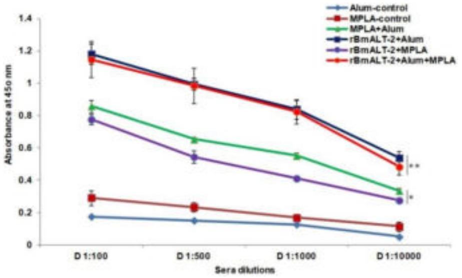

Figure 1: Antibody titer was determined by ELISA, ten days after the last booster dose. Mastomys immunized with r*Bm*ALT-2+Alum, r*Bm*ALT-2+MPLA, r*Bm*ALT-2+Alum+MPLA, MPLA+Alum and control (Alum and MPLA). Each data point represents Mean±SD (n=5-7 per group). *$p<0.05$; ** $p<0.001$ analyzed by Kruskal–Wallis test.

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 r*Bm*ALT-2 alone or in combination with MPLA adjuvant formulation were analysed using indirect ELISA. Our results showed that, Mastomys immunized r*Bm*ALT-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, r*Bm*ALT-2 immunized Mastomys showed higher level of IgG2a ($p<0.001$) compared to r*Bm*ALT-2+Alum+MPLA immunized group and control groups. Antigen specific IgG2a and IgG2b antibodies predominantly observed in Mastomys immunized with r*Bm*ALT-2+MPLA or r*Bm*ALT-2+Alum+MPLA followed with increased level of IgG1 and IgG3 antibodies compared to control groups (Fig. 2).

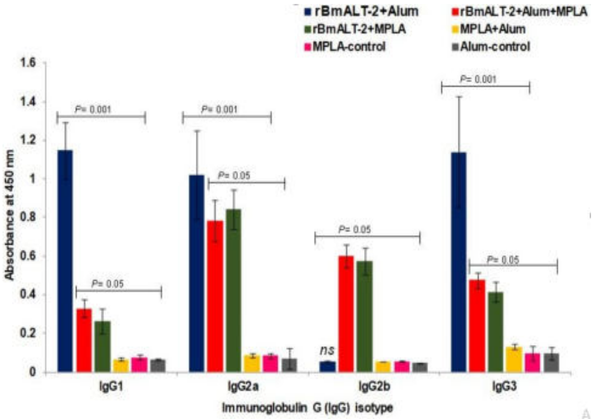

Figure 2: Levels of antigen specific IgG isotypes in the sera of mastomys were measured using an ELISA. Mastomys immunized with r*Bm*ALT-2+Alum, r*Bm*ALT-2+MPLA, r*Bm*ALT-2+Alum+MPLA, MPLA+Alum and control (Alum and MPLA). Each data bar represents Mean ± SD (n=5-7 per group). *$p<0.05$; ** $p<0.001$ analyzed by Kruskal–Wallis test followed by Bonferroni correction test.

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 (Veerapathran *et al*., 2009; Veerapathran *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. r*Bm*ALT-2 plus Alum immunized Mastomys induced significant death of L3 larvae (80.90%; $p< 0.05$) compared to r*Bm*ALT-2 plus MPLA immunized Mastomys sera (60.98%). Whereas, r*Bm*ALT-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 r*Bm*ALT-2 plus Alum challenged with *B. malayi* parasites gave highest protection (79.79%; $p< 0.05$) compared to r*Bm*ALT-2+Alum+MPLA or r*Bm*ALT-2+MPLA (Table 1)

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

Vaccination groups	% cytotoxicity (Mean ± SD)	
	In vitro ADCC assay (%)⁋	In vivo micropore assay (%)⁋⁋
Alum-control	11.11±0.00	11.56±5.53
MPLA-control	23.63±5.14	13.79±3.14
MPLA+Alum	31.66±2.35a	28.44±6.50 a,b
rBmALT-2 +Alum+ MPLA	66.81±4.49 a,b,c	65.96±2.69 a,b,c
rBmALT-2 +MPLA	60.98±3.74 a,b,c	59.84±3.13 a,b,c
rBmALT-2 +Alum	80.90±1.28 a,b,c,e	79.79±4.64 a,b,c,d,e

