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Safety, toxicity and immunogenicity of a malaria vaccine based on the circumsporozoite protein (FMP013) with the adjuvant army liposome formulation containing QS21 (ALFQ)

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ABSTRACT

Antibodies to Circumsporozoite protein (CSP) confer protection against controlled human malaria infection (CHMI) caused by the parasite *Plasmodium falciparum*. Although CSP is highly immunogenic, it does not induce long lasting protection and efforts to improve CSP-specific immunological memory and duration of protection are underway. We have previously reported that the clinical grade CSP vaccine FMP013 was immunogenic and protective against malaria challenge in mice when combined with the Army Liposomal Formulation adjuvant containing immune modulators 3D-PHAD™ and QS21 (ALFQ). To move forward with clinical evaluation, we now report the safety, toxicity and immunogenicity of clinical grade FMP013 and ALFQ in Rhesus macaques. Three groups of Rhesus (n = 6) received half or full human dose of FMP013 + ALFQ on a 0-1-2 month schedule, which showed mild local site reactions with no hematologic derangements in red blood cell homeostasis, liver function or kidney function. Immunization induced a transient systemic inflammatory response, including elevated white blood cell counts, mild fever, and a few incidences of elevated creatine kinase, receding to normal range by day 7 post vaccination. Optimal immunogenicity in Rhesus was observed using a 1 mL ALFQ + 20 μg FMP013 dose. Doubling the FMP013 antigen dose to 40 μg had no effect while halving the ALFQ adjuvant dose to 0.5 mL lowered immunogenicity. Similar to data generated in mice, FMP013 + ALFQ induced serum antibodies that reacted to all regions of the CSP molecule and a Th1-biased cytokine response in Rhesus. Rhesus antibody response to FMP013 + ALFQ was found to be non-inferior to historical benchmarks including that of RTS,S + AS01 in humans. A four-dose GLP toxicity study in rabbits confirmed no local site reactions and transient systemic inflammation associated with ALFQ adjuvant administration. These safety and immunogenicity data support the clinical progression and testing of FMP013 + ALFQ in a CHMI trial in the near future.

1. Introduction

Malaria caused by the parasite *Plasmodium falciparum* kills hundreds of thousands of children annually, and emergence of multi-drug-resistant strains indicates an urgent need for an efficacious vaccine for those living in endemic areas [1]. A vaccine is also needed by those who travel to malaria-endemic areas, such as tourists and troops. The most promising malaria vaccines to-date have targeted the circumsporozoite protein (CSP), present abundantly on the surface of the mosquito-transmissible sporozoite stage of the parasite. Antibodies against various regions of the CSP molecule have been associated with protection against malaria

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challenge in mice and humans [2–5]. The first generation of CSP vaccines contained soluble protein or peptides combined with Aluminum-based adjuvants. Human trials with these first-generation CSP vaccines showed low levels of inconsistent protection against controlled human malaria infection (CHMI) [6] and naturally transmitted malaria [7]. A significant and reproducible improvement in efficacy was observed when a hepatitis B particle based CSP vaccine, RTS,S, was combined with a rationally designed molecular adjuvant, AS02 (GlaxoSmithkline Vaccines, Belgium) [8]. Oil-based AS02 has subsequently been replaced by a liposomal formulation (AS01) [9,10], though both adjuvant formulations contain two key immune-stimulators: monophosphoryl lipid A (MPLA), which is a potent TLR4 agonist derived from bacterial membrane, and QS21, a saponin extracted from the bark of *Quillaja saponaria.* QS-21 can activate the NOD-like receptor P3 (NLRP3) inflammasome complex present within the antigen presenting cell cytosol [11], resulting in a potent Th1-biased immune response. These two immune-modulators synergize to induce NK cells and CD8+T cells to produce a burst of IFNγ in the draining lymph nodes [12]. Based on extensive Phase-3 trials in Africa, a pediatric formulation of RTS,S has been approved for Phase 4 trials in three African countries under the trade name Mosquirix® [13]. While the AS01 adjuvant was key to the success of RTS,S, AS01 was never combined with a soluble CSP in humans to determine if soluble immunogens could be as protective as RTS,S if adjuvanted with AS01. Soluble protein vaccines are relatively simple to scale-up and often more cost-effective to manufacture and stabilize for storage than particulate vaccines. Indeed, a soluble protein vaccine adjuvanted with AS01, "Shingrix", was recently licensed by GlaxoSmithKline (GSK), showing >90% efficacy against herpes zoster virus in adults [14,15].

The CSP molecules on the parasite contain tetra-peptide repeating units (NANP) flanked by a conserved amino terminal region (N-term) and a polymorphic, cysteine-rich carboxy-terminal region (C-term). The RTS,S vaccine antigen does not contain the N-term sequence. While RTS,S confers sterile protection against the homologous 3D7 strain parasite in controlled infection, *i.e.* CHMI, the efficacy of RTS,S against diverse parasite populations in the field remains below 50% [4,13]. Protection against CHMI appears to be enhanced if the interval between boosts is increased and booster dose is decreased [16], but it remains to be seen if this delayed and fractional dose schedule will improve RTS,S mediated protection in the field. Another possible strategy to improve a vaccine is immune-broadening aimed at enhancing the immunogenicity of functional, but less dominant, protective epitopes. Several B- and T-cell epitopes that have been mapped to the N-term and C-term of CSP that are now being targeted for immune-broadening [5,17–19]. As a step in that direction, the Walter Reed Army Institute of Research (WRAIR) Malaria Vaccine Branch has produced a nearly full-length recombinant CSP vaccine: Falciparum Malaria Vaccine-013 (FMP013) [20]. FMP013 was expressed and purified from *E. coli* and contains the central repeat (19 NANP+3 NVDP), C-term and N-term regions of CSP. A cGMP lot of FMP013 was produced and has now met the purity and stability criteria for human vaccines [20]. FMP013 was tested in mice with a variety of available investigational and commercial adjuvants including a particle delivery platform [21]. Army liposomal formulation ALFQ, which contains QS21 along with a synthetic MPLA analog (3D-PHAD®, Avanti Polar Lipids) emerged as the most potent adjuvant for FMP013 [22,23]. In mice, three doses of 2.5 μg FMP013 in 50 μl ALFQ containing 20 μg 3D PHAD™ and 10 μg QS21 induced high IgG antibody titers, high IgG2c antibodies that conferred sterile *in vivo* protection against transgenic *P. berghei* parasites expressing a functional copy of *P. falciparum* CSP. FMP013 + ALFQ also augmented the numbers of splenic germinal center-derived activated B-cells, antibody secreting cells, CD4+T-cells and antigen-specific IFN-γ producing cells [23].

