Immunogenicity of Self-Associated Aggregates and Chemically Cross-Linked Conjugates of the 42 kDa Plasmodium falciparum Merozoite Surface Protein-1

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Abstract

Self-associated protein aggregates or cross-linked protein conjugates are, in general, more immunogenic than oligomeric or monomeric forms. In particular, the immunogenicity in mice of a recombinant malaria transmission blocking vaccine candidate, the ookinete specific Plasmodium falciparum 25 kDa protein (Pfs25), was increased more than 1000-fold when evaluated as a chemical cross-linked protein-protein conjugate as compared to a formulated monomer. Whether alternative approaches using protein complexes improve the immunogenicity of other recombinant malaria vaccine candidates is worth assessing. In this work, the immunogenicity of the recombinant 42 kDa processed form of the P. falciparum merozoite surface protein 1 (MSP142) was evaluated as a self-associated, non-covalent aggregate and as a chemical cross-linked protein-protein conjugate to ExoProtein A, which is a recombinant detoxified form of Pseudomonas aeruginosa exotoxin A. MSP142 conjugates were prepared and characterized biochemically and biophysically to determine their molar mass in solution and stoichiometry, when relevant. The immunogenicity of the MSP142 self-associated aggregates, cross-linked chemical conjugates and monomers were compared in BALB/c mice after adsorption to aluminum hydroxide adjuvant, and in one instance in association with the TLR9 agonist CPG7909 with an aluminum hydroxide formulation. Antibody titers were assessed by ELISA. Unlike observations made for Pfs25, no significant enhancement in MSP142 specific antibody titers was observed for any conjugate as compared to the formulated monomer or dimer, except for the addition of the TLR9 agonist CPG7909. Clearly, enhancing the immunogenicity of a recombinant protein vaccine candidate by the formation of protein complexes must be established on an empirical basis.

Introduction

Chemical conjugation is widely used to make haptons such as peptides and polysaccharides immunogenic. This is particularly significant for the development of several important human vaccines against polysaccharide moieties such as Hemophilus influenzae type b, Streptococcus pneumoniae, Neisseria meningitidis and Salmonella enterica serovar Typhi [1,2,3,4]. Chemical conjugation can also be used on some proteins that are poor immunogens in order to enhance their immunogenicity. Conjugation effectively enhances the immunogenicity of the Plasmodium falciparum Pfs25, a transmission blocking malaria vaccine candidate, when recombinant Pfs25 was conjugated either to carrier proteins such as the outer-membrane protein complex of Neisseria meningitidis or ExoProtein A of Pseudomonas aeruginosa, (a detoxified form of exotoxin A from P. aeruginosa) or to itself (self-conjugation) [5,6,7]. Significant enhancement of Pfs25-specific antibody responses induced in both mice and rhesus monkeys was achieved. Similar results were observed when two other malaria antigens of P. falciparum, Pfs28 and AMA1, were conjugated to the ExoProtein A [7,8]. In addition to enhancing immunogenicity, conjugation may overcome the restriction of host genetic backgrounds of vaccinees. While the single MSP119 of P. yoelii failed to induce specific antibody responses in mice expressing H-2s major histocompatibility complex haplotype, its conjugate coupled to diphtheria toxoid induced functional antibody responses in these mice [9].

The P. falciparum merozoite surface protein 1 (MSP1) is considered an important candidate for a vaccine approach targeting clinical disease or more specifically erythrocytic stage parasites. MSP1 is synthesized during blood stage development as
a precursor with a molecular mass of ~200 kDa, and later undergoes post-translational proteolytic processing. The proteolytic processing produces a C-terminal 42 kDa fragment (MSP142) which is subsequently processed to 33 kDa and 19 kDa [10,11]. Although inhibitory antibodies of MSP142 are principally directed toward the 19 kDa fragment [12], the T cell epitopes on the 33 kDa MSP1 fragment enhance the immunogenicity and protective efficacy of the recombinant MSP142 in non-human primates [13] and humans [14].

