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Improving the Efficacy of a Prophylactic Vaccine Formulation against Lymphatic Filariasis

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Abstract

Mass drug administration (MDA) is the current strategy for interrupting the transmission of lymphatic filariasis (LF) infection and control of the disease in endemic areas. However, subject noncompliance has resulted in the presence of several 'transmission hotspots' in the endemic regions threatening the reemergence of LF. This situation is further complicated by the fact that the drugs used in MDA are not effective against adult LF worms, a major concern for the control strategy. Thus, there is clearly a need for an effective and sustainable approach to control LF. Prophylactic vaccine combined with targeted treatment of infected patients and vector control is suggested as a more sustainable strategy to eliminate LF infection from endemic regions. A multivalent vaccine (r*Bm*HAT) developed in our laboratory confered about 90% protection in rodents. However, when we tested the r*Bm*HAT vaccine along with alum in rhesus macaques only about 40% protection was achieved and the immune response obtained was Th2 biased. In an attempt to improve the vaccine, in this study we tested two vaccine antigens (r*Bm*HAT and r*Bm*HAX) along with two adjuvant formulations [alum+GLA (AL019) and mannosylated chitosan (MCA)] in a mouse model. Our results show that r*Bm*HAT is a better vaccine antigen than r*Bm*HAX. Combination of r*Bm*HAT with AL019 or MCA adjuvants gave 94% and 88% protection respectively against challenge infections. Immunized animals developed antigen specific memory T cells that secreted significant levels of IL-4, IFN-γ and IL-17 suggesting the generation of a balanced Th1/Th2 responses following immunization. A major advantage of MCA adjuvant is that the vaccine booster doses can be administered orally. These studies thus showed that r*Bm*HAT is a better vaccine antigen and can be given in combination with AL019 or MCA adjuvant to obtain excellent results.

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Conflict of interest

There are no conflicts of interest for any of the authors.

Ethical approval: Use of animal in this study was approved by the Animal Care Committee of the University of Illinois, Rockford. The study followed the National Institutes of Health guidelines for the care and use of Laboratory animals.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Use of animal in this study was approved by the Animal Care Committee of the University of Illinois, Rockford. The study followed the National Institutes of Health guidelines for the care and use of Laboratory animals. This article does not contain any studies with human participants performed by any of the authors.

Keywords

TLR-4 agonist; MCA; alum; vaccine; adjuvant; lymphatic filariasis; *Brugia malayi*

Introduction

Lymphatic filariasis (LF) is a mosquito transmitted tropical parasitic infection caused mainly by three species of filarial parasites, *Wuchereria bancrofti, Brugia malayi* and *B. timori*. According to the World Health Organization (WHO) currently about 73 countries are considered endemic for LF (WHO 2007). The Global Programme to Eliminate Lymphatic Filariasis (GPELF) was launched in 2000 to eliminate LF by 2020 from the endemic regions using annual mass drug administration (MDA) as a preventive chemotherapy strategy (WHO 2007; WHO 2016). This approach has significantly reduced the incidence of LF in several countries. In fact, China and the Republic of Korea have already declared eliminating LF from these regions in 2007 and 2008 (WHO 2007). However, majority of the endemic regions still face significant roadblocks in stopping the transmission and elimination of LF. Treatment with drugs alone is not effective as a prophylaxis against LF. There is a need for a more sustained approach such as a prophylactic vaccination to stop transmission and eliminate LF from the endemic areas (Dakshinamoorthy et al. 2013c; Jambulingam et al. 2016; Harris et al. 2017). Our laboratory and others have identified several potential vaccine antigens that are shown to confer significant protection against challenge infections in experimental animals (Samykutty et al. 2010; Dakshinamoorthy et al. 2013b, Dakshinamoorthy et al. 2013c; Arumugam et al. 2014). One of our recent trials in non-human primates using a trivalent fusion protein vaccine (r*Bm*HAT) showed that approximately 40% protection could be achieved in vaccinated animals against a challenge infection (Dakshinamoorthy et al. 2012). In these studies we used alum as an adjuvant and the immune responses were predominantly biased towards IgG1/IL-4. However, in naturally immune endemic normal individuals and in immunized rodents the protective immune responses were correlated with balanced Th1/Th2 responses (Dakshinamoorthy et al. 2013a; Dakshinamoorthy et al. 2013c; Dakshinamoorthy et al. 2014). Therefore, there is a need to improve the current vaccine formulation so that a balanced Th1/Th2 response can be achieved following immunization with enhanced protection.

Adjuvants play an important role in enhancing the potency of an antigen and polarizing the immune responses to Th1 or Th2 (Di Pasquale et al. 2015). Among these, alum is the most commonly used adjuvant in the human and veterinary vaccines (Gupta 1998; Marrack et al. 2009). Alum is known to polarize the immune response to a Th2 bias (Marrach et al. 2009). Several other adjuvants such as the monophosphoryl lipid A (Casella and Mitchell 2008), imidazoquinolines (Steinhagen et al. 2010), poly(I:C) (Tewari et al. 2010), CpG motifs (Mohan et al. 2013) and glucopyranosyl lipid A (Coler et al. 2011) are also extensively tested. These adjuvants recognize specific pattern recognizing receptors on immune cells activating both innate and adaptive immune responses against the vaccine antigen (Schnare et al. 2001). Adjuvants containing TLR4 agonist can promote both Th1 and Th2 biased responses towards vaccine antigens (Bortolatto et al. 2008; Didierlaurent et al. 2009; Fox et al. 2010; Arias et al. 2012; Goulopoulou et al. 2016). One of our recent studies showed that