While mouse models frequently are the first animal platform for early down-selection and detailed immunology of investigational vaccines, data from these models have not translated to development of human malaria vaccines. The Rhesus monkey (*Macaca mulatta*) model has repeatedly proven as a much better predictor of human responses. Most notably, the AS01 adjuvant was first found to be superior to AS02 in Rhesus and this was later confirmed in humans [9,10,24]. The Rhesus model also mirrored human immune responses against an adenovirus-RTS,S prime-boost vaccine [25,26] and an irradiated sporozoite vaccine [27]. Furthermore, the safety and toxicological data on the AS0X series of adjuvants was initially collected in Rhesus [24,28–31]. The Rhesus model has therefore remained on the critical path of down-selecting CSP vaccines in preclinical models at WRAIR.

Here we tested the safety and immunogenicity of FMP013 formulated with ALFQ in Rhesus monkeys. The 1 mL adjuvant dose containing 200 μg 3D PHAD™ and 100 μg QS21 was at 10X the dose shown to be safe in mice [23]. GSK's AS01 dose adjuvant (Shingrix™ vaccine) contains 50 μg QS21, hence ALFQ was also tested at half dose. FMP013 doses of 40 μg and 20 μg tested here was based on previous experience in Rhesus [32]. We report that FMP013 formulated in ALFQ was safe in the Rhesus model and a GLP toxicology study in rabbit model substantiated these findings The immunogenicity of FMP013 met the go-criteria based on historical published data on RTS,S vaccine in humans. An investigational new drug (IND) application for a clinical trial of the FMP013 + ALFQ vaccine is being filed with the U.S. Food and Drug Administration based on these findings.

2. 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. Rhesus

Colony-bred adult rhesus macaques of Indian origin (*Macaca mulatta*), were housed at the WRAIR animal facility and used under the IACUC-approved protocol number 16-MVD-25L. Macaques were naïve and had never been used in an experimental study. All animals were quarantined for a period of 4 weeks and free from any overt clinical signs of illness and deemed to be in good health, testing negative for Macacine herpesvirus 1, measles, Simian Retrovirus, Simian Immunodeficiency Virus, Simian T-cell Leukemia Virus and tuberculin skin test. Animals were pair-housed in a controlled environment as previously described [32], in accordance with WRAIR Veterinary Service Programs standard operating procedures.

2.3. Rhesus vaccination

Three groups of 6 Rhesus macaques were given FMP013 and ALFQ corresponding to proposed human doses, according to Table 1. Sample size of n = 6 was determined to give a statistical power of 0.84 to discern >2-fold difference in serum antibody titer using an online power calculator (http://www.stat.uiowa.edu/

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Table 1
Rhesus vaccine antigen and adjuvant dose by group.

Groups	Dose 1	Dose 2	Dose 3
LO Ag LO Adj	20 μg CSP 0.5 mL ALFQ	20 μg CSP 0.5 mL ALFQ	20 μg CSP 0.5 mL ALFQ
LO Ag HI Adj	20 μg CSP 1 mL ALFQ	20 μg CSP 1 mL ALFQ	20 μg CSP 1 mL ALFQ
HI Ag HI Adj	40 μg CSP 1 mL ALFQ	40 μg CSP 1 mL ALFQ	40 μg CSP 1 mL ALFQ

~rlenth/Power). Vials of lyophilized cGMP grade FMP013 were suspended in 0.4 mL sterile water and brought up to 2X desired concentration with PBS, then combined with an equal volume of cGMP grade ALFQ (Table 1). The formulation was rolled on a rotary platform for 1 h ± 20 m before administration. Macaques were anesthetized with Ketamine HCl at 5–10 mg/kg in combination with acepromazine at 0.05–0.1 mg/kg. A small patch was shaved and vaccines were administered intramuscularly (IM) in the outer thigh muscle. Injection sites were alternated between right and left thigh for each administration day. Rhesus macaques were bled 2 weeks prior to the first dose and at 2 weeks after each vaccination for serology.

2.4. Rhesus safety and tolerability assessment

Animals were examined on days 0, 1, 3, and 7 after each immunization. Fever cutoff was set at any temperature exceeding two times the standard deviation above the 6-month average rectal temperature for each individual animal. We inspected the injection site at each time point for skin warmth, erythema, swelling, muscle induration, ulceration, abscess, or any other abnormalities around the vaccine site. The grading scales for local skin reactions were: 0, absent; 1, mild; 2, moderate; 3, severe [24]. Two ml of blood was collected from the femoral vein for complete blood count (CBC) and serum chemistry at each time point (assessed parameters are listed in the supplement).

2.5. ELISA

Direct and avidity ELISA were performed by the International Malaria Serology Reference Center (WRAIR, Silver Spring, MD) against the full-length CSP, $(NANP)_6$ peptide or a C-term CSP peptide (PF16; [33]) exactly as described by Regules et al. [16]. As a result the secondary antibody used for the Rhesus ELISA was heterologous, HRP-conjugated anti-human IgG (Southern Biotech, Birmingham, AL). For a region-specific ELISA, the protocols were similar to above except the plates were coated with GST fusion proteins representing the N-term, repeat or C-term of CSP. Rabbit ELISA required the use of anti-rabbit IgG H + L secondary antibody (Southern Biotech, Birmingham, AL). ELISA titer was defined as the serum dilution that resulted in optical density (OD) of 1.0 as predicted by a 4-parameter curve fitting equation (Biotek, Winooski, VT). Avidity ELISA was conducted with nearly full-length FMP013 (FL) as reported previously [16].

2.6. IgG purification

Protein G (Thermo Fisher Scientific Waltham, MA) was used for IgG purification using 3 mL serum from 1 monkey in each group. Purified IgG was dialyzed against PBS, concentrated using Amicon® Centrifugal Filter Units (Millipore Sigma), and quantified by optical density measurement at 280 nm.

2.7. Inhibition of liver stage development assay (ILSDA)

Purified rhesus IgG was tested by ILSDA [34]. Briefly, the NF54 strain of *Plasmodium falciparum* (*Pf*) sporozoites obtained from salivary gland dissections of infected *Anopheles* mosquitoes were mixed with a positive control monoclonal antibody NFS1 or polyclonal Rhesus IgG at a final concentration of 160 μg/ml and incubated at room temperature for 20 min. The sporozoite-antibody mixtures were then introduced into the wells containing cryopreserved human hepatocytes (BioIVT, Baltimore, MD) and incubated at 37 °C for 3 h to allow sporozoites to infect hepatocytes, washed to remove non-invaded sporozoites, and incubated at 37 °C for 96 h. *Pf* 18 s rRNA levels were quantified to determine the level of inhibition of liver stage development by quantitative real-time PCR (qRT-PCR) analysis.