Several formulated MSP142-based recombinant proteins of *P. falciparum* have been tested in *Aotus* monkeys [13,15,16,17,18] as well as in humans [19,20,21]. Protection against a lethal parasite challenge in *Aotus* monkeys has been reported, and is generally associated with a high level of MSP142-specific antibody titers using Freund’s adjuvant [17,18]. In contrast, in a phase 1 human trial a recombinant MSP142/Alhydrogel™ vaccine formulation induced only a weak antigen-specific antibody response [19]. Various efforts have been made to enhance the immunogenicity and/or improve the efficacy of MSP142-based vaccines, including the addition of toll-like receptor (TLR) agonists to the formulation [20,21,22], and the construction of chimeric proteins that replace the MSP133 fragment either with other malarial antigens [23,24] or adjuvanting protein fragments [25,26,27,28]. Only the use of TLR agonists in vaccine formulations has subsequently been evaluated in human clinical trials, showing enhanced antibody responses [20,21].

In this study, we evaluated whether the immunogenicity of MSP142 in mice is enhanced when presented as 1) a self-associated aggregated protein or 2) chemically conjugated to a carrier protein formulated on Alhydrogel with or without CPG 7909, a synthetic B type CpG-ODN (unmethylated oligodeoxynucleotide containing cytosine-guanosine (CpG) dinucleotide motifs). In contrast to previously reported findings for Pb85 [5,6,7], neither self-association nor chemical conjugation to ExoProtein A (EPA) enhanced the immunogenicity of recombinant MSP142 in mice.

### Materials and Methods

#### Ethics Statement

Rodent studies were carried out in compliance with the National Institutes of Health guidelines and an animal care and use committee-approved protocol.

**MSP142.**

**Antigens and Carrier Protein**

MSP142-FUP and MSP142-FVO are two allelic forms of recombinant *P. falciparum* MSP142, with an E-KNG or Q-KNG MSP19 phenotype, respectively. The recombinant MSP142 proteins were expressed in *Escherichia coli*, refolded, purified and characterized as previously described [16,29]. The MSP133 fragment of MSP142-FUP contains a single unpaired cysteine residue, which is absent in the MSP133 fragment of the FVO allele that provided an unpaired sulfhydryl group for the conjugation of the MSP142-FUP to a carrier protein modified by maleimide groups. The aggregated MSP142-FVO protein was produced following the reported purification process, except the S30 reverse-phase chromatography step was replaced with a hydrophilic interaction chromatography (HIC) step following the refold by rapid dilution. The HIC step used a Phenyl 650 M (GE Healthcare, Piscataway, NJ) column equilibrated in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 12.2 M NaCl, 1 M Urea at 200 cm/h. Sodium chloride crystals were added to the rapid dilution refolded protein to make a 1.2 M final concentration and loaded onto the Phenyl 650 M at 200 cm/h after which unbound proteins were washed from the column using equilibration solution. The MSP142-FVO protein eluted from the column with 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 M Urea was pooled and polished using a Superdex 200 size exclusion column (GE Healthcare, Piscataway, NJ) equilibrated with PBS with 0.02% polysorbate 80, pH 7.4. The EPA was used as a carrier protein in this study, was produced in an *E. coli* expression system, as previously described [7].

#### Maleimide Modification

Two chemical linkers, N-[6-maleimidocaproyloxy]sulfosuccinimide ester (Sulfo-EMCS) and succinimidyl-[N-maleimidopropionamido]-diethylenglycol] ester (NHS-PEO₂-Maleimide) (Pierce Inc., Rockford, IL), containing hydrocarbon and polyethylene glycol spacers, respectively, were used to modify EPA. These two chemical linkers are heterobifunctional cross-linkers with an N-hydroxysuccinimide (NHS) ester and a maleimide group at each of their termini. The maleimide-reaction pH was fixed at 7.2 and the EPA concentration was fixed at 2 mg/mL. Three other parameters: reaction time, reaction temperature and chemical linker concentration were optimized by employing a model using a three-level Box-Behnken design and the JMP statistical software (SAS Institute, Inc., Cary, NC). The final parameters used for these conditions were a reaction time of 60 min, a reaction temperature at 22°C and linker concentration of 2 mM.

The EPA was buffer exchanged to PBS-E (1× PBS, 5 mM EDTA, pH 7.2) using 5 kDa MWCO spin filter (Millipore, Billerica, MA). The sulfo-EMCS dissolved in PBS-E and NHS-PEO₂-Maleimide dissolved in Dimethylsulfoxide were added to the EPA, respectively. The mixtures were incubated under the defined conditions with gentle shaking. At the end of the reaction, stop solution (1 M Tris-HCl pH 7.4) was added to a final concentration of 20 mM and then the buffer was immediately exchanged to PBS-E. The maleimide modified EPA (maleimide-EPA) was characterized by reversed-phase HPLC and by maleimide measurement.