including alum plus a synthetic TLR-4 as an adjuvant for r*Bm*HATαc promoted a Th1/Th2 biased response (Dakshinamoorthy et al. 2013a). Similarly, mannosylated chitosan adjuvant (MCA) is successfully used as an adjuvant to target the mannose receptors on macrophages to stimulate a Th1 biased immune response against the vaccine antigens (Jiang et al. 2008; Carroll et al. 2016). In this study we attempted to evaluate two different adjuvants; alum + GLA (GLA-SE is an emulsion that is not adsorbed to alum, AL019) obtained from Infectious Disease Research Institute (IDRI) and MCA obtained from Pacific GeneTech Inc for their ability to promote Th1/Th2 biased response following vaccination with r*Bm*HAT in mice and determine if a higher percentage of protection can be achieved following a challenge infection with *B. malayi* third stage larvae (L3). The second aspect of the study was to use a different vaccine antigen, r*Bm*HAX. The only difference here is that we replaced the tetraspanin large extracellular loop (TSP) sequence in the r*Bm*HAT with thoredoxin peroxide-2 (TPX-2) to make the r*Bm*HAX multivalent construct. The rationale for this switch is based on our previous publication, where we show that TPX-2 is a potent inducer of Th1 responses (Gnanasekar et al. 2004; Anand et al. 2008) and the percent of protection conferred with r*Bm*TPX-2 is better than r*Bm*TSP (Anand et al. 2008; Dakshinamoorthy et al. 2013b). In the present study we evaluated the vaccine potential of r*Bm*HAX along with AL019 and MCA adjuvants.

Material and methods

Animals and parasites

B. malayi infective third stage larvae (L3) were obtained from the NIAID/NIH Filariasis Research Reagent Resource Centre (University of Georgia, Athens, GA) and Balb/c mice were purchased from Taconic biosciences (Hudson, NY).

Adjuvants

Alum plus synthetic TLR4 agonist GLA (AL019) was purchased from Infectious Disease Research Institute, Seattle, WA and Mannosylated Chitosan (MCA) was a gift from Pacific GeneTech, Hong Kong.

Construction of multivalent gene sequence

Multivalent gene sequences of *bmhat* (consisting of *bmhsp12.6, bmalt-2* and *bmtsp*) and *bmhax* (consisting of *bmhap12.6, bmalt-2 and bmtpx2*) were constructed at Genscript (Piscataway, NJ) using published gene sequences (Dakshinamoorthy et al. 2013c; Dakshinamoorthy et al. 2014).

Cloning, expression and purification of recombinant multivalent fusion proteins

Genscript supplied the sequences in pUC57 vector. The genes were amplified using forward CG**GGATCC**ATGGAAGAAAAGGTAGTG and reverse CG**GAATTC**TCAATCTTTTTGAGATGAAT primers for B*m*HAT and forward CG**GGATCC**ATGGAAGAAAAGGTAGTG and reverse CCC**GAATTC**TTAATGTTTCTCAAAATATGCTTT primers for *Bm*HAX with restriction sites for *BamHI* and *EcoRI*. The PCR amplified products were cloned into pRSETA expression vector, transformed into competent BL21 (DE3) *E. coli* cells for expression of

the recombinant proteins with 6X histidine tag as described previously (Dakshinamoorthy et al. 2013c). Recombinant fusion proteins were purified using immobilized metal affinity Ni^+ charged sepharose column (GE Healthcare Life Sciences, Pittsburg, PA) and eluted with 50 mM–300 mM imidazole. Endotoxin in the final purified protein preparation was removed using an endotoxin removal column (Thermo Fisher Scientific, Rockford, IL). The expression and purity of recombinant proteins were checked in 12% SDS PAGE gel and western blot using anti-his antibodies (Qiagen, Valencia, CA). Protein concentration was determined using a Bradford reagent (Thermo Fisher Scientific).

Immunization of Balb/c mice

Six weeks old male Balb/c mice were randomly divided into six groups with five mice per group. Two groups of mice were immunized three times at two weeks' interval with 15 μg of purified r*Bm*HAT plus 10 μg of one of the adjuvant formulation (AL019 or MCA) and two groups of mice were immunized with 15 μg of r*Bm*HAX plus 10 μg of one of the adjuvant formulation (AL019 or MCA). Two groups of mice served as AL019 or MCA controls. All immunizations with AL019 adjuvants were given s/c route. However, for immunization with MCA, the first immunization was given s/c and booster second and third immunizations were given by oral gavage (Instech, Plymouth meeting, PA). Blood samples were collected by submandibular bleeding (Golde et al. 2005) on day -2, 0, 14, 28 and 42. Sera was separated and stored at −80°C.

Titer of antigen-specific IgG

Titer of antigen specific IgG antibodies was determined by an indirect ELISA as described previously (Dakshinamoorthy et al. 2013c). IgG antibodies in diluted sera samples (1:100, 1:500, 1:1,000, 1:2,000, 1:4,000 and 1:8,000) were detected using biotin-labeled goat anti mouse IgG (BioLegend, San Diego, CA) and color developed using streptavidin conjugated horse radish peroxidase (HRP) and 1-step Ultra TMB-ELISA substrate (Thermo Fisher Scientific). The reaction was stopped using 0.16M H_2SO_4 and optical density at 450 nm was read in a BioTek Synergy2 ELISA reader.