⁋In *in vitro* antibody dependent cellular cytotoxicity induced against *B. malayi* L3 peritoneal exudate cells (PECs) were incubated with the pooled sera from the Mastomys immunized with r*Bm*ALT-2+Alum, r*Bm*ALT-2+MPLA, r*Bm*ALT-2+Alum+MPLA, MPLA+Alum and control (Alum and MPLA) and L3 larvae *in vitro*. After 48 h, the total live and dead worms were counted. Values represent Mean±SD of two wells (n=5-7 per group).

⁋⁋*In vivo* cytotoxicity assay against *B. malayi* L3 Mastomys immunized with r*Bm*ALT-2+Alum, r*Bm*ALT-2+MPLA, r*Bm*ALT-2+Alum+MPLA, MPLA+Alum and control (Alum and MPLA) were challenged intraperitoneally with *B. malayi* L3s. After 48 h, live L3 were recovered from the micropore chambers implanted into the peritoneal cavity of Mastomys, the total live and dead worms were counted. Values represent the Mean % cytotoxicity and standard error (n=5-7 per group).

[a,b,c] Statistically significant ($p<0.05$) compared to control (Alum and MPLA) and MPLA+Alum group

[d,e] Statistically significant ($p<0.001$) compared to r*Bm*ALT-2+MPLA and r*Bm*ALT-2+Alum+MPLA group

Data were analysed by Kruskal–Wallis test followed by Bonferroni correction test.

Live *B. malayi* L3 larvae

Dead *B. malayi* L3 larvae

Dead *B. malayi* L3 larvae with cells attached

Figure 3: Photomicrograph of *B.malayi* infective L3 larvae recovered after 48 h from micropore chamber assay (a) no cells adhered to the larvae and the larvae were active collected from control group of Mastomys (b) Several cells were found attached to the dead *B.malayi* L3 larvae in the vaccinated Mastomys (c) high resolution of figure (b) showing cells attached to the dead larvae. The clusters of cells adhered throughout the surface of the larva causing death of the larvae.

3.5 Spleen cells from vaccinated animals showed an antigen-specific recall response:

Spleen cells from 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 r*Bm*ALT-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, r*Bm*ALT-2+Alum and MPLA+Alum groups were pulsed with r*Bm*ALT-2 antigen, showed high proliferation (S.I =3.36±0.44; 5.99±2.02). Whereas, Mastomys immunized with r*Bm*ALT-2+Alum+MPLA or r*Bm*ALT-2+MPLA groups showed decrease in proliferation (Fig. 4).

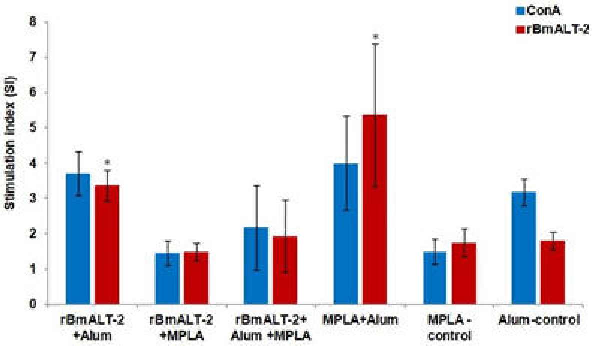

Figure 4: Splenocytes proliferation assay: Splenocytes from Mastomys with r*Bm*ALT-2+Alum, r*Bm*ALT-2+MPLA, r*Bm*ALT-2+Alum+MPLA, MPLA+Alum and control (Alum and MPLA) were cultured *in vitro* and re-stimulated with either ConA or with r*Bm*ALT-2 and the effect on the splenocytes proliferation was checked by MTS assay after 48 h. The data presented is a mean stimulation index (S.I.) ± SD (n=5-7 per group). *$p<0.05$ compared to Control-alum group as analyzed by Kruskal–Wallis test.

