Multivalent Vaccine Formulation with BmVAL-1 and BmALT-2 Confer Significant Protection against Challenge Infections with *Brugia malayi* in Mice and Jirds

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Purpose: Lymphatic filariasis is a mosquito borne infection affecting 120 million people in 83 different countries. Despite several setbacks, mass drug administration is fully underway in several parts of the world to eradicate this infection by year 2020. Even though drug alone is highly efficient in treating this infection, long term sustainable prophylaxis needs effective vaccine. Unfortunately there are no vaccines available to control this infection in human and animals despite the fact that several potential candidate vaccine antigens have been identified by several laboratories. *Brugia malayi* Vespid venom Allergen homologue-Like protein (BmVAL-1) and *B. malayi* Abundant Larval Transcript (BmALT-2) are two of the most promising vaccine candidates. In this study we have evaluated various vaccination regimens consisting of DNA and protein antigens and evaluated the potential of monovalent and multivalent vaccine formulations in mice and jird animal models.

Methods: Mice and jirds were vaccinated with monovalent DNA preparations of *BmVAL-1* or *BmALT-2* in pVAX-1 vector or monovalent protein preparations of rBmVAL-1 and rBmALT-2 in alum using a homologous or heterologous prime boost approach. These vaccine regimens were then compared with a multivalent vaccine formulation consisting of DNA or hybrid protein formulation of the two antigens. Challenge experiments were performed with *B. malayi* L3 in mice and jirds to evaluate the degree of protection and immunological parameters were determined in mice and human to elucidate the characteristics of the protective immune responses.

Results: Results presented in this study show that vaccination with monovalent BmVAL-1 vaccine confers from 39% (DNA vaccine) protection to 54% (DNA prime and protein boost) protection in mice. Similar degree of protection was observed in jirds (50% to 52% protection). Monovalent BmAT-2 afforded 51% to 75% protection in mice and 58% to 79% protection in jirds. When we tested a multivalent formulation of BmVAL-1 and BmALT-2, there was 57% to 82% protection in mice and 77% to 85% protection in jirds. Heterologous prime boost approach using the multivalent vaccine gave the highest degree of protection in both mice and jirds. Serological analysis in mice showed that BmVAL-1 vaccination induced an IgG1, IgG2a and IgG3 antibody response, whereas, BmALT-2 vaccination predominantly induced an IgG1 and IgG3 antibody response. Cytokine responses of antigen responding cells in the spleen secreted predominantly IFN-γ and IL-5 in response to BmVAL-1 and IL-4 and IL-5 in response to BmALT-2.

Conclusion: In conclusion, results presented in this study show that a multivalent vaccine formulation of BmVAL-1 and BmALT-2 when given as a prime boost regimen gave significant protection against lymphatic filariasis caused by *B. malayi* in mice and jirds. Since putatively immune EN subjects also carry protective antibodies against BmVAL-1 and BmALT-2, there is a great potential for developing this multivalent formulation as a prophylactic vaccine against *B. malayi* for human and veterinary use.

INTRODUCTION

Human lymphatic filariasis is a mosquito borne disease caused mainly by two filarial parasites, *Brugia malayi* and *Wuchereria bancrofti*. The disease affects over 120 million people living in over 83 different countries in the tropical and subtropical regions of the world.[1] Diethylcarbamazine (DEC) is an effective drug against this infection.[2,3] Mainly this drug clears the microfilaria stage of the parasite thereby preventing transmission. A combination of DEC with Albendazole or Ivermectin is currently used as a drug of choice for multidrug administration (MDA) strategy to control this infection in endemic areas.[3] The ultimate goal is to eliminate the disease by 2020. MDA strategy is showing high success in several parts of the world. Despite its initial success, some endemic regions have already started facing significant difficulties in implementing the MDA program due to political unrest or lack of compliance.[4-7] To be effective MDA need to be repeated annually at least for 5 years (WHO). Vector control measures are not as successful as treating the patient for controlling the infection. However, since vector population is very high in these regions, multiple treatments with MDA are necessary for consistent protection. Multiple administrations of drugs, especially albendazole in the MDA regimen raises concern because of the potential for drug resistance. In fact, resistance to benzimidazole group of drugs has already been reported in *B. malayi* and *W. bancrofti*.[8] Thus, repeated administration of albendazole each year in the endemic region may not an ideal situation. A more practicable and sustainable approach to prevent this infection in a large population would be a prophylactic vaccination possibly with simultaneous mass

screening and treating infected individuals with DEC or combination drugs such as that used in MDA.

Protective immunity against lymphatic filarial parasite has been demonstrated in both human and animals.[9,10] Certain individuals living in the endemic areas under the same environmental conditions as the infected individuals do not show any symptoms of the disease, but carry high titer of antibodies against the filarial parasites in their sera. Previous studies from our laboratory showed that these circulating antibodies can participate in the killing of infective third stage (L3) *B. malayi* larvae *in vitro* through an antibody dependent cell cytotoxicity (ADCC) mechanism.[11] Similarly, animal studies also showed that vaccination with irradiated third stage larvae (L3) of *B. malayi* confer significant protection against challenge infections.[9] These findings provided strong evidence that protective immunity against *B. malayi* and *W. bancrofti* can be induced in human and animals. However, identifying the host protective antigens and the development of a suitable vaccine against lymphatic filariasis has been severely hampered by the complicated life cycle of the parasite and the difficulty in maintaining *W. bancrofti* life cycle stages under laboratory conditions. Despite these difficulties several potential candidate vaccine antigens are reported from many laboratories.[11-14] Completion of the *B. malayi* genome substantially boosted the vaccine antigen discovery.

Using a phage display-based iterative screening of a *B. malayi* L3 cDNA library with immune human sera, our laboratory previously showed that *B. malayi* vespid venom allergen homologue-like protein (BmVAL-1) and *B. malayi* Abundant Larval Transcript -

2 (BmALT-2) are potential vaccine candidates. [13] Vaccine potential of both BmVAL-1 (BmVAL-1) and ALT-2 has already been reported previously by other groups.[15-18] Thus, the powerful phage display-based parasite cDNA expression library screening confirmed previous reports and narrowed down the candidate vaccine antigens to VAL-1 and ALT-2. VAL-1 belongs to a family of proteins called *Ancylostoma caninum* secreted proteins or the ASP.[19] VAL-1 homologues have been reported from *A. duodenale, Necator americanus*, *Onchocerca volvulus* and *Meloidogyne incognita*.[20-22] Although VAL-1 and ALT-2 are excellent vaccine candidates, their functional role in the parasite is not fully understood.

Conventional methods of immunization with recombinant proteins require co-administration with adjuvants and usually have to be given in multiple doses. Moreover, there is a need for cold chain to transport and store the protein vaccine. These pose significant problems especially in tropical regions of the world where the disease is highly prevalent. Immunization with DNA vaccine can overcome the need for such requirements associated with conventional protein immunization. In addition, DNA vaccines are easy to prepare and can be stored with relative ease, eliminating the need for a cold chain. More importantly, DNA based vaccine can induce high level of protective immune responses and elicit both cellular and humoral immune responses as demonstrated against several pathogens.[23] Finally, multiple DNAs can be easily constructed into the vaccine vector to generate a multivalent DNA vaccine, which is a great advantage over protein vaccine. A recent study by Anand et al[30] show that BmALT-2 and BmVAH when given as as a cocktail vaccine can confer significant protection. In

this manuscript we present data that evaluates the vaccine potential of a multivalent combination of BmVAL-1 and BmALT-2 as a potential DNA vaccine against lymphatic filariasis.