Levels of antigen-specific antibody isotypes in the sera of mice

Levels of antigen specific IgG1, IgG2a, IgG2b, IgG3, IgE, IgM and IgA antibodies against r*Bm*HAT or r*Bm*HAX were determined in the sera of mice using an indirect ELISA as described above using respective isotype-specific HRP labeled antibodies.

Analysis of vaccine-induced protection in mice

Vaccine-induced protection was determined by surgically implanting a micropore chamber containing 20 *B. malayi* L3 into the peritoneal cavity of mouse as described previously (Abraham et al. 1989; Dakshinamoorthy et al. 2013a). 72h after implanting, contents of each chamber were examined using a light microscope at 400X for larval viability as described previously (Joseph and Ramaswamy 2013). Larvae that appeared transparent and straight with no movement were counted as dead. Live larvae were active, coiled and translucent. Percentage protection was calculated using the formula: (Number of dead parasites/Number of recovered parasites) X 100.

Splenocyte proliferation and flow cytometric analysis

Two weeks after the last immunization, spleens were collected and single cell suspension was prepared. Cells at a concentration of 1×10^6/ml were incubated with 5mM CFSE (BioLegend) in the dark for 20 minutes at 37°C. After washing the cells were stimulated for five days with 1μg/ml of the respective antigen. Cells treated with concanavalin-A or media alone remained as controls. Following incubation cells were stained with APC labeled anti-CD3 antibody (BioLegend) and the proliferating T cell populations were determined on a BD FACScalibur flow cytometer and data analyzed using cell quest software v6.1.2.

Flow cytometric analysis for cell surface markers

1×10^6/ml of splenocytes stimulated with 1 μg/ml of respective antigens (r*Bm*HAT or r*Bm*HAX), ConA or media alone were stained with combinations of CD3-APC/CCR7-FITC/CD62LPE and analyzed on a BD FACScalibur flow cytometer after gating the cells for CD3. Subpopulation of cells that are double positive for CCR7 and CD62L were counted as central memory T cells (T_{CM}) and cells double negative for CCR7 and CD62L were counted as effector memory T cells (T_{EM}). Data was analyzed using cell quest software v6.1.2.

Secreted levels of cytokines in the culture supernatants of antigen-stimulated splenocytes

1×10^6 splenocytes in 1 ml were stimulated with 1μg of r*Bm*HAT or r*Bm*HAX for 72h at 37°C. Culture supernatants were collected and secreted levels of IFNγ, IL-2, IL-4, IL-10 and IL-17 were determined using a Cytokine Bead Array (BD Biosciences) as described previously (Dakshinamoorthy et al. 2013a).

Statistical analysis

GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA) was used to analyze the data. Comparison between two individual data points was made using Student's t-test. For multiple comparisons, one way ANOVA was used along with the Tukey-Kramer and/or Dunnet's post-test wherever appropriate. For cytokine analysis two-way ANOVA was used with Bonferronipost test. A probability (P) value of <0.05 was considered statistically significant.

Results

Cloning and expression of multivalent fusion proteins

Purified recombinant proteins separated on an SDS PAGE gel showed a prominent band at 39 kDa for r*Bm*HAT and a prominent band at 52 kDa for r*Bm*HAX (Fig 1). Endotoxin levels in the final purified preparations were <10 EU/μg of the vaccine protein.

Immunization with r*Bm*HAT along with AL019 or MCA gave the highest level of protection against challenge infections

In vivo challenge experiments showed that compared to AL019 adjuvant controls that had only 5±11.2% larval death, mice immunized with r*Bm*HAT plus AL019 showed 94±13.4% ($p<0.0001$) larval death and mice immunized with r*Bm*HAX plus AL019 showed 67.5±17% ($p<0.0001$) larval death (Fig 2a). Comparison between r*Bm*HAT and r*Bm*HAX

immunization showed that significantly higher protection ($p<0.0236$) was observed in r*Bm*HAT immunized animals (Fig 2a). Most of the dead larva had cells attached to their surface (Fig 2b). The number of cells attached to the larval surface varied, some L3 had very few cells attached to the anterior or posterior end, whereas, some L3 were totally covered with the cells. In this study, we did not evaluate the type of cells that were attached to the L3s.

Similarly, when MCA was used as the adjuvant for r*Bm*HAT or r*Bm*HAX immunizations, 87.88±14.42% ($p<0.0001$) and 55.5±22.8% ($p<0.0006$) larval death respectively were observed compared to MCA adjuvant controls where no larval death occurred (0±0%). Larval death in r*Bm*HAT plus MCA immunized mice was significantly high ($p<0.0332$) compared to those in r*Bm*HAX plus MCA immunized mice. These results thus show that r*Bm*HAT is a better vaccine antigen than r*Bm*HAX. AL019 was found to be a better adjuvant for r*Bm*HAT immunizations in mice. The most interesting finding in this study was the significant protection that we observed when MCA was used as the adjuvant along with r*Bm*HAT, clearly showing that oral booster doses of MCA adjuvanated r*Bm*HAT were immunogenic.

