A Vaccine Composed of Antigen Fused to a Ligand for a Receptor on Immature Dendritic Cells Elicits Protective Antibody Responses to Malaria Sporozoites in Infant Macaques

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Abstract

Infants and young children are the groups at greatest risk for severe disease and mortality following acquisition of *Plasmodium falciparum* infection. Recent large clinical trials with a candidate malaria vaccine have demonstrated very limited protection of only short duration. We have previously demonstrated in mice that a protein vaccine in which the chemokine macrophage inflammatory protein 3α is genetically fused to the minimally truncated circumsporozoite protein of *P. falciparum* (MCSP*)* elicits extended high level protection against sporozoite challenge in a mouse model system. In the current study we determined that the research grade formulation of a clinically approved vaccine adjuvant, MF59, elicited greater antibody responses in mice than the previously employed adjuvant, poly(I:C). Use of the MF59 vaccine also expanded the range of IgG subtypes elicited by vaccination. Two immunizations of infant rhesus macaques at one and two months of age with the MCSP/MF59 regimen elicited antibody concentrations that were sustained for 18 weeks at levels that provided passive protection of mice challenged with large sporozoite inocula. The efficacy of this vaccine in a relevant non-human primate model indicates its potential usefulness for the analogous high risk human population.

The World Health Organization reported in 2017 that after many years of decline in malaria incidence, the number of estimated cases increased from 211 million in 2015 to 216 million in 2016 [1]. This increase, and emerging evidence of increased resistance to antimalarial drugs [2,3], indicate the continued need for cost-effective strategies to control the epidemic. The development of an effective malaria vaccine would obviously address this need. However, despite clear evidence from animal model systems that sufficient concentrations of specific antibody can protect against malaria infection, candidate vaccines tested in human populations have failed to achieve and sustain the levels of protection expected from an effective vaccine. The inadequacy of the observed protection has been particularly apparent in the infants and young children who are at greatest risk of severe malaria infection [4].

We have previously reported that combining the experimental adjuvant polyinosine-polycytidylic acid (poly (I:C)) with a vaccine platform targeting the circumsporozoite protein of the *Plasmodium falciparum* parasite (PfCSP) yielded a protective response that, in a mouse challenge model system, was sustained for 22 weeks following the final immunization, which was the latest time point tested. Protection sustained over that period of time had not previously been reported in malaria mouse challenge models. Our vaccine platform consists of a protein in which the chemokine Macrophage Inflammatory Protein 3 Alpha (MIP3α), also known as Chemokine (C-C Motif) Ligand 20 (CCL20) has been genetically fused to the PfCSP antigen. This fusion product has two functions: 1) To target the vaccine antigen to the C-C Motif Chemokine Receptor 6 (CCR6) protein present on the surface of the immature dendritic cells (iDC) that initiate the adaptive immune response [5,6] and 2) To attract immune cells to the site of immunization [7,8]. Previous studies demonstrated marked

enhancement of the antibody response compared to that observed with vaccine constructs not employing the chemokine component Luo, 2014 #2978;Luo, 2017 #3226].

In the current study we have compared in the mouse model system the immune responses and protection observed using clinically approved adjuvants in addition to the the poly (I:C) adjuvant previously demonstrated to be effective. These studies indicated that the clinically approved adjuvants elicited more profound antibody responses than poly (I:C). We then examined in a pilot study the ability of this vaccine construct used with a clinically approved adjuvant to elicit protective immune responses in one and six month old macaques, representing the age groups in humans most vulnerable to severe malaria infection. Our studies indicate that this vaccine construct is highly immunogenic in infant and juvenile macaques.

2. Materials and Methods

2.1 Animals

Six- to eight-week-old female C57BL/6 (H-2b) mice were purchased from Charles River Laboratory (Charles River Laboratories, Inc, Wilmington, MA) and maintained in a pathogen-free micro-isolation facility in accordance with the National Institutes of Health guidelines for the humane use of laboratory animals. One and six months Rhesus Macaques were housed at the Johns Hopkins University Research Farm. All experimental procedures involving mice and monkeys were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University and determined to be in accordance with the guidelines outlined in the Animal Welfare Act, federal regulations, and the Guide for the Care and Use of Laboratory Animals.

