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Immunological evaluation of fusion protein of *Brugia malayi* abundant larval protein transcript-2 (BmALT-2) and Tuftsin in experimental mice model

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ABSTRACT

Introduction: Filariasis, a neglected tropical helminth disease needs vaccine besides mass drug administration for its successful eradication.

Methods: An attempt was made to produce a fusion protein (P-TUFT-ALT-2) of abundant larval transcript protein-2 and Tuftsin to enhance its immunogenicity. The fusion construct was expressed in *Pichia pastoris*, a nonexpensive commercial expression system. This study focused on the evaluation of immunological response produced by P-TUFT-ALT-2 in Balb/c mice.

Result and discussion: P-TUFT-ALT-2 showed an enhanced IgG peak titre compared to *E. coli* expressed E-ALT-2 and *P. pastoris* expressed P-ALT-2. IgG2b, IgG2a and IgG1 production were predominant indicating a balanced Th1/Th2 response. P-TUFT-ALT-2 also induced about 28% and 9.5% higher splenocyte proliferation over control and E-ALT-2 respectively. Splenocytes produced predominant IFN-γ followed by IL-5, IL-2 and IL-10 specifying a balanced Th1/Th2 response. P-TUFT-ALT-2 showed 55% to 80% with an average of 65% cytotoxicity in *B. malayi* L3 larvae in *in vitro* ADCC assay.

Conclusion: This experiment validates P-TUFT-ALT-2 as a potential vaccine candidate for human lymphatic filariasis.

1. Introduction

Lymphatic filariasis or famously called 'elephantiasis' is a neglected tropical parasitic disease characterized by enlargement of limbs of the body. It is caused by mosquito-borne *Wuchereria bancrofti* and *Brugia malayi*. In 2000, World Health Organization introduced the 'Global Program to Eliminate Lymphatic Filariasis' (GPELF) with mass drug administration (MDA) using albendazole, diethylcarbamazine and ivermectin in single or two-doses (Dreyer et al., 1996; Emilio, 2008; Gyapong et al., 2005; Jayakody et al., 1993; Molyneux and Taylor, 2001; Noroes et al., 1997; World Health Organization, 2000). These drugs have a microfilaricidal effect only, without any impact on adult worms even after prolonged treatment. They also pose various physical problems due to

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their undesirable side effects. Thus, there is a need to develop suitable prophylactic agents, such as vaccines, for the successful elimination of human lymphatic filariasis by multiple elimination strategies (Grieve et al., 1995; Price and Kieny, 2001; Selkirk et al., 1992; Vercruysse et al., 2007). Although vaccines against multi-cellular organisms such as *Plasmodium chabaudi* and *P. falcifarum* (Burns et al., 2003; Shi et al., 1999) have been developed, no suitable vaccine currently exists for filarial parasites. Earlier studies proposed BmALT-2 of *B. malayi* as a promising vaccine candidate with 74–76% protection in animal models (Anand et al., 2008; Gomez-Escobar et al., 2005; Gregory et al., 2000; Madhumathi et al., 2016; Murray et al., 2001; Ramachandran et al., 2004; Thirugnanam et al., 2007). BmALT-2 is the most abundant of the L3-expressed stage-specific novel proteins according to various research reports (Ben-Wen et al., 2012; Gomez-Escobar et al., 2005). The cDNA sequence encoding this protein is available in the GenBank database under accession number U84723 (Gregory et al., 2000).

Early activation of phagocytosis induced by many stimulating factors can prevent bacterial and fungal infections. Tuftsin enhances immunogenicity of an antigenic protein by targeting it to macrophages and dendritic cells to produce stronger humoral and cellular immune response. Many researchers have already used Tuftsin with various antigens in vaccine development for malaria, leprosy, HIV *etc.* (Gokulan et al., 1999; Khare et al., 1992; Kumar et al., 1995). The phagocytotic activity of macrophages was also shown to be drastically enhanced by Tuftsin-based fusion proteins, which suppressed the growth of human epidermoid carcinoma (Liu et al., 2014a).

The methylotrophic yeast, *Pichia pastoris* has been developed as a commercially important host for the production of heterologous proteins (Buckholz and Gleeson, 1991; Cereghino et al., 2001; Cregg et al., 2000). A large number of proteins like Cattle Tick vaccine (Canales et al., 1997), endo-β-1,4-mannase (Vu et al., 2012), hepatitis B surface antigen (Bo et al., 2005; Cregg et al., 1987) have been successfully produced in *P. pastoris* expression system. So far, most of the filarial proteins have been expressed in *E. coli* system. VAH, ALT-2 (P-ALT-2) and ALT-2 fused with Tuftsin (P-TUFT-ALT-2) were expressed in *P. pastoris* earlier in our lab (Paul et al., 2017; Paul et al., 2018a; Prince, 2010). Moreover, *P. pastoris* expressed P-TUFT-ALT-2 showed higher PBMC proliferation with elevated cytokines and immune-dominant transcription factors and better reactivity with human clinical sera sample of natural infection (Paul et al., 2018a).