2.8. IL-2/IFNγ Fluorospot assay

Antigen-specific interferon (IFN)-γ and interleukin 2 (IL-2) cytokine-secreting T cells were measured by Fluorospot (Ucytech Biosciences, Utrecht, Netherlands) following the manufacturer's instructions. Monkey anti-CD3 mAb (Mabtech Inc., Cincinnati, OH) was used as an internal positive control. Each well was treated with 25 μl CD28 and CD49d (BD Biosciences, San Diego, CA) cell stimulants, 25 μl of antigen and 50 μl of cells (2.5×10^5/well). Plates were incubated at 37 °C, 5% CO_2, 100% humidity for 40–48 h. Fluorospot plates were analyzed using the Autoimmun Diagnostica (AID) GmbH Fluorospot reader (Strassberg, Germany) equipped with filters for FITC (excitation 490 nm/emission 510 nm) and Cy3 (excitation 550 nm/emission 570 nm).

2.9. Mesoscale cytokine analysis

PBMC (pre-immune and 4wP3) were thawed and stimulated for 48 h with CSP peptide pools (1 μg/mL) or Fl CSP (5 μg/mL). The cytokine in the culture supernatants were measured using the Meso Scale Discovery 10-plex NHP pro-inflammatory panel kit to quantitate the amounts of IL-2, IFN-γ, IL-12/IL-23p40 (Th1); IL-4, IL-5 (Th2); TNF-α, IL-1β, IL-6, IL-8 (pro-inflammatory), and IL-10 (immunomodulatory) according to the manufacturer's protocol. Plates were read using a MESO QuickPlex SQ120 (Meso Scale Diagnostics, Rockville, MD).

2.10. Rabbit toxicology

The repeat dose rabbit toxicity study of FMP013 vaccine (Lot#1891) and ALFQ adjuvant (Lot#1974) was conducted at SNBL USA, Ltd. (Everett, WA) according to Good Laboratory Practice regulations (GLP). New Zealand White rabbits aged 13–16 weeks (Charles River Laboratories) were randomized to four groups in a weight-stratified manner to achieve similar dosing group body weight distributions (Table 2) and acclimated for 14 days. Lyophilized FMP013 was reconstituted with sterile water for injection and diluted to 40 μg/mL with either saline or ALFQ, mixed at medium speed on a bottle roller for 1 h at room temperature and used

Table 2
Rabbit vaccine antigen and adjuvant dose by group.

Groups	Dose 1	Dose 2	Dose 3	Dose 4
1	Saline	Saline	Saline	Saline
2	1 mL ALFQ	1 mL ALFQ	1 mL ALFQ	1 mL ALFQ
3	40 μg CSP	40 μg CSP	40 μg CSP	40 μg CSP
4	40 μg CSP 1 mL ALFQ	40 μg CSP 1 mL ALFQ	40 μg CSP 1 mL ALFQ	40 μg CSP 1 mL ALFQ

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Table 3
Rabbit bleed schedule.

Study Day	0	1	3	8	14	27	28	40	41	42	43	45	51	56	70
Vaccination	X				X		X			X					
Chemistry and count	X	X	X					X			X	X			X
C-reactive protein	X		X	X					X			X	X		X
Serology						X				X				X	X
Necropsy												X			X

within 3 h. Four groups of 10 male and 10 female rabbits were dosed IM on study days 0, 14, 28, and 42 (Table 2) alternating between right and left quadriceps for each administration day. Samples of each administered formulation were tested by SDS-PAGE for homogeneity, compatibility and stability assessments.

2.11. Rabbit toxicology readouts

For each animal, clinical observations were performed twice daily beginning on the second day of acclimation, with additional clinical observations performed on dosing days (1 h post dose administration). Dermal draize scoring of injection sites was performed prior to each dose administration and at 3 and 24 h post dose administration. Body temperature was assessed twice during acclimation and on dosing days at the following time points: pre-dose, 4 h post dose, and 24 h post dose. Body weight was assessed twice during acclimation, on study days 0 (pre-dose) and 3, and then weekly thereafter. Food consumption was estimated daily. Ophthalmological evaluations were performed during acclimation and during the week prior to necropsy. Blood collections for hematology were performed according to the schedule in Table 3. A terminal necropsy was performed with half of the animals in each group (5 males and 5 females) on study day 45 and a recovery necropsy was performed with the remaining animals on study day 70. Macroscopic examination was performed at necropsy. Organs were weighed and relative weights calculated as percentages of final body weight and brain weight. For histology, tissues were collected, fixed, processed and stained, and examined microscopically.

2.12. Protein analysis

Reconstituted vials of FMP013 + ALFQ were frozen following rabbit vaccination and analyzed for homogeneity and integrity. A 10 μl aliquot was applied on to a 4–12% acrylamide gel and analyzed by electrophoresis and silver staining.

2.13. Statistical analysis

Rhesus immunological data was analyzed as the mean group value for each parameter. ELISA titers were log transformed and analyzed by ANOVA with Tukey's correction (GraphPad Prism software, La Jolla, CA). Rabbit toxicology was analyzed using Provantis software (InStem, Philadelphia, PA). Briefly, homogeneity of variance was assessed using Bartlett's test on both raw and log-transformed data, followed by a Kruskal-Wallis's H test and Dunn's modification to Steel's test if the results were significant. If the Bartlett's test was not statistically significant, a one way analysis of variance (ANOVA) was applied, followed by a Dunnett's test to determine significant differences between the saline control and each test group. Statistical significance is indicated on figures: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or **** ($p < 0.0001$).

3. Results

3.1. Safety and reactogenicity in Rhesus

As indicated in Table 1, Rhesus macaques (n = 6) were assigned to the following 3 groups based on low (LO) or high (HI) antigen and adjuvant dose (Ag and Adj, respectively): LO Ag + LO Adj, LO Ag + HI Adj and HI Ag + HI Adj. The vaccinated animals were closely monitored for adverse events during the study. No major vaccine-related effects on body weight or food consumption were observed during the entire course of the study. Local reactions at the vaccine site were scored on a 0–3 scale and a mean score was calculated for each group (Fig. 1A). Compared to the 0.5 mL ALFQ dose group (LO Ag + LO Adj; blue bars), the local reactions such as skin warmth, erythema, muscle swelling and muscle induration were more commonly associated with 1 mL adjuvant groups (red[2] and green bars in Fig. 1A). No apparent wounds, ulcers or abscesses were noted and local reactions were considered mild and self-resolving by day 7 post vaccination. Two macaques in the HI Ag + HI Adj group developed an ecchymosis skin reaction around the vaccine site on day 3 post dose 2, which cleared by day 7. Anesthetized animals before vaccinations showed a baseline body temperature between 101.6 and 104.5°F. Out of 54 vaccinations, we recorded 26 episodes of fever (Fig. 1B); five episodes were >104°F, though none exceeded 104.5°F, and all fevers spontaneously resolved by day 7 post vaccination. The LO Ag + HI Adj group had the most frequent fever events overall, most notably post 1st and 2nd vaccinations (red bars, Fig. 1B).