Immune correlates of protection after immunization

Mice immunized with r*Bm*HAT or r*Bm*HAX developed high titer of antigen-specific IgG antibodies—Titer of IgG antibodies against each of the recombinant antigens was determined in the sera of immunized mice using an indirect ELISA. Results showed that high titer of antigen-specific IgG antibodies were generated in all vaccinated mice irrespective of the adjuvant used. AL019 adjuvanated r*Bm*HAT and AL019 adjuvanated r*Bm*HAX gave the highest IgG titer of 1:8,000 (Fig 3). Despite the fact that booster doses of MCA adjuvanated r*Bm*HAT or MCA adjuvanated r*Bm*HAX were given orally, these mice also developed significant titer (1:4,000) of antigen-specific IgG antibodies. Antigen-specific IgG titer was significantly high ($p<0.0318$) in r*Bm*HAT plus AL019 immunized animals compared to the other vaccinated groups. These findings thus suggested that any of the two adjuvants (AL019 or MCA) could be used along with the recombinant antigens to trigger significant antigen-specific IgG antibody titers in mice.

Antigen-specific IgG1, IgG2a, IgG2b antibodies were elevated in the sera of vaccinated mice—To determine the pattern of humoral immune response generated following vaccination, we determined the levels of antigen-specific IgG1, IgG2a, IgG2b, IgG3, IgM, IgE and IgA antibodies in the sera of mice vaccinated with r*Bm*HAT or r*Bm*HAX in combination with different adjuvant formulations. Our results showed that compared to preimmune sera samples, antigen-specific IgG1 ($p<0.0001$), IgG2a ($p<0.0001$) and IgG2b ($p<0.0138$) antibodies were significantly high in the sera of all vaccinated animals (Fig 4). Levels of IgG1 did not show any significant differences between the vaccinated groups (Fig 4). However, levels of IgG2a ($p<0.0016$) and IgG2b ($p<0.0016$) was significantly high in r*Bm*HAT plus AL019 immunized animals compared to r*Bm*HAT plus MCA immunized animals suggesting that AL019 may be a slightly better compared to MCA in promoting a balanced Th1/Th2 humoral response to r*Bm*HAT in mice. Nevertheless, both the adjuvants can clearly promote a balanced Th1/Th2 response in mice. Levels of IgM, IgE

and IgA antibodies were not significantly different from the controls in all vaccinated animals (Fig 4).

Spleen cells from vaccinated animals showed antigen specific recall response—Spleen cells from all vaccinated animals proliferated significantly ($P<0.001$) in response to the antigen stimulation compared to the adjuvant control groups. Spleen cells from the vaccinated group (r*Bm*HAT+AL019, r*Bm*HAT+MCA, r*Bm*HAX+AL019 and r*Bm*HAX +MCA) divided three times as indicated by 4 clear CFSE peaks in all the vaccinated mice compared to the unstimulated and adjuvant controls, which had only 1 CFSE peak (data not shown). These results suggest that antigen-responding cells were present in the spleen of vaccinated mice.

Antigen-responding spleen cells of vaccinated animals predominantly secreted both Th1 and Th2 cytokines—Secreted levels of cytokines in the culture supernatants of antigen-stimulated spleen cells were measured using a cytokine bead array. Our results show that cells from all vaccinated animals secreted significantly high ($p<0.0001$) levels of IL-2, IL-4, IL-10, IL-17 and IFN-γ compared to controls (Fig 5). Cells from rBmHAT plus AL019 secreted significant ($p<0.0001$) levels of IL-4, IFN-γ, IL-10 and IL-17. Cells from r*Bm*HAX plus AL019 immunized mice predominantly secreted IL-4 and levels of IL-10 and IL-17 were not significant compared to the controls. MCA was more efficient in promoting IFN-γ secreting cells irrespective of the antigen used. These results confirmed our results from the antibody responses that both AL019 and MCA can promote balanced Th1/Th2 cytokine responses to vaccine antigens in immunized mice.

Immunization with r*Bm*HAT generated T_{EM} cells in the spleen—Spleen cells were incubated with 1 μg/ml of r*Bm*HAT or r*Bm*HAX for 5 days at 37°C. Following incubation the cells were stained with CD3/CCR7/CD62L and evaluated in a flow cytometer. Cells were first gated for CD3 and the CCR7+ and CD62L+ sub population of cells within the CD3 was determined. Majority of the CD3 cells were $CD62L^{low}$ and $CCR7^{low}$ suggesting effector memory T cells. Compared to AL019 or MCA controls, r*Bm*HAT and r*Bm*HAX immunized animals had significantly high ($p<0.0001$) number of T_{EM} and T_{CM} cells. r*Bm*HAT immunized animals had significantly high number of T_{EM} ($p<0.0061$) and T_{CM} ($p<0.0061$) cells compared to r*Bm*HAX immunized animals in their spleen (Fig 6).

Discussion

An effective prophylactic vaccine against LF can support the effort towards control and total elimination of LF from a community (Ramaswamy 2016). Subjects living in an endemic region can develop natural immunity and carry protective antibodies against the infective stage of LF. These naturally immune individuals are called ‘Endemic Normals (EN)” (Day 1991). Using a phage-display based cDNA expression library of the parasite, sera of EN subjects were screened to identify several potential vaccine antigens that recognized the protective antibodies (Gnanasekar et al. 2004). Subsequent evaluation of these antigens especially as a multivalent formulation (r*Bm*HAT) gave the highest (92%) rate of protection in rodent models (Thirugnanam et al. 2007; Samykutty et al. 2010; Kalyanasundaram and Balumuri 2011; Dakshinamoorthy et al. 2013a; Dakshinamoorthy et al. 2013c; Arumugam

et al. 2014). However, the same vaccine with alum adjuvant gave only about 40% protection in rhesus macaques (Dakshinamoorthy et al. 2014) and the immune response generated was predominantly a Th2 biased with little or no Th1 response. Thus, there is a critical need to improve the vaccine formulation. Results presented in this study show that inclusion of alum + GLA (AL019) or MCA as an adjuvant for r*Bm*HAT promoted balanced Th1/Th2 responses and significantly improved the vaccine-induced protection.