2.2 Construction, expression, production and purification of recombinant proteins

Recombinant *P. falciparum* CSP (3D7) and CSP fused with human MIP-3α proteins were prepared as previously described [8]. The codon-optimized CSP DNA sequence containing deletions of the N-terminal 20 aa signaling sequence and the C-terminal 23

aa anchor region fused with human MIP-3α was used to express MIP-3α-CSP chimeric protein (MCSP). Twenty-two NANP repeats were included in the truncated CSP. CSP and MCSP sequences were cloned into pET-47b (Novagen Inc., Madison, WI) fused with 6XHis. The proteins were expressed in BL21 DE3 competent cells (New England Biolabs, Ipswich, MA). Protein purification was undertaken using nickel-affinity chromatography (Qiagen, Valencia, CA, USA) and endotoxin was removed by two-phase extraction with Triton X-114 [9]. Protein concentration was measured by Bradford assay (BioRad, Hercules, CA). Endotoxin concentration was determined using the ToxinSensorTM Chromogenic LAL Endotoxin Assay Kit (Genscript, NJ). All protein used for immunization had final endotoxin levels below 10EU/ml.

2.3 Animal immunization

C57BL/6 mice were immunized with 20 μg (50 μl) purified MCSP in PBS with the adjuvant polyinosinic-polycytitdlic acid (Poly (I:C)), Aluminium hydroxide gel (Alum), , or the squalene-based oil-in-water nano-emulsion (MF59, available in research grade as Addavax). All adjuvants were obtained from Invivogen, San Diego, CA. For Poly (I:C), Alum or MF59 formulation, 50 μg adjuvant was mixed with 50 μl antigen, and the final volume ratio was 1:1. The mixture was administered intramuscularly in both anterior tibialis muscles in a total volume of 50 μl per leg. Mice were immunized twice at a 3 week interval. Rhesus Macaques were immunized with 50 μg or 250 μg purified MCSP in 250 μl PBS. The antigen was mixed with same volume of MF59 and administered intramuscularly in both leg muscles in a total volume of 250 μl per leg. Macaques were immunized according to schedules described in the Results section.

2.4 Antibody titer by ELISA

Humoral immune responses to the immunodominant B cell epitope of the relevant CSP protein were measured using a previously described method [Luo, 2014 #. Briefly,

ELISA plates were coated with 2 μg of recombinant PfCSP overnight at room temperature (RT). Plates were blocked with 2% BSA for 30 min at RT and then serially diluted serum samples were added and incubated for 2 h. After washing six times, peroxidase labeled goat anti-mouse IgG or goat anti-human IgG antibodies (Santa Cruz Biotechnology Inc, Dallas, TX) was added at a dilution of 1:4,000 and incubated at RT for 1 h. After washing six times, ABTS Peroxidase substrate (KPL, Gaithersburg, MD) was added for development and incubated for 1 h. The data were collected using the Synergy HT (BioTek Instruments, Inc, Winooski, VT). The endpoint ELISA titer is reported as the highest dilution of serum at which the average absorbance was twice the value obtained using pre-immunization serum.

2.5 Parasites for challenge

Transgenic *P. berghei* sporozoites [Persson, 2002 #3069] were used for challenge. Sporozoites were obtained by hand dissection of salivary glands of *Anopheles stephensi* mosquitoes maintained in the Johns Hopkins Malaria Research Institute insectary. The isolated sporozoites were suspended in HBSS medium containing 1% normal mouse serum. Challenges to evaluate vaccine effect on hepatic parasite load were accomplished by tail vein injection of 5×10^3 sporozoites. In one experiment, mice were challenged with exposure to five infectious mosquitoes for 10 minutes.