Blood was collected on days 1, 3 and 7 after each vaccination and toxicological changes were recorded. Supplementary data shows plots of group means for data on blood chemistry (Fig S1, S2 and S3) and blood cell count parameters (Fig S4, S5, S6), as well as the upper/lower limit and average baseline values for each parameter. Numerical values (mean ± SD) are shown in Table S1–S3. Specifically, we saw no significant elevations in key biochemical and enzymatic markers of liver or kidney toxicity following FMP013 + ALFQ vaccinations. Increased creatine kinase (CK) on day 1 and 3 after vaccination was observed and the impact was highest in the HI Ag + HI Adj group (Fig. 2A and Fig. S1–3). Elevation of CK in any group did not reach statistical significance over baseline due to a wide range and several high CK values that were observed before the 1st vaccination; nevertheless, most macaques showed CK values returning to normal by day 7 post vaccination (Fig. 2A and Fig. S1–3). Hematological cell count revealed a normal RBC count, hemoglobin and hematocrit level in all groups throughout the study (Fig. 2B and Fig. S4–6). In contrast, a significant increase in WBCs, neutrophils and monocytes was attributed to the vaccine, most frequently seen on day 1 post 1st and 2nd vaccine (Fig. 2C-E, Fig. S4–6). Likewise, a trend of transient increase in platelet counts was observed on day 7 post each vaccination in all groups (Fig. 2F and Fig S6). A sporadic and transient increase in eosinophils was observed both before and during the vaccination period, most likely due to a prior whipworm (gastrointestinal

[2] For interpretation of color in Fig. 1, the reader is referred to the web version of this article.

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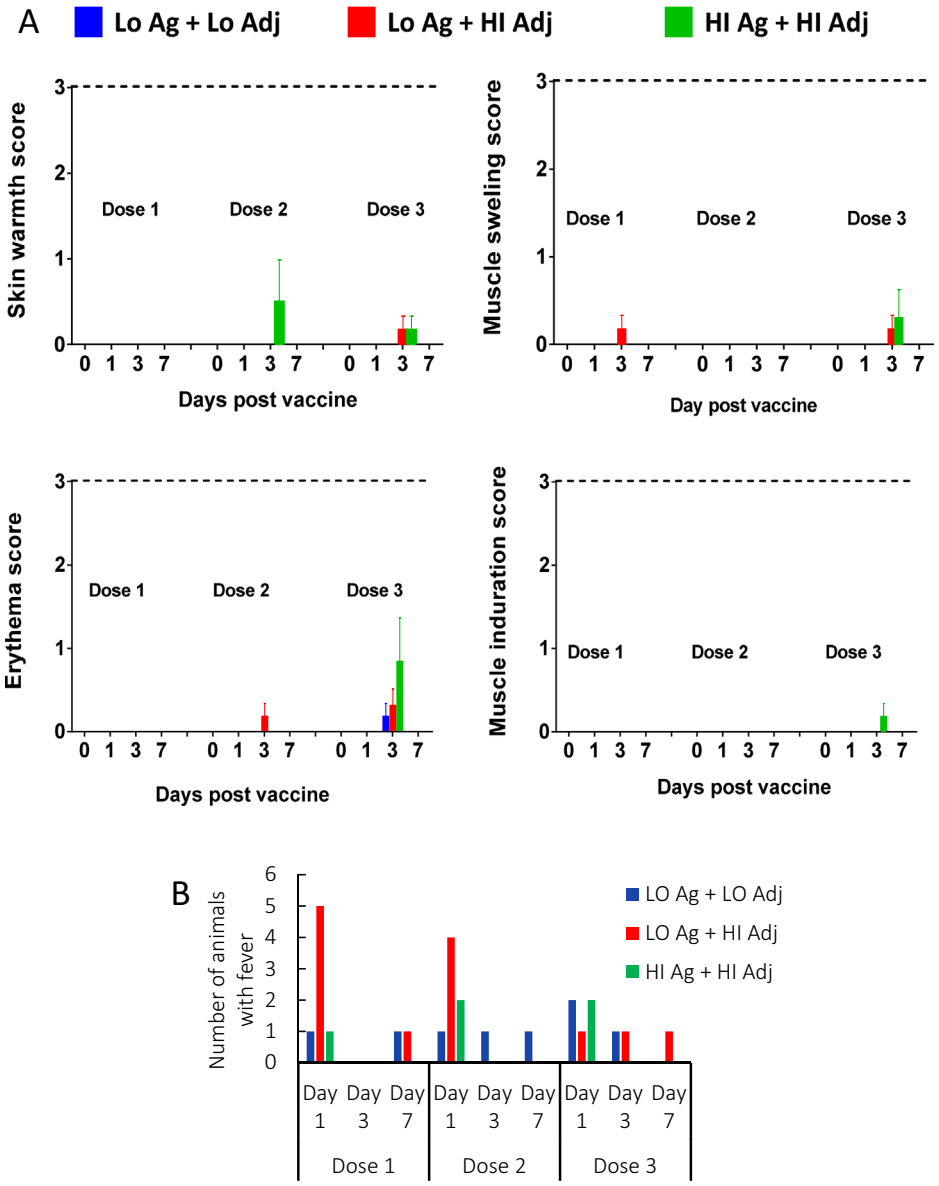

Fig. 1. Local reactions and fevers in Rhesus macaques vaccinated with FMP013 + ALFQ. (A) Group local adverse events score (Mean + SEM for n = 6); dotted line represents max possible score. Bars were omitted if no reaction was observed. No group mean score was significantly above 0, as determined by a one-tailed one sample *t*-test. (B) Number of macaques that developed a fever on day 1, 3, and 7 after each of 3 doses.

parasite) infection that was treated a week before the study began (Fig S4–6). All blood cell count elevations that were statistically significant and vaccine related (WBC, neutrophils, monocytes and platelets) returned to normal before the next vaccination time-point. Having observed only mild and transient reactions and no serious safety signals, we concluded that both the high and low dose FMP013 + ALFQ vaccines were safe and well-tolerated in the Rhesus model.