3.6 Splenocytes of vaccinated Mastomys predominantly secreted both Th1 and Th2 cytokines:

The secreted levels of cytokines in culture supernatants of r*Bm*ALT-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, r*Bm*ALT+MPLA or r*Bm*ALT+Alum+MPLA immunized Mastomys secreted very high level of IL-10 cytokine ($p<0.05$) compared to r*Bm*ALT-2+Alum and control groups (Table 2). The IL-10 levels of MPLA control group were higher than r*Bm*ALT-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

Groups	IFN-γ (pg/ml)*	IL-4 (pg/ml)*	IL-10 (pg/ml)*
rBmALT-2+Alum	1512.07±469.56 ^{a,b,c,d,e}	1507.16±65.56 ^{a,b,c,d,e}	508.57±101.01^{ns}
rBmALT-2+Alum +MPLA	736.97±184.87 ^{a,b}	955.64±36.73 ^{a,b}	2076.19±170.01 ^{a,b,c}
rBmALT-2+MPLA	672.90±214.88 ^{a,b}	979.35±42.83 ^{a,b}	1932.52±35.51 ^{a,b,c}
MPLA+Alum	661.36±146.16 ^{a,b}	964.01±62.58 ^{a,b}	555.65±110.22 ^{ns}
MPLA-control	182.97±144.52	535.02±168.25	675.37±194.77
Alum-control	276.56±47.52	555.52±73.92	599.73±57. 84

*Each data represents the Mean ± SD value of cytokine levels (pg/ml) in culture supernatants of spleen cells, from mastomys immunized with the r*Bm*ALT-2+Alum, r*Bm*ALT-2+MPLA, r*Bm*ALT-2+Alum+MPLA and stimulated *in vitro* with r*Bm*ALT-2 antigens.

^{a,b,c}Statistically significant ($p<0.05$) compared to MPLA+Alum and control (MPLA or Alum) groups