2.6 Real-time PCR for liver stage parasites

Approximately 40 hours after sporozoite challenge, livers of the mice were harvested and homogenized, and RNA was extracted. After reverse transcription (Applied Biosystems, Foster city, CA), Real-time PCR was used for the detection and quantification of the liver stage of Plasmodium parasites. Two pairs of specific primers were designed to amplify the parasite 18s rRNA sequence. The forward primer was 5'-TGGGAGATTGGTTTTGACGTTTATGT-3' and the reverse primer was 5'-AAGCATTAAATAAAGCGAATACATCCTTAC-3'. Values were normalized against

measurements of mouse actin mRNA in the same samples. The primers used for mouse actin were as follows: 5'-GTCCCTCACCCTCCCAAAAG-3' (forward) and 5'-GCTGCCTCAACACCTCAACCC-3' (reverse). The reactions were performed in a final volume of 20 µl using SYBR green PCR Master Mix (2X) from Applied Biosystems and processed with ABI StepOne Real-time PCR system (Applied Biosystems).

2.7 Passive antibody or serum transfer

Rhesus Macaques were immunized with 50 or 250 µg MCSP and 250 µl MF59 at the indicated frequencies and intervals (see Results Section). Four weeks after the second immunization, macaque blood was collected and serum was isolated. All samples were pooled and some of the sera were used to obtain purified IgG, using protein A affinity chromatography (GE Healthcare, NJ). IgG from pooled samples that were collected from the macaques before immunization was purified as the control for passive antibody transfer. C57BL/6 mice were injected I.V. with 200µl purified anti-CSP IgG or immune sera or control IgG or sera. Thirty minutes following injection of antibody or sera, mice were inoculated via tail vein with 5×10^3 transgenic *P. berghei* sporozoites. Liver stage sporozoite copy number was measured 48h following infection.

2.8 Statistical analysis

Linear regression analysis was used to compare the antibody concentration curves from mice receiving different adjuvants. An ANOVA analysis was used to compare antibody concentrations at given time points and liver sporozoite copy numbers following challenge. Prism statistical analysis and graphing software (GraphPad Software, Inc., San Diego, CA) was used for all studies.

Results

Evaluating the efficicacy of different adjuvants in enhancing the anti-CSP humoral immune response. While vaccine adjuvant development is an active research area, only

alum and MF59 have been approved by the US FDA for use in commonly employed parenterally administered vaccines. We first compared in C57Bl/6 mice the CSP-specific humoral immune responses elicited by the MCSP vaccine in combination with these two adjuvants and the previously studied poly (I:C) [8]. The results (Figure 1) indicate that the most profound response is elicited by MF59, with a mean OD_{405} value significantly greater than that observed with poly (I:C) at all time points after the priming immunization ($p<0.001$ for all points). However only at time points at or beyond 13 weeks after the primary immunization was the MF59 response significantly greater than that observed with use of the alum adjuvant ($p\leq0.05$).

We next examined the impact of the different adjuvants on the IgG subtype of the elicited antibody response (Figure 2). For each adjuvant there were significant differences in the magnitude of the responses among the different subclasses ($p<0.05$), except for IgG3 where the alum and poly(I:C) responses were statistically equivalent ($p=0.12$). Although the responses observed with the use of each of the different adjuvants was statistically distinct, the most striking difference among the adjuvants was the markedly reduced IgG2b and IgG2c responses observed with use of the alum adjuvant.

Efficacy of MIP3α-CSP vaccine combined with different adjuvants in protecting mice from mosquito bite challenge. To characterize at 22 weeks post-the final of two immunizations the protective efficacy of the different combinations of adjuvant and vaccine, immunized or non-immune C57Bl6 mice were challenged by exposure for 10 minutes to 5 *Anopheles stephensi* mosquitoes infected with transgenic *P. berghei* sporozoites. Sporozoite copy numbers determined by qRT-PCR indicated no statistically significant difference among the adjuvant groups in the protective efficacy elicited (Figure 3). However all groups receiving vaccine plus adjuvant had highly

significant reductions of at least 78% in the liver sporozoite copy number compared to unimmunized mice.