MATERIALS AND METHODS

2.1. Sera

Sera samples used in this study were from archived samples stored at the Mahatma Gandhi Institute of Medical Sciences, Sevagram, India. These samples were collected as part of epidemiological surveys in and around Wardha, an area endemic for lymphatic filariasis. Use of human subjects in this study and the protocols and the consent forms were approved by the Institutional Research Board of the University of Illinois, Rockford and Mahatma Gandhi Institute of Medical Sciences, Sevagram, India.

No demographic data was available to this study except that the sera samples were classified into microfilaremic (MF), chronic pathology (CP) or Endemic normals (EN) based on the detection of circulating parasites, parasite antigens or by evaluating clinical symptoms of lymphatic filariasis. Circulating microfilariae were detected in the blood of subjects as described previously.[11,13] The presence of circulating antigen was detected using an Og4C3 kit and a WbSXP-based enzyme-linked immunosorbent assay (ELISA). Subjects with no circulating antigen or microfilariae were classified as EN, whereas subjects with circulating microfilariae and/or circulating antigen, as detected by ELISA, were considered as MF. Subjects showing lymphedema and other visible clinical

symptoms of filariasis were grouped into CP. Control non-endemic normal (NEN) sera were collected at the University of Illinois Clinic at Rockford, IL.

2.2. Parasites

Brugia malayi L3s were obtained from the NIAID/NIH Filariasis Research Reagent Resource Center (FR3) at the University of Georgia, Athens, GA

2.3. Construction of monovalent and multivalent DNA vaccines:

To prepare monovalent vaccine, codon optimized *BmVAL-1* (Acc: AF042088) or *Bmalt-2* (Acc: U84723) genes were cloned into the eukaryotic expression vector pVAX1 (Invitrogen, Carlsbad, CA) using insert specific primers.[13,24] To prepare multivalent vaccine, codon optimized *BmVAL-1* gene was first cloned into pVAX1 vector with no stop codon using already published primer sequences with a pst I site. Codon optimized *Bmalt-2* gene was then inserted into this clone using gene specific primers. PCR parameters for all the constructs were: 94°C denaturation for 30 s, 50°C primer annealing for 30 s, 72°C primer extension for 30 s for 30 cycles; a final extension of 5 min was performed at 72° C. Insert DNA was finally sequenced to ensure authenticity of the cloned nucleotide sequence on both strands. Plasmids were maintained and propagated in *E.coli* Top10F′ cells. Plasmids were purified using endotoxin free plasmid extraction kit (Qiagen, Valencia, CA). DNA was analyzed by agarose gel electrophoresis and quantified in a spectrophotometer (OD 260/280, ratio>1.8).

2.4. Expression and Purification of recombinant proteins

Recombinant BmVAL-1 and rBmALT-2 were expressed in pRSET-A vector and purified using an immobilized cobalt metal affinity column chromatography as described previously from our laboratory.[17,18] Endotoxin in the recombinant preparations were removed by passing the recombinant proteins through polymyxin B affinity columns (Thermo Fisher Scientific, Rockford, IL) and the levels of endotoxin in the final preparations were determined using an E-TOXATE kit (Sigma, St Louis, MO) as per manufacturer's instructions. Endotoxin levels in the final preparations (0.005 EU/ml) were below detection limits in these recombinant protein preparations.

2.5. Immunoreactivity of the various human sera

To determine if the human sera samples carries antibodies against BmVAL-1 or BmALT-2, we performed an ELISA as described before.[11,13] For isotype specific ELISA, alkaline phosphatase conjugated goat anti-human IgG1, anti-human IgG2, anti-human IgG3, and anti-human IgG4 antibodies (Sigma) were used as the secondary antibodies.

2.6. Immunization protocol for mice and jirds

Six-week old male Balb/c mice and 35-40 gm outbred male mangolian gerbils (jirds) purchased from Charles River Laboratories (Wilmington, MA) were used in these experiments. Animals were treated as per the guidelines in the Guide for the Care and Use of Laboratory Animals. Humane use of animals in this study and the protocol was approved by the IACUC committee at the College of Medicine, University of Illinois

Rockford. The reason for using two different animal models to test the vaccine formulations is that the *B. malayi* parasite does not mature into adults in mouse, so vaccine-induced protection against the L3 stages can be evaluated in the mouse model. In addition, significant immunological parameters can be measured in mice. Conversely, *B. malayi* parasite develops into mature adult worms in jirds. Therefore, we can evaluate vaccine-induced protection against adult worm establishment in jirds.

There were three sets of experiments (1) monovalent BmVAL-1 vaccination, (2) monovalent BmALT-2 vaccination and (3) multivalent mVAL-1/BmALT-2 vaccination. Each set of experiment had four groups (a) DNA prime plus DNA boost (homologous), (b) protein prime plus protein boost (homologous), (c) DNA prime plus protein boost (heterologous) and pVAX plus alum controls. Each group consisted of ten (10) animals each. All animals were immunized subcutaneously with codon optimized DNA (100 μg) in 50 μl volume or with recombinant protein (150 μg) plus alum in 50 μl volume. Control group received 100 μg of pVAX1 blank vector or 50 μl of alum. Blood samples were collected at frequent intervals, sera separated and stored at -80^0C. Protocol used for immunizing mice and jirds are schematically represented in Figure 1.

2. 7. Protection studies in mice:

Challenge studies were conducted in mice by surgically implanting twenty *B. malayi* live infective L3s into the peritoneal cavity in a micropore chamber as described previously.[11,25] Aseptic conditions were followed for the surgical procedures. 48 hours after implantation, chambers were recovered from the peritoneal cavity and viability of

the larvae was determined under a light microscope. The percentage of protection was expressed as the number of dead parasites ÷ number of total parasites recovered x 100.

2.8. Splenocyte proliferation and cytokine assays

Single cell suspension of spleen cells (0.5x 10^6 cells per well suspended in 200µl media) were prepared from each mouse and cultured in triplicate wells with either (1) 1µg/ml rBmVAL-1, (2) 1µg/ml rBmALT-2, (3) 1µg/ml rBmVAL-1+BmALT-2, (4) a non-specific recombinant protein (1µg/ml of *Schistosoma mansoni* G-binding protein) or (5) were left unstimulated in the media. All cells were incubated for 3 days at 37^0C with 5% CO_2. After 3 days, 3H-Thymidine (0.5 lCi per well, Amersham Biosciences) was added to each well and further incubated. Cells were harvested 16 h later and 3 H-thymidine uptake was measured in a liquid scintillation counter and expressed as stimulation index (SI) = (counts per min of stimulated cultures counts per min of unstimulated cultures). Cell culture supernatants collected from the above spleen cultures were assayed for IFN-γ, IL-4, IL-5 and IL-10 using an ELISA kit purchased from eBioscience Inc., (San Diego, CA).