We were able to express and purify both r*Bm*HAT and r*Bm*HAX with minimal endotoxin contamination. Following immunization, both the antigens elicited significant levels of IgG antibodies irrespective of the adjuvant used for immunization. Vaccine antigen plus AL019 was given subcutaneously. However, for immunizations with MCA adjuvant, only the first dose of immunization was given subcutaneously. The rest of the booster doses were given orally. Despite giving the booster immunization as an oral dose, comparable levels of IgG antibody titer was generated in these vaccinated animals. These findings were similar to those observed by Carroll et al. (2016), who also demonstrated induction of significant cellular immunity and type 1 interferons following the use of MCA as an adjuvant in mice. Ability to deliver the vaccine orally is potentially a major advantage of using MCA as adjuvant especially in clinical setting where the vaccine booster doses can be given orally to children. Further studies are needed to determine if all the immunization doses with MCA adjuvant can be given orally and still achieve the high IgG antibody titer. Protection studies showed that r*Bm*HAT is a better vaccine antigen than r*Bm*HAX (94% vs 67%). Compared to our previous studies, inclusion of AL019 as an adjuvant for r*Bm*HAT immunization was found to be slightly better (94% vs 92% protection) (Samykutty et al. 2010, Dakshinamoorthy et al. 2013a; Dakshinamoorthy et al. 2013c). We did not include alum adjuvant group in this study. Nevertheless, nearly all our previous studies used alum as an adjuvant for r*Bm*HAT and consistently we observed approximately 94% protection against challenge infection in the mouse model. The present study showed that protection obtained with the r*Bm*HAT + AL019 formulation was comparable or slightly better to the r*Bm*HAT + Alum formulation. As far as the vaccine-induced immune responses, r*Bm*HAT + AL019 were considerably better in inducing a balanced Th1/Th2 responses compared to our previously reported (Dakshinamoorthy et al. 2014) vaccine-induced immune responses with r*Bm*HAT + Alum. One of our earlier vaccination trails in the mouse model suggested that significant protection can be achieved in the mouse model with the vaccine protein alone and no adjuvant (Dakshinamoorthy et al. 2014). However, when we tested the protein alone formulation in the rhesus macaque, all five animals became positive (unpublished data) indicating that the protein alone formulation is not protective in non-human primates. Given these findings, we decided not to pursue the ‘no adjuvant’ formulation for further vaccine development.

Several of our previous studies showed that the mechanism of parasite killing in r*Bm*HAT vaccinated animals involve antibody dependent cell mediated cytotoxicity (ADCC) mechanism. Thus, both cells and antibodies are critical for the killing of larvae. In this study also we observed numerous cells attached to the surface of the dead larvae confirming our previous observations (Dakshinamoorthy et al. 2013c; Dakshinamoorthy et al. 2014). In this study we did not analyze the larvae bound cell population, however, one of our ongoing studies show that the bound cells are largely macrophages and produce activation products

such as myeloperoxidase. Larvae incubated in sera samples from control animals had no cells attached to them.

Analyses of the antigen-specific antibody responses generated following vaccination with r*Bm*HAT showed that IgG1, IgG2a and IgG2b isotype were predominant suggesting a balanced Th1 and Th2 responses. These findings were further confirmed when the cytokine responses of spleen cells were analyzed. Spleen cells from vaccinated animals secreted significant amounts of IL-4 and IFN-γ in response to r*Bm*HAT stimulation. This observation further confirmed the generation of a balanced Th1/Th2 response to the antigens in the vaccinated animals. Increases in IL-17 have been shown to be critical for the vaccine-induced protection in several systems (Lin et al. 2010, Habets et al. 2016). Thus, an increase in the levels of secreted IL-17 in the culture supernatants of spleen cells from vaccinated animals suggests a role for IL-17 secreting cells in the vaccine-induced protection to r*Bm*HAT. The spleen T cell population from vaccinated animals also contained both effector memory and central memory T cells phenotypes. Taken together these findings suggest that significant humoral and cellular protective immune responses were generated against the vaccine antigens. Our studies also confirmed that AL019 and MCA adjuvants can promote a balanced Th1/Th2 response to vaccine antigens in mice. Our previous vaccination trials using r*Bm*HAT in macaque gave only ~40% protection (Dakshnamoorthy et al. 2014). These poor results were attributed to lack of induction of Th1 responses following vaccination. The fact that both AL019 and MCA can promote balanced Th1/Th2 responses suggests that any of these two adjuvants can be tested in the macaque model to improve the vaccine-induced protection to r*Bm*HAT. Previous studies showed that vaccination using r*Bm*HAT was safe in the macaque model and the monkeys did not develop any IgE responses to r*Bm*HAT Dakshinamoorthy et al. 2014). In the current vaccination trial also there was no IgE responses to the vaccine antigens in the mice when given along with AL019 or MCA adjuvant.

In conclusion, our present study showed that including AL019 or MCA as adjuvant along with r*Bm*HAT vaccination significantly improved the rate of protection and the immune responses generated were a balanced Th1/Th2 response. Further studies are planned to test the r*Bm*HAT plus AL019 or MCA adjuvant in the macaque model.