Generally, mice are used as semi-permissive model for protection studies against infectious organisms. The jirds or *Meriones unguiculatus* and *Mastomys coucha* have been established as permissive host for filarial research (Lok and Abraham, 1992). Hence, the Balb/c mice were used for the evaluation of the prophylactic efficacy of the vaccine constructs. Mice model was the most convenient due to their easy availability and solid immunological characterization. In the present study, we evaluated the *E. coli* expressed E-ALT-2 along with *P. pastoris* expressed P-ALT-2 and P-TUFT-ALT-2 in Balb/c mice.

2. Materials and methods

2.1. Mice immunization ad sera collection

The animal used in this study was approved by the Institutional Animal Ethical Committee (IAEC) of Anna University approved (CBT/AU/IAEC/2013/03) under the guidelines of committee for the purpose of control and supervision on experiments on animals (CPCSEA), Chennai, India. Two months-old BALB/c (H-2d) mice were procured from T. Sivamani Lab Animal Breeders, Chennai and housed under standard laboratory conditions with food and water in the animal house facility at Centre for Biotechnology, Anna University, Chennai. Animals were split into four groups with six animals in each group. E-ALT-2, P-ALT-2 and P-TUFT-ALT-2 fusion protein were obtained from our lab (Paul et al., 2018b). One group was immunized intraperitoneally with 30 μg of P-TUFT-ALT-2 per animal in 100 μL of 0.5 M PBS along with equal volume of alum (1.3%) as adjuvant. Other two groups were immunized with 30 μg of E-ALT-2 and P-ALT-2 respectively per animal in similar manner. The control group was injected with alum only. Each group was vaccinated at day 0, and one booster dose of each antigen was given on day 14. Blood was collected from the vein tail at 0th, 14th, 21st, 28th, 35th and 42nd day. Pooled sera was collected from immunized and control animals' blood and stored at −20 °C for further study.

2.2. Analysis of antibody titre and isotypes

Antibody titre against E-ALT-2, P-ALT-2 and P-TUFT-ALT-2 was tested by indirect ELISA. The 96-well microtitre plates (Nunc, Denmark) were coated with 100 μL of E-ALT-2, P-ALT-2 and P-TUFT-ALT-2 (100 ng/well) prepared in 0.1 M bicarbonate buffer, pH 9.6, separately and incubated overnight at 4 °C followed by blocking with 5% skimmed milk. The plates were then washed with PBST. Subsequently, they were incubated with 100 μL of pooled sera in a serial two-fold dilution (1:500–128,000) for 1 h 30 min at 37 °C. Similar dilutions were prepared for pre-immuned and alum immunized pooled sera. After three washes with phosphate buffered saline with 0.5% tween-20 (PBST), the plates were incubated again with goat anti-mouse IgG ALP conjugate (1:1000) for 1 h at 37 °C. The plates were washed and colour was developed using *p*-nitrophenyl phosphate substrate (1 mg/mL) in substrate buffer (100 mM Tris–Cl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Absorbance was read at 405 nm. The mean OD ± 3SD of the pre-immune serum was taken as cut-off value for determining antibody titre. The highest dilution of the antiserum that showed an OD value above the cut-off value was taken as the antibody titre. The mice sera which showed maximum total IgG titre were diluted (1:100) from different vaccinated groups for estimating IgG isotypes. The diluted sera were subjected to incubation with ALP conjugated rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3 and IgM (Pierce Biotechnology, Rockford, IL, USA) for 1 h at 37 °C. The absorbance was read at 405 nm.

2.3. Splenocyte proliferation assay

Immunized mice were sacrificed on day 60, and the spleens were removed aseptically (Anand et al., 2008; Anand et al., 2011). Splenocytes were separated and washed twice with fresh culture medium RPMI 1640 (Gibco, Langley, OK, USA). Lysis buffer (0.1% ammonium chloride) was added to the pellet to remove the RBC's, and the cells were counted. Single-cell suspension of pooled spleen cells was prepared from both immunized and control Balb/c mice. Approximately 0.2×10^6 cells/mL suspended in RPMI 1640 supplemented with 10% heat-inactivated foetal bovine serum (FBS), gentamicin (80 mg/mL) (Ranbaxy Laboratories, India), 25 mM HEPES (USB; Amersham Pharmacia, UK) and 2 mM glutamine (USB; Amersham Pharmacia) were cultured in triplicate in 96-well plates (100 μL/well). Pooled cells cultured in triplicate wells were stimulated with protein (1 μg/well) or positive control Con A (1 μg/well) *in vitro*. The cells with medium alone were used as unstimulated controls. The cultures were incubated for a total of 72 h with Alamar blue incubation for the last 8 h and the absorbance was taken at 570 nm (Ausiello et al., 1986). Proliferative responses were expressed as stimulation index (SI), calculated as cells stimulated with antigen divided by unstimulated cells. All cultures were taken in triplicates and the proliferation results expressed as mean S.I. ± SEM.