3.2. Immunogenicity and fine specificity

Serum was collected two weeks after each dose and antibody acquisition against FL (full-length CSP), C-term and NANP repeat antigens was determined by ELISA (Fig. 3A, B, C respectively). All monkeys seroconverted after the 1st vaccination and subsequent vaccinations boosted titers. FL and C-term antibody profiles were similar, but the repeat NANP ELISA revealed suboptimal boosting after 2nd and 3rd vaccination with 0.5 mL ALFQ (Fig. 3C, blue line). Overall, the 20 μg FMP013 + 1 mL ALFQ dose (LO Ag + HI Adj) showed the best antibody acquisition profile over 3 vaccinations (Fig. 3A, B, C, red lines). Group antibody titers were statistically analyzed at 2 weeks post 3rd vaccination (Fig. 3D, E, F). The 0.5 mL ALFQ group showed a trend towards lower mean FL, NANP and C-term responses compared to the 1 mL ALFQ groups, although this effect was not statistically significant (Fig. 3D, E, F; blue bars). Differences in antibody responses were not significant between 20 μg and 40 μg FMP013 doses with 1 mL adjuvant (red *vs.* green bars), although the 20 μg antigen dose appeared to perform slightly better. Since FMP013 antigen contains the N-term, repeat and C-term of CSP, we next mapped region-specific ELISA titers using GST fusion proteins representing the N-term, repeat or C-term regions of CSP. Antibodies to all three regions of CSP were induced by FMP013 + ALFQ, with the highest numerical titers observed against the C-term region and lowest titers against the N-term region (Fig. 3G). Antibody avidity measured by urea wash against the FL antigen showed no difference between groups

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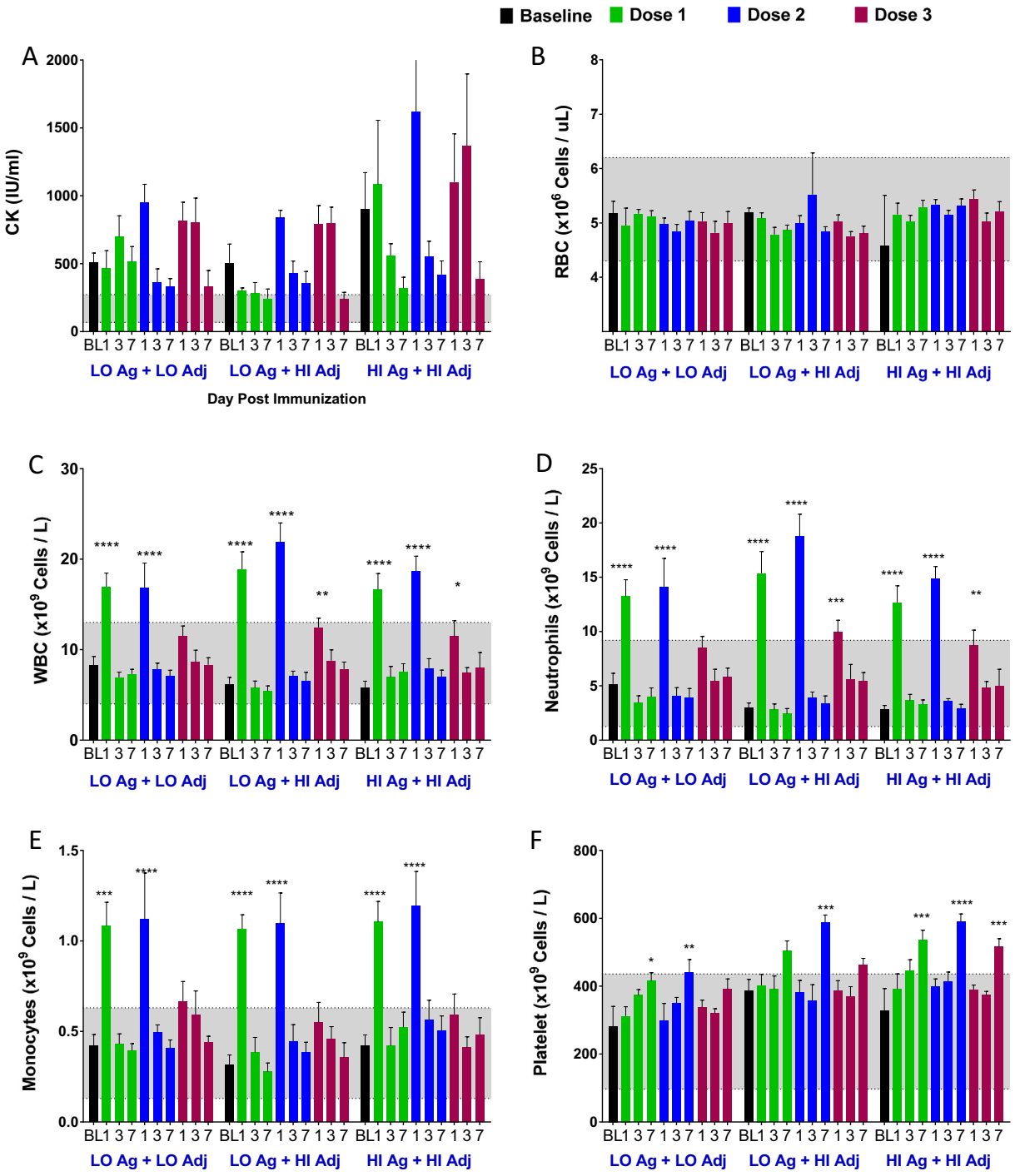

Fig. 2. Blood chemistry and complete blood count changes for Rhesus macaques vaccinated with FMP013 + ALFQ. Group Mean + SEM (n = 6) (A) creatine kinase levels, (B) red blood cell count, (C) WBC, (D) neutrophil, (E) monocyte and (F) platelet cell counts on days 1, 3, and 7 after each of 3 vaccinations (green, blue, and red bars in sequential order). Mean baseline (BL) for each group and reference range (shaded area) are shown. Significance above baseline was determined using ANOVA and Dunnett's correction for multiple comparisons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3H). In addition to potent antibody response against the repeat region, FMP013 induced a broad range of anti-CSP responses, against C-term and N-term epitopes.