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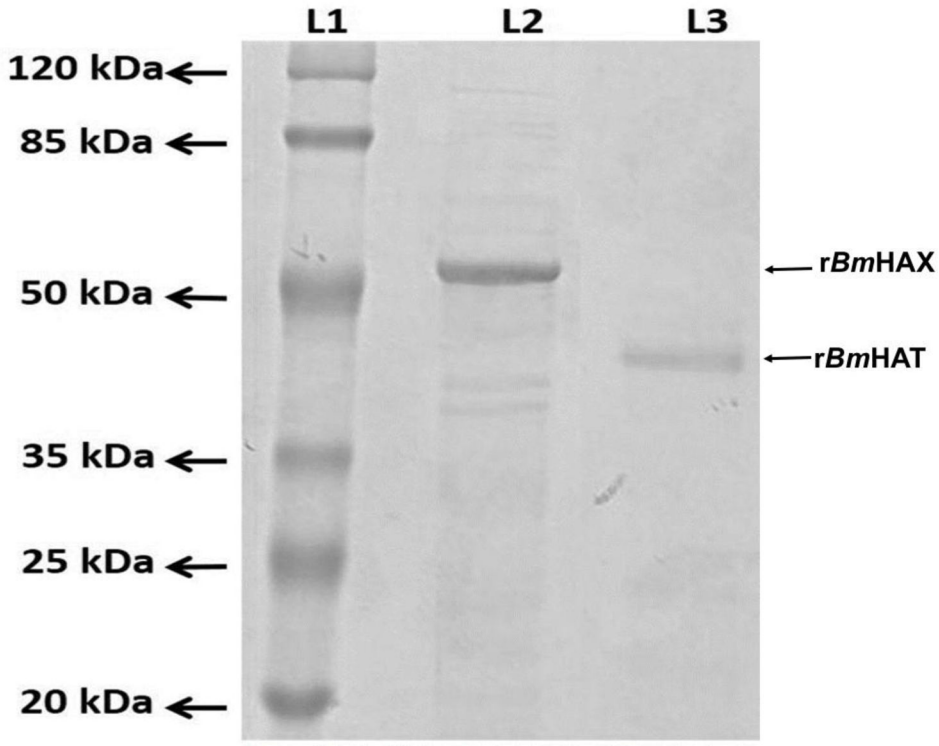

Figure 1. Expression of r*Bm*HAX and r*Bm*HAT

The multivalent genes *bmhax* (1320 bp) and *bmhat* (957 bp) were amplified and cloned into the expression vector pRSETA and successfully expressed in *E. coli* BL21 (DE3). Both the expressed proteins were purified by immobilized metal affinity chromatography (IMAC) and purity confirmed by western blot analysis using anti-His antibodies. Purified r*Bm*HAX and r*Bm*HAT were separated on a 12% SDS PAGE gel. Lane 1- Molecular weight marker, Lane 2- r*Bm*HAX (a prominent band at 52 kDa) and Lane 3 - r*Bm*HAT (a prominent band at 39 kDa).

