RESEARCH ARTICLE

Maintaining immunogenicity of blood stage and sexual stage subunit malaria vaccines when formulated in combination

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Abstract

Background

Eradication of Plasmodium falciparum malaria will likely require a multivalent vaccine, but the development of a highly efficacious subunit-based formulation has been challenging. We previously showed that production and immunogenicity of two leading vaccine targets, PfMSP119 (blood-stage) and PfS25 (sexual stage), could be enhanced upon genetic fusion to merozoite surface protein 8 (PfMSP8). Here, we sought to optimize a PfS25-based formulation for use in combination with rPfMSP1/8 with the goal of maintaining the immunogenicity of each subunit.

Methods

Comparative mouse studies were conducted to assess the effects of adjuvant selection (Alhydrogel vs. glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE)) and antigen dose (2.5 vs. 0.5 μg) on the induction of anti-PfS25 immune responses. The antibody response (magnitude, IgG subclass profile, and transmission-reducing activity (TRA)) and cellular responses (proliferation, cytokine production) generated in response to each formulation were assessed. Similarly, immunogenicity of a bivalent vaccine containing rPfMSP1/8 and rPfS25/8 was evaluated.

Results

Alum-based formulations elicited strong and comparable humoral and cellular responses regardless of antigen form (unfused rPfS25 or chimeric rPfS25/8) or dose. In contrast, GLA-SE based formulations elicited differential responses as a function of both parameters, with 2.5 μg of rPfS25/8 inducing the highest titers of functional anti-PfS25 antibodies. Based on these data, chimeric rPfS25/8 was selected and tested in a bivalent formulation with rPfMSP1/8. Strong antibody titers against PfS25 and PfMSP119 domains were induced with GLA-SE based formulations, with no indication of antigenic competition.
Conclusions

We were able to generate an immunogenic bivalent vaccine designed to target multiple parasite stages that could reduce both clinical disease and parasite transmission. The use of the same PfMSP8 carrier for two different vaccine components was effective in this bivalent formulation. As such, the incorporation of additional protective targets fused to the PfMSP8 carrier into the formulation should be feasible, further broadening the protective response.

Introduction

Significant strides have been made in the control and treatment of malaria since the year 2000. However, there has been a rise in drug-resistant parasites and insecticide-resistant mosquitoes, and progress towards elimination has stalled over recent years. Development of additional tools, including highly efficacious vaccines, would greatly aid efforts to further decrease clinical disease and mortality due to Plasmodium falciparum. Considering the suboptimal protection afforded by single antigen vaccines such as RTS,S [1–3], it is likely that induction of broad responses against multiple targets will be required to achieve adequate efficacy. While the ideal vaccine would induce sterilizing immunity, a more attainable, yet still impactful goal, may be the development of a multistage vaccine capable of reducing both the severity of clinical disease and parasite transmission rates.

One strategy being pursued for the rational development of a multivalent subunit malaria vaccine requires the production of high-quality and potent recombinant immunogens that can be successfully combined into a single formulation while adequately maintaining the protective effect of each component. These have been challenges for the field, as many protective targets are structurally complex and difficult to produce properly in recombinant form. Furthermore, antigenic competition has been observed with various formulations that incorporated multiple pre-erythrocytic and/or blood-stage antigens [4–6]. We developed a strategy to help facilitate this process and address the issues of vaccine production, folding and immunogenicity while minimizing antigenic competition, through the use of merozoite surface protein 8 (PfMSP8) as a malaria-specific carrier protein.

Antibodies directed against conformational epitopes within the C-terminal epidermal growth factor-like domains of P. falciparum merozoite surface protein 1 (PfMSP1) are highly protective in rodent and non-human primate models of malaria [7–14]. However, in clinical trials of PfMSP142, efficacy was limited due, in part, to suboptimal immunogenicity and epitope polymorphism [15–21]. Our early studies in the P. yoelii rodent model pointed to the potential of MSP8 as a vaccine carrier to avoid antigenic competition, to enhance the production, folding and immunogenicity while minimizing antigenic competition, through the use of merozoite surface protein 8 (PfMSP8) as a malaria-specific carrier protein.