2.9. BmVAL-1 and BmALT-2 specific IgG antibodies in the sera of immunized mice

Titer of anti-BmVAL-1 and anti-BmALT-2 specific antibodies was determined in the sera of immunized mice using an ELISA as described previously.[11,13] Pre immune sera served as controls. HRP conjugated goat anti-mouse IgG was used as the secondary antibody (Thermo Fisher Scientific) for mouse assay. OPD (Sigma) was used as the substrate and optical density (OD) was measured at 405nm.

Anti-BmVAL-1 and anti-BmALT-2 specific IgG1, IgG2a, IgG2b, IgG3 and IgG4 antibodies were determined in the sera of mouse using a mouse antibody isotyping kit purchased from Thermo Fisher Scientific. All ELISAs were performed as per the manufacturer's recommendation and absorbance was read at 405nm. Respective HRP-labeled goat anti-IgG isotype antibody was used as the secondary antibodies and color was developed using OPD substrate.

2.10. Challenge studies in jirds

Jirds were challenged with 100 *B. malayi* L3s and worm establishment was determined on day 95 after challenge as described previously.[26] Jirds are permissive hosts for *B. malayi* and the worms mature into adult males and females in about 75 days. Presence of mature worms in the control group of jirds was confirmed by demonstrating microfilariae in their blood on day 80 after challenge. Percent reduction in the worm establishment was calculated using the formula: average number of worms recovered from control worms – average number of worms recovered from vaccinated animals / average number of worms recovered from control animals x 100.

2.11. Statistical analysis

Statistical analysis was performed using Sigmastat program (Jandel Scientific, San Rafel, California) and Statview (SAS Institute, Cary, NC.) software. Wilcoxon signed rank test was used to compare paired data; comparison between the groups was performed using the Mann-Whitney U test. p value of $p<0.05$ was considered statistically significant.

3. RESULTS

3.1. EN individuals carry high titer of antibodies against BmVAL-1 and BmALT-2

Significant anti-BmVAL-1 and anti-BmALT-2 IgG antibodies were present in the sera of EN subjects compared to MF subjects ($p < 0.01$) and CP subjects ($p < 0.005$) (Figure 2A). NEN subjects did not carry IgG antibodies against both the antigens. Subsequent analysis of the IgG isotype of antibodies in the sera of EN subjects showed that antiBmVAL-1 and antiBmALT-2 antibodies were predominantly of IgG1 and IgG3 isotypes (Figure 2B).

3.2. High titer of antibody responses in the sera of immunized mice:

Previous studies from our laboratory showed that mice vaccinated with *B. malayi* antigens elicited significant host protective IgG antibodies[11,13,24]. Therefore, in this study, we mainly focused on measuring IgG antibody titers in the sera of immunized mice. Monovalent immunization with *BmVAL-1* (Figure 3A) and monovalent immunization with *Bmalt-2* (Figure 3B) both elicited significant ($p< 0.005$) titers of anti-BmVAL-1 and anti-BmALT-2 IgG antibodies in the sera of mice. Compared to controls, the prime boost immunized group gave the maximum titer of antibodies followed by protein immunized and DNA immunized groups (Figure 3). Immunization with the multivalent vaccine formulation (*BmVAL-1 + BmALT-2*) also elicited significant IgG antibody titers against both rBmVAL-1 and rBmALT-2 (Figure 3C) and the titers were comparable suggesting that the antigens do not interfere each other or compete for dominance. An interesting finding was that the multivalent vaccine elicited significantly higher ($p< 0.001$) titer of IgG antibodies in mice compared to any of the monovalent vaccines (Figure 3). These

finding suggested that the two antigens in the multivalent formulation synergistically increase the vaccine-induced antibody responses.

Overall, protein vaccination elicited higher titer of IgG antibodies compared to DNA vaccines suggesting that protein vaccinations are highly immunogenic (Figure 3). This may be partially due to the adjuvant used along with the protein vaccine. Another observation was that a heterologous prime boost approach gave a higher seroconversion than homologous prime boost approach (Figure 3). Thus, overall heterologous prime boost approach appeared to stimulate the highest titer of antibodies.

IgG antibody subset analysis showed that BmVAL-1 vaccination elicited primarily IgG1 and IgG2a isotype of antibodies, whereas, BmALT-2 vaccination induced IgG1, IgG2a and IgG3 isotype of antigen-specific antibody responses (Figure 4). Antigen-specific IgG4 antibody response was not evident. The prime boost approach significantly amplified the IgG isotype responses. Following multivalent vaccination regimen IgG1, IgG2a and IgG3 subset of antigen specific antibodies were present in the sera of mouse (Figure 4).

3.3. Antigen-specific responses in the spleen of mice

Spleen cells from immunized mice stimulated with either rBmVAL-1 or rBmALT-2 proliferated significantly (SI 10.8 $\pm$ 1.1 and SI 14.6 $\pm$ 1.2 respectively) compared to the media control (SI 2.1$\pm$0.9). Spleen cells from mice immunized with the multivalent construct responded to both rBmVAL-1 (SI 18.9 $\pm$ 2.6) and rBmALT-2 (SI 23.5 $\pm$ 3.1)

suggesting that a strong recall cellular response is generated to both BmVAL-1 and BmALT-2 following vaccination with the multivalent construct.

3.4. Cytokine analysis from proliferated culture supernatants

To identify the cytokine profile of the antigen-responding cells, we collected the culture supernatant of mouse spleen cells stimulated with respective antigen (rBmVAL-1 or rBmALT-2) and measured the level of IFN-γ, IL-4, IL-5 and IL-10. These results showed that significant levels of IL-5 and IFN-γ are secreted by the spleen cells in response to rBmVAL-1. Spleen cells stimulated with rBmALT-2 predominantly secreted IL-4 and IL-5 (Figure 5).

3.5. Multivalent vaccine induces significant protection in mice and jirds:

Above results show that significant IgG antibodies are elicited following vaccination with monovalent and multivalent vaccine preparations. To test if the immune responses elicited following vaccination is protective, we challenged vaccinated animals with live third stage infective larvae (L3) of *B. malayi*. Since the parasites do not reach to maturity in mice, we used a standard micropore chamber challenge method.[13] These studies showed that 39% to 74% protection can be achieved in mice following immunization with monovalent vaccine (Table 1). Protein vaccination gave better results than DNA vaccination. The prime boost regimen gave the best results overall. Vaccination with BmALT-2 gave higher percent of protection compared to BmVAL-1. Similarly, multivalent vaccination regimen gave the 57% to 82% protection compared to the monovalent vaccination regimen. These finding suggested that BmVAL-1 and BmALT-2

synergistically enhance the protective immune responses in vaccinated animals when given as a multivalent vaccine (Table 1).

Analysis of the thick blood smear prepared from the control group of jirds on day 80 after challenge showed that all five jirds were positive for microfilaria, whereas, microfilaria were not detected in the peripheral blood of vaccinated jirds (data not shown). Fifteen (15) days later we sacrificed the animals and counted the male and female worms in the peritoneal, pelvic and pleural cavities and compared the results between controls and vaccinated groups (Table 2). Findings from vaccination of jirds also confirmed that the multivalent prime boost regimen gave the highest rate of protection. No female worms were recovered from the multivalent vaccinated animals.