2.4. Cytokine response in immunized mice

Residual splenocyte cells were set up for cytokine assay. The spleen cells were thoroughly washed and cultured in 24-well flat-bottomed tissue culture plates (Costar) at 2×10^6 cells mL^{-1} in 1 mL of RPMI 1640 with 10% heat-inactivated fetal bovine serum and 80 μg mL^{-1} gentamycin. The cells were stimulated as described previously followed by incubation for 72 h. The supernatants were collected by centrifugation and stored at −80 °C. ELISA (Pierce Biotechnology, Rockford, IL) was performed to measure IFN-γ, IL-2, IL-4, IL-5 and IL-10 from different culture supernatants as per manufacturer's protocol. ELISA plates were incubated with 100 μL of supernatants collected from 72 h old culture (IL-2, IL-4, IL-5 and IL-10 estimation) and 96 h old culture (IFN-γ estimation) followed by incubation at 4 °C overnight. The plates were washed with wash buffer (PBS with 0.05% of Tween-20) thrice. Then they were incubated with blocking buffer (5% skimmed milk in PBS) for 2 h at room temperature. Then another wash was performed followed by incubation with rabbit anti-mouse IL-2, IL-4, IL-5, IL-10 or IFN-γ in PBS for 2 h at room temperature. After washing, the wells were further incubated with diluted (1:200) streptavidin-HRP conjugate for 1 h. The wells were incubated with 100 μL of the substrate tetramethyl benzidine (TMB/H_2O_2) after final washing. The reaction was stopped after 20 min by adding 50 μL/well of 1 N H_2SO_4. The absorbance was measured at 450 nm wavelength. The standard curves were run simultaneously with standard concentrations of cytokines and data expressed in pg mL^{-1} after deducting the values of unstimulated cultures.

2.5. ADCC assay

An *in vitro* ADCC assay was performed against *B. malayi* L3 larvae by sera of mice immunized with recombinant antigen as described previously (Chandrashekar et al., 1990; Mahalakshmi et al., 2014). Pre-immune sera samples were used as negative control. In brief, 11–15 L3 larvae in 50 μL of RPMI 1640 media supplemented with 10% inactivated FBS were incubated with 100 μL of 0.2×10^6 peritoneal exudate cells from normal BALB/c mice and 50 μL of E-ALT-2, P-ALT-2 or P-TUFT-ALT-2 sera collected on 28th day of the immunization in 96-well flat-bottomed culture plate for 48 h at 37 °C in 5% CO_2. Viability of larvae was determined under light microscope after 48 h of incubation. Parasites that were limpid and straight were considered as dead and bent active parasites as live. The results were expressed as the ratio of dead or immobile parasites to total number of parasites recovered within experimental group.

2.6. Statistical analysis

All the statistical analysis were performed using GRAPHPAD PRISM software version 6.0 (GraphPad Software Inc., CA, USA). Two-way ANOVA with Kruskal-Wallis test was performed ($P < 0.0001$) for total IgG and with Friedman test was performed ($P < 0.0001$) for antibody sub-types. The data value is represented as mean with SD or SEM. A probability (P) value ≤0.05 was considered statistically significant.

3. Result

3.1. Antibody titre and isotypes in immunized mice

The antibody titre and isotype antibodies induced by antigen proteins in BALB/c mice were used to carry out for experimental validation of B cell responses. Various *in vivo* and *in vitro* studies have demonstrated the role of different antibodies in immunity to filarial nematodes. IgE is actively involved in L3 and adult worm death in the mouse model. IgM plays an important role in host protection by reacting strongly with the surface antigens of *Brugia* L3 (Xu et al., 2011). The Total IgG peak titers in BALB/c mice model immunized by E-ALT-2, P-ALT-2 and P-TUFT-ALT-2 were 32,000, 16,000 and 64,000 ($P < 0.0001$) at 28th day after immunization (Fig. 1a). The determination of isotypes and sub-types of antibodies is essential to draw a proper immunological response by vaccine candidates. The isotype and sub-type profile showed a predominantly significant ($P < 0.0001$) level of IgG2b (1.37 ± 0.06), IgG1 (0.97 ± 0.04) and IgG2a (0.68 ± 0.02) in E-ALT-2 immunized mice. The P-ALT-2 showed IgG1 (0.71 ± 0.01) followed

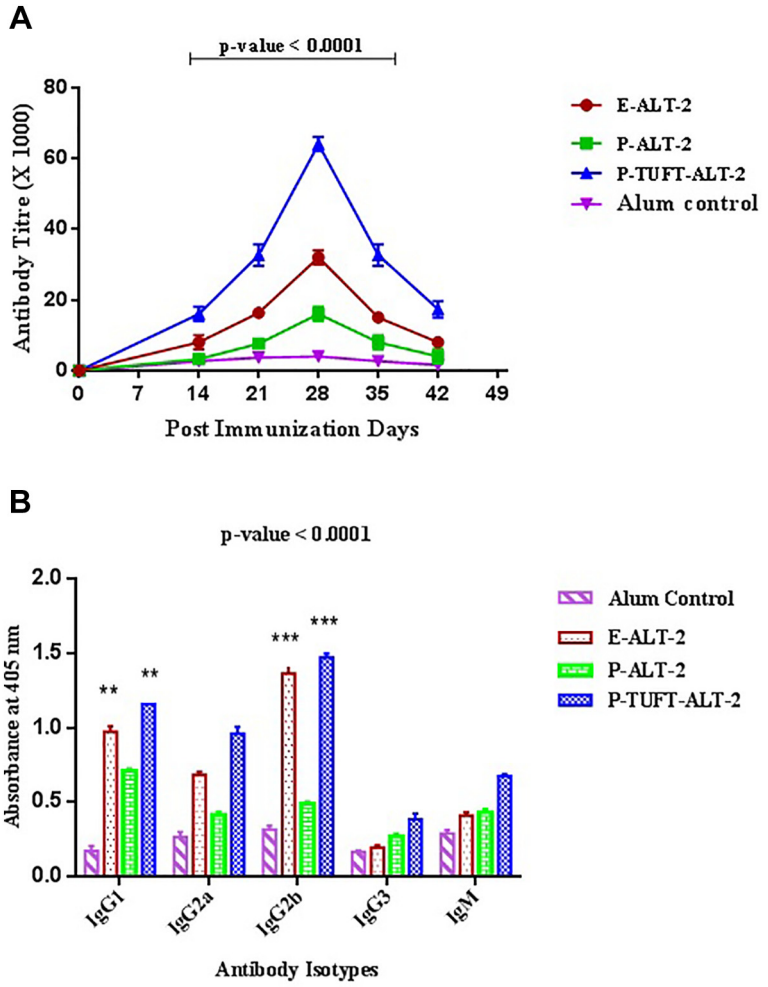

Fig. 1. a) Total IgG Titre in immunized mice: the total antibody induced by the recombinant proteins, at different intervals post immunization in mice model (n = 6 per group) was assessed by indirect ELISA. Mice were immunized with 30 μg of protein intraperitoneally and blood was collected at different intervals. Serum from the mice immunized with alum alone was taken as negative control. Data is presented as mean ± SD. Two-way ANOVA with Kruskal-Wallis test was performed ($P < 0.0001$). b) Isotypes and subtypes of antibody titre in mice: Isotype ELISA was carried out to measure the levels of antibody subtypes elicited by the proteins in mice using class or subclass specific antibodies. Data is represented as mean absorbance ± SEM of proteins with the respective antisera. Antisera of alum immunized mice were used as control. Friedman test was performed ($P < 0.0001$).

by IgG2b (0.49 ± 0.01) and IgG2a (0.41 ± 0.03). The P-TUFT-ALT-2 induced the similar pattern of antibody isotype level like E-ALT-2, but the amount of all isotypes was much higher (1.47 ± 0.02, 0.96 ± 0.03 and 1.16 ± 0.04 respectively for IgG2b, IgG1 and IgG2a) than E-ALT-2 and P-ALT-2 response. IgG3 level was very less in all of E-ALT-2, P-ALT-2 and P-TUFT-ALT-2 (0.20 ± 0.02, 0.27 ± 0.02 and 0.39 ± 0.02 respectively). IgM was measured as higher in P-TUFT-ALT-2 (0.67 ± 0.01) than E-ALT-2 (0.41 ± 0.02) and P-ALT-2 (0.43 ± 0.02). The IgG1, IgG2a, IgG2b, IgG3 and IgM level in alum control were 0.17 ± 0.02, 0.26 ± 0.02, 0.32 ± 0.02, 0.16 ± 0.01 and 0.29 ± 0.01 (Fig. 1b). Both IgG1 (20) and IgM (39–40) have been identified in vaccine induced protection in experimental filariasis.

3.2. Splenocyte proliferation assay

The cellular response was also studied by the ability of the protein to stimulate the spleen lymphocytes of mice immunized with respective protein, which leads to the proliferation of T cells. The splenocytes of P-TUFT-ALT-2 immunized mice showed significantly ($P < 0.03$) high proliferation response (S.I.) to respective antigen (4.27 ± 0.05) than the same by E-ALT-2 (3.90 ± 0.01) and P-ALT-2 (3.53 ± 0.05). The mean S.I. of splenocyte proliferation in alum control immunized by P-TUFT-ALT-2, E-ALT-2 and P-ALT-2 were 1.45 ± 0.04, 1.74 ± 0.04 and 1.34 ± 0.05 respectively The mean S.I. value for ConA in P-TUFT-ALT-2, E-ALT-2, P-ALT-2 and alum immunized mice was measured as 4.63 ± 0.06, 4.58 ± 0.09, 5.02 ± 0.11 and 4.11 ± 0.05 (Fig. 2).

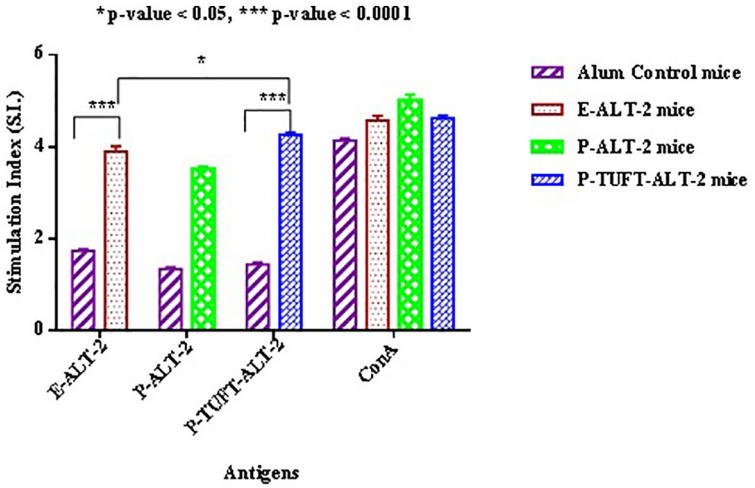

Fig. 2. Splenocyte from immunized mice proliferated in response to corresponding antigen: single cell suspension of spleen cells (0.2×10^6) from vaccinated and control mice were stimulated with the respective protein antigens or conA compared to that of the alum control mice. The data is represented as mean stimulation index (S.I.) of six mice ± SEM. The asterisk on top of the bars indicates a significantly high S.I. value compared to P-TUFT-ALT-2 *vs.* E-ALT-2 ($P < 0.05$) and control cells ($P < 0.0001$).

3.3. Cytokine analysis

Cytokine analysis is essential to find out the Th1 and Th2 mediated immune response by vaccine antigens. The cytokine analysis showed that E-ALT-2 exhibited higher IL-2 and IL-5 cytokine level (106.80 ± 0.81 pg mL^{-1} and 176.43 ± 3.43 pg mL^{-1}), whereas P-ALT-2 showed 38.18 ± 0.35 pg mL^{-1} and 64.09 ± 0.30 pg mL^{-1} respectively. A significant rise of these cytokines ($P < 0.001$) was observed in the culture supernatants stimulated with P-TUFT-ALT-2 (131.67 ± 1.45 and 245.67 ± 4.65 pg mL^{-1}). The amount of IL-10 was also higher in E-ALT-2, P-ALT-2 and P-TUFT-ALT-2 (145.14 ± 2.36, 77.06 ± 0.52 and 134.63 ± 2.52 pg mL^{-1} respectively). However, there was no significant change of IL-4 levels. The IFN-γ level was highest among all cytokines, and was predominant in E-ALT-2 (2492.13 ± 30.48 pg mL^{-1}) followed by P-TUFT-ALT-2 (1818.90 ± 12.15 pg mL^{-1}) and P-ALT-2 (708.66 ± 9.15 pg mL^{-1}) (Table 1).

3.4. ADCC assay

Earlier studies showed that protective antibodies can kill filarial parasites by ADCC-mediated mechanism (Chandrashekar et al., 1990; Mahalakshmi et al., 2014). In the *in vitro* cytotoxicity assay, anti-P-TUFT-ALT-2 and anti-E-ALT-2 sera illustrated higher cytotoxicity as a number of PECs adhered to the straight and dead *B. malayi* L3 larvae. The E-ALT-2 exhibited 75.00 ± 8.33% cytotoxicity against L3 infective larvae compare to alum control (18.18 ± 1.66%), whereas P-ALT-2 exhibited 27.03 ± 7.86% cytotoxicity. The result also showed ~~the~~ cytotoxicity ranging from 55 to 80%, conferred by P-TUFT-ALT-2, on L3 larvae with an average of 64.52 ± 13.40%. The difference was statistically significant (Fig. 3 & Table 2).

4. Discussion

Despite extensive research, no potential vaccine for filarial parasite is available in the market. The present study attempts to develop an effective filarial vaccine produced conjugated with Tuftsin immunopotentiator in *P. pastoris*. Fusion proteins have been used as vaccines in different studies with great success. Tuftsin was examined with many antigens in vaccine development namely leprosy, malaria, HIV *etc.* (Gokulan et al., 1999; Khare et al., 1992; Kumar et al., 1995). Tuftsin covalently conjugated to fullerene C exhibited significant enhancement of phagocytosis, chemotaxis activities (Xu et al., 2011). The phagocytotic activity

Table 1
Analysis of cytokine levels (pg mL^{-1}) in Balb/c mice: the cytokine concentrations were estimated in culture supernatants of spleen cells from Balb/c immunized and stimulated *in vitro* with the corresponding antigens. Experiments were performed in triplicates and the data are represented as mean concentration ± SD.

Animal groups	Cytokines (pg mL^{-1})					Th1/Th2 type response
	IL-2	IL-4	IL-5	IL-10	IFN-γ	
Alum	25.15 ± 0.32	18.32 ± 0.75	21.05 ± 0.46	18.12 ± 0.62	28.54 ± 0.89	
E-ALT-2	106.80 ± 0.81	25.55 ± 0.27	176.43 ± 0.43	145.14 ± 0.36	2492.13 ± 30.48	Th1/Th2 balanced
P-ALT-2	38.18 ± 0.35	8.71 ± 0.09	64.09 ± 0.30	77.06 ± 0.52	708.66 ± 9.15	Th1/Th2 balanced
P-TUFT-ALT-2	131.67 ± 1.45	23.31 ± 0.49	245.67 ± 4.65	134.63 ± 2.52	1818.90 ± 12.15	Th1/Th2 balanced

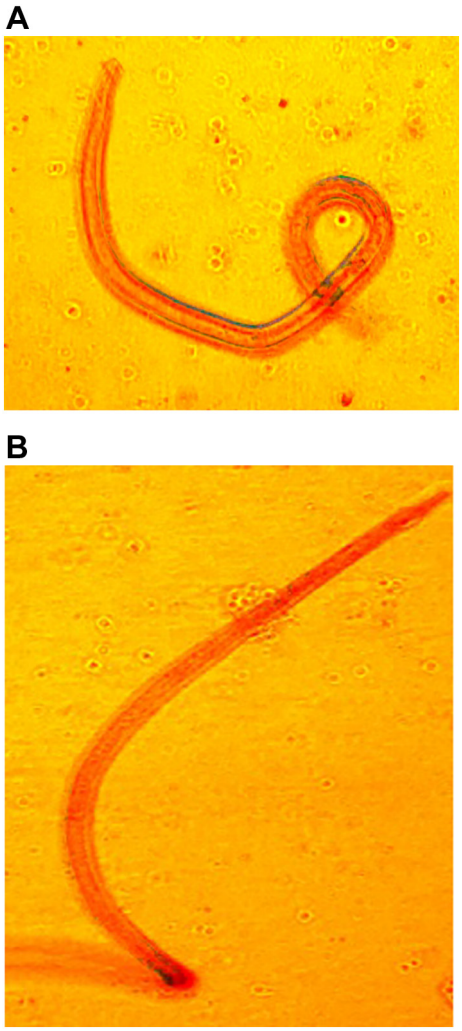

Fig. 3. Photomicrograph of *B. malayi* L3 recovered from cultures after antibody-dependent cellular cytotoxicity (ADCC) assay: (a) L3 incubated in prebleed sera and PECs. There are no PECs adhered to the larva and the larva was active. (b) L3 incubated with mice immunized sera and PECs. The cluster of cells adhered on the surface of larvae.

of macrophages was drastically enhanced by Tuftsin-based fusion proteins, which caused the suppression of human epidermoid carcinoma growth (Liu et al., 2012). A novel Tuftsin derivative with four Tuftsin peptides effectively prolonged the survival time of tumor-resected mice (An et al., 2014). Tuftsin has already been recognized as one of the most potential immunopotentiators to control infectious diseases (Nishioka et al., 1981).

The total IgG titre in this experiment suggested that P-TUFT-ALT-2 induced more IgG titre than E-ALT-2 and P-ALT-2. The IgG peak titre was reduced in P-ALT-2 than E-ALT-2. The enhancement of mean peak titre by P-TUFT-ALT-2 suggests its ability to stimulate B cells significantly owing to its immunomodulatory nature. The Tuftsin might have enhanced its immunogenicity as observed in P-TUFT-ALT-2. Higher antibody titres could be associated with protective efficacy of the vaccine candidate as suggested by the previous studies (Lawrence, 2001). The antibody-dependent cell-mediated cytotoxicity (ADCC), which needs specific antibodies, is believed to be one of the principal immunological mechanisms for clearing circulating parasites (Chandrashekar et al., 1990).