Immunogenicity of MCSP vaccine and MF59 adjuvant in one month old macaques. Although protection achieved by vaccination of C57Bl/6 mice challenged at 22 weeks did not differ significantly between the MF59 and alum adjuvants that are used in FDA approved vaccines, the consistently higher antibody concentrations achieved with MF59 and its ability to elicit a broader subclass response led to its selection for use in subsequent studies. One issue in vaccine development is whether responses in mice can be recapitulated in the clinical setting. Non-human primates, particularly Old World primates such as rhesus macaques, have immune systems that are most homologous to humans [10-14].. To address the immunogenicity of this vaccine construct in a setting relevant to the groups at high risk from malaria, we initiated an immunization protocol in infant, one month old, and juvenile, six month old, macaques, using one of two dosing regimens. In each of the age groups two macaques received a priming 50 µg dose of the MCSP vaccine construct and 250 µg of the MF59 adjuvant followed by two subsequent immunizations with the same dosing at monthly intervals. The two other macaques in each age group macaques received a priming immunization with 250 µg of vaccine and 250 µg of the MF59 adjuvant followed one month later by a second immunization using the same vaccine and adjuvant dose. Figure 4 displays the antibody responses, expressed as reciprocal ELISA titers, maintained over 25 weeks post the initial immunization. All of the infant macaques, independent of the immunization regimen, maintained protective concentrations of antibody, as demonstrated in the following section, for 22 weeks after the initiation of the immunization regimen.

Passive protection by immune macaque sera in a mouse challenge model. To evaluate the protective capability of the different antibody concentrations achieved in the Rhesus macaques, immune macaque sera or purified immune macaque IgG was passively transferred to naïve C57Bl/6 mice that were challenged via intravenous inoculation with transgenic *P. berghei* sporozoites expressing *P. falciparum* CSP. This challenge model was used because there are no macaque pre-erythrocytic challenge models for *P. falciparum*. The IgG or sera were administered in volumes and concentrations to achieve in the mice a titer equivalent to the titers achieved in macaques. Typically an intravenous injection of 200 µl of macaque IgG or sera of a given concentration resulted in an 8-fold dilution of that concentration in the sera of the mice (data not shown). Control mice received pre-immune macaque sera or IgG in volumes equivalent to that received by recipients of immune sera or IgG. As indicated in Figure 5, the mice with reciprocal anti-CSP ELISA titers as low as 64,000 still had significant reduction in their liver sporozoite load.

Discussion

In the current study, we have examined in C57Bl/6 mice and infant and juvenile Rhesus macaques the efficacy of a malaria vaccine platform that had previously demonstrated, using a different adjuvant, extended protection efficacy in a murine malaria challenge system. A distinctive feature of the vaccine platform is its fusion of vaccine antigen to the chemokine MIP3α, which serves as the ligand for CCR6, expressed predominantly on iDC. The MF59 adjuvant selected for study in the macaques was chosen based on studies in the mouse model system and the fact that it has been approved as an adjuvant for clinical use with other vaccines by the US FDA.

The vaccine construct and adjuvant used in these studies target the earliest steps in the encounter between the host immune system and the foreign antigen. Those steps include the attraction of antigen presenting cells to the site of immunization, attributable to the chemoattractant properties of both MF59 [15] and MIP3α [16], and the efficient targeting of antigen to the CCR6 receptor on iDC that are typically poorly represented at immunization sites, despite the use of adjuvants [17-20]. Previous studies with this vaccine used with the adjuvant poly(I:C) have demonstrated the independent chemoattractant activity of each component, but also synergistic chemoattractant ability when both the MIP3α construct and the adjuvant were employed [8,20].

Studies of the mechanism of action of MF59 have shown that it also promotes the differentiation of monocytes into cells that bear the iDC phenotype and that it promotes release of chemokines such as CCL2 (MCP-1), CCL3 (MIP-1α), or CCL4 (MIP-1β) that would be expected to attract additional iDC to the vaccination site. Further, MF59 enhanced expression of CCR7, which is implicated in the migration of DC from peripheral tissues to lymph nodes [15]. These specific immunoenhancing activities of MF59 position it well to complement the activity of an iDC targeting vaccine. While the combination of MF59 and the MCSP construct did not, under the experimental conditions of this study, elicit higher levels of protection in the mouse model system compared to vaccine plus other adjuvants, the antibody concentrations attained were uniformly higher than those attained with use of alum or poly (I:C) and those differences may be relevant in the clinical setting.