#### Conjugation

The purified MSP142-FUP protein was reprocessed prior to conjugation using a preparative SEC column (16×60 mm, Superdex 200) equilibrated with PBS-EU (1× PBS, 1 mM EDTA, 5 M urea, pH 7.2) in order to remove the 0.2% polysorbate 80 present in the protein solution and expose the single unpaired cysteine residue on the MSP133 fragment for conjugation. The conjugation conditions were 22°C for 1 hour with gentle shaking in PBS-EU. MSP142-FUP and EPA-maleimide were mixed based on an equal number of moles of free sulfhydryl and maleimide groups. The MSP142-FUP-EPA conjugate was loaded on a SEC column (16/60 Superdex 200) equilibrated with PBS-A (1× PBS, 0.5 M arginine, pH 7.2). The peak elution fractions containing MSP142-FUP-EPA conjugates were selected and pooled based on a Coomassie blue stained SDS-PAGE gel.

#### Characterization of Recombinant Protein Intermediates and Conjugates

##### A. SDS-PAGE and Western blotting

The EPA conjugates of MSP142-FUP were characterized by Coomassie blue stained SDS-PAGE under non-reduced condition [29]. Western-blot was performed as described previously using antigen specific monoclonal antibodies AD223 and 13E3-53 [30].

##### B. Maleimide measurement

Maleimide groups were measured using Ellman’s reaction (indirect) as per manufacturer’s instructions (Pierce Inc., Rockford, IL). Briefly, the maleimide-EPA samples were titrated with the solution of Cysteine.
Hydrochloride Monohydrate. After the addition of Ellman’s reagent to each reaction, the absorbance was read at 405 nm. Based on the cysteine consumed in the reaction, the concentration of maleimide in the maleimide-EPA sample was determined relative to the standard curve of the cysteine with increasing concentrations. The number of maleimide groups added onto the EPA was obtained by dividing the moles of maleimide by the moles of EPA.

C. Composition analysis of MSP142-FUP-EPA conjugate by amino acid analysis. Amino acid analysis was performed by the W.M. Keck Facility at Yale University. These results were used to calculate the molar ratios of MSP142-FUP to EPA (average conjugation ratio) [31] and the concentrations of MSP142-FUP molecules.

D. Reversed Phase-HPLC. Recombinant EPA was characterized, pre- and post-modification with maleimide groups, on an analytical 2.1 x 250 mm C4 column (GraceVydac, Hesperia CA) connected to a Waters 2695 HPLC system (Waters, Milford, MA). The column was equilibrated in 95% acetonitrile +0.1% TFA (trifluoroacetic acid) and the proteins eluted in a gradient of 40% to 58% acetonitrile +0.1% TFA over 36 minutes at a flow rate of 0.2 mL/minute.

E. SEC-MALS-HPLC. SEC-MALS-HPLC [7,29] was performed on a Waters 2695 HPLC system, with an in-line Wyatt Dawn EOS light scattering detector, a quasi-elastic light scattering detector (QELS) and an Optilab refractive index detector (Santa Barbara, CA). The MSP142-FVO self-associated aggregates or MSP142-FUP-EPA conjugate were analyzed on a G4000SWxl size exclusion column (Tosoh Biosciences, Montgomeryville, PA) equilibrated with the following solution: 1.04 mM K2HPO4, 2.97 mM Na2HPO4, 308 mM NaCl, and 0.02% azide, pH 7.4 at a flow rate of 0.5 mL/minute.

F. Endotoxin level measurement. Endotoxin levels of the various antigens were measured using *Limulus* amoebocyte lysate in a 96-well plate with chromogenic reagents and PyroSoft software (Associates of Cape Cod Inc., East Falmouth, MA) before administration. The endotoxin values were all less than 41 EU/mg of recombinant MSP142.

Animal Studies and Serological Assays

Antigens were formulated on 1600 mols CPG 7909 formulations of MSP142-FUP monomer and two types of MSP142-FUP-EPA conjugates were compared at the doses of 5 and 13 μg.