3.3. Comparison to historical immunogenicity data

We showed previously that Rhesus macaques (n = 5) vaccinated with 25 μg FMP013 in 0.5 mL Aluminum Hydroxide (containing 0.15 mg Al^{+3}) or 25 μg FMP013 covalently linked to a Qβ phage particle in 0.5 mL Aluminum Hydroxide produced antibody responses against the NANP repeat antigen [32]. We were able to compare the NANP titers from this past study to the present study, since both data were obtained by the International Malaria Serology Reference Center using a standardized ELISA protocol (Fig. 3F, grey bars). Mean NANP titer of the LO Ag + HI Adj and HI Ag + HI Adj groups were statistically superior (>3-fold) to FMP013 in Alum or FMP013 linked to Qβ particles. In another study, Regules *et al.* reported titers for RTS,S + AS01 vaccinated humans (standard dose

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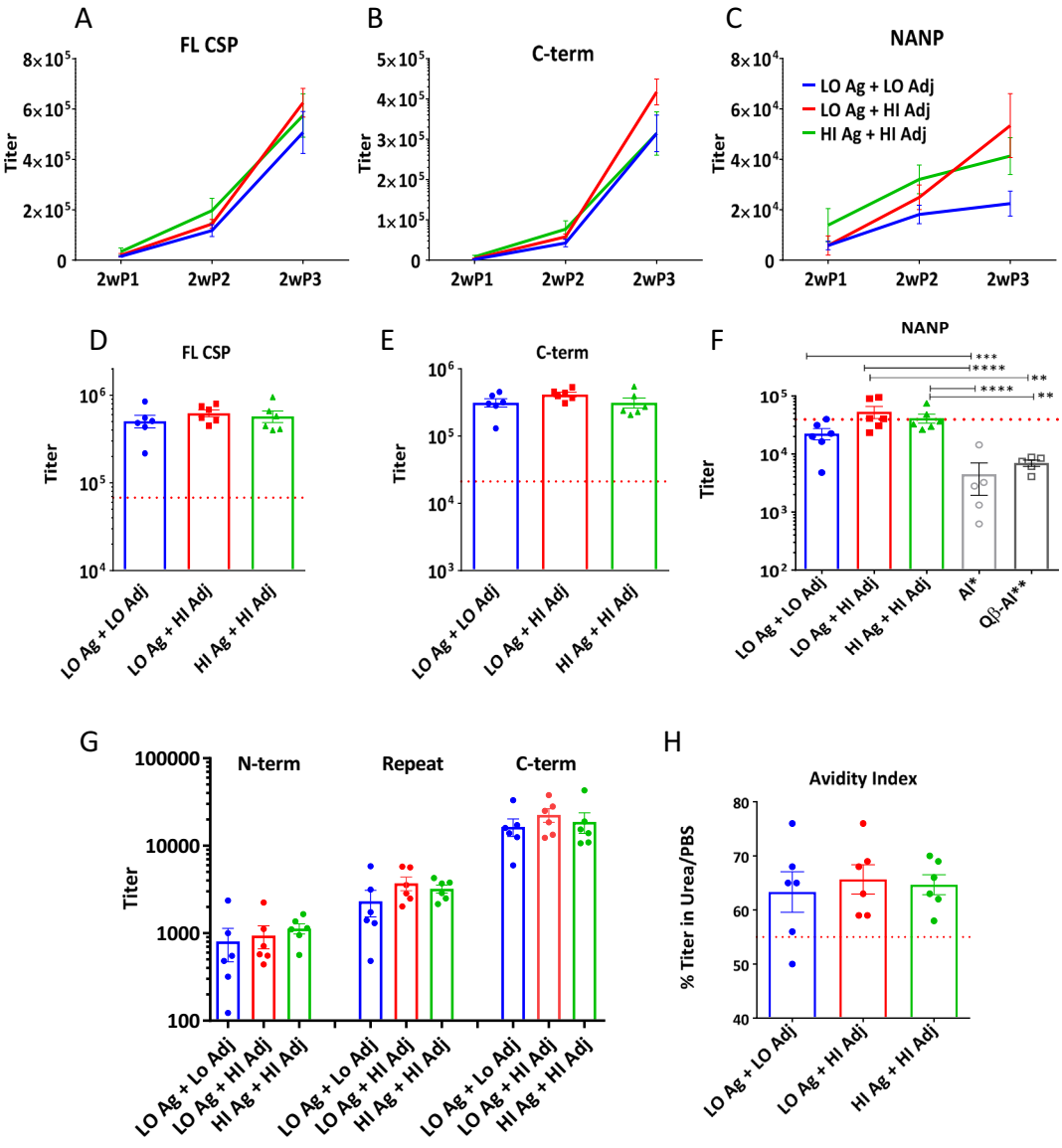

Fig. 3. Antibody profile induced by FMP013 + ALFQ vaccination of Rhesus macaques. Kinetics of antibody acquisition are shown ELISA titer (OD = 1) against (A) FL CSP, (B) C-term or (C) NANP plate antigen 2 weeks after each vaccine dose. (D-F) Comparison of ELISA titer at 2 weeks post 3rd vaccination. (G) ELISA titers against the N-term, Repeat and C-term regions of CSP measured using GST fusion coat antigens. (H) Antibody avidity index at 2 weeks post 3rd vaccination. Al* and Qβ-Al** NANP titers (F) refer to historical data obtained from macaques receiving FMP013 vaccine adjuvanted in Aluminium hydroxide or vaccinated with FMP013 conjugated to Qβ phage and formulated in Aluminium hydroxide. Red dotted lines represent historical mean ELISA titer or avidity against RTS,S + AS01 obtained in humans reported by Regules *et al.* Statistical significance was determined by ANOVA and Tukey's correction for multiple comparisons. Bars represent group mean ± SEM (n = 6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

at 0–1-2 month interval) as part of the MAL071 clinical study [16]. Since identical human ELISA protocols were used in both studies, it was encouraging to note that the mean FL titers, C-term titers and antibody avidity of FMP013 + ALFQ in Rhesus were numerically greater than RTS,S + AS01 in humans, with comparable NANP titers (red dotted lines in Fig. 3D, E, F, H). A major caveat of this observation is that direct numerical comparisons of ELISA titers may be confounded by differences in the cross-reactivity of secondary anti-human antibodies to Rhesus immunoglobulin.

3.4. Functional activity

While FMP013 + ALFQ induces protective immunity in mice [23], there are no transgenic parasites available to challenge Rhesus monkeys vaccinated with *P. falciparum* CSP vaccines. Alternatively, IgG from 3 monkeys (one from each group) was purified and tested in an inhibition of sporozoite development assay (ILSDA) using *P. falciparum* sporozoites and primary human hepatocytes [34]. The assay was performed at ~1:75 dilution of whole serum, considering Rhesus monkeys have ~12 mg IgG per ml serum [35]. The assay was repeated 3 times to account for variability in the quality of primary human hepatocytes, the quality of sporozoite isolation and non-specific inhibition by pre-immune IgG. IgG from one animal in the LO Ag + HI Adj group animal showed ~2 log reduction in mean parasite 18 s rRNA burden that was significantly higher than pre-immune serum inhibition (Fig. 4A, shaded bar). An ELISA revealed that the relative NANP titer of this inhibitory IgG sample was about 2-fold higher than the non-inhibitory samples (Fig. 4B).