Antibodies directed against conformational epitopes within the C-terminal epidermal growth factor-like domains of P. falciparum merozoite surface protein 1 (PfMSP1) are highly protective in rodent and non-human primate models of malaria [7–14]. However, in clinical trials of PfMSP142, efficacy was limited due, in part, to suboptimal immunogenicity and epitope polymorphism [15–21]. Our early studies in the P. yoelii rodent model pointed to the potential of MSP8 as a vaccine carrier to avoid antigenic competition, to enhance the production of PyMSP19-specific antibodies and to provide solid [22] and durable [23] protection against lethal P. yoelii malaria. Therefore, we tested the utility of this approach for P. falciparum. PfMSP8 was engineered to be highly expressed, properly folded and easily purified using an E. coli expression system [24]. To assess the ability of PfMSP8 to enhance the production, folding and immunogenicity of PfMSP19, a chimeric antigen containing rPfMSP19 genetically fused to the N-terminus PfMSP8 was generated [25]. The resulting fusion protein, rPfMSP19, i) was expressed and purified in high yield, bearing proper conformation of the PfMSP19 domain, ii) induced a predominant PfMSP8-specific T cell response, iii) elicited high titers of antigen-specific antibodies in inbred and outbred mice, rabbits and non-human primates, which were cross-reactive with PfMSP19 from the FVO and 3D7 strains of P. falciparum, and iv) could be formulated with diverse adjuvants to stimulate production of anti-
*Pf*MSP1<sub>19</sub> antibodies that potently inhibited the *in vitro* growth of *P. falciparum* blood-stage parasites. Using a similar strategy, we have also reported success utilizing *Pf*MSP8 as a carrier for a second blood-stage target, *P. falciparum* merozoite surface protein 2 (*Pf*MSP2), to elicit antibodies that opsonize merozoites for phagocytosis [26].

*Pf*s25 is a highly conserved, 25 kDa glycosylphosphatidylinositol (GPI) anchored surface protein expressed exclusively during the sexual stages of the parasite life cycle within the mosquito midgut [27]. It is well established that vaccine-induced antibodies directed against conformational epitopes within the four EGF-like domains of *Pf*s25 are able to block sexual stage development within the vector, effectively preventing parasite transmission [28–32]. This induction of transmission-blocking immunity has been demonstrated in mouse models, non-human primates and human subjects. However, similar to *Pf*MSP1, it has been difficult to produce sufficient quantities of high quality recombinant *Pf*s25 bearing proper conformation using common expression systems. Thus far, clinical trials conducted on *Pf*s25-based candidates have resulted in suboptimal immunogenicity and durability of vaccine induced responses [33–36]. To begin to address these issues, we produced a chimeric r*Pf*s25-r*Pf*MSP8 fusion protein as well as unfused, mature r*Pf*s25 [37]. r*Pf*s25 was purified with a modest yield but required denaturation and renaturation procedures to obtain the correct conformation. In contrast, r*Pf*s25/8 was purified in higher yield without the need for refolding. Both antigens were immunogenic in rabbits, inducing IgG that bound native, macrogamete-associated *Pf*s25 and exhibited potent transmission-reducing activity in a standard membrane feeding assay (SMFA).

Here, we sought to systematically assess the relative immunogenicity of these *Pf*s25-based vaccines as a function of several formulation parameters including adjuvant selection and antigen dose, with the ultimate goal of selecting an optimized *Pf*s25-based antigen for incorporation into a multivalent vaccine. We tested the influence of two distinct human-compatible adjuvants on the anti-*Pf*s25 responses. Alhydrogel (Alum), a safe and widely used adjuvant for childhood vaccines, has been shown to enhance humoral immunity and skew immune responses toward a Th<sub>2</sub> profile with production of IL-5 and IgG1 antibodies [38, 39]. In contrast, GLA-SE is a two-component adjuvant that contains glucopyranosyl lipid adjuvant (GLA), a synthetic TLR4 agonist, in a stable squalene-in-water emulsion (SE). GLA-SE shifts responses toward a Th<sub>1</sub> profile characterized by increased production of IFNγ and TNFα with a more diverse IgG subclass profile featuring increased levels of the cytophilic IgG2a/c in mice [40]. As a next step in building a multistage vaccine, the *Pf*s25/8 and *Pf*MSP1/8 vaccines were tested in combination to i) assess the potential for antigenic competition, ii) select an optimal adjuvant for the bivalent formulation, and iii) determine the impact of concurrent immunization with two subunit vaccines fused to the same carrier protein.