4. DISCUSSION

Developing a vaccine against lymphatic filariasis has been challenging due to the complex life cycle of the parasite and the difficulty in obtaining enough parasite materials for research, especially of *W. bancrofti*. Our laboratory recently developed a phage expression library of different stages of *W. bancrofti* and *B. malayi*.[13] Extensive screening of this library with sera from immune individuals yielded several potential candidate antigens. Many of these antigens show significant sequence identity and cross reactivity between *W. bancrofti* and *B. malayi*. In this manuscript we analyzed two of the leading vaccine candidates, BmVAL-1 and BmALT-2. Both these antigens were significantly recognized by all EN sera and thus are potential vaccine candidates. Results presented in this study show that multivalent vaccine formulation that combines these two antigens is highly immunogenic than a monovalent vaccine regimen of each antigen separately. Similarly, a heterologous prime boost approach using DNA prime and protein boost gave significantly higher protection compared to homologous prime boost approach.

Results from our studies show that immunization of mice with BmVAL-1 and BmALT-2 as a multivalent prime boost regimen conferred 82% protection. Since the worms do not mature into adult worms in mice a micropore chamber method was used to evaluate protection. This is a well established method to evaluate protection against *B. malayi* in mice.[24,25,27] The parasite develops into mature adults in jirds. Thus, parasite establishment can be evaluated as a determinant of protection in jirds. Vaccination in jirds also confirmed our results in mice. Multivalent vaccination using a prime boost regimen in

jirds also conferred that nearly 85% protection can be achieved. While sterile immunity will be ideal, it is often very difficult to achieve that level of protection with a prophylactic vaccine for any parasitic infection. Nevertheless, close to 85% protection should substantially reduce the incidence of the infection when coupled with treatment of the infected individuals at a much faster rate than multiple application of MDA alone.

The vaccine candidates BmVAL-1 and BmALT-2 are the highly expressed proteins in the third stage larvae of *B. malayi*.[28] Because of their abundance these proteins are easily available to the host immune system. Previous immunization studies using either of these proteins show that vaccination with each of them can confer significant protection in mouse and jird models.[16-18,24]

B. malayi Vespid venom allergen homologue-like protein (BmVAH or BmVAL-1) or *Ancylostoma* secreted protein (ASP), or Sperm Coating protein-like protein (SCP) are all a family of SCP domain containing proteins that are highly immunogenic and are expressed by a number of nematode parasites. [13,18-20,29,30] Among these ASP has been extensively evaluated for its vaccine potential.[19,20] Vaccination studies using BmVAL-1 in lymphatic filariasis model show that significant protection can be achieved in mice against challenge infections.[18,30] Although the true function of filarial VAL-1 is not fully understood, VAL-1 homologous are believed to be involved in the establishment of infection and transition to parasitism, as they are very abundant in the excretory–secretory (ES) material of infective larvae.[19] *O. volvulus* ASP has angiogenic property[22] suggesting that VAL-1 may have potential role in the pathogenesis. VAL-1 also has

significant sequence homology with venom allergens from vespid wasps (Ag5) and fire ants (Ag3).[29] Study by Visser et al.[31\]show that VAL-1 and its homologue may have a role in the reproductive development of the male *Ostertagia* worms. Thus, VAL-1 homologue appears to be important for the establishment and survival of the parasite in the host[19] and may have a possible role in immune evasion.[28] Based on the reports to date, BmVAL-1 appears to be an ideal candidate for vaccine development against lymphatic filariasis.

Another vaccine candidate used in this study is BmALT-2. BmALT-2 and the closely related BmALT-1 together represent about 5% of the total *B. malayi* L3 cDNA indicating that they are the most abundant proteins in this parasite.[16] BmALT-1 and BmALT-2 are also abundant in the protein secretions of the infective L3.[32] Immunization with Bm-ALT-1 or BmALT-2 can confer approximately 75-76% reduction in worm load.[16,17] This is probably the only filarial recombinant antigen that can afford this high protection rate.[16] The mechanism of this high protection is not known. However, it is believed that these proteins may play a significant role in host immunomodulation.[28] Significant amounts of BmALT proteins are shown to be released from the glandular stockpiles of L3 larvae. Similar to BmVAL-1 vaccination results, our results with BmALT-2 vaccination also showed that BmALT-2 heterologous vaccination regimen is highly protective than monovalent or homologous regimens.

Since both BmVAL-1 and BmALT-2 conferred significant protection, we combined the two antigens as a multivalent formulation and compared the homologous and

heterologous vaccine regimens. These studies showed that a multivalent heterologous vaccine regimen using *BmVAL-1/BmALT-2* DNA to prime and BmVAL-1/BmALT-2 protein to boost gave the best protection results. Our findings also showed that combining BmVAL-1 with BmALT-2 can synergistically increase the protection efficiency of both the antigens. Following multivalent vaccination, immunized mice developed comparable titer of antigen-specific antibodies against both the antigen suggesting that BmVAL-1 and BmALT-2 do not interfere in the immunogenicity of the antigens. Thus, the multivalent combination of BmVAL-1 and BmALT-2 is an excellent choice for further vaccine development.

A major contributor to this synergistic effect of these two antigens may be that both the antigens selectively promoted IgG1 and IgG3 antibody responses in EN subjects although the antibody responses in mouse appear to be largely IgG1, IgG2a and IgG3. Human IgG1 and IgG3 are cytophilic antibodies and can fix complement. Both IgG1 and IgG3 can also bind to FcγRI on the surface of various effector cells that when activated can potentially kill *B. malayi* larvae.[11,13,33,34] Our previous studies show that antibodies in the sera of endemic normal individuals can participate in the killing of *B. malayi* L3 through an antibody dependent cell cytotoxicity (ADCC) mechanism.[11,24] Thus, the antigen-specific IgG1 and IgG3 subtype of antibodies that we observed against both BmVAL-1 and BmALT-2 in the sera of putatively immune EN subjects may have some functional significance. Mouse studies show that IgG1, IgG2a and IgG3 antibody isotypes were increased following vaccination. The human counterparts of murine IgG subclasses are based on similarities in biological and functional activities. Murine IgG2a and IgG2b and

human IgG1 and IgG3 share the ability to fix complement and bind to protein antigens, whereas, murine IgG1 and human IgG4 are considered to be similar because of their property of binding to mast cells. Murine IgG3 and human IgG2 both recognize predominantly carbohydrate epitopes. All mouse subtypes can participate in ADCC.[35] Mouse IgG3 and IgG2a immune complexes can bind strongly to FcγRI and trigger receptor mediated responses[36] suggesting that these two isotypes may play a significant role in the vaccine-induced protection against *B. malayi*. Mouse IgG3 however is an early effector molecule of the immune system and appears early in the immune responses independently of T cell help. Thus, all three subtypes of IgG antibodies that we observed in vaccinated animals may have a significantly role in the vaccine-induced protection in mice.