3.5. T-cell responses

PBMCs collected at 4 weeks post 3rd vaccination were stimulated by a pool of overlapping CSP peptides (Megapool) or the

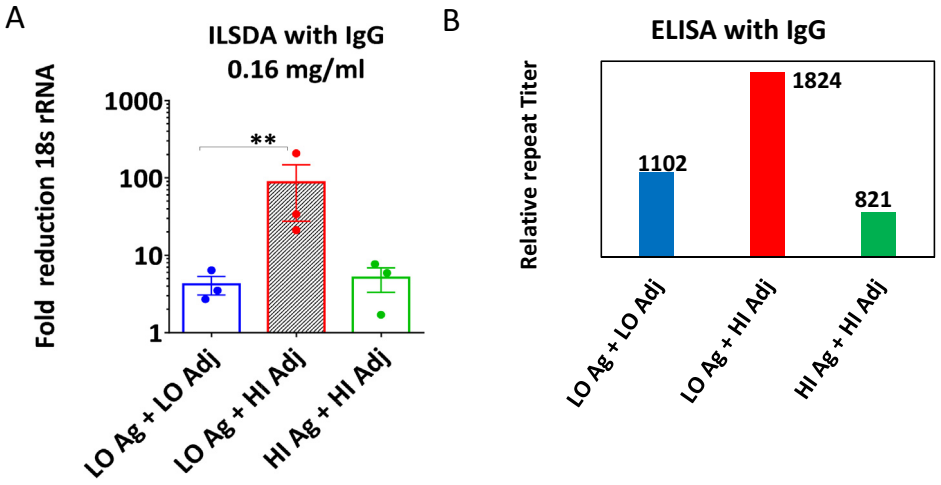

Fig. 4. (A) Functionality of purified Rhesus IgG assessed in an inhibition of liver stage development (ILSDA) assay, measured by fold reduction in *P. falciparum* sporozoite 18 s RNA using 0.16 mg/ml total IgG purified from one animal in each group (~1:75 dilution of whole serum). This assay was performed in triplicate in three independent experiments. Shaded bar represents significantly higher inhibition activity than pre-immune serum (B) Relative NANP titer obtained from ELISA of the purified IgG used in (A).

nearly full-length FMP013 protein (FL-CSP). The frequency of CSP-specific cytokine producing cells was then determined by Fluorospot. Significant IL-2 and IFN-γ producing cells were detected, though IFN-γ responses failed to achieve statistical significance over baseline pre-immune PBMCs (Fig. 5). No antigen or adjuvant dose effects were observed between groups. A Th1-biased T-cell response was further confirmed in vaccinated Rhesus by a 10-plex MesoScale assay (Fig. S7). Cytokine analysis shows a predominant TH1 profile (IL-2, TNF-a, IL-12/23p40) with some contribution by IL-5 (TH2, mediating antibody responses) and IL-6 (pro-inflammatory, supporting B cell responses). The IL-2 responses were highest in the HI/HI group regardless of the in vitro stimulation (FL-CSP *vs.* CSP peptides (Fig. S7).

3.6. Rabbit toxicology

To supplement safety data from the Rhesus trial, FMP013 and ALFQ were tested in four groups of 10 male and 10 female New Zealand White rabbits according to Table 2. Whole blood and serum samples were collected for toxicology readouts following the bleed schedules shown in Table 3. Following four doses of 40 μg FMP013 + 1 mL ALFQ administered at 2 week intervals, no injection site reactions, no change in body weight, no temperature elevation and no reduction in food consumption was noted. On day 1 after the 1st and 4th vaccine, a statistically significant increase in mean neutrophil counts was observed for ALFQ and FMP013 + ALFQ but not in saline and FMP013 groups (Fig. S8A), returning to normal levels at subsequent assessment time point. Coagulation assessment showed ALFQ administration caused elevated fibrinogen on day 1 and day 3 post vaccination in ALFQ and FMP013 + ALFQ groups, also returning to normal at the subsequent assessment time point (Fig. S8B). A broad range of CRP concentrations were found in the rabbits, including moderately high values observed during the acclimation period prior to 1st vaccination. CRP concentrations trended higher on day 3 post 1st and 4th vaccine in ALFQ or FMP013 + ALFQ groups (Fig. S8C). One animal in the FMP013 alone group also demonstrated higher than normal CRP at 28 days post 4th vaccination; however similar elevations were observed during acclimation period.

Results of terminal procedures and pathology demonstrated no abnormal observations or differences between groups by gross pathology or organ weight. At terminal necropsy (study day 45), histological assessments of the injection sites demonstrated increased incidence and severity of subcutaneous or muscular inflammatory cell infiltration in ALFQ and FMP013 + ALFQ groups. At recovery necropsy (study day 70), most of these findings had resolved and thus considered reversible. A few animals in the saline and FMP013 alone groups also demonstrated some inflammatory cell infiltration at injection sites during the terminal necropsy, though no animals in either group demonstrated such findings during the recovery necropsy. Based on the evaluated parameters, results of the repeat dose rabbit toxicity study

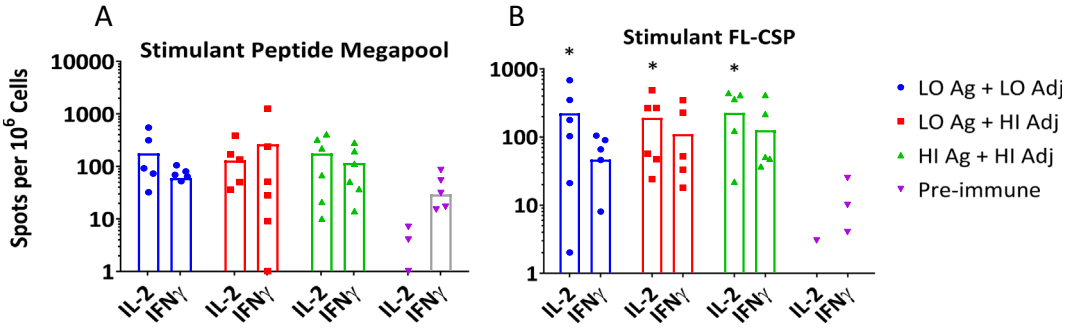

Fig. 5. T-cell response induced by FMP013 + ALFQ vaccination of Rhesus macaques. T-cell response determined by IL-2 and IFN-γ Flourospot at 4 weeks post 3rd vaccination. Rhesus PBMCs were stimulated with either (A) a peptide pool spanning the full length CSP sequence or (B) FL CSP vaccine antigen. Bars represent mean + SEM spots formed per million cells (n = 6). Cellular responses significantly above pre-immune baseline are shown using ANOVA and Dunnett's correction for multiple comparisons.