**Materials and methods**

**Mice and immunizations**

Five-week-old, male CB6F1/J mice (BALB/c x C57BL/6) or male and female outbred CD1 mice were obtained from The Jackson Laboratory and Charles River Laboratories, respectively. Mice were maintained in the Animal Care Facility of Drexel College of Medicine under specific-pathogen-free conditions. All animal studies were designed, reviewed, approved and conducted in accordance with the Institutional Animal Care and Use Committee of Drexel University College of Medicine (protocol # 20308). For comparative immunogenicity studies in CB6F1/J mice, groups (n = 5) were immunized with 0.5 μg/dose (low) or 2.5 μg/dose (high) of purified r*Pf*s25, r*Pf*s25/8, r*Pf*MSP8 or an admixture of r*Pf*s25 + r*Pf*MSP8 (0.5 μg or 2.5 μg of each antigen/dose). Production and purification of recombinant antigens have been previously
reported [37]. Antigens were formulated in either 2% Alhydrogel adjuvant (500 μg/dose; Invi-voGen, San Diego, CA) or GLA-SE (5 μg/dose, Infectious Disease Research Institute, Seattle, WA). Additional control groups received adjuvant alone. For the bivalent vaccine study, groups of CD1 mice (n = 10; 5 male and 5 female) were immunized subcutaneously with 2.5 μg/dose of purified rPfs25/8, rPfMSP1/8, an admixture of rPfMSP1/8 + rPfs25/8 (2.5 μg of each antigen/dose) or adjuvant alone. Antigens were formulated, as above, with Alum or GLA-SE as adjuvant. For assessment of antibody responses, mice were immunized subcutane-ously, three times at 4-week intervals. Sera samples were collected three weeks following the first two immunizations and 4 weeks following the final immunization. For assessment of T cell responses, mice were immunized subcutaneously three times at 4-week intervals. Follow- ing an 8–10 week rest, mice received an additional boost by intraperitoneal (i.p.) injection to increase trafficking of antigen-specific T cells to the spleen. Splenocytes were harvested 2 weeks following the i.p. boost.

**Antigen-specific T cell analysis**

**Splenocyte preparation.** Harvested spleens were processed into single cell suspensions in sterile complete medium consisting of RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 2 mM L-glutamine, 0.5 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 1X streptomycin/penicillin (Corning Costar Corporation, Cambridge, MA), 10 μg/ml of Polymyxin B (Sigma-Aldrich) and 10% heat-inactivated Benchmark™ fetal bovine serum (Gemini Bio Products, Sacramento, CA). Cellular debris was removed from suspensions by filtration through Falcon 70 μm cell strainers (Thermo Fisher Scientific). RBCs were lysed using ACK lysis buffer (Thermo Fisher Scientific), and the quantification of viable splenocytes was deter- mined by microscopy following trypan blue staining (Thermo Fisher Scientific).

**T cell proliferation assay.** To measure antigen-specific proliferative responses, spleno-cytes (5 mice/group) were plated in 96-well round-bottomed Falcon plates (Thermo Fisher Scientific) at a concentration of 2 x 10^5 cells/well. Cells from each mouse were stimulated in triplicate in RMPI complete medium containing 10 μg/ml of rPfs25, rPfs25/8, or rPfMSP8 antigens. Additional sets of wells from each mouse were stimulated in triplicate with Concanavalin A (Sigma-Aldrich; 1 μg/ml) or left unstimulated to serve as positive and negative controls, respectively. Plates were incubated at 37°C in 5% CO₂ for 96 hours, and pulsed with methyl [³H]-thymidine (1 μCi/well; 70–90 Ci/mmol; PerkinElmer, Inc., Waltham, MA) for the final 18 hours. Cells were harvested onto glass fiber filters using an automatic cell harvester (PerkinElmer, Inc.). Incorporation of [³H]-thymidine was quantified by liquid scintillation counting (PerkinElmer, Inc.). The stimulation indices were calculated for each animal as the mean counts per minute of each stimulated condition divided by the mean counts per minute of the corresponding unstimulated condition.

**Cytokine production.** For the quantification of secreted cytokines induced by antigen-specific stimulation, splenocytes were plated in 96-well round-bottomed Falcon plates at a concentration of 5 x 10^5 cells/well and stimulated as described above for 96 hours. Culture supernatants were transferred to new plates and stored at -80°C. Custom magnetic Luminex® assay kits (R&D Systems, Minneapolis, MN) were used for the quantification of IL-2, IL-4, IL-5, TNFα and IFNγ in cell supernatants according to the manufacturer’s protocol, utilizing a Luminex 200 analyzer and xPONENT3.1 software. Based on a standard curve, concentrations (pg/ml) of each analyte were calculated for all samples and final, antigen-specific concentra- tions were determined by subtracting out the background levels in corresponding unstimu-lated conditions.
Determination of antigen-specific antibody titers

**Enzyme-linked immunosorbent assay (ELISA).** Sera collected from all experimental and control mice following each immunization were analyzed for antigen-specific IgG by ELISA as previously described [25]. Briefly, plates coated with 0.25 μg/well of rPfs25, rPfs25/8 or rPfMSP8 were incubated with two-fold dilutions of mouse sera for two hours at room temperature. Bound antibodies were detected by HRP-conjugated rabbit anti-mouse IgG (0.08 μg/ml; ThermoFisher Scientific) and ABTS [2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] as substrate. A$_{405}$ values between 0.1 and 1 were plotted and titers were calculated as the reciprocal of the dilution yielding an A$_{405}$ of 0.5. A high titer pool of sera obtained from rPfs25/8-immunized mice was included on every plate to normalize data between plates.