BmVAL-1 and BmALT-2 antigen-specific cells were present among the spleen cell population of the immunized animals as demonstrated by the recall responses. These cells in response to the antigens secreted IL-4, IL-5 and IFN-γ. Thus there was a mixed Th-1/Th2 response elicited in animals vaccinated with heterologous multivalent vaccine. Predominantly the IFN-γ response was in response to BmVAL-1, whereas, BmALT-2 largely induced an IL-4/IL-5 response. In the murine system, gamma interferon produced by Th1 cells induces IgG2a and IgG3 *in vitro*[37]; interleukin-4 (IL-4) produced by Th2 selectively stimulates IgG1 and IgE.

In conclusion, results presented in this study show that a multivalent combination of BmVAL-1 and BmALT-2 is an excellent vaccine for lymphatic filariasis caused by *B.*

malayi. Since putatively immune EN subjects also carry IgG1 and IgG3 antibodies against BmVAL-1 and BmALT-2 there is a great potential for developing this multivalent formulation as a prophylactic vaccine against *B. malayi* for human and veterinary use.

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Figure caption

Figure 1. Schematic presentation of the vaccination protocol used in mice and jirds.

Figure 2. Figure 2A. Titer of BmVAL-1 specific IgG antibodies **(A)** and BmALT-2 specific IgG antibodies **(B)** in the sera of human subjects**.** Titer of antigen specific IgG antibodies were determined in sera samples from MF (n=20), CP (n=20) and EN (n=20) subjects using an ELISA. Sera samples from NEN subjects served as controls. Each spot represent sera sample from one individual. The cut off value (mean OD$\pm$ 3 times SD of NEN sera) is indicated by a line drawn parallel to X-axis. **Figure 2B.** Titer of IgG isotype specific antibodies against rBmVAL-1 (**C**) and rBmALT-2 (**D**) in the sera samples of endemic normal subjects**.** Values were determined by specific ELISA for each isotype. Each spot represent sera sample from one individual. The IgG isotype antibody patterns against BmVAL-1 and BmALT-2 were comparable in the sera samples. N=20.

Figure 3. Titer of anti-VAL-1 IgG and anti-ALT-2 IgG antibodies in the sera of immunized mice after 4 immunizations at two weeks interval. IgG levels were measured using an ELISA. **DNA** – DNA vaccinated group, **Protein** – Protein vaccinated group, **Prime boost** – DNA Prime protein boost vaccinated group and **Control** – vector plus alum controls. **Panel A**: Sera collected from mice immunized with monovalent BmVAL-1 vaccine. **Panel B**: Sera collected from mice immunized with monovalent BmALT-2 vaccine. **Panel C**: Sera collected from mice immunized with BmVAL-1/BmALT-2 multivalent vaccine. Dotted lines values for anti-BmVAL-1 IgG, solid lines values for

anti-BmALT-2 IgG. N=10. Data represent results from one of two experiments with comparable results.

Figure 4. Levels of antiVAL-1 and anti-ALT-2 IgG isotype of antibodies were measured in the sera of immunized mice two weeks after the last immunization. (A) Monovalent BmVAL-1 vaccinated mice, (B) Monovalent BmALT-2 vaccinated mice and (C) multivalent vaccinated mice. N=10. *Significant $p < 0.01$ compared to vector plus alum control group.

Figure 5. Cytokine responses in the culture supernatants of spleen cells stimulated for 72 hrs with rBmVAL-1 (A) rBmALT-2 (B) or rBmVAL-1-BmALT-2 hybrid protein (C) were measured using an ELISA. Concentrations of each cytokines are represented as pg/ml. spleen cells from vector/alum injected mice served as control. Additional controls include cells incubated in media alone and cells stimulated with a non-specific recombinant protein (SmGBF). Spleen cells from the prime boost group were used for the last two control groups. N=10. * Significance $p < 0.01$ compared to control cells**.**

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Figures and Tables

Table 1: Percent larval death (protection) in mice challenged with 20 *B. malayi* L3.

Vaccination Groups	**Mean ± SD live L3s**	**Percent protection**
pVAXBmVAL-1 DNA monovalent homologous	12.2 ± 4.5	39.0% ± 1.7%**
rBmVAL-1 protein monovalent homologous	10.4 ± 3.1	48.0% ± 2.1%*
pVAXBmVAL-1 DNA plus rBmVAL-1 monovalent heterologous	9.2 ± 2.2	54.0% ± 3.1%*
pVAXBmALT-2 DNA monovalent homologous	9.8 ± 2.1	51.0% ± 2.5%*
rBmALT-2 protein monovalent homologous	7.0 ± 1.1	65.0% ± 4.2%*
pVAXBmALT-2 DNA plus rBmALT-2 monovalent heterologous	5.1 ± 0.5	74.5% ± 3.1%*
pVAXBmVAL-1/ALT-2 DNA multivalent homologous	8.6 ± 0.1	57.0% ± 2.2%*
rBmVAL-1/rBmALT-2 protein multivalent homologous	5.2 ± 1.1	74.0% ± 3.3%*
pVAXBmVAL-1/BmALT-2 DNA plus rBmVAL-1/rBmALT-2 multivalent heterologous	4.4 ± 0.4	82.0% ± 2.2%*
pVAX + Alum control	20 ± 0	0%

Significance - * $p < 0.01$, ** $p < 0.05$ compared to control

Table 2: Percent worm reduction (protection) in jirds challenged with 100 *B. malayi* L3. Values were calculated as the percent reduction in worm establishment compared to control jirds that had a total of 39 ± 7 worms established.

Vaccination Groups	**Percent protection**
pVAXBmVAL-1 DNA monovalent homologous	50 ± 3.7%
rBmVAL-1 protein monovalent homologous	40.0 ± 3.1%
pVAXBmVAL-1 DNA plus rBmVAL-1 monovalent heterologous	52.4 ± 2.5%
pVAXBmALT-2 DNA monovalent homologous	58.3 ± 2.1%
rBmALT-2 protein monovalent homologous	72.0 ± 5.5%
pVAXBmALT-2 DNA plus rBmALT-2 monovalent heterologous	78.5 ± 3.2%
pVAXBmVAL-1/ALT-2 DNA multivalent homologous	77.1 ± 2.0%
rBmVAL-1/rBmALT-2 protein multivalent homologous	79.9 ± 3.5%
pVAXBmVAL-1/BmALT-2 DNA plus rBmVAL-1/rBmALT-2 multivalent heterologous	85.0 ± 1.4%
pVAX + Alum control	0%

Significance - $p < 0.01$ compared to control

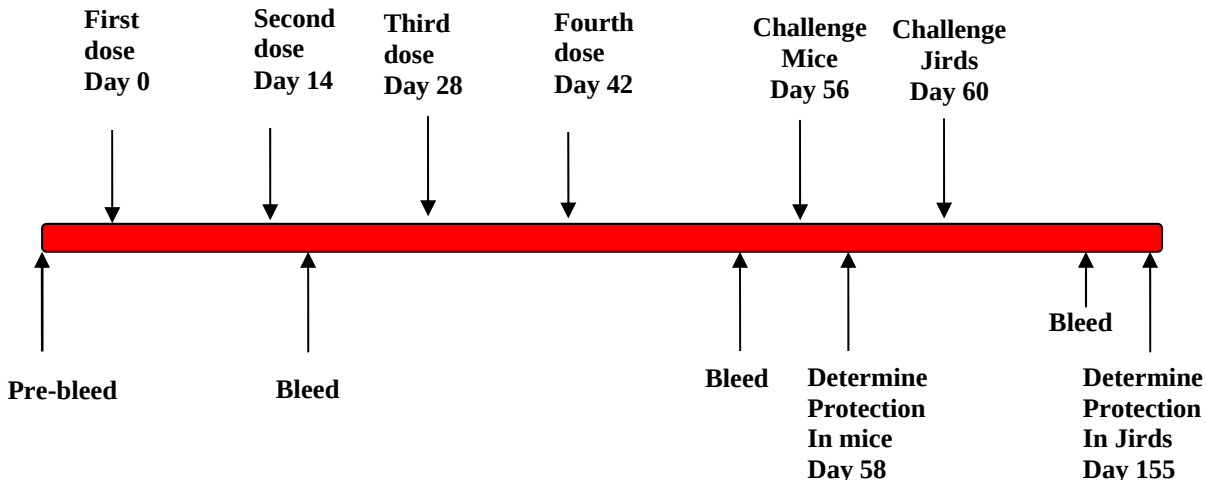

Figure 1. Schematic representation of the vaccination protocol used in mice and jirds

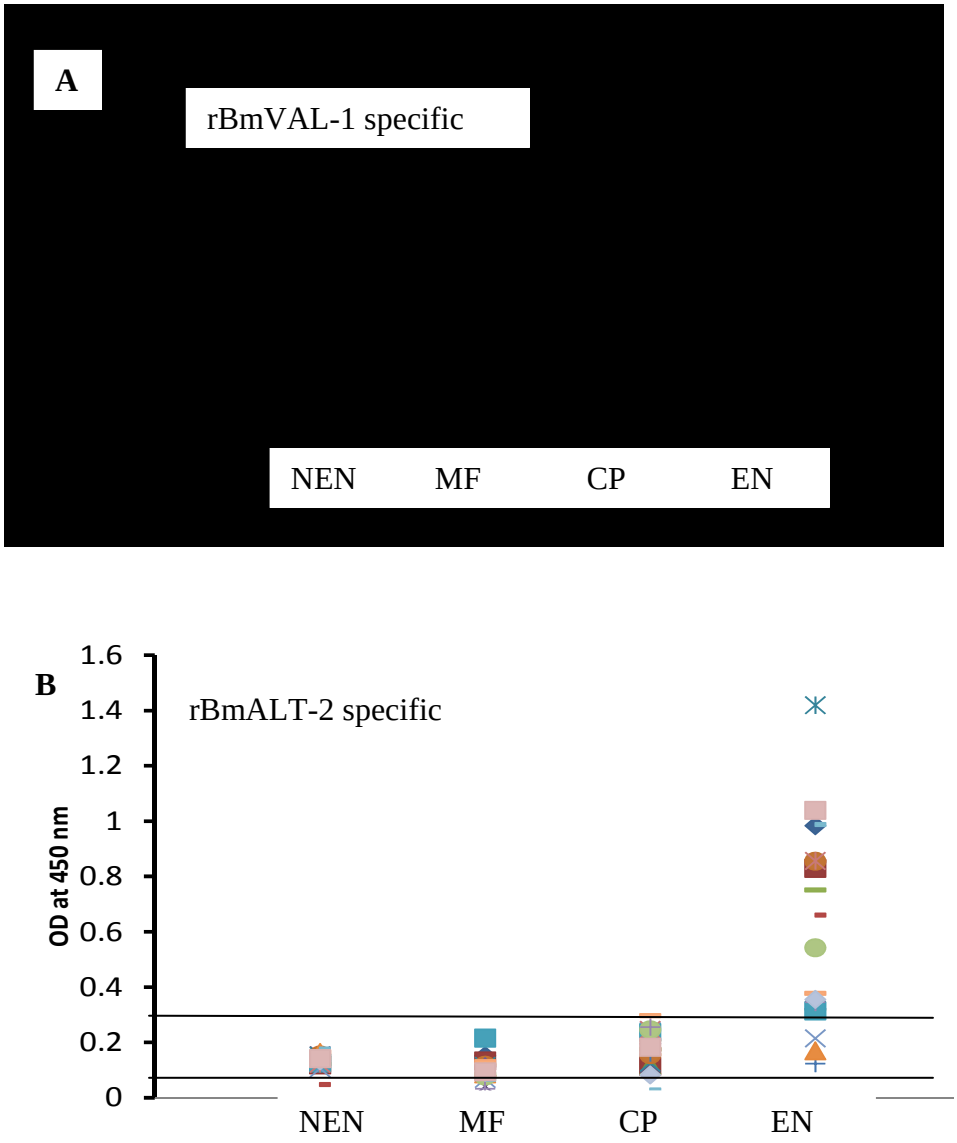

Figure 2A. Titer of BmVAL-1 specific IgG antibodies (A) and BmALT-2 specific IgG antibodies (B) in the sera of human subjects. Titer of antigen specific IgG antibodies were determined in sera samples from MF (n=20), CP (n=20) and EN (n=20) subjects using an ELISA. Sera samples from NEN subjects served as controls. Each spot represent sera sample from one individual. The cut off value (mean OD± 3 times SD of NEN sera) is indicated by a line drawn parallel to X-axis.