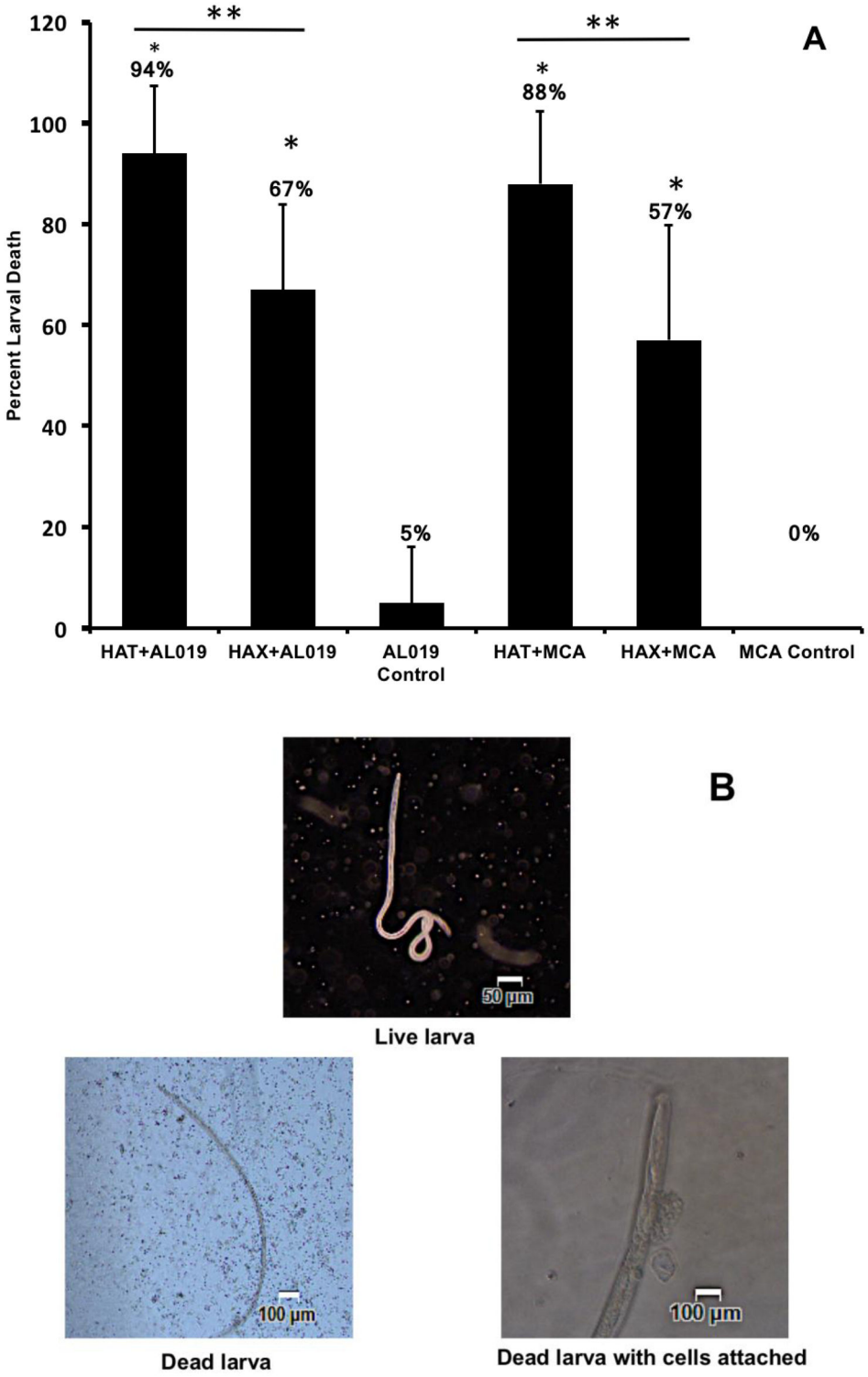

Figure 2. Percent protection in vaccinated animals was calculated by determining the percent larval death

Figure 2A. Approximately 20 live *B. malayi* L3s sealed in a micropore chamber was surgically implanted into the peritoneal cavity of mice. 72 hrs after implanting the chambers

were removed and the number of live and dead larvae was counted and percent larval death determined. Compared to the controls, there was significant larval death in vaccinated animals (Fig 2A). The highest percent of larval death (expressed as protection) was observed in mice immunized with r*Bm*HAT. Comparison between r*Bm*HAT and r*Bm*HAX immunization plus AL019 adjuvant showed that significantly higher protection ($p<0.0236$) was observed in r*Bm*HAT + AL019 immunized animals. Similarly, larval death in r*Bm*HAT plus MCA immunized mice was significantly higher ($p<0.0332$) compared to those in r*Bm*HAX plus MCA immunized mice. ***Figure 2B.*** Several cells were found attached to the dead larvae in the vaccinated animals (bottom panel). However, no cells were found attached to the live larvae collected from control animals. Magnification bars are indicated in each photomicrograph. n=10, statistically significant *$p<0.0001$ and **$p<0.0006$.

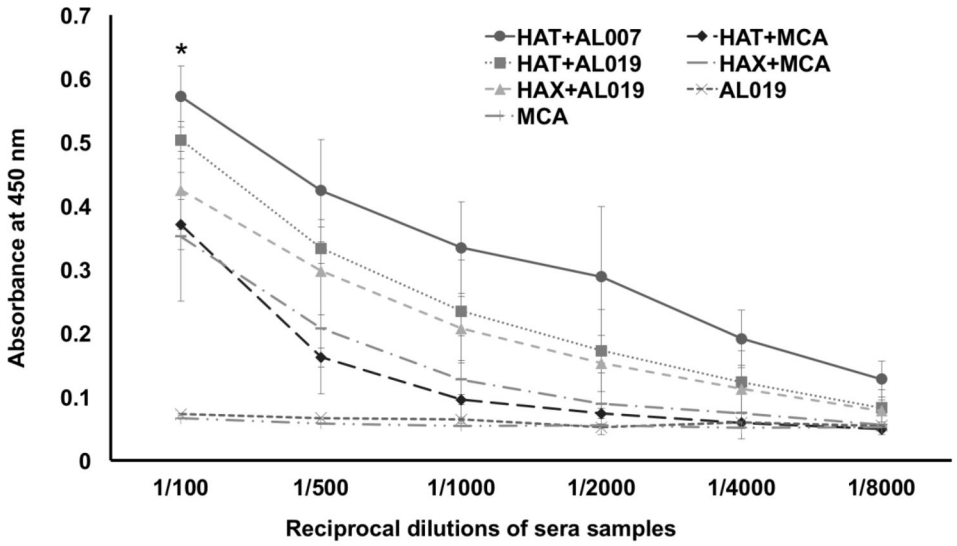

Figure 3. Titer of antigen-specific IgG antibodies in the sera of immunized mice

An indirect ELISA was used to determine the titer of anti-r*Bm*HAT and anti-r*Bm*HAX IgG antibodies in the sera of immunized animals. Pre-immune sera and sera from control mice were used as the baseline controls in these assays. Results showed that vaccination with r*Bm*HAT and r*Bm*HAX induced high titer of IgG antibodies. AL019 adjuvant appeared to be slightly better adjuvant than MCA in promoting antigen-specific IgG responses. It is interesting to note that the booster doses of MCA adjuvanated r*Bm*HAT and r*Bm*HAX were given orally, yet comparable titers of IgG antibodies were generated in immunized mice. n=10 *significant $p<0.01$.