**IgG subclass profiles of antigen-specific antibodies.** To determine the IgG subclass profiles of antigen-specific IgG, tertiary immunization serum from each mouse was titered, as described above, in wells coated with rPfs25/8. Bound antibodies were detected by HRP-conjugated rabbit anti-mouse IgG specific for subtypes IgG1, IgG2a, IgG2b, IgG2c and IgG3 (Southern Biotech, Inc., Birmingham, AL) followed by ABTS substrate. To generate a standard curve, each plate included wells coated with 2-fold dilutions of subtype-specific mouse myeloma immunoglobulin at known concentrations. Here, IgG subclass quantities in sera are reported as units/ml (U/ml) where 1 U/ml is equivalent to the signal obtained with 1 μg/ml of purified myeloma protein.

Standard membrane feeding assay

The transmission-reducing activity (TRA) of IgG antibodies induced by each vaccine formulation was measured by a Standard Membrane Feeding Assay (SMFA) using cultured *P. falciparum* NF54 gametocytes and *Anopheles stephensi* mosquitoes, as previously described [41]. Pools of protein G-purified, vaccine-induced IgG (750 μg/ml) were mixed with stage V gametocytes and fed to *A. stephensi* mosquitoes through a membrane feeding apparatus. Mosquitoes were kept for 8 days prior to dissection to quantify midgut oocysts. Percent inhibition of mean oocyst intensity was calculated relative to adjuvant control IgG. The best estimate of % inhibition in mean oocyst density (% TRA), the 95% confidence interval, and the *p*-value (whether the observed %TRA is significantly different from no inhibition) of each test sample were calculated using a zero-inflated negative binomial model [42].

Statistical analysis

All statistical analyses conducted in this study were nonparametric. To assess T cell responses (proliferation and cytokine production) in antigen-immunized groups relative to the corresponding control group, a Kruskal-Wallis test was conducted. To assess boosting of antigen-specific IgG responses within the same animals following each immunization, a Friedman’s test for multiple repeated samples followed by a Dunn’s post hoc test was utilized. For analysis of final anti-Pfs25 titers induced by immunization with rPfs25, rPfs25 + rPfMSP8, or rPfs25/8 in the different dose and adjuvant formulations, a Kruskal-Wallis test followed by a Dunn’s post hoc test was performed. Instances in which two unrelated groups were directly compared, Mann-Whitney *U* tests were used. In all cases, differences with a probability (*p*) value of <0.05 were considered significant.

Results

The relative immunogenicity of the two recombinant *Pfs25*-based vaccines, unfused *Pfs25* and chimeric *rPfs25/8*, was compared as a function of various vaccine parameters including
antigen form (rPfs25, rPfs25/8 or an admixture of rPfs25 + rPfMSP8), antigen dose and adjuvant selection. Antibody responses induced by each vaccine formulation were measured with respect to magnitude, specificity, IgG subclass and functionality. In addition, the phenotype and specificity of vaccine-induced T cells were assessed.

Pfs25 and PfMSP8 domains elicit antigen-specific T cell responses

The domain specificity of T cell responses elicited by immunization with rPfs25 and rPfMSP8 antigens was determined. To this end, splenocytes from immunized animals were harvested and stimulated in vitro with rPfs25, rPfMSP8, or rPfs25/8 antigens. Additional sets were stimulated with Concanavalin A (Con A) or left unstimulated to serve as positive and negative controls, respectively. Proliferative responses of antigen-specific T cells were determined using a standard [3H]-thymidine incorporation assay.

As shown in Fig 1(A)–1(D), T cells from all groups immunized with any formulation containing rPfMSP8 (rPfs25 + PfMSP8 admixture, rPfs25/8, rPfMSP8) demonstrated similar and strong proliferative responses when stimulated with rPfMSP8; all were significantly higher than those observed by cells from corresponding adjuvant control mice. This was true irrespective of antigen dose or adjuvant. Similarly, T cells from groups immunized with Pfs25-containing formulations (rPfs25 alone, rPfs25 + PfMSP8 admixture, rPfs25/8) demonstrated similar and specific proliferative responses when stimulated with rPfs25 that were significantly higher than corresponding control mice. Again, this was true irrespective of antigen dose or adjuvant, with one exception. Proliferation of T cells from mice immunized with 2.5 μg of rPfs25/8 formulated with GLA-SE and stimulated in vitro with rPfs25 was low and not significantly different relative to adjuvant control mice (Fig 1C). This could potentially be due to a shift of the T cell response toward epitopes in rPfMSP8 at the higher dose. With Alum-based formulations, the slightly higher than expected proliferation of cells from rPfