

Liposomes containing monophosphoryl lipid A and QS-21 serve as an effective adjuvant for soluble circumsporozoite protein malaria vaccine FMP013



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ARTICLE INFO

Article history:

Received 7 November 2016

Received in revised form 3 May 2017

Accepted 24 May 2017

Available online 7 June 2017

Keywords:

Adjuvant

Malaria

Vaccine

Liposomes

ALFQ

Monophosphoryl lipid A

QS-21

Plasmodium falciparum

Circumsporozoite protein

FMP013

ABSTRACT

Malaria caused by *Plasmodium falciparum* continues to threaten millions of people living in the tropical parts of the world. A vaccine that confers sterile and life-long protection remains elusive despite more than 30 years of effort and resources invested in solving this problem. Antibodies to a malaria vaccine candidate circumsporozoite protein (CSP) can block invasion and can protect humans against malaria. We have manufactured the Falciparum Malaria Protein-013 (FMP013) vaccine based on the nearly full-length *P. falciparum* CSP 3D7 strain sequence. We report here immunogenicity and challenge data on FMP013 antigen in C57BL/6 mice formulated with two novel adjuvants of the Army Liposome Formulation (ALF) series and a commercially available adjuvant Montanide ISA 720 (Montanide) as a control. ALF is a liposomal adjuvant containing a synthetic monophosphoryl lipid A (3D-PHAD[®]). In our study, FMP013 was adjuvanted with ALF alone, ALF containing aluminum hydroxide (ALFA) or ALF containing QS-21 (ALFQ). Adjuvants ALF and ALFA induced similar antibody titers and protection against transgenic parasite challenge that were comparable to Montanide. ALFQ was superior to the other three adjuvants as it induced higher antibody titers with improved boosting after the third immunization, higher serum IgG2c titers, and enhanced protection. FMP013 + ALFQ also augmented the numbers of splenic germinal center-derived activated B-cells and antibody secreting cells compared to Montanide. Further, FMP013 + ALFQ induced antigen-specific IFN- γ ELISPOT activity, CD4⁺ T-cells and a T_H1-biased cytokine profile. These results demonstrate that soluble CSP can induce a potent and sterile protective immune response when formulated with the QS-21 containing adjuvant ALFQ. Comparative mouse immunogenicity data presented here were used as the progression criteria for an ongoing non-human primate study and a regulatory toxicology study in preparation for a controlled human malaria infection (CHMI) trial.

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1. Introduction

The sporozoite stage of *P. falciparum* is coated with the circumsporozoite protein (CSP), which is critical for hepatocyte invasion [1,2]. Antibodies to the CSP central repeat region, (NANP)_n, can effectively block invasion [3] and are believed to associate with

protection against Controlled Human Malaria Infection (CHMI) [4]. Several early CHMI trials showed low level and inconsistent protection was conferred by aluminum hydroxide-adjuvanted CSP vaccines [5–7]. The first highly protective recombinant CSP vaccine was a hepatitis B particle fusion protein, RTS,S, formulated in an oil-in-water adjuvant (AS02) containing bacterial membrane monophosphoryl lipid A (MPLA) and QS-21, a triterpene glucoside compound derived from the *Quillaja saponaria* tree [8]. Subsequently, RTS,S formulated with a liposomal adjuvant containing

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MPLA and QS-21 (AS01), showed further improvement in protection against CHMI [4]. Several Phase 2 and Phase 3 field trials have since shown AS01 to be safe in adults and children and that optimization of the vaccine schedule and dose may further augment RTS,S protection [9,10].

Despite the success against CHMI, the RTS,S + AS01 vaccine-induced protection in endemic areas has remained low (30–50% range) [9,11–13]. Protection wanes over time and one report suggests negative efficacy in RTS,S vaccinees after a long-term follow-up [14]. Antigenic escape by non-vaccine strain parasites has been cited as a possible cause of low RTS,S efficacy [15]. Measures attempting to counter the limited field efficacy of RTS,S could be to include blood stage antigens [16], transmission blocking antigens [17] and prime-boost approaches that improve the CD4+ and CD8+ T-cell responses [18,19]. A vaccine that ultimately eliminates malaria may be a combination of multiple delivery platforms and contain antigens of both *P. falciparum* and *P. vivax* [20]. However, to build upon the partial success of CSP-based vaccines, researchers need unfettered access to RTS,S or another CSP vaccine that can reproducibly protect humans against CHMI. As a step in that direction, WRAIR Malaria Vaccine Branch has developed the Falciparum Malaria Vaccine-013 (FMP013), a soluble, *E. coli*-derived, nearly full-length CSP which contains 19 NANP and 3 NVDP repeats, as well as the C-terminal and the N-terminal regions. The N-terminal region of CSP is not included in RTS,S, although several B and T cell epitopes of CSP have been mapped to this region along with a functional protease cleavage site [1,2,21–23]. FMP013 antigen has met all of the purity and stability criteria for advancing to CHMI studies [24], and we are in the process of down-selecting a suitable human-use adjuvant to be combined with FMP013.

Molecular adjuvants are designed to directly target innate and adaptive immune pathways [25]. MPLA is a ligand for Toll-like-receptor 4 (TLR4) and can activate a signaling cascade terminating at transcription factors NF- κ B and IRF-7 (MyD88 pathway) as well as IRF-3 (TRIF pathway) [26]. QS-21 can activate the NOD-like receptor P3 (NLRP3) inflammasome complex present within the APC cytosol [27]. Among other pathways, aluminum adsorption of the antigen can activate the NLRP3 inflammasome complex [28], but unlike QS-21, aluminum salts induce primarily a T_H2 response [29]. The WRAIR Antigen and Adjuvant Research Laboratory has developed the Army Liposome Formulation (ALF) series of adjuvants. ALF is based on small unilamellar liposomes, 50–100 nm diameter, containing a synthetic MPLA derivative, 3D-PHAD[®] (Avanti Polar Lipids). ALF formulations with a recombinant HIV-1 envelope protein have been characterized in mice [30]. Here, FMP013 was tested with ALF, ALF containing QS-21 (ALFQ) or ALF containing aluminum hydroxide (ALFA). The data presented here provide the rationale for continued evaluation of FMP013 with ALFQ in Rhesus macaques and in a future human vaccine trial.

2. Materials and methods

2.1. Ethics statement

Research was conducted under an IACUC-approved animal use protocol in an AAALACi accredited facility in compliance with the Animal Welfare Act and other federal statutes and regulations

relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition.