Enzyme-linked immunosorbent assay (ELISA) was performed on each individual mouse serum to measure the antibody titer following a standardized protocol [32,34]. A Mann-Whitney U Test was performed in the first mouse study to test for significant differences of antibody titers between the two groups receiving different kinds of antigens at each dose level. If the P value was less than 0.05, the differences were considered significant. In the second study, Kruskal-Wallis One-Way ANOVA was performed among the groups at each dose level on any of three sera collection days. If the P value was less than 0.025, a post hoc analysis of Student-Newman-Keuls was performed. If the P value of Student-Newman-Keuls was less than 0.05, the differences were considered significant. For the IgG subclass analysis, ELISA was performed on the pooled sera of each group, which were pooled based on the equivalent ELISA unit of each individual serum [32,35].

Results

Preparation and Characterization of MSP142-FVO Self-associated Aggregate and MSP142-FUP-EPA Conjugates

A MSP142–FVO self-associated aggregate was produced while developing a modified purification process that aimed to replace usage of a Source 30 pilot-scale reversed phase column with a hydrophobic interaction column in order to avoid the use of organic solutions during pilot-scale manufacturing. The molar mass of the MSP142-FVO aggregate in an aqueous solution was determined by SEC-MALS-HPLC to be approximately 1.7 MDa for 96% of the total peak area as compared to a purified form following a standard procedure, which yielded 72% dimers and 28% multimers with a molar mass of approximately 80–100 kDa and 0.700–1MDa, respectively (Figure 1A). Analysis of the same protein lots by Coomassie blue stained SDS-PAGE analysis under reduced and non-reduced conditions showed that a predominant band at approximately 42 kDa was observed, which is consistent with the expected mass of 42,173 Da. Based on protein mobility in the presence of SDS, the solution state of the self-associated aggregate appeared to be primarily due to hydrophobic or ionic interactions, and not due to disulfide bond formation between different forms of MSP142-FVO (Fig. 1B).

To determine the best conditions for linker addition, a Surface Response Mode (RSM) was used to pinpoint the maximum or minimum condition for three factors considered key for process development: temperature, pH and linker concentration. To evaluate these variables together, a Box-Behnken’s RSM was used to determine the optimum condition for the maleimide modification reaction based on the following conditions: temperature (22–26°C), Sulfo-EMC concentration (1.0–4.0 mM) and reaction time (30–90 minutes). All three factors were statistically significant, p<0.05, and played an important role in the number of modifications observed based on the Ellman’s Reaction. However, due to process development constraints, and tolerability of temperature and pH to marked linker substitution, the following conditions were used: reaction temperature 22°C, linker concentration of 2 mM, and reaction time 60 min (Figure 2A). To test the validity of the Box-Behnken’s RSM, the residual plot was investigated. Figure 2B shows the Ellman’s reaction was randomly dispersed around the horizontal axis with no trend, which is consistent of a linear regression model. Furthermore, the fitted model accounted for (R-squared) 96% of the variation in Ellman’s reaction. We were interested in only main factors in the RSM and did not account for squared terms, interactions or synergistic effects. Further process optimization, using the Box-Behnken
RSM, could address higher order terms, and surface response model curvatures.

Batches of chemical cross-linked MSP142-FUP-EPA conjugates were prepared using two different maleimide cross-linkers Sulfo-EMCS and NHS-PEO2-maleimide. The composition of each linker indicates that the solubility properties may be different, even though this did not appear to impact the solubility of the conjugates (data not shown). Recombinant EPAAPA and EPAPEO, modified by Sulfo-EMCS and NHS-PEO2-maleimide, respectively, were prepared and the addition of the bifunctional linkers was monitored by RP-HPLC analysis. The retention times as well as the peak shape of the modified EPA shifted with the addition of the maleimide groups onto the protein (Figure 3A). The extent of the shift in retention time appeared dependent on the number and physical properties of the chemical modifiers (Figure 3A and data not shown). The number of maleimide groups added on EPA was assessed by titrating with cysteine and measuring the absorbance at 405 nm (Figure 3B). The titration curves of two batches of EPAAPA and one batch of EPAPEO were similar (Figure 3B). Based on the titration curves and the standard curve of cysteine, the number of maleimide groups added onto the EPA was calculated to be 5.0 and 4.7 for the two batches of EPAAPA and 5.0 for the EPAPEO.