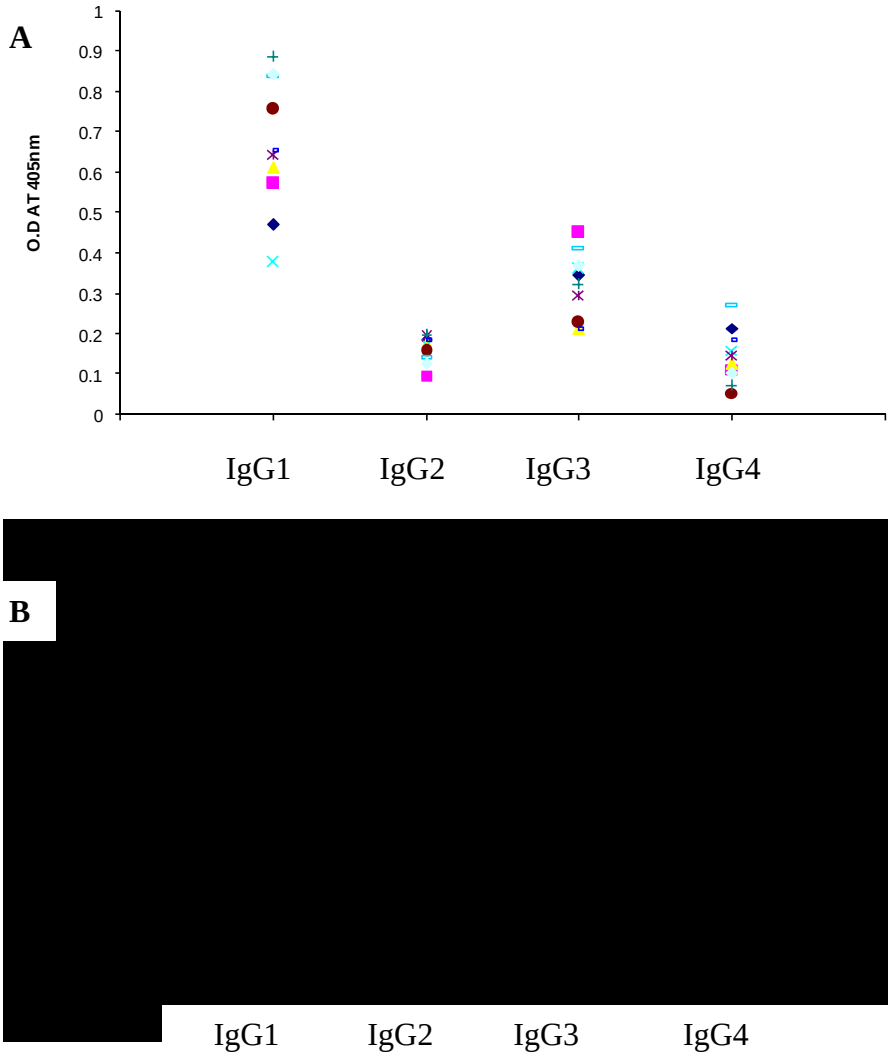

Figure 2B. Titer of IgG isotype specific antibodies against rBmVAL-1 (**A**) and rBmALT-2 (**B**) in the sera samples of endemic normal subjects**.** Values were determined by specific ELISA for each isotype. Each spot represent sera sample from one individual. The IgG isotype antibody patterns against BmVAL-1 and BmALT-2 were comparable in the sera samples. N=20.

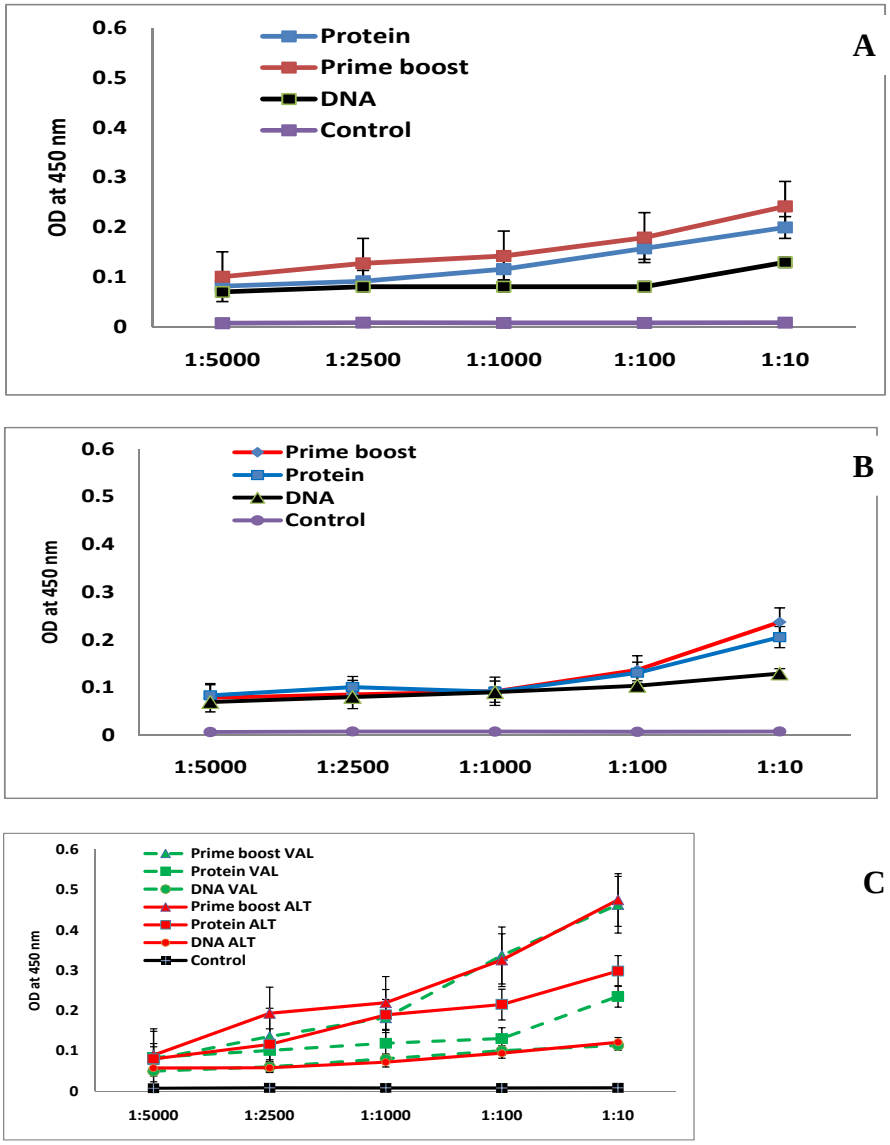

Figure 3. Titer of anti-VAL-1 IgG and anti-ALT-2 IgG antibodies in the sera of immunized mice after 4 immunizations at two weeks interval. IgG levels were measured using an ELISA. **DNA** – DNA vaccinated group, **Protein** – Protein vaccinated group, **Prime boost** – DNA Prime protein boost vaccinated group and **Control** – vector plus alum controls. **Panel A**: Sera collected from mice immunized with monovalent BmVAL-1 vaccine. **Panel B**: Sera collected from mice immunized with monovalent BmALT-2 vaccine. **Panel C**: Sera collected from mice immunized with BmVAL-1/BmALT-2 multivalent vaccine. Dotted lines values for anti-BmVAL-1 IgG, solid lines values for anti-BmALT-2 IgG. N=10. Data represent results from one of two experiments with comparable results.