2.2. Preparation of liposomes

Dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), cholesterol and synthetic monophosphoryl lipid A (MPLA) derivative 3-deacyl monophosphoryl lipid A (3D-PHAD[®]) were obtained from Avanti Polar Lipids (Alabaster, AL). ALF contained DMPC:DMPG phospholipids (9:1 M ratio), cholesterol (43 mol%) and 3D-PHAD[®] (0.26 mM). For ALFQ liposomes, the cholesterol concentration was adjusted to 55 mol%. Briefly, multilamellar liposomes were formed using the lipid deposition method by combining DMPC and cholesterol (both in chloroform), DMPG and 3D-PHAD[®] (in chloroform:methanol; 9:1 v/v) [31]. Multilamellar liposomes were then microfluidized (LV1 instrument, Microfluidics, Westwood, MA) to yield small unilamellar liposomes, which were sterile filtered and stored in lyophilized form at +4 °C. Final cholesterol concentration was quantified by colorimetric assay [32].

2.3. Preparation of vaccine formulations

FMP013 was cGMP-grade nearly full-length recombinant 3D7 strain *P. falciparum* CSP expressed and purified from *E. coli* [24]. A total of 2.5 μ g of FMP013 was present in each vaccine dose. Liposomal formulation compositions are summarized in Table 1. For ALF formulation, lyophilized FMP013 was reconstituted and added to dried liposomes. For ALFA formulation, reconstituted FMP013 was mixed with Alhydrogel (Brenntag Biosector, Frederikssund, Denmark) before adding to dried liposomes. For ALFQ, QS-21 (Desert King International, San Diego, CA) was mixed with small unilamellar liposomes before adding FMP013. Montanide formulations containing 70% Montanide ISA 720 VG (SEPPIC Inc., Fairfield, NJ) and 30% antigen (v/v) were vigorously vortexed for 25 min and emulsification was confirmed by a water surface dispersion test.

2.4. Protein and liposome analysis

Particle size dispersion was measured on a Zetasizer Nano S (Malvern, Worcestershire, United Kingdom). Thermal stability was assessed by incubating the formulations at +37 °C (kinetic stability) or room temperature and analyzing the samples at different time-points by SDS-PAGE followed by silver staining (Pierce Silver Stain Kit, Thermo Fisher Scientific, Waltham, MA). Western blot was performed to stain CSP specific degradation products using polyclonal mouse anti-CSP (1:2500) essentially as described previously [33].

2.5. Immunization of mice and challenge

Female C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were immunized intramuscularly (IM) with 50 μ l of the vaccines by injection in alternate rear thighs at 0, 3, and 6 weeks. The animals were bled three weeks after the first and second immunizations and two weeks after the third immunization. Protective

Table 1
Amount (μ g) of each component in 50 μ L dose of ALF, ALFA, and ALFQ formulated vaccines administered to mice. The contents listed for ALFQ apply to both ALFQ-B and ALFQ-L.

Group	CSP (μ g)	DMPC (μ g)	DMPG (μ g)	Cholesterol (μ g)	MPLA (μ g)	Aluminum Hydroxide (μ g)	QS21 (μ g)
ALF	2.5	70	7.9	33.4	20	–	–
ALFA	2.5	70	7.9	33.4	20	30	–
ALFQ	2.5	700	79	541	20	–	10

efficacy of vaccines was assessed using transgenic *P. berghei* (Tr-Pb) sporozoites expressing a functional full-length *P. falciparum* CSP gene [2]. Animals were challenged 15 days after the last immunization with 100 μ l intravenous (IV) injection of 3000 Tr-Pb sporozoites into the caudal vein, as described by Porter et al. [33]. Blood-stage parasitemia was detected by microscopy of giemsa-stained thin blood smear. Animals were considered protected if parasitemia was not detected during a two-week observation period following challenge. Ten naïve mice were included in each study to verify a 100% infection rate of the transgenic parasite.

2.6. Antibody ELISA

Full-length CSP (FL; 200 ng/well) or NANP peptide (100 ng/well) in PBS were coated on Immulon 2HB 96-well microtiter plates (Thermo Scientific, Rochester, NY) and ELISA was performed essentially as described previously [24,34]. Epitope-mapping peptides were coated on Immulon 4HBX plates (200 ng/well). CSP-based peptides used for mapping were pep1: LKKNSRSLGENDDGNNED NEKLKPKHKKLQPADGNPDP; pep2: DGNNED-NEKLKPKHKKLQKQ; pep3: KKKQPADGNPDPNANPNVDP NANPNVDPNANPNVDPNANP; pep4: WSPCSVTCNGIQVRIKPG SANKPKDELIDYANDIEKKICKMEKCSS. Titer was calculated as the dilution that resulted in $OD_{415} = 1.000$ using Gen5™ 4-parameter nonlinear regression (BioTek, Winooski, VT). HRP-linked goat anti-mouse IgG1 and anti-mouse IgG2c (SouthernBiotech, Birmingham, AL) were used for detection of subclass-specific serum IgG. For avidity ELISA, an additional step where either PBS or urea (6 M urea for FL CSP avidity, 2 M for NANP avidity) were incubated for 10 min after the primary antibody incubation. Avidity index was defined as the titer after urea incubation divided by titer after PBS incubation $\times 100$.

2.7. Germinal center B-cell and antibody secreting cell (ASC) analysis

Cell suspension from spleens were prepared as described [35]. For phenotypic analysis, pooled cells were stained with mAb specific for CD19 (1D3), CD95 (Jo2), and GL7 (GL7) (BD Biosciences, Mountain View, CA), counted by FACS (BD Biosciences) and data was analyzed using FlowJo 10 software (Tree Star, Ashland, OR). For detecting CSP-specific IgG antibody secreting cells (ASC) an ELISPOT was conducted. Splenocytes in complete RPMI containing 10% fetal bovine albumin and 10⁻⁶% v/v 2-mercaptoethanol, 1 mM glutamine, 1 mM pyruvate, and Gibco™ MEM non-essential amino acids were plated on 10 μ g/ml FL CSP coated 96-well Multi-Screen HTS IP plates (EMD Millipore, Billerica, MA) and incubated for 4 h at +37 °C. ASC were detected by sequential incubation with biotinylated rabbit anti-mouse IgG (0.5 μ g/mL; Southern Biotech, Birmingham, AL) overnight at +4 °C, NeutrAvidin horseradish peroxidase (1:1000; Thermo Scientific, Rockford, IL) for 1 h at room temperature, and filtered 3,3'-diaminobenzidine substrate (Sigma-Aldrich, St. Louis, MO) in 0.03% v/v hydrogen peroxide. Spots were counted using an AID ELISPOT Reader and software (Autoimmun Diagnostika, Columbia, MD). For a subsequent experiment (Fig. 5D), the frequency of CSP-specific B cells was determined by ELISPOT analysis following the instructions of the manufacturer (U-CyTech Utrecht, NL), i.e., splenocytes were polyclonally activated with R848 and IL-2 for two days, cell number determined, and then plated on CSP-coated ELISPOT plates for 6 h at 37 °C.

2.8. T-cell analysis by flow cytometry

Cell suspensions of mouse splenocytes were prepared essentially as described [35]. Stimulator cells were prepared by pulsing EL4 (Clone TIB-39, ATCC, Manassas, VA) suspension cells with pep-

tide pools of 15mers overlapping by 11 amino acids covering the PfCSP protein sequence. The peptides pools were “N-term pool” (aa 1–107; 24 peptides total), “repeat pool” (aa 97–283; 12 peptides total, only unique 15mers in this repeat sequence included), and “C-term pool” (aa 273–397; 29 peptides total) [36,37]. A “Mega pool” was made from all 65 peptides. T-cell analysis was performed on freshly isolated splenocytes from individual mice (n = 5) stimulated with peptide pulsed EL4 cells. Briefly, 1×10^6 splenocytes from individual mice and 1.5×10^5 pulsed EL4 stimulator cells were incubated for 6 h at 37 °C in 5% CO₂. BD Golgi Plug™ (BD Bioscience) was added 1 h into the incubation to block cytokine release and stored at 4 °C overnight. The samples were stained for viability using the LIVE/DEAD® Fixable Blue Dead Cell Stain Kit for UV excitation from Molecular Probes® (Life Technologies, Grand Island, NY) and blocked for non-specific staining using Mouse BD Fc Block™ (BD Biosciences). The samples were surface-stained with the following antibodies (fluorochrome): CD4 – RM4-5 (BD Horizon V500), CD3e – 500A2 (Alexa 700) (BD Biosciences), KLRG1-2F1 (PerCP-eFluor710) (eBioscience San Diego, CA), CD8a – 53-6.7 (BV785), CD127 – A7R34 (BV421) and CD27-LG.3A10 (APC) (Biolegend, San Diego, CA). Following separate fixation and permeabilization steps, the samples were stained intracellularly with the following fluorochrome-labeled antibodies: CD44-IM7 (Cy7PE), CD8a – 53-6.7(BV785), TNF- α – MP6-XT22 (BV605) (Biolegend), CD3e – 500A2 (Alexa 700), IFN- γ – XMG1.2 (Alexa 488) and IL-2 – JES6-5H4 (PE) (BD Biosciences). The data were acquired using a LSR II (BD Biosciences) and analyzed using FlowJo 10 software (Tree Star, Ashland, OR). The gating scheme for T-cell analysis has been detailed in Supplementary Fig. 1. A small positive threshold was established to account for negative data in a non-biased manner after background subtraction from non-pulsed EL4 samples [38]. The criterion for inclusion in the dataset was a frequency greater than the mean +2SD frequency of the ALFQ adjuvant control group.

2.9. IFN- γ ELISPOT

IFN- γ ELISPOT responses were assessed with fresh splenocytes in group pools (5 mice/group) in quadruplicate wells. Splenocyte group pools were plated at 400,000 cells/well with 135,000 peptide-pulsed EL4 stimulator cells/well on multiscreen MAHAS4510 plates (EMD Millipore) coated with 1 μ g/well of a rat anti-mouse IFN- γ antibody (Clone R4-6A2, BD Biosciences, San Jose, CA). PMA/Ionomycin-stimulated splenocytes served as assay controls. The plates were incubated at 37 °C in 5% CO₂ for 40 h and biotinylated rat anti-mouse IFN- γ antibody (clone XMG1.2, BD Biosciences) was added at 1 μ g/ml for 3 h at room temperature, followed with 1:800 dilution of peroxidase-labeled streptavidin (KPL, Gaithersburg, MD). Spots were developed and counted as above.

2.10. Cytokine profiling by pro-inflammatory panel kit

Freshly prepared splenocytes were stimulated with CSP-peptide pools described above at 1.25 μ g/mL final concentration for 48 h. Meso Scale Discovery's 10-plex mouse pro-inflammatory panel kit (IL1 β , KC, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IFN- γ , TNF- α) was used to analyze culture supernatants according to the manufacturer's protocol. Plates were read using a MESO Quick-plex SQ120 (Meso Scale Diagnostics, Rockville, MD).

2.11. Statistics

FL, NANP and subclass ELISA data were log transformed. For data sets with multiple groups, data was analyzed by ANOVA and p-values were corrected by Tukey's multiple comparisons test

using GraphPad Prism software (La Jolla, CA). Comparisons between two groups were made using two-tailed T-test. Statistically significant difference in group means was indicated in figures as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. After parasite challenge, Dunnett's method was used to establish significant delay in patency over naïve control mice and Fisher's exact test was used to determine significant differences in protection between vaccinated groups.

3. Results

3.1. Comparative immunogenicity and efficacy of liposomal adjuvants

In order to down-select an adjuvant, FMP013 was formulated in three liposomal preparations and a commercially available oil-based adjuvant Montanide ISA 720 VG (Montanide) was used as a control. The composition of the liposomal formulations, ALF, ALFA, and ALFQ, are detailed in Table 1. The FMP013 cGMP product is stored as frozen bulk protein (B) and in lyophilized form (L). ALFQ formulations with both bulk (ALFQ-B) and lyophilized FMP013 (ALFQ-L) were evaluated while ALF and ALFA were tested with only the lyophilized FMP013. Following each of 3 vaccinations

in C57BL/6 mice ($n = 8$), mice were monitored 1–2 times daily for local and systemic adverse reactions at the vaccine site, significant weight loss, scruffiness of the coat and lethargy, with none detected during the observation period. ELISA conducted against the full-length CSP antigen (FL) and the NANP repeat peptide showed that Montanide group mice sero-converted after the first dose; the second dose boosted antibody titers; however, no further boosting was observed post-third dose (Fig. 1A). ALF and ALFA FL titers did not differ significantly from Montanide throughout the course of the experiment, while ALFQ-B and ALFQ-L induced higher FL titers than Montanide post-second and post-third dose (Fig. 1A). ALFQ-L group FL titer was 4-fold higher than Montanide ($P = 0.0001$), 3-fold higher than ALFA ($P = 0.0007$) and 5-fold higher than ALF ($P < 0.0001$) post-third dose (Fig. 1B). In our studies, the ALFQ-B and ALFQ-L performed similarly in this experiment and a repeat experiment (data not shown). The NANP titer profiles for the five vaccine formulations (Fig. 1C) were similar to the FL profiles. ALFQ-B and ALFQ-L NANP titers were both ~5-fold higher than Montanide ($P = 0.01$ for both comparisons) and no difference between ALF, ALFA and Montanide NANP titers was observed post-third dose (Fig. 1D). To evaluate the protective efficacy of the vaccines, mice were challenged 2 weeks after the third dose with

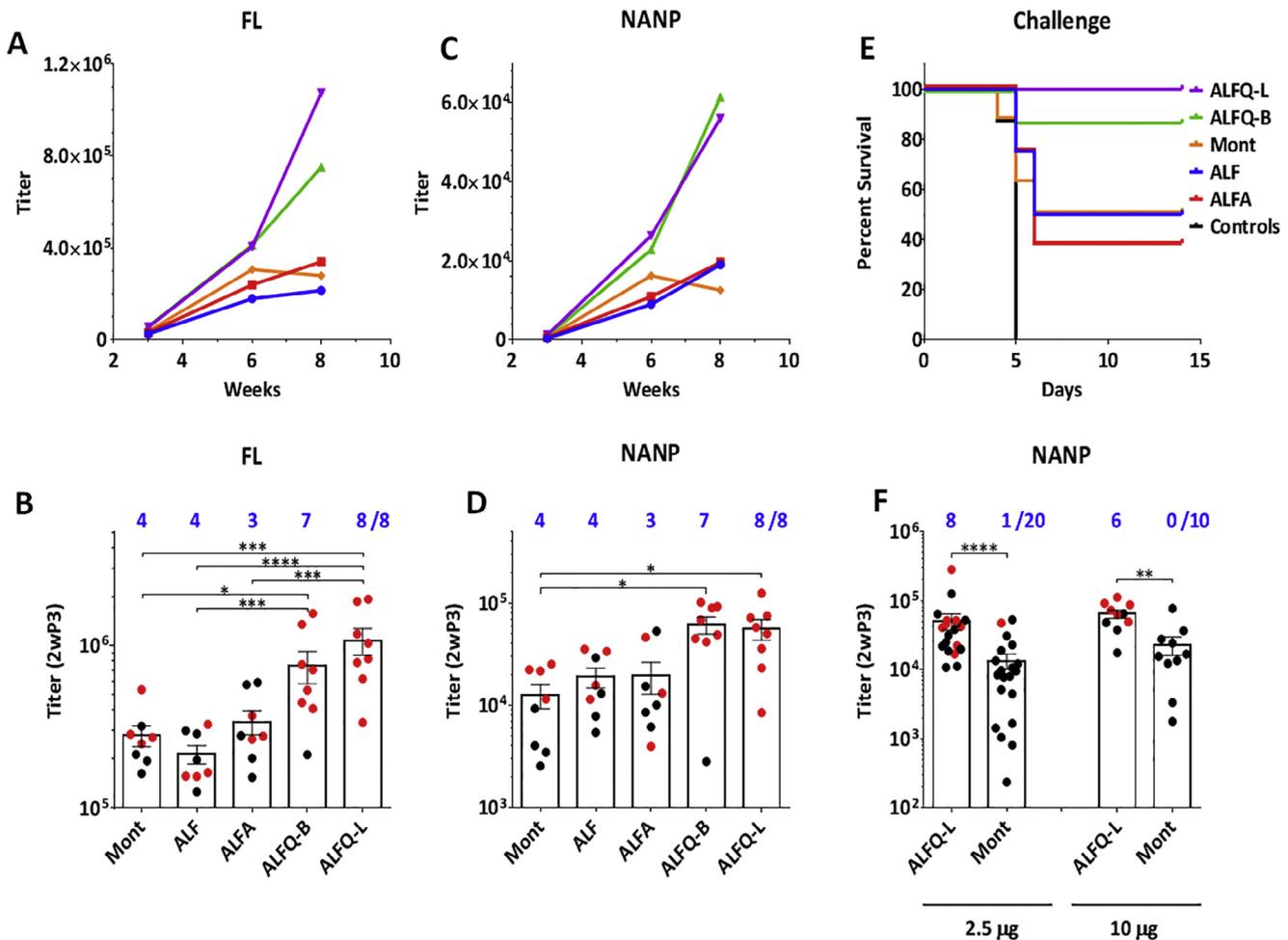


Fig. 1. Comparative immunogenicity and efficacy of liposomal adjuvants. (A) Group mean ($n = 8$) full-length (FL) CSP titer during 8-week vaccination period. (B) FL titer and protection status of individual mice at 2 weeks post-third dose. (C) Group mean NANP titer during 8-week vaccination period. (D) NANP titer and protection status of individual mice at 2 weeks post-third dose. (E) Kaplan-Meier survival following transgenic *Plasmodium berghei* (Tr-Pb) challenge. Mice were considered protected if no blood-stage parasites were observed during the two-week follow-up. (F) NANP titer and protection status of individual mice at 2 weeks post-third dose for repeat challenge studies at 2.5 μg FMP013 ($n = 20$) or 10 μg FMP013 ($n = 10$). Number of protected and total challenged mice in blue. Red symbols: protected; black symbols: non-protected mice. P-values in (B), (D) are for Tukey's test for multiple comparisons of log transformed titers. P-values in (F) are for individual two-sided T-tests on log transformed titers. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. Open bars are group means, whiskers represent SEM.

sporozoites of transgenic strain *P. berghei* (Tr-Pb). Parasitemia was observed in all naïve control mice by day 5 (Fig. 1E). Montanide showed 50% sterile protection (4/8), while ALFQ-B protected 83% (7/8) and ALFQ-L protected 100% of mice (8/8). ALF and ALFA protected similarly to Montanide, at 50% and 38% (4/8 and 3/8) respectively.

ALFQ and Montanide adjuvants were further compared in two subsequent challenge studies. Whereas the previous experiment compared multiple groups, the direct comparison of only two groups allowed for more statistical power to discern differences in protection between ALFQ and Montanide adjuvanted FMP013. In both studies, three doses of 2.5 µg FMP013 (n = 10) were tested and the combined NANP and protection data was analyzed (Fig. 1F, left bars). ALFQ showed higher NANP response ($P < 0.0001$) and higher protection (40% vs. 5%, $P = 0.02$) compared to Montanide (Fig. 1F). In one of the studies, we also compared ALFQ and Montanide at 10 µg FMP013 dose (n = 10). At this elevated dose, higher NANP titers ($P = 0.002$) and protection (60% vs. 0%, $P = 0.01$) were again observed in the ALFQ group compared to Montanide (Fig. 1F, right). We also examined the slide positivity data for time-to-blood stage patency (Supplementary Fig. 2). Indeed the 2.5 µg and the 10 µg dose groups of CSP + ALFQ group both showed approximately a one day increase in time to patency as compared to the naïve controls ($p = 0.004$ and $p = 0.005$ for 2.5 µg and 10 µg, respectively), while Montanide showed no delay compared to the naïve controls. In order to rule out any non-specific protective role of the adjuvant, 10 mice per group were vaccinated with 3 doses, 3 weeks apart, with 2.5 µg CSP + ALFQ and 10 mice received equivalent volumes of ALFQ adjuvant without antigen. None of the adjuvant controls were protected while 3 of 10 CSP + ALFQ vaccinated mice were protected in this experiment. Despite the observed variability in sterile protection outcomes between experiments, the CSP + ALFQ vaccinated groups reproducibly showed high titer, excellent boosting and a varying degree of sterile protection against transgenic parasite challenge.

3.2. Subclasses and avidity

Sera from the first challenge experiment collected 2 weeks after the third dose were analyzed for IgG subclasses (Fig. 2A). While IgG1 responses of the ALFA group were higher than ALF ($P = 0.04$), no other significant difference in IgG1 levels were observed across adjuvants. In sharp contrast to the IgG1 data, ALFQ-L IgG2c responses were 8-fold higher than Montanide ($P < 0.0001$), 7-fold higher than ALF ($P = 0.0008$) and 9-fold higher than ALFA ($P < 0.0001$) (Fig. 2A). IgG2c responses of the ALFQ-B group were similarly higher than the other groups. The serum antibody avidity against FL and NANP antigens were measured, but no significant differences between adjuvant groups were observed (Fig. 2B). Elevated levels of IgG2c antibodies suggested a T_H1 biased immune response was induced by ALFQ.

3.3. Region-specific ELISA

CSP contains conserved motifs Region I and Region II (Fig. 3A) that are believed to be of functional significance *vis-a-vis* hepatocyte binding and invasion [1,2]. In particular, residues around Region I have been shown to be conserved proteolytic cleavage sites, and are targeted by an invasion inhibitory antibody, 5D5 [23]. To determine if ALFQ formulation enhanced immunogenicity to these key epitopes, a mapping ELISA was conducted on the ALFQ-L and control Montanide group sera from the first mouse study. ELISA against Region I-spanning peptides (pep1, pep2, and pep3) and a Region II-spanning peptide (pep4) showed higher antibody binding in the ALFQ group as compared to Montanide (Fig. 3B). These data, combined with the FL and NANP ELISA data,

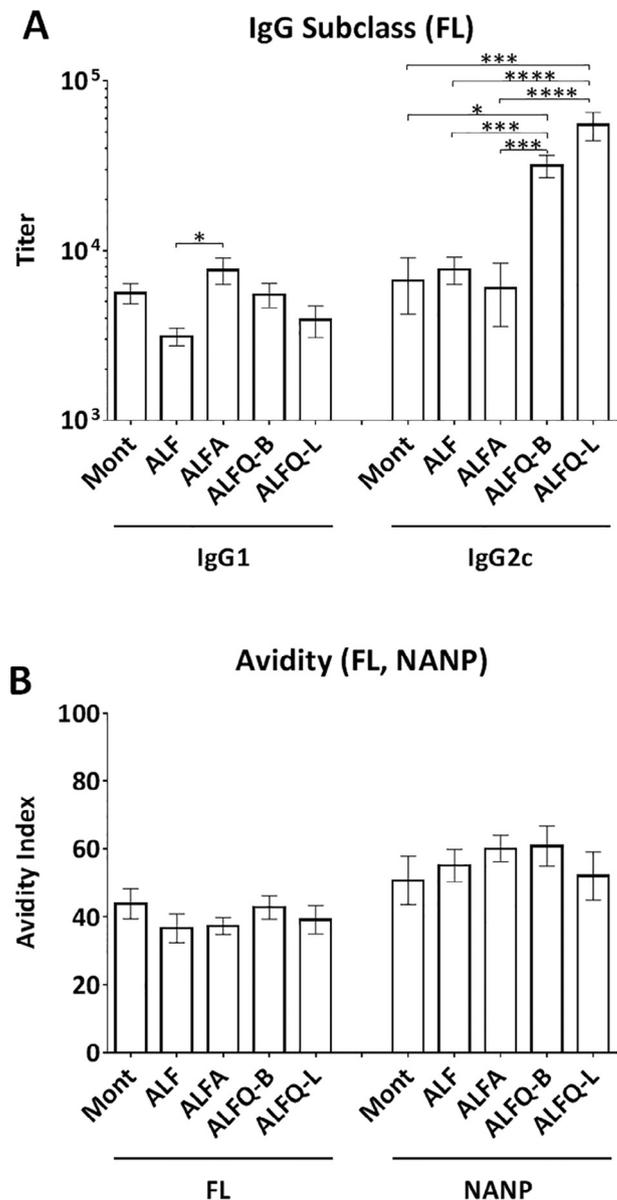


Fig. 2. Subclasses and Avidity. (A) Mean anti-FL CSP IgG1 (left) and IgG2c (right) titers (n = 8) from first challenge study at 2 weeks post-third dose. (B) Mean avidity index values from FL (left) and NANP (right) avidity ELISA. P-values are for Tukey's test for multiple comparisons of log transformed titers. Open bars are group means, whiskers represent SEM.

show an overall increase in titers across the CSP molecule by the ALFQ adjuvant.

3.4. B-cell activation

To investigate the ability of ALFQ-adjuvanted FMP013 to activate B-cells, groups of 6 mice were administered ALFQ-L- or Montanide-adjuvanted FMP013. Two weeks post-first dose, splenocytes were harvested (n = 2) and stained for phenotypic markers of activated germinal center (GC)-derived B cells (GL7 and CD95). Cells were gated for CD19 and the CD19 + GL7 + CD95 + cells were quantified by flow-cytometry (Fig. 4A). As compared to the naïve animals, the percentage of CD19 + GL7 + CD95 + cells were 2-fold higher in the Montanide group and 14-fold higher in the ALFQ group (Fig. 4A). Furthermore, CSP-specific antibody secreting cells (ASC) were quantified in pooled

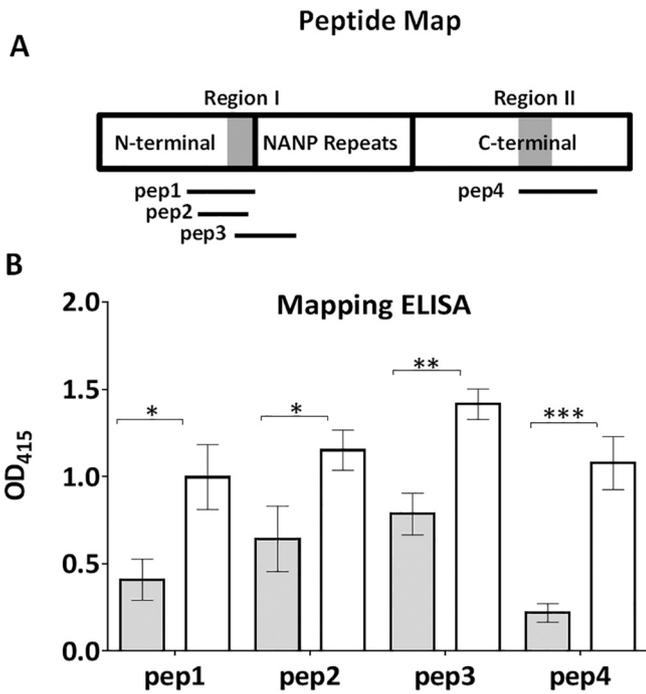


Fig. 3. Region-specific ELISA. (A) Location of Region I and Region II and location of peptides spanning the CSP primary structure are shown with respect to the N-terminal, C-terminal and the NANP repeats. (B) Group mean (n = 8) absorbance at 415 nm against each peptide at a serum dilution factor of 1:1000 for Montanide (filled bars) and ALFQ-L (open bars) from the first study.

splenocytes using a B-cell ELISPOT at 3 weeks after the first dose and 3 weeks after a second dose (n = 2) (Fig. 4B). The ALFQ group showed a higher number of splenocyte-derived ASC than the Montanide group at both the post-first and post-second dose time points. Together these data show improved antibodies and protection induced by ALFQ associated with improved B-cell activation and ASC formation in the spleen.

3.5. T-cell activation

Five mice per group were immunized three times at three week intervals with 2.5 µg of bulk FMP013 in either Montanide or ALFQ.

Control mice received ALFQ alone with PBS. Two weeks after the third dose, splenocytes from mice were harvested, stimulated with the antigen, and evaluated for T-cell activity by IFN-γ ELISPOT (Fig. 5A), flow-cytometric determination of the frequency of IFN-γ, TNF-α and IL2 producing CD4+ and CD8+ T-cells (Fig. 5B) and quantification of secreted cytokines by a multiplex pro-inflammatory panel kit (Fig. 5C). IFN-γ ELISPOT revealed that the number of CSP-specific IFN-γ producing splenocytes was higher in the FMP013 + ALFQ vaccinated mice as compared to FMP013 + Montanide (Fig. 5A). ELISPOT activity was mainly focused to the repeat and lesser to the C-term region. Flow-cytometric analysis confirmed that the ALFQ group showed a higher frequency of CD4 + T-cells that were positive for IFN-γ or TNF-α as compared to mice vaccinated with FMP013 + Montanide (Fig. 5B). This CD4 + T-cell response was also highly focused towards the repeat region of CSP. Remarkably, no cytokine positive CD8+ T-cells were detected (data not shown). In an effort to profile the immune responses induced by the various vaccine formulations, 10 different cytokines were quantified by a pro-inflammatory panel kit. The FMP013 + ALFQ group splenocytes produced higher levels of IFN-γ, IL-2 and TNF-α as compared to FMP013 + Montanide, which only produced low levels of IL-2 (Fig. 5C). In all three assays described above, the ALFQ adjuvant control mice showed no CSP-specific T-cell activity. Overall, these data along with the antibody subclass profile established that FMP013 + ALFQ induced a T_H1 biased response. In these groups of mice, CSP-specific ASC frequency was also determined using a B-cell ELISPOT assay against FL CSP (Fig. 5D) and similar to the data in Fig. 4B, the CSP + ALFQ induced a significant 5-fold higher frequency than Montanide (P = 0.005) and 9-fold higher than ALFQ control mice (P = 0.002).

3.6. Antigen stability and particle size analysis

The stability and integrity of the FMP013 antigen formulated in ALFQ were studied at room temperature and at +37 °C (kinetic stability). At room temperature, no band broadening or degradation was observed in PBS or ALFQ for up to 4 h on a silver stained gel (Fig. 6A upper left panels). At the 8 h time-point, some weakening of FMP013 band intensity was noted in PBS, but not in ALFQ. Indeed, FMP013 was stable in ALFQ for up to 96 h, as was confirmed by the corresponding CSP-specific western blot (Fig. 6A lower left panels). FMP013 was also found to be stable for 96 h

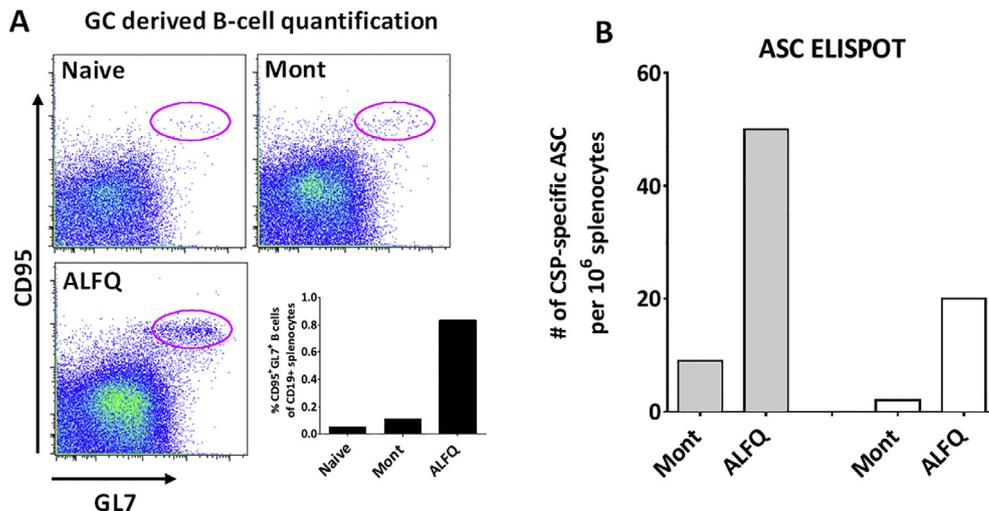


Fig. 4. B-cell activation. (A) Representative scatter plots of splenic GL7+ (x-axis) CD95+ (y-axis) GC-derived B-cells within the total CD19+ B-cell population (pink oval) at 2 weeks post-first dose. Mean percentage (n = 2) of GL7⁺CD95⁺ B-cells within the total CD19+ B-cell population are plotted in the bar graph. (B) Mean number (n = 2) of splenic CSP-specific ASC detected by ELISPOT three weeks post-first (gray bars) and post-second (open bars) dose.