The EPAAPA and EPAPEO carriers were conjugated with MSP142-FUP and the resultant conjugates were purified by preparative SEC. The SEC elution fractions were analyzed by Coomassie blue stained SDS-PAGE and relevant fractions were pooled (Figure 4A). Analysis of the mobility of the conjugates by Coomassie blue stained SDS-PAGE indicated that the conjugation products were chemically cross-linked complex protein-protein mixtures with ratios ranging from 1:1 MSP142-FUP per carrier to approximately 6:1. The predominant conjugated forms

Figure 1. SEC-HPLC-MALS and Coomassie blue stained SDS-PAGE gel analysis of aggregated MSP142-FVO. (A) Size exclusion chromatography with multi-angle light scattering and (B) SDS-PAGE analysis run on a 4–20% gradient Tris–glycine polyacrylamide gel under non-reducing (NR) and reducing (R) conditions.

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Figure 2. Multi-parameter analysis of linker substitution. Response curves for the analysis of temperature, modifier concentration and time on the linker substitution of EPA using a Box-Behnken model (A). Residual plot of the Ellmans’ reaction showing there is no systematic pattern (B). A linear regression model was used for analysis given the following assumptions: relationships between dependent and independent variables are linear, no serial correlation, the response variables are normally distributed, and have the same variance.

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appeared at a ratio of 3:1 and 4:1 (see Figure 4A asterisks). A
minor quantity of unreacted MSP142-FUP was observed in the
SEC pool. The average conjugation ratio for MSP142-FUP-
EPAAPA and MSP142-FUP-EPAPEO by amino acid analysis was
3.9 and 3.8, respectively. Thus the results obtained by mobility
on SDS-PAGE and amino acid analysis were consistent for the
ratio of MSP142-FUP and EPA by mass ratios and molar ratios.
Considering that the unreacted MSP142-FUP was not completely
removed from either conjugate, the average conjugation ratios
should be slightly lower than the reported values. Analysis by
SEC-MALS-HPLC showed the presence of three populations or
peaks for each conjugate with the second peak representing the
major conjugated form with the major peak consisting of 80% of
total protein for the MSP142-FUP-EPAAPA and 81% of the total
protein for the MSP142-FUP-EPAPEO (Figure 4B). The weighted
average masses of the MSP142-FUP-EPAAPA were $1.1 \times 10^7$, $1.6
\times 10^6$ and $1.4 \times 10^5$ Da, respectively, whereas the weighted
average masses for the MSP142-FUP-EPAPEO of three peaks

Figure 3. Characterization of chemically modified EPA. Analysis of un-modified EPA, EPAAPA batch 1, EPAAPA batch 2 and EPAPEO by RP-HPLC
(A), and titration analysis of EPAAPA batch 1, EPAAPA batch 2 and EPAPEO (B) are shown.
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Figure 4. Characterization of MSP142-FUP-EPA conjugates. Panel (A) Coomassie blue stained SDS-PAGE gel analysis of maleimide-EPA (lanes 1
and 5); monomeric MSP142-FUP (lanes 2 and 6), un-purified conjugation mixture of MSP142-FUP-EPAAPA (lane 3), bulk purified MSP142-FUP-EPAAPA
(lane 4); un-purified conjugation mixture of MSP142-FUP-EPAPEO (lane 7) and bulk purified MSP142-FUP-EPAPEO (lane 8). The asterisks indicate the
conjugates with 3:1 and 4:1 ratio. Panel (B) SEC-HPLC-MALS analysis. Solid and dashed lines represent absorbance at 280 nm and molecular mass for
MSP142-FUP-EPAPEO and MSP142-FUP-EPAAPA, respectively. The capital letters A, B and C indicate the profile peaks of each conjugate.
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were $1.1 \times 10^7$, $1.3 \times 10^6$ and $1.1 \times 10^5$ Da (Figure 4B, A–C, respectively). Since the predominant weighted average mass for each conjugate by SEC-MALS-HPLC was greater than the ratios determined by Coomassie blue stained SDS-PAGE, this indicates that a non-covalent association exists in the presence of the 0.5 M arginine, which was used to stabilize the solubility of the conjugates. The conjugates in this form were stable to freeze-thaw by analytical SEC-MALS-HPLC and were filterable through a 0.22 μm filter (data not shown). Two conformation-dependent monoclonal antibodies, AD223 and 13E3-53 [30], were used to assess the structural integrity of the conjugated MSP142-FUP proteins by Western blot analysis. Each protein band observed by Coomassie blue stained SDS-PAGE appeared to be recognized by both monoclonal antibodies (data not shown), demonstrating that the conformational structure of the 19 kDa fragment of MSP142-FUP remained intact during the process of conjugation.