Further, vaccination with the MF59 adjuvant also resulted in significantly higher concentrations of IgG2b and IgG2c in the sera of immunized mice, particularly when compared to alum, the other clinically approved adjuvant. There are conflicting data on the importance of IgG isotype in resistance to malaria infection. Aucun *et al.* showed that elevated levels of human IgG2 specific for blood stage antigens were associated

with resistance to malaria infection in Burkina Faso [21], while Aribot *et al.* found that only concentrations of IgG3 specific for blood stage antigens were associated with reduced risk of infection in Senegal [22]. Studies with the RTS,S/AS01E vaccine in Mozambique and Ghana found protection against sporozoite mediated infection positively associated with concentrations of IgG1 and IgG3 antibodies specific for vaccine antigens [23]. These human antibody isotypes are important in mediating antibody-dependent cellular cytotoxicity and complement dependent killing, as are the mouse IgG 2b and 2c isotypes elicited by MF59, but not alum, in the current study. While this finding suggests further justification for the use of the MF59 adjuvant in subsequent vaccine trials, Potocnjak *et al.* found in mouse challenge models that monovalent fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen could protect mice against sporozoite challenge at concentrations roughly equivalent to protective concentrations of fully intact antibodies [24]. Whether the broader isotype response achieved with MF59 provides additional protection will, therefore, require further study.

A recent study of responses in humans to a candidate HIV-1 vaccine found that MF59 elicited higher responses in human infants than those observed in adults [25]. This observation is consistent with our findings. Even with the small sample size of our pilot study, the maximum antibody response obtained seven weeks after the primary immunization, three weeks after the second immunization, was significantly higher in macaques initially immunized at one month of age compared to those initially immunized at six months of age ($p=0.03$). Importantly the HIV infant immunization study demonstrated that the higher responses observed in infants, while unexplained, was not simply attributable to differences in the per body weight dose of vaccine. Two different dosing regimens were used in our macaque immunization studies, but across both age groups dose did not significantly affect the magnitude of the antibody response at 7

weeks (p=0.13), suggesting that an immunogenicity threshold had been reached with the 50 µg vaccine dose.

Macaques were chosen for our nonhuman primate study because their immune ontogeny more closely parallels that of humans than does that of rodents or that of the New World monkeys that have been used as non-human primate challenge models of malaria bloodstream infection [10-14]. A recent study comparing the immunogenicity of two formulations of a candidate sporozoite-targeted vaccine demonstrated that different conclusions might be reached using mouse vs macaque models for analysis of CSP immunogenicity [26]. Pre-erythrocytic stage *P. falciparum* challenge models using macaques have been unsuccessful, and efforts to develop *Aotus* monkey based challenge systems have also proved problematic. Reports from two laboratories indicated success with sporozoite transmission in this species [27,28]. However, for sporozoite transmission to Aotus monkeys, splenectomy is required and, according to these reports, successful transmission occurs with varying frequency, achieves highly variable levels of parasitemia, has wide variation in the pre-patent period, is frequently accompanied by clearing of parasitemia in the non-immune host and requires use of parasites that have been obtained from relatively recent human infection for best results [27]. No studies using this model have been published since 2009.

To evaluate the protective efficacy of the antibody concentrations attained with macaque immunization we determined the quantity of passively transferred IgG or serum to be passively administered to duplicate the concentrations in macaques. Purified and concentrated IgG was required to reproduce in the mice the higher concentrations observed in the macaques. Passively immunized mice were challenged with inocula of transgenic *P. berghei* sporozoites, far exceeding that which would be encountered in the clinical setting, to ensure liver infection at a level that would facilitate comparison of the efficacy of the different concentrations.

The results indicate that this vaccine formulation, which employs clinically approved adjuvants, can elicit protective concentrations of antibody in infant and juvenile macaques. Further studies are required to identify the immunization protocols that will be optimal for eliciting sustained protective immunity in this immunological setting, which closely parallels that of humans at greatest risk for adverse outcomes following malaria infection.