^{d,e}Statistically significant ($p<0.001$) compared to r*Bm*ALT-2+MPLA and r*Bm*ALT-2+Alum+MPLA 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: i) 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 (Critchley *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 an potential vaccine against LF (Gnanasekar *et al*., 2004; Thirugnanam *et al*., 2007; Veerapathran *et al*., 2009; Samykutty *et al*., 2010; Kalyanasundaram and Balumuri 2011; Dakshinamoorthy & Kalyanasundaram 2013, Immanuel *et al*., 2017). *Brugia malayi* ALT-2, a novel antigen in Jirds 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 r*Bm*ALT-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, QS-21, 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 r*Bm*ALT-2 antigen has been used with MPLA as an adjuvant with or without Alum, generate high antibody response. Mastomys vaccinated with r*Bm*ALT-2+Alum generated antigen-specific IgG1, IgG2a and IgG3 antibodies suggesting Th1 and Th2 responses our results correlates with previous studies (Sharmila *et al*. 2011). Whereas, r*Bm*ALT-2+MPLA and r*Bm*ALT-2+Alum+MPLA 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 cells. The splenocyte proliferation assay demonstrated reduced stimulation index in r*Bm*ALT-2+MPLA and r*Bm*ALT-2+Alum+MPLA vaccinated group when compared to r*Bm*ALT-2+Alum and control groups, which could be associated to various *in vivo* factors our results in harmony with Aparnaa and Kaliraj 2014. The spleen cells from Mastomys vaccinated with r*Bm*ALT-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 r*Bm*ALT-2+MPLA and r*Bm*ALT-2+Alum+MPLA immunized group, compared to r*Bm*ALT-2+Alum and control group propose that *Bm*ALT-2 may be responsible for the down regulation of IFN-γ, possibly due to the immunomodulatory effect of *Bm*ALT-2 (Kurniawan *et al*. 1993; Ravichandran *et al*. 1997; Aparana and Kaliraj 2014). Several studies reported that the up-regulation of IL-10 resulted in downregulation of IL-12 and IFN-γ cytokines and macrophage activation eventually reducing both parasite killing and toxicity to the local environment (Bogdan *et al*., 1991; Oswald *et al*., 1992; 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) and enhancing cell-mediated immunity (Tomai and Johnson 1989) whereas, Alum predominately uphold a Th-2 type antibody-response (Glenny *et al*., 1926; Brewer 2006; McKee *et al*., 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 r*Bm*ALT-2 vaccinated animals (Gnanasekar *et al*., 2004; Thirugnanam *et al*., 2007; Veerapathran *et al*., 2009; Dakshinamoorthy *et al*., 2013). 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 r*Bm*ALT-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*., 2001). Whereas, Mastomys immunized with r*Bm*ALT-2+MPLA and r*Bm*ALT-2+Alum+MPLA showed 61% and 67% cytotoxicity, respectively. The diminished levels of cytotoxicity in these groups may be attributed due to up-regulation of IL-10 cytokine. Several studies showed that, IL-10 attenuated the macrophage effector function by inhibiting its nitric oxide production, which appear to be critical for the killing of both intra and extra cellular parasites such as *Toxoplasma gondii*, *Trypanosoma cruzi*, *Leishmania donovani* (Bogdan *et al*., 1991; Kane and Mosser 2001; Oswald *et al*., 1992) and extracelluar larval stage of *Schistosoma mansoni* (Silva *et al*., 1992; Wilson *et al*., 2011). Both antibody- and complement-mediated effector mechanisms have been shown to be involved in inducing cytotoxicity to the microfilaria and infective L3 larvae *in vitro* (Sim *et al*., 1982). The microscopic observation of dead larvae showed numerous cells adhered to their surface (Gnanasekar *et al*., 2004; Dakshinamoorthy *et al*., 2013). In this study, we did not examine the larva-bound population of cells; however, Khatri *et al*., (2018) shows that the bound cells are mainly macrophages and secreted myeloperoxidase (MPO) as activation products. Larvae incubated in serum samples from control animals had no cells attached to them.

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 r*Bm*ALT-2+Alum immunized Mastomys provide highest protection (79.79%; $p<0.001$) compared to r*Bm*ALT-2+MPLA and r*Bm*ALT-2+Alum+MPLA showed 59.84% and 65.96% 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 Balumuri 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; Babayan *et al*., 2005).

5. CONCLUSIONS

Conclusively, the *in vitro* and *in vivo* experiments results showed that Mastomys immunized with r*Bm*ALT-2+Alum as adjuvant have better protective efficacy when compared with Mastomys immunized with r*Bm*ALT-2+MPLA or r*Bm*ALT-2+Alum+MPLA. The vaccine candidate r*Bm*ALT-2 when administered with MPLA showed significantly increased level of IL-10 cytokine in both r*Bm*ALT-2+MPLA or r*Bm*ALT-2+Alum+MPLA immunized Mastomys groups reflecting the inhibitory effect of IFN-γ and IL-4 cytokines. Whereas, r*Bm*ALT-2+Alum immunized group showed elevated level of IFN-γ and IL-4 cytokines and diminutive levels of IL-10. This study proved again the immunomodulatory role of ALT-2 in combination with MPLA as adjuvant formulation as an attempt to enhance the cellular immune response. However, the use of MPLA as adjuvant increase level of IL-10 cytokine and decrease of IFN-γ and IL-4 secretion, suggesting a inhibitory effect on protective immune responses as reflected in in vivo protective immune response experiment, showing almost 20% decrease in protection. In order to identify the specific TLR along with co-stimulatory signals more insight is required, which might play an essential role in the hyporesponsiveness.

Conflict of Interest: There is no conflict of interest.

Ethical Approval: All authors hereby declare that "Principles of laboratory animal care" (NIH 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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