Assessment of Immunogenicity of Aggregated, Chemically Conjugated or Dimeric Recombinant MSP142

To evaluate whether the MSP142-FVO self-associated aggregate or MSP142-FUP-EPA conjugates could enhance antigen specific antibody responses compared to the predominately monomeric or dimeric forms, two mouse studies were performed with the immunogens formulated on Alhydrogel, and in the case of the MSP142-FUP-EPA conjugate with or without CPG 7909. The antibody titers of each mouse serum were measured by ELISA. In the first study, the antibody titers induced by aggregated or unconjugated MSP142-FUP formulated on Alhydrogel, and in the case of the MSP142-FUP-EPA conjugate with or without CPG 7909. The antibody titers of each mouse serum were measured by ELISA. In the second study, the antibody titers induced by the formulations without CPG 7909 at both doses of 5 µg and 15 µg on all three serum collection days (Table 2). However, at the 5 µg dose, the MSP142-FUP-EPAAPA conjugate consistently had a higher response when formulated without CPG 7909 and the MSP142-FUP monomer consistently had a higher response when formulated with CPG 7909. When the comparison was performed between the formulations with and without CPG 7909, significant differences were observed. Antibody titers induced by the formulations with CPG 7909 were significantly higher than those induced by the formulations without CPG 7909 at both doses of 5 and 15 µg on all three serum collection days (Table 2). On day 42, the differences in antibody titers reached 31–83-fold higher at the dose of 5 µg and 18–18-fold higher at the dose of 15 µg.

IgG subclass analysis was performed on the pooled sera of the mouse groups to characterize the type of antibody responses induced by the formulations with and without CPG 7909. The Alhydrogel formulations of both conjugated and unconjugated MSP142-FUP with CPG 7909 predominately induced an IgG1 response (data not shown). With the addition of CPG 7909 to the Alhydrogel formulations, an IgG2a subclass level was greatly increased. Both IgG1 and IgG2a were the predominant IgG subclasses in these immune sera. The IgG1 to IgG2a ratios from

Table 1. Anti-MSP142-FVO antibody titers in mice.

| Anti-MSP142-FVO units* (Geometric mean ± SEM) | Dose 1 µg | Dose 3 µg | Dose 10 µg |
|---------------------------------------------|-----------|-----------|------------|
| MSP142-FVO dimer                            | 715 ± 2890| 6297 ± 4940| 23667 ± 5022|
| MSP142-FVO aggregate                        | 2444 ± 4950| 4973 ± 8357| 57936 ± 7457|

*The antibody titers were compared at any of three doses levels with Mann-Whitney U Test. No significant differences were presented as the P values were all more than 0.05.

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Table 2. Anti-MSP142-FUP antibody titers in mice.

| Anti-MSP142-FUP units (Geometric mean ± SEM)* | Dose 5 µg | | Dose 15 µg | |
|-----------------------------------------------|-----------|---|-----------|---|
| | Day 42 | Day 56 | Day 70 | Day 42 | Day 56 | Day 70 |
| MSP142-FUP monomer                            | 1309 ± 2109| 1570 ± 2538| 1241 ± 1882| 7588 ± 4477| 9493 ± 4050| 8369 ± 4306|
| MSP142-FUP-EPAAPA                              | 1770 ± 2701| 2102 ± 3281| 1827 ± 2369| 5685 ± 2116| 5627 ± 2630| 4275 ± 2335|
| MSP142-FUP-EPAAPA CPG 7909                    | 1009 ± 1716| 1288 ± 1665| 1062 ± 2023| 8064 ± 3029| 7744 ± 3732| 4881 ± 1692|
| MSP142-FUP monomer CPG 7909                  | 109075 ± 21780| 89277 ± 20008| 44237 ± 4929| 107850 ± 13162| 118401 ± 13938| 52794 ± 5243|
| MSP142-FUP-EPAAPA CPG 7909                   | 54327 ± 10650| 45206 ± 9532| 21341 ± 5183| 103919 ± 17292| 59705 ± 8829| 33042 ± 4018|
| MSP142-FUP-EPAAPA CPG 7909                   | 73995 ± 15475| 33363 ± 10701| 24742 ± 6113| 108278 ± 15234| 75767 ± 11789| 34766 ± 3467|