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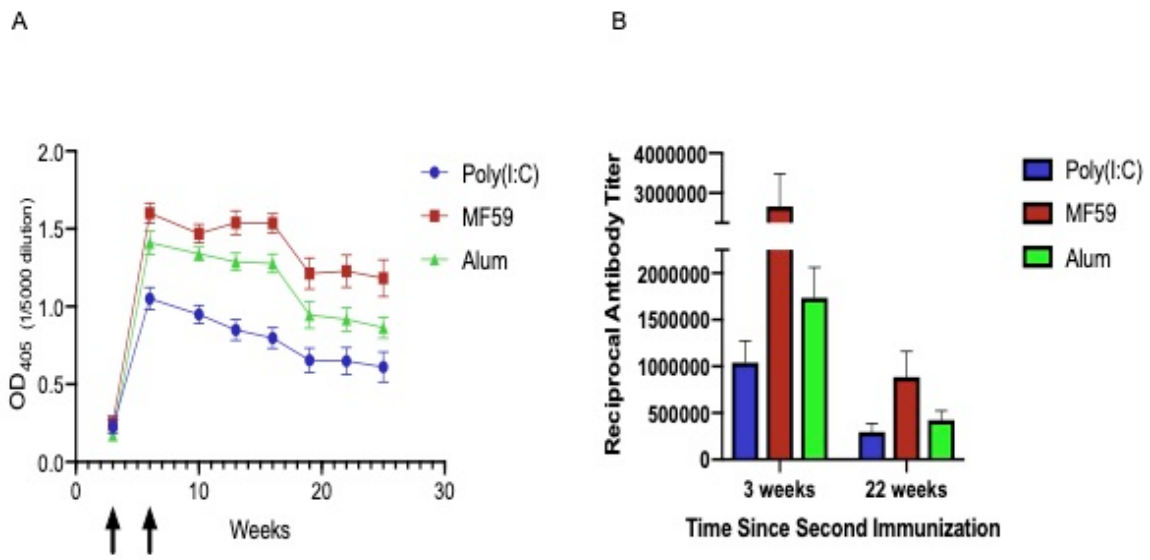

Fig.1 Relative concentrations and slope of decline after two immunizations of anti-CSP antibody with use of different adjuvants. Groups of 15 C57BL/6 mice were immunized twice with 20 µg MCSP plus the indicated adjuvants at a three week interval. Mice were bled at the indicated time points after the last immunization to determine specific antibody levels. Values shown represent absorbance in an ELISA assay at OD_{405}nm after a 1/5000 serum dilution (A) and the reciprocal of the endpoint ELISA titer at 3 weeks and 22 weeks after the last immunization (B). The endpoint titer is reported as the highest dilution of serum at which the absorbance was twice the value obtained using pre-immunization serum. By linear regression analysis of antibody response curves in (A), the slopes of decline in antibody concentrations from the six week time point for all of the adjuvants do not differ significantly ($p>0.05$) from each other, although the difference in slopes between MF59 and poly (I:C) approach significance ($p=.0538$). However, the y intercept for antibody concentration curves differ significantly ($p<0.0001$) between all groups. The O.D. levels using MF59 is significantly greater in individual comparisons between MF59 and alum or poly (I:C) at all time points after 13 weeks

($p \leq 0.05$) (A). In Figure (B) only the difference in antibody titers between MF59 and poly (I:C) is significant ($p \leq 0.03$) at both 3 and 22 weeks after the final immunization.