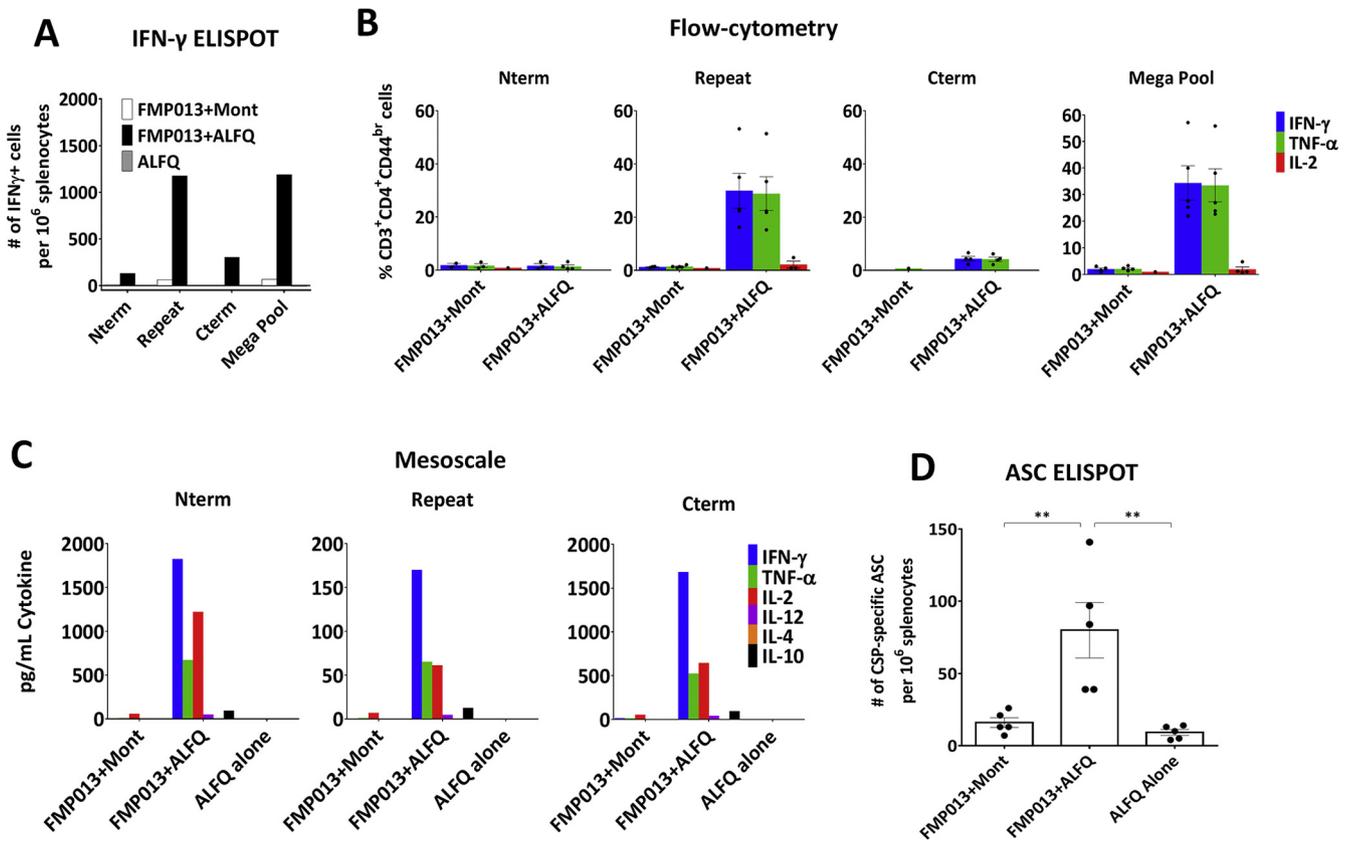


Fig. 5. T-cell activation. Splenocytes ($n = 5$ mice) from FMP013 immunized mice were stimulated by N-term, repeat, C-term peptides or a mega pool of all CSP peptides. (A) Mean of spot forming CSP-specific IFN γ + cells in quadruplicate wells from pooled splenocytes. (B) Flow-cytometric analysis (individual mice) of T-cells shown as mean \pm SEM frequency of viable CD3 $^{+}$ CD4 $^{+}$ CD44 br splenocytes expressing IFN- γ , TNF- α , or IL-2. Cytokine positivity was determined when the frequency of positive events exceeded mean \pm 2SD of the ALFQ adjuvant control group. (C) Secreted cytokines analyzed by a multiplex proinflammatory panel kit. Plot shows cytokines whose levels were detected above background. (D) Mean \pm SEM ($n = 5$) of splenic CSP-specific ASC detected by ELISPOT. P-values are for Tukey's test for multiple comparisons; $^{**}P < 0.01$.

at +37 °C in a kinetic stability assay (Fig. 6A, right panels). Analysis by dynamic light scattering (DLS) determined the average particle size of the liposomes in ALFQ adjuvant to be 55 ± 13 nm (Fig. 6B). After the addition of QS-21 and FMP013, the liposome size increased and the profile became more poly-dispersed. This particle profile of ALFQ formulated FMP013 vaccine was stable for at least 8 h at room temperature. These data suggest that formulation in ALFQ does not modify or accelerate degradation of the antigen at ambient and physiological temperatures.

4. Discussion

Malaria vaccine development has long suffered from limited access and availability of human-use adjuvants. The most advanced CSP based vaccine RTS,S is formulated in a proprietary adjuvant system AS01 (GlaxoSmithKline), thus to address the problem of cost-of-goods, freely accessible alternatives to RTS,S and AS01 are urgently needed [39]. The immunological readouts of RTS,S are mostly reported in human studies and the various components of the RTS,S vaccine are not individually available for direct comparisons with new vaccine formulations. Therefore, we propose to use mouse immunogenicity and protection results as the basis for advancing FMP013 + ALFQ formulation to the human CHMI model, where the immunological benchmarks and protective outcomes of RTS,S are well established.

Dendouga et al. have suggested that synergistic action of MPLA and QS-21 in AS01 is critical for the enhancement of antibodies against glycoprotein E of varicella-zoster virus in mice [40]. In the current study, the individual contributions of MPLA and QS21

could not be discerned, but ALFQ showed a clear enhancement of both the B- and T-cell responses. Compared to Montanide, the antibody reactivity of epitopes throughout the CSP sequence was increased, suggesting that humoral immune-enhancement was not directed to a specific region within CSP. GC-derived B-cells and CSP-specific ASC were increased by ALFQ as compared to Montanide, suggesting that ALFQ may be inducing superior germinal center and ASC formation. Furthermore, secondary and tertiary immune responses following repeated immunizations have been attributed to memory lymphocytes [41] and higher levels of boosting seen in the ALFQ group may reflect a superior memory response. ALFQ also induced higher IgG2c antibody titers and a detailed immune-profiling of splenocytes confirmed a clear T $_H$ 1 biased immune response was induced by ALFQ. TLR agonist adjuvants that induced a T $_H$ 1 biased response have been shown in the past to contribute to higher protection in this mouse model [24].

ALFQ outperformed Montanide, a widely used adjuvant known to induce high antibody production of investigational malaria vaccine candidates in animals [42–45] and has also been used in humans [46,47]. Our laboratory has tested FMP013 with a variety of available adjuvants, including Glucopyranosyl Lipid A (TLR4 agonist), R848 or 3M-051 (TLR7/8 agonists), aluminum hydroxide, and particulate formulations with Q β [24,34,48]. While only a head-to-head comparison can establish superiority, FMP013 + ALFQ antibody titers and protection reported here were among the highest observed with FMP013 in our laboratory.