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demonstrated that intramuscular administration of FMP013 alone or in combination with ALFQ was well tolerated by study animals without any adverse effects.

3.7. Rabbit immunogenicity and stability of the formulation

Sera from male and female rabbits (n = 10) were pooled separately and analyzed for seroconversion (Fig. S9). No antibodies were detected in saline and ALFQ groups; FMP013 alone was minimally immunogenic (~1000 endpoint titer) but FMP013 + ALFQ showed about one log higher titers and boosting post 2nd and 3rd vaccination. FMP013 antigen analyzed by SDS-PAGE after formulation in ALFQ migrated as a single band on silver stained gel, confirming that the antigen did not breakdown or aggregate after the 4 h storage at room temperature during vaccination of rabbits (Fig. S10). Overall, the observations of FMP013 + ALFQ obtained from study in rabbits correlate the safety and immunogenicity data from Rhesus macaques. Combined, these outcomes support FMP013 + ALFQ proceeding to clinical trial.

4. Discussion

Vaccination of Rhesus macaques with FMP013 + ALFQ did not induce ulceration or abbesses at the vaccine site, with only transient local reactions notable, particularly in the groups receiving 1 mL of ALFQ. This included an episode of ecchymosis observed at the vaccine site in two macaques at day 3 post 2nd vaccination. Ecchymosis is an acute inflammatory response that could be elicited by immune-modulators in ALFQ, though ecchymosis can often result from needle injury during vaccination. We believe that latter was the case because 1 mL ALFQ containing vaccines have been administered in multiple studies more than 100 times in Rhesus of Indian and Chinese origin (including this study) with no recurrence of vaccine site ecchymosis (unpublished data). Additionally, no ecchymosis reaction or any other local reaction was observed in rabbits after four 1 mL administrations of the vaccine. In our studies, administration of 1 mL ALFQ caused only mild and transient local reactions that are not uncommon for vaccines currently approved for use in adults and infants.

Transient elevation of white blood cell counts (WBC), particularly neutrophil counts, was observed in both Rhesus and rabbit models. A similar elevation of WBC has been reported for other adjuvants, including AS01 in Rhesus [36–38]. We also report a trend towards increased platelet counts in Rhesus. While increased platelet counts could suggest mild hemotoxicity or an inflammatory response to the vaccine, this observation was almost exclusively noted at the last scheduled phlebotomy after each vaccination (day 7), suggesting that this could be due to multiple phlebotomies within a short time period. Regardless of the cause, the platelet count abnormalities resolved before the next vaccination time-point and, most importantly, the hematocrit, RBC count, and hemoglobin remained stable in all studied macaques and rabbits. The current dataset further confirms that the unique cholesterol and lipid ratio of ALFQ reduces RBC lysis caused by free QS-21, as has previously been shown [39]. Eosinophil levels were higher than normal after vaccination in some of the macaques both before and during the vaccination period. Eosinophil elevation is a marker of hypersensitivity and its elevation could be associated with a previously treated parasitic worm infection in the colony. Overall, the cellular changes in blood observed after FMP013 + ALFQ administration in Rhesus and rabbits were transient and they normalized prior to the next vaccination time-point. Elevation of cellular markers of inflammation is caused by a robust innate immune response to vaccination which may lead to a strong adaptive response required for protective immunity against malaria [40].

No evidence of gross toxicity with respect to elevation in liver enzymes or kidney function panel was apparent following FMP013 + ALFQ administration in rabbits and Rhesus. The levels of fibrinogen and CRP were transiently elevated in rabbits after ALFQ administration, both of which are acute-phase proteins that elevate during systemic inflammation [41]. ALFQ administration caused CK elevation and transient fevers in Rhesus, both of which are markers of tissue damage and have been also reported to elevate following AS01 administration in Rhesus [24,30]. Overall, a transient systemic inflammation was confirmed by cellular and biochemical excursions in both Rhesus and rabbits, most likely linked to the potent immune-modulators 3D-PHAD® or QS21 in ALFQ.

The 20 μg FMP013 + 1 mL ALFQ dose (containing 200 μg 3D-PHAD® + 100 μg QS21) showed the best immunogenicity and functional antibody profile that was also superior to previously reported immunogenicity of FMP013 in Aluminum Hydroxide and as a Qβ particle conjugate [32] We also show numerical non-inferiority of 20 μg FMP013 + 1 mL ALFQ induced titers in Rhesus to the historical RTS,S + AS01B induced titers in humans [16]. The 0.5 mL AS01B volume contains 50 μg MPLA and 50 μg QS21 which has been safely administered to adults and children in RTS,S and Shingrix™ vaccine trials [14] and MPLA dose up to 200 μg has been safely tested in humans [42]. The safety data obtained from two animal models with a relatively high dose of immune-modulators in ALFQ reaffirms our hypothesis that using a specific lipid and cholesterol ratio in ALFQ can reduce QS21-mediated cell lysis [39] and other toxic adverse effects. With only minor and transient adverse effects observed in Rhesus and rabbits, we proposed that 20 μg FMP013 + 1 mL ALFQ be the full-human dose of this vaccine.

Rhesus data confirm previous mouse data that high titers of antibodies could be induced by FMP013 + ALFQ vaccine [23]. We also showed previously that soluble FMP013 conjugated to virus-like particle Qβ specifically enhanced the NANP repeat responses [32 21]. Here, soluble FMP013 + ALFQ induced high repeat and C-term responses along with measurable N-term responses. Epitopes outside of the repeat region of CSP may be important to induce protection. The C-term appears to have a protective role in RTS,S field trials in humans [4] and monoclonal antibodies against a protease cleavage site [5] and a junctional epitope [43] in the N-term have protected mice. Since the N-term is absent in RTS,S, the epitope broadening reported using nearly full-length soluble FMP013 may augment the degree and longevity of protection. Furthermore, cost-effectiveness of manufacturing and stability of soluble proteins could be critical for disease elimination efforts against malaria. Regulatory approvals are being sought for the conduct of a CHMI trial with FMP013 + ALFQ in the near future.

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Disclaimer

Material has been reviewed by the Walter Reed Army Institute of Research and the US Agency for International Development. There is no objection to its presentation and/or publication. The

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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, the Department of Defense, or the US Agency for International Development.

Ethics approval

Research was conducted under an 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.

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Declaration of Competing Interest

SD and the US Army hold the patent on FMP013 antigen. SD, ZB and CA have filed for a patent on FMP013 + ALFQ formulation.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.05.059.

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