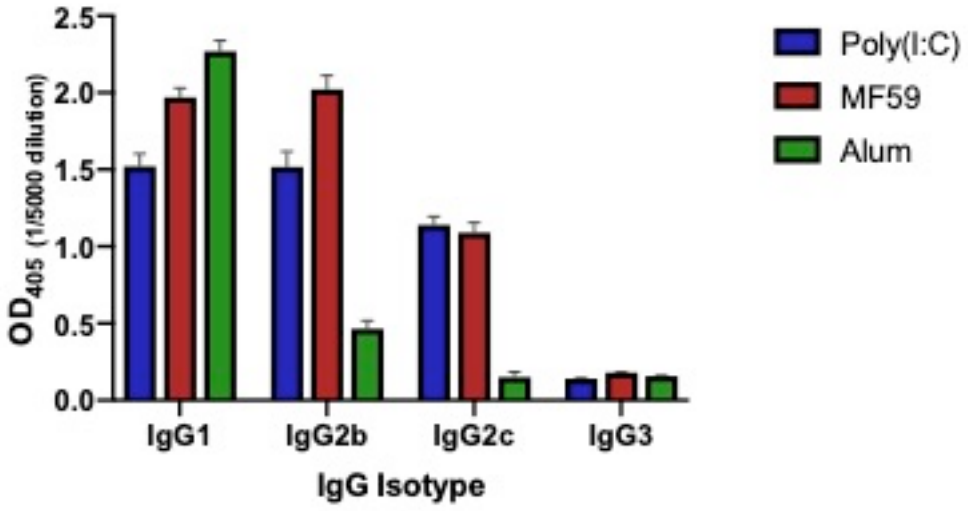

Fig. 2 Generation of anti-CSP antibody of different isotypes after immunization with different adjuvants. C57BL/6 mice were immunized twice with 20 µg MCSP plus different adjuvants at a three week interval. Mouse sera were obtained 3 weeks after the last immunization for ELISA, performed as in Fig. 1, except that isotype specific secondary antibodies were used to determine concentrations of IgG1, IgG2b, IgG2c and IgG3. Within each subtype the results comparing the different adjuvants differ significantly ($p \leq 0.02$) for all comparisons except MF59 vs. poly (I:C) for IgG2c, which did not differ significantly.

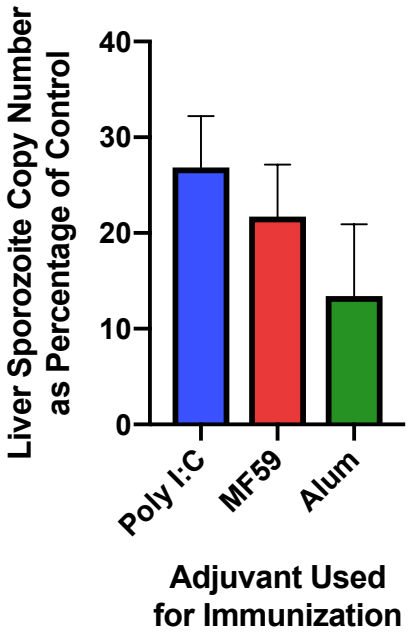

Fig. 3 Protection of immunized C57Bl/6 mice immunized with different adjuvants from challenge by bites of mosquitoes carrying transgenic *P. berghei.* C57BL/6 mice were immunized twice with 20 µg MCSP plus different adjuvants at a three week interval. Twenty-two weeks after the final immunization, mice were exposed for 10 minutes to 5 mosquitos infected with transgenic *P. berghei* sporozoites. Parasite-specific rRNA levels in the liver were determined by quantitative RT-PCR on samples obtained 48 hours post challenge. All qPCR results were normalized against the expression of mouse β-actin. Percentage reduction in liver sporozoite copy numbers did not differ significantly among immunized mice ($p>0.5$) and all immunized groups differed significantly from control mice ($p<0.001$).