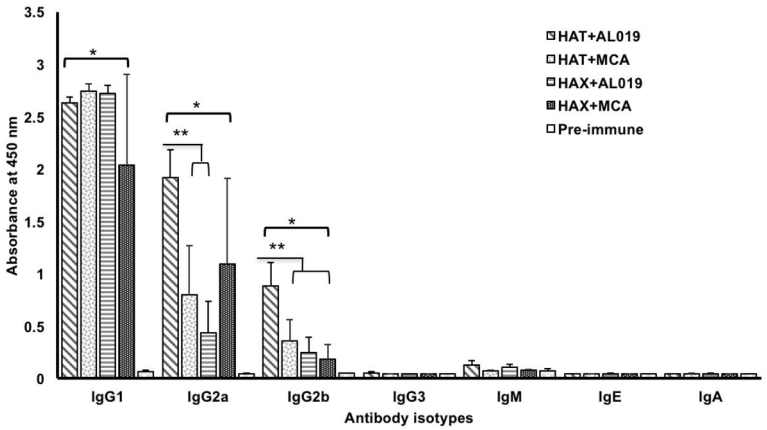

Figure 4. Levels of antigen-specific antibody isotypes

in the sera of immunized mice were determined using an indirect ELISA. Results show that significant levels of antigen-specific IgG1>IgG2a>IgG2b antibodies (in that order of abundance) were present in the sera of all immunized mice compared to the adjuvant controls. Levels of IgG1 were not significant between the vaccinated groups. However, levels of IgG2a and IgG2b were significantly high in r*Bm*HAT plus AL019 immunized animals compared to r*Bm*HAT plus MCA immunized animals. The most predominant antibody isotypes were IgG1, IgG2a and IgG2b suggesting that a balanced Th1/Th2 response was generated following immunization with the antigens. Levels of IgE, IgA and IgM were not significantly different from the controls. n=10. *Statistically significant $p<0.0001$, ** $p<0.0016$.

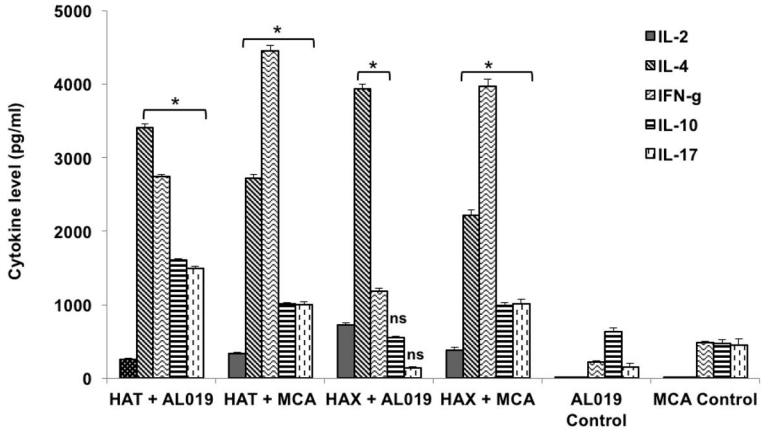

Figure 5. Levels of secreted cytokines in the culture supernatants of spleen cells

stimulated with respective vaccine antigens. 1×10^6 spleen cells were stimulated with 1 μg/ml of r*Bm*HAT or r*Bm*HAX for 72 hrs at 37°C. Levels of secreted cytokines in the culture supernatants were determined using a BD cytokine bead array. Results show that spleen cells from vaccinated mice secreted significantly high levels of both IL-4 and IFN-γ compared to respective AL019 or MCA controls. MCA was more efficient in promoting IFN-γ secreting cells irrespective of the antigen used. However, cells from mice immunized with r*Bm*HAX plus AL019 predominantly secreted IL-4. Levels of secreted IL-17 and IL-10 were also high in the culture supernatants of spleen cells from r*Bm*HAT (with AL019 or MCA) and r*Bm*HAX plus MCA vaccinated animals. These results suggested that spleen cells from both r*Bm*HAT and r*Bm*HAX vaccinated animals secreted cytokines with a balanced Th1/Th2 cytokine pattern irrespective of the adjuvants used. n=5, *Significant $p<0.0001$ compared to adjuvant controls. **ns**. not significant.

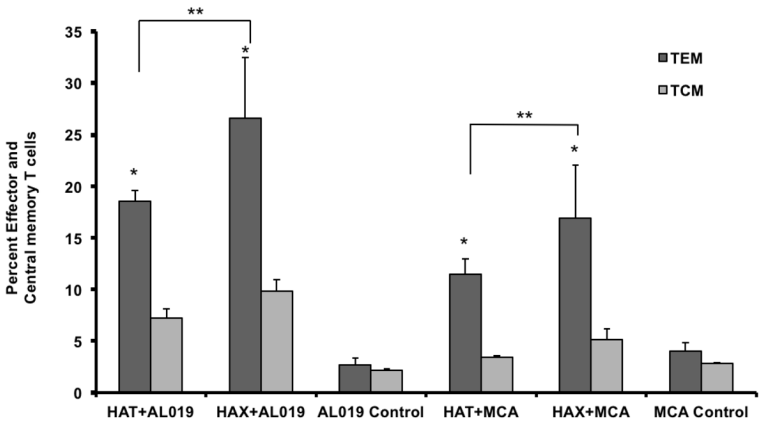

Figure 6. Memory T cell populations

in the spleen of mice vaccinated with r*Bm*HAT or r*Bm*HAX. Isolated spleen cell were stimulated with respective antigen for five days and stained with CD3, CD62L and CCR7 and was evaluated by flow cytometry. CD3+CD62L-CCR7- cell population were counted as effector memory T cells and CD3+ CD62L+CCR7+ cells were counted as central memory T cells. Significant numbers of T_{EM} and T_{CM} T cell population were present in the spleen of vaccinated animals. r*Bm*HAT immunized animals had slightly higher number of memory cells than r*Bm*HAX immunized animals. n=5. *Statistically significant ($p<0.001$) compared to controls, **Significant ($p<0.0.0061$) between the vaccinated groups.