*The antibody titers were compared at two dose levels on any of three serum collection days with Kruskal-Wallis One-Way ANOVA. As the P values were all less than 0.025, the post hoc analysis of Student-Newman-Keuls was performed. The differences between the groups of conjugated and unconjugated immunogens were not significant (MSP142-FUP-EPAAPA vs. MSP142-FUP monomer and MSP142-FUP-EPAAPA CPG 7909 or MSP142-FUP-EPAAPA CPG 7909 vs. MSP142-FUP monomer CPG 7909) (P>0.05), whereas the differences between the groups with and without CPG 7909 were significant (MSP142-FUP monomer CPG 7909 vs. MSP142-FUP-EPAAPA CPG 7909 and MSP142-FUP-EPAAPA vs. MSP142-FUP-EPAAPA CPG 7909 vs. MSP142-FUP monomer CPG 7909) (P>0.05).

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day 42 to day 70 were different between the groups of conjugated and unconjugated MSP142-FUP. The ratios decreased in the sera elicited by the conjugated MSP142-FUP, indicating that in those sera the IgG1 waned more quickly than the IgG2a (Table 3).

### Discussion

An effective malaria vaccine is urgently needed to augment existing control measures for individuals living in malarial endemic areas. Unfortunately, several investigative malaria vaccines tested in phase 1 or 2 trials have not induced antibody levels that have warranted further development [19,36,37,38]. Self-assembled virus like particles [39] including the leading malaria vaccine RTS,S that contains the circumsporozoite protein fused with the hepatitis B surface antigen are capable of inducing protective responses in humans. RTS,S has protected about 50% of vaccinees for a duration of 12–18 months in Phase 2 trials [40,41], and in a recent Phase 3 trial [42]. In preclinical studies, a non-human primate immunogenicity study demonstrated that recombinant Pf625H protein, a mimic of the sexual stage specific protein Pf625, conjugated to the outer membrane complex of *N. meningitidis* increased antibody titers to a greater degree than their monomeric forms and increased the apparent duration of the antigen specific antibodies [5]. Similar observations of an increase in antibody titers have been made when recombinant Pf625H was conjugated to a different carrier protein i.e., EPA or to itself [6,7].

Unfortunately, based on the results reported here, whether recombinant MSP142 was presented as a uniform non-covalently associated aggregate or chemically cross-linked to EPA no significant increase in antibody responses were observed in mice (Tables 1 and 2). The basis for the differences in antibody responses observed for recombinant Pfs25 conjugates and those associated aggregate or chemically cross-linked to EPA no protein-protein coupling. Further investigation is required to understand the benefit of this protein-protein conjugation strategy for the development of investigational malaria vaccines or other vaccines.

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### Author Contributions

Conceived and designed the experiments: FQ GEDM LBM CAL LHM DLN. Performed the experiments: FQ KR YZ RLS JAA KMR LL. Analyzed the data: FQ KR VN DZ DLN. Contributed reagents/materials/analysis tools: VN DZ. Wrote the paper: FQ DLN.

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In summary, neither recombinant MSP142 self-associated aggregates nor chemical cross-linked conjugates enhanced the immunogenicity of the MSP142 compared to monomeric or oligomeric forms of the antigen. The addition of CPG 7909 into the Alhydrogel formulation of the MSP142-EPA conjugates significantly enhanced the specific antibody levels in mice as compared to the formulation using Alhydrogel as a single adjuvant. Even though the MSP142-EPA conjugates failed to enhance MSP142 immunogenicity, the conjugation procedure including the use of design of experiments may be used as a platform for development of other protein-protein conjugates through chemical modification of both antigen and carrier, or use of innate or genetically engineered free sulphydryl groups for protein-protein coupling. Further investigation is required to understand the benefit of this protein-protein conjugation strategy for the development of investigational malaria vaccines or other vaccines.

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**Table 3.** IgG subclass analysis of MSP142-FUP-specific mouse antibodies elicited by the formulations with CpG 7909.

| IgG1 : IgG2a ratio | Dose 5 μg | Dose 15 μg |
|--------------------|----------|-----------|
| Day 42 Day 56 Day 70 | Day 42 Day 56 Day 70 |
| MSP142-FUP, CPG 7909 | 1.28 1.23 1.26 | 1.04 1.01 1.11 |
| MSP142-FUP-EPAAPA, CPG 7909 | 0.88 0.77 0.69 | 0.70 0.58 0.51 |
| MSP142-FUP-EPAAPA, CPG 7909 | 0.73 0.71 0.50 | 0.71 0.62 0.57 |

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