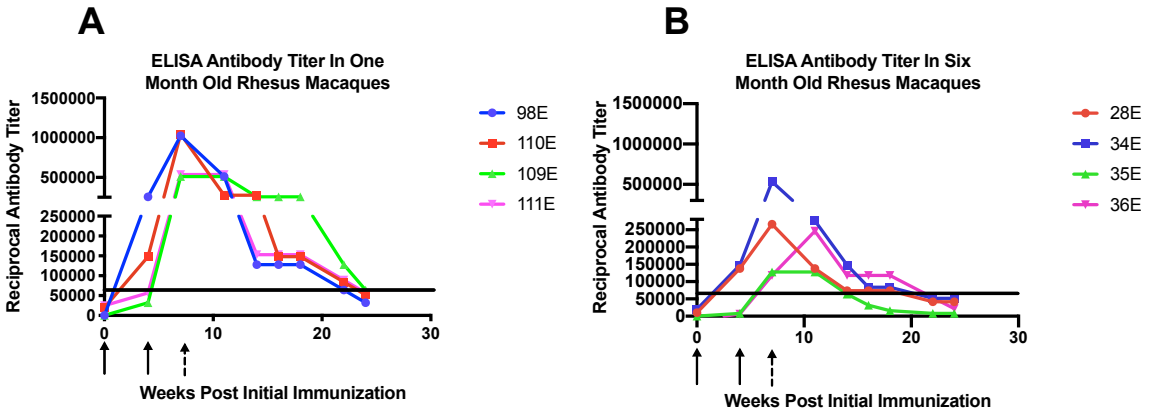

Fig.4. Response of One and Six Month Old Rhesus Macaques to Different MCSP + MF59 Immunization Regimens. A) One month old macaques 98E and 110E received two immunizations with 250 µg MCSP + 250 µg MF59, indicated by solid arrows. One month old macaques 109E and 111E received three immunizations with 50 µg MCSP + 250 µg MF59 (solid arrows for first two immunizations and broken arrow for the third immunization). B) Six month old macaques received similar regimens: 28E and 34E received two immunizations with 250 µg MCSP + 250 µg MF59, indicated by solid arrows. Macaques 35E and 36E received three immunizations with 50 µg MCSP + 250 µg MF59 (solid arrows for first two immunizations and broken arrow for the third immunization). For both figures, the solid black line indicates reciprocal antibody titer associated with protection in C57Bl/6 mice passively administered sera from immunized macaques . Positive titer = 2x the pre-immunization control. At 7 weeks after the primary immunization, at the point of greatest response, the antibody concentrations were significantly higher in the macaques initially immunized at one month of age compared to those initially immunized at 6 months of age (p=0.03).

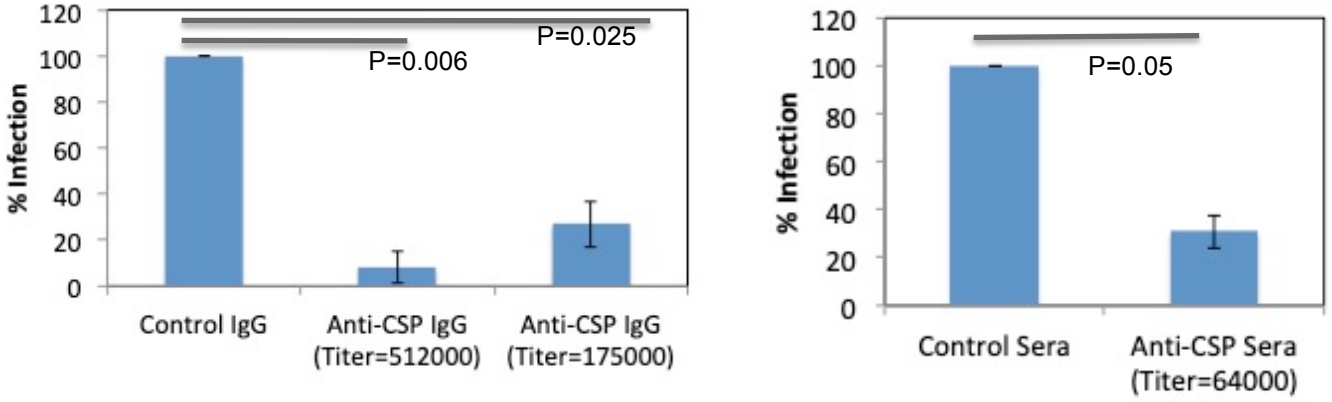

Fig. 5 Protection of C57Bl/6 mouse recipients of CSP-immune sera from immunized infant macaques against intravenous challenge with transgenic *P. berghei expressing P. falciparum CSP* C57BL/6 mice were injected i.v. with anti-*Pf* CSP IgG (A) that was purified from sera of MCSP/MF59 immunized monkeys or (B) sera of MCSP/MF59 immunized monkeys. Groups of control mice were injected with IgG purified from sera obtained from macaques prior to immunization or with directly with the pre-immunization sera. 30 minutes following injection of IgG or sera, mice were infected with 5×10^3 transgenic *P. berghei* sporozoites expressing Pf CSP by i.v. injection. Parasite-specific 18S rRNA levels in the liver were determined by qPCR on samples obtained 48 h post challenge. The average of 18S rRNA level in control groups was set as 100%.