Transgenic parasite challenge allows for preliminary functional comparison of human vaccines in mice. In the CHMI model, 5

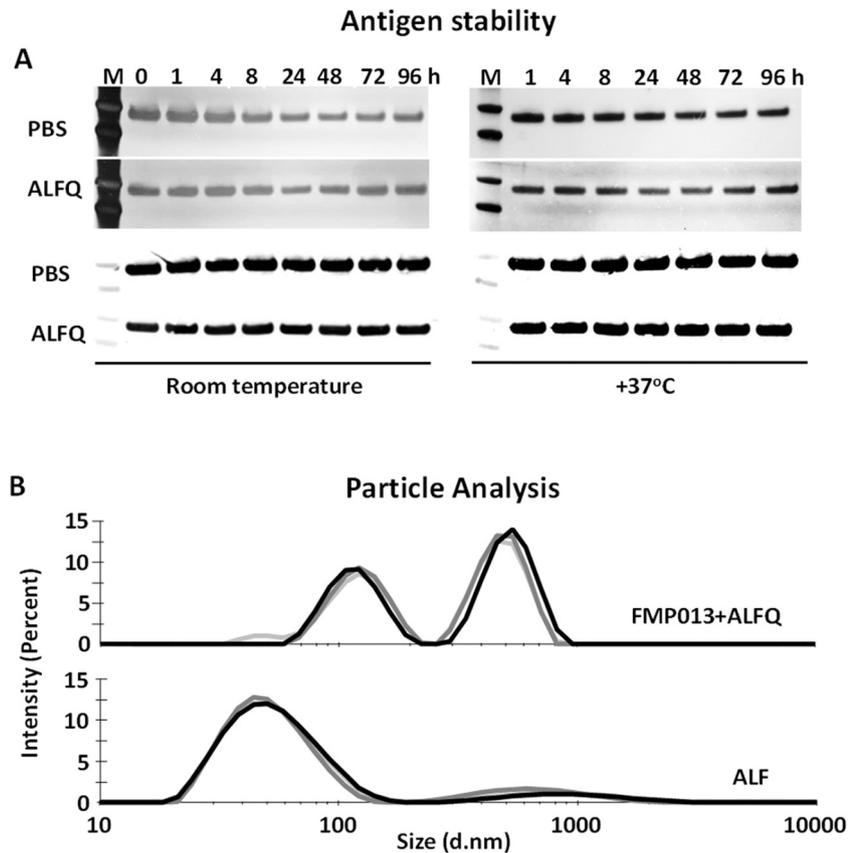


Fig. 6. Antigen stability and particle size analysis. (A) Silver stain (top panels) and western blots (bottom panels) of FMP013 formulated with either ALFQ or PBS control incubated at room temperature or 37 °C for 96 h. (B) Dynamic light scatter (DLS) profile of ALFQ with FMP013 and QS-21 added (top) or no antigen or QS-21 added (bottom); y-axis: relative intensity of scattered light; x-axis: diameter (d) in nanometers. Each line in light gray, gray, or black represents one of three independent reads of the DLS profile.

infected mosquito bites deliver approximately ≤ 500 sporozoites into a human [49,50]; by contrast, each challenged mouse received 3000 sporozoites intravenously and we used the most stringent outcome (sterile immunity) as a measure of protection rather than a reduction in parasite RNA levels in the liver [51,52]. The ALFQ-adjuvanted FMP013 protected 100% mice in one experiment; however, lower protection was observed in subsequent experiments. Despite the variability between experiments, the FMP013 + ALFQ groups repeatedly showed a degree of sterile protection that was higher than FMP013 + Montanide in every experiment. The variability could be due to a difference in adjuvant batches or sporozoite viability. Mice that did become infected in the FMP013 + ALFQ group showed an approximately 1 day delay in blood stage patency as compared to the controls while this was not observed for the mice in the FMP013 + Montanide group. Although we did not measure liver stage burden by PCR, it has been reported that a 1 day delay in time-to-patency correlates with >90% inhibition of liver parasite burden [53]. The high-specificity of the protection outcome has been established by four previous reports where NANP titers have positively associated with protection [24,33,34,48] while adjuvant-alone controls of ALFQ (this report), GLA/SE [24] and Montanide [33] did not protect against challenge at 2 weeks post third vaccine dose. While it remains to be proven if rodent models can predict human responses to CSP vaccines, we used a combination of immunogenicity and sterile protection outcomes to down-select the ALFQ adjuvant in mice.

The effective adult dose of AS01B contains 50 μg of QS-21 and 50 μg of MPLA [4,54]. While MPLA up to 2200 μg per dose has been safely administered to humans [55], the dose of QS-21 has been limited by its hemolytic and cytotoxic effects [27,56]. Mouse stud-

ies have shown that vaccines containing a lower dose of QS-21 than AS01B (e.g., AS01E and AS01F) show reduced immunogenicity [40]. We have shown that increasing the cholesterol concentration to 55 mol% while using saturated fatty acyl phospholipids in the liposomes can greatly reduce *in vitro* hemolytic activity of QS-21 [32]. The ALFQ adult dose is currently projected to contain 100 μg QS-21 and 200 μg 3D-PHAD[®], and while the difference in body size between mice and humans make it difficult to directly predict a human dose, 10 μg QS-21 and 20 μg 3D-PHAD[®] (one-tenth of human dose) were found to be generally safe and effective in mice. A rhesus study and a rabbit toxicity study are currently underway to assess the systemic and local toxicity of a full human dose of cGMP-grade ALFQ in combination with cGMP-grade FMP013. Toxicity data from rabbits and rhesus will be used to determine if the proposed dose of QS-21 and 3D-PHAD[®] in ALFQ may be safe in humans.

Adjuvants are often known to modify structure and antigen stability after formulation [57,58]. Thermal stability and a long shelf-life are critical for any malaria vaccine to be used in elimination efforts. Recombinant protein based vaccines are highly thermostable, compared to the whole sporozoite vaccine which requires ultra-low temperature storage methods [59,60]. Further work is underway on the long-term stability of FMP013 + ALFQ with the aim to eliminate the bedside mixing of antigen and adjuvant.

Lastly, particulate vaccines are believed to work better than soluble proteins [34,61–64]. We argue that FMP013 + ALFQ is highly immunogenic and protective in mice, and a soluble protein CSP vaccine with MPLA- and QS-21-containing adjuvant remains to be tested in humans. Indeed, a soluble protein vaccine adjuvanted with AS01 has recently shown remarkable efficacy in a Phase 3 trial

against herpes zoster virus [65]. A future CHMI study will enable us to determine if a potent molecular adjuvant like ALFQ would allow FMP013 vaccination to protect humans against malaria.

Conflict of interest

SD holds a patent on FMP013 vaccine; ZB and CA hold patent on the ALFQ adjuvant.

Disclaimer

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense or those of the US Agency for International Development.

Acknowledgements

The funding for this work was provided by the USAID Malaria Vaccine Development Program. This work was supported through a Cooperative Agreement Award (no. W81XWH-07-2-067) between the Henry M. Jackson Foundation for the Advancement of Military Medicine and the U.S. Army Medical Research and Materiel Command (MRMC), as well as in part by an appointment to the Postgraduate Research Participation Program at the Walter Reed Army Institute of Research administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and MRMC. We thank LTC James Moon, David Lanar, April Sikaffy, Tanisha Robinson, Farhat Khan and Lisa Dlugosz for help with product characterization, assays, laboratory support and helpful discussions. We thank Lorraine Soisson, Carter Diggs and the United States Agency for International Development Malaria Vaccine Program for advice and funding support.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2017.05.070>.

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