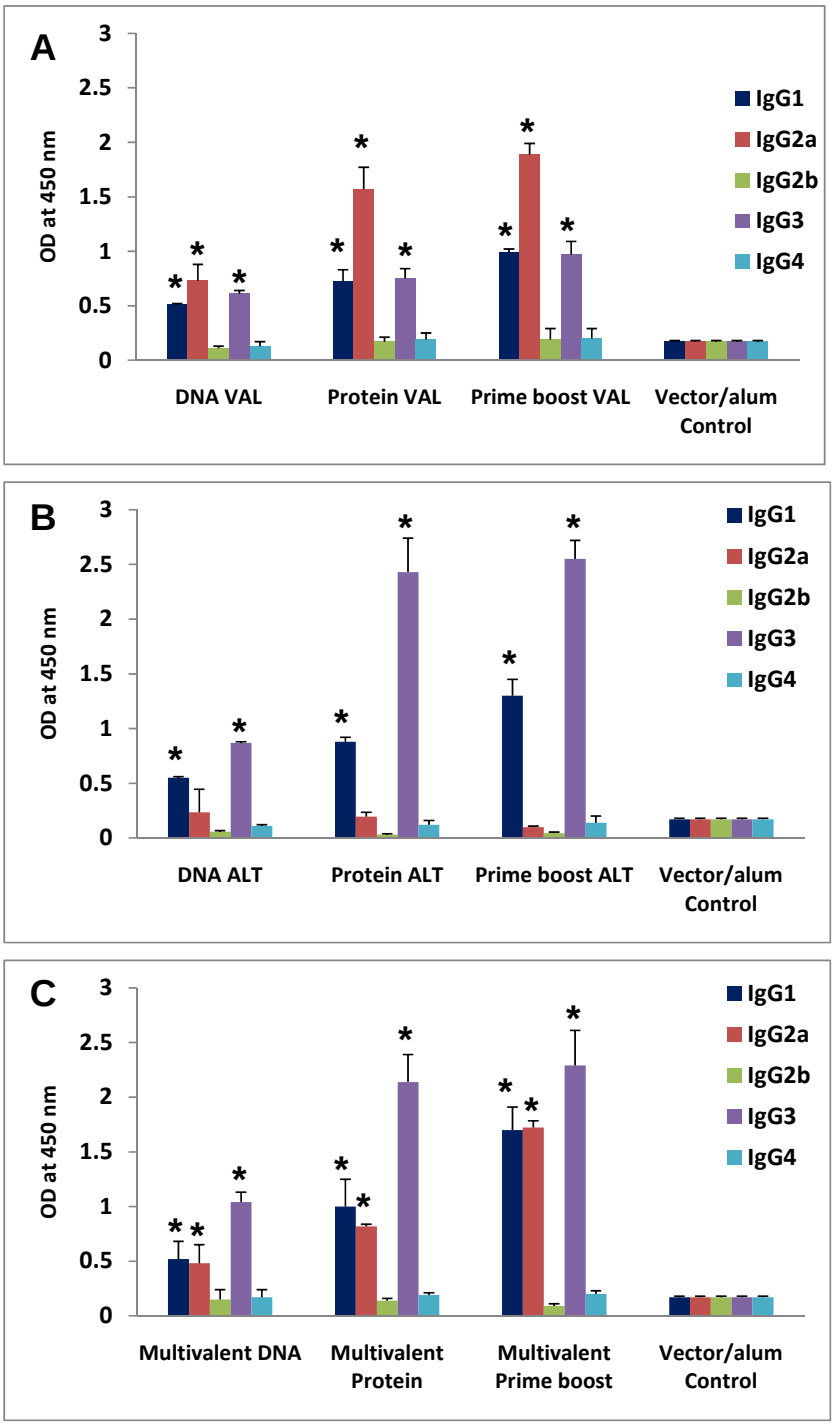

Figure 4. Levels of antiVAL-1 and anti-ALT-2 IgG isotype of antibodies were measured in the sera of immunized mice two weeks after the last immunization. (A) Monovalent BmVAL-1 vaccinated mice, (B) Monovalent BmALT-2 vaccinated mice and (C) multivalent vaccinated mice. N=10. *Significant $p < 0.01$ compared to vector plus alum control group.

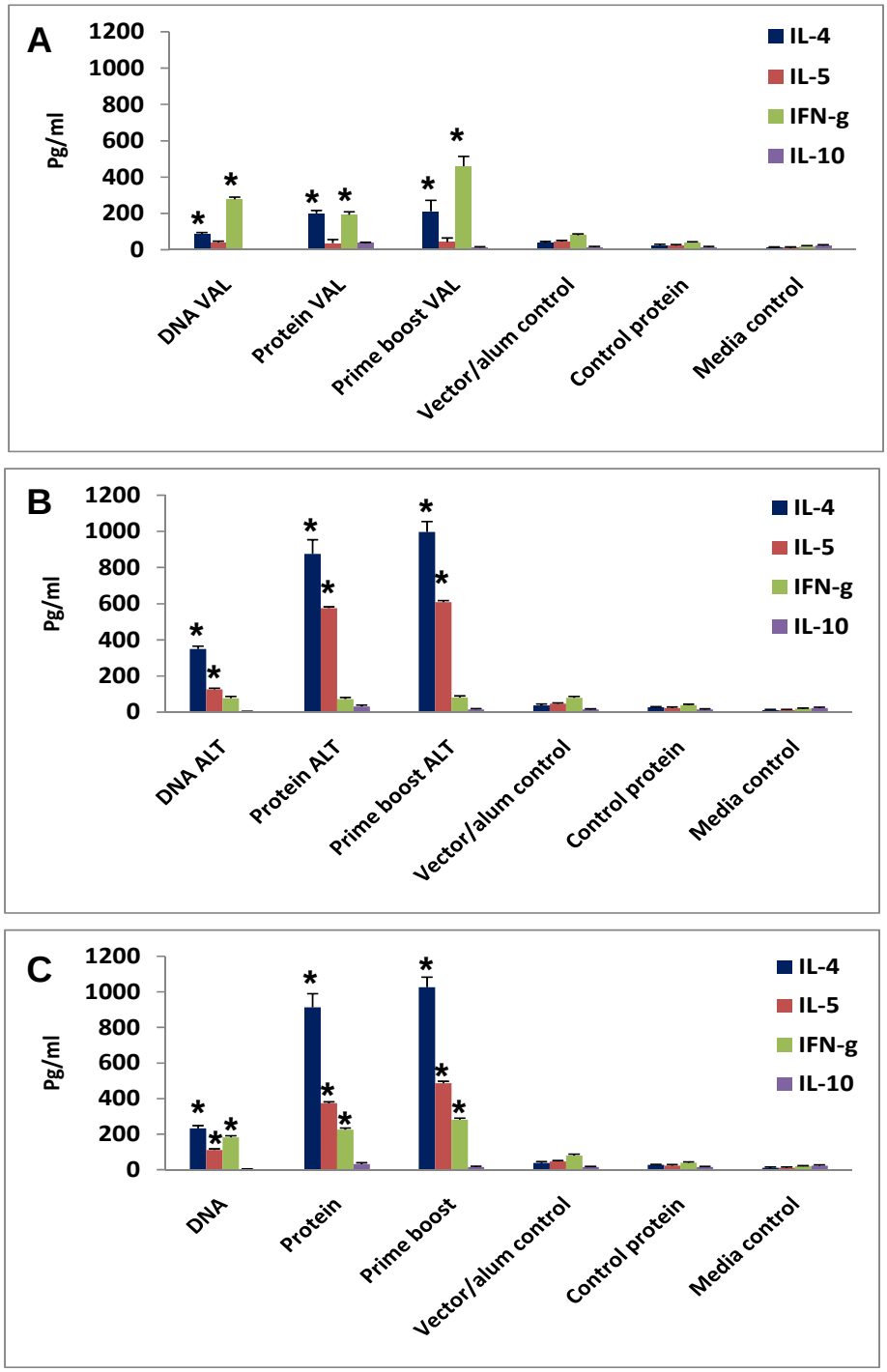

Figure 5. Cytokine responses in the culture supernatants of spleen cells stimulated for 72 hrs with rBmVAL-1 (**A**) rBmALT-2 (**B**) or rBmVAL-1-BmALT-2 hybrid protein (**C**) were measured using an ELISA. Concentrations of each cytokines are represented as pg/ml. spleen cells from vector/alum injected mice served as control. Additional controls include cells incubated in media alone and cells stimulated with a non-specific recombinant protein (SmGBF). Spleen cells from the prime boost group were used for the last two control groups. N=10. * Significance $p < 0.01$ compared to control cells.