Table 2
Antibody-dependent cellular cytotoxicity (ADCC) against *Brugia malayi* L3 larvae: percentage reduction data represents the mean ± SD value of sera sample in triplicates from each group. The percentage reduction was compared to control sera.

Groups	Live L3	Dead L3	Percentage reduction with SD
Prebleed	14	0	
Alum control	18	4	18.18 ± 1.66
E-ALT-2	8	24	75.00 ± 8.33
P-ALT-2	27	10	27.03 ± 7.86
P-TUFT-ALT-2	11	20	64.52 ± 13.40

The isotype antibody profile showed a balanced Th1/Th2 as both E-ALT-2 and P-TUFT-ALT-2 showed higher IgG2b, IgG2a and IgG1 response. P-ALT-2 couldn't enhance the immunological response at par with the other two proteins. The classical complement pathway is activated mostly by IgM followed by IgG2a, IgG2b and IgG3 in mice, while IgG1 is a poor activator (Klaus et al., 1979; Neuberger and Rajewsky, 1981; Rizzo et al., 1992). Our results also indicate high levels of IgG2b followed by IgM, which could activate the classical complement system and enhance the innate immunity of the host. Th2-type response shows the production of IgG1 antibody, whereas IgG2a antibody is produced in Th1 response. Murine IgG2a and IgG2b isotypes have the ability to fix complement by binding to protein antigens, while IgG1 binds to mast cells in ADCC (Akiyama et al., 1984; Sim et al., 1982). IgG1 mediates antibody-dependent cell-mediated cytotoxicity (ADCC) whereas IgG2a isotype mediates complement-dependent cytotoxicity (CDC). The Fc region of the antibody binds to Fc receptor (FccRs) on the cell surface of immune effector cells such as macrophages and natural killers and causes the phagocytosis or lysis of the target by ADCC mechanism. On other hand, the antibody kills the targeted cells ~~in~~ through CDC by activating the complement system at the cell surface. Both antibody and complement-mediated effector mechanisms have been shown to be involved in inducing cytotoxicity to the microfilaria and infective larvae *in vitro* (LeGoff et al., 2000). Also, IgG3 isotype which is involved with ADCC mechanism was found to be increased ($P < 0.05$) (Akiyama et al., 1984). Therefore, the enhanced multifaceted immunological responses like cellular, humoral, ADCC *etc.* by P-TUFT-ALT-2 fusion protein were considered as its immunomodulatory activity over E-ALT-2 and P-ALT-2.

T cells are required for host protection in a mouse model of *Brugia malayi* infection. Our data shows the presence of T epitope in the three proteins- E-LAT-2, P-ALT-2 or P-TUFT-ALT-2 recognized in mice. The splenocyte proliferation showed that there was 28% higher proliferative response, when cells were stimulated with P-TUFT-ALT-2 compared to E-ALT-2 and control groups immunized with alum. The epitopes were recognized by the Th cells indicating a strong cellular response elicited by the antigen. Thus, P-TUFT-ALT-2 elicited increased production of IL-2, IL-5 and IFN-γ in cytokine analysis possibly triggering a mixed Th1/Th2 response. But higher level of IL-10 was also observed in all cases indicating a regulatory control over immune response. The higher IL-10 level showed the profile towards Th1/Treg along with levels of IFN-γ. Increase in production of IL-5 is also a constructive lead as earlier studies indicate that IL-5 alone has important consequences for protective immunity to filarial nematode model systems. Also, the production of IL-5 was essential for protection to filariasis induced by irradiated L3 larvae in a permissive murine model (Babu et al., 2000; Martin et al., 2000). Recent studies in various animal models of filarial diseases have revealed the Th1-type responses in host protection against filarial infection and IFN-γ for the clearance of *B. malayi* in mice (Mohr et al., 2010). In the murine system, IFN-γ produced by Th1 cells induces predominantly IgG2a, while IL-5 produced by Th2 selectively stimulates IgG1 and IgM; this also correlates well with our observation (Mizoguchi et al., 1999). In one study, the Tuftsin-based fusion proteins such as LDP-TF and Ec-LDP-TF drastically enhanced the phagocytotic activity of macrophages and distinctly suppressed the growth of human epidermoid carcinoma A431 xenograft in athymic mice by 84.2% with up-regulated expression of TNF-α and IFN-γ (Liu et al., 2014b). In a similar study, Dakshinamoorthy et al. reported high titers of IgG1 antibodies in macaques and significantly high levels of IFN-c secreted by PBMC in response to a multivalent fusion protein vaccine (rBmHAT) consisting of small heat shock proteins 12.6 (HSP12.6), large extracellular domain of tetraspanin (TSP LEL), and abundant larval transcript-2 (ALT-2) (Dakshinamoorthy et al., 2014). In a study, CHIK-VLPs, Chikungunya vaccine showed a high level of IgG2a and IgG1 subtypes was identified suggestive of balanced Th1/Th2 response and higher level of TNF-α, IL-10 along with the substantial level of IL-2, IL-4, and IFN-γ indicating a balanced Th1/Th2 response (Saraswat et al., 2016).

The vaccine must be capable of inducing antibodies that are protective and neutralizes the pathogenic organism for effective prophylaxis (Kerekes et al., 2001). Earlier studies showed that protective antibodies can kill filarial parasites by an ADCC-mediated mechanism (Chandrashekar et al., 1990; Mahalakshmi et al., 2014). In a study, Tuftsin enhanced the serum-specific IgG and IgA antibodies against hepatitis A virus (HAV) and hepatitis E virus (HEV) with higher levels of IFN-γ-secreting splenocytes (Th1 response) and the ratio of CD4+/CD8+ T cells indicating stronger humoral and cellular immune responses (Gao et al., 2015). The heat-inactivated whole *P. pastoris* expressing N-PbCS RNPs reduced a significant number of parasites in C57Bl/6 mice (Jacob et al., 2014). In the *in vitro* cytotoxicity assay, anti-P-TUFT-ALT-2 and anti-E-ALT-2 sera showed higher cytotoxicity against *B. malayi* L3 larvae. The E-ALT-2 exhibited 75% cytotoxicity compare to alum control (18.18 ± 1.66%), whereas P-ALT-2 exhibited 27.03% cytotoxicity which was enhanced up to 65% by P-TUFT-ALT-2. Vaccination with a fusion protein of Bm-103 and Bm-RAL-2 or concurrently, induced a significant worm reduction of 61% and 42% in gerbils respectively (Arumugam et al., 2016). The prophylactic efficacy of Wb20/22 protein without acidic domain (WOAD) conferred 62.26% protection which was higher than 49.82% of Wb20/22 and 54.78% of Wb20/22 without signal sequence (WOSS) (Aparnaa et al., 2014). Anugraha et al. reported the prophylactic efficacy of the filarial epitope protein (FEP), a chimera of selective epitopes from thioredoxin (TRX), several epitopes from transglutaminase (TGA) and abundant larval transcript-2 (ALT-2) indicating a significant protection (69.5%) against filarial L3 larvae in jirds (Anugraha et al., 2015). The cytotoxicity conferred by P-TUFT-ALT-2 ranging from 55 to 80% on L3 larvae with an average of 64.52 ± 13.40% is comparable to that of protection result of the well-established recombinant protein vaccine candidate such as abundant larval transcript (74%) for lymphatic filariasis (Gregory et al., 2000; Ramachandran et al., 2004).

5. Conclusion

Lymphatic filariasis is the second leading neglected tropical parasitic disease with the long-term permanent physical disability. So, besides developing effective drug that will kill all the stages of the parasites, there is also a need to develop suitable prophylactic agents such as a vaccine for the successful elimination of human lymphatic filariasis by multiple elimination strategies. Mice model is the most convenient due to their easy availability and well defined immunological characterization. The total IgG peak titre was enhanced in BALB/c mice immunized by P-TUFT-ALT-2. The isotype profile showed predominantly significant level of

IgG2b followed by IgG1 and IgG2a indicating a balanced Th1/Th2 response. The splenocyte proliferation showed that there was 9.5% higher proliferative response, when cells were stimulated with P-TUFT-ALT-2 compared to E-ALT-2. The significant rise of IL-2, IL-5 and IFN-γ cytokines was also observed in the culture supernatants stimulated with P-TUFT-ALT-2. Moreover, it also exhibited higher cytotoxicity (55 to 80%) to L3 larvae as shown by the *in vitro* ADCC assay. Therefore, P-TUFT-ALT-2 may be a considered as a potential vaccine candidate for human lymphatic filariasis.

Declaration of conflicting interests

The Authors declare that there is no conflict of interest.

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Authors' contributions

Dr. P. Kaliraj, Dr. Reddy and Mr. R. Paul were involved in study design, data analysis and writing the manuscript of this study. Mr. Paul and Mr. Ilamaran performed all mice experiments including splenocyte proliferation assay. Mr. Paul, Mr. Khatri and Mr. Amdare were involved in ADCC assay. All authors read and approved the final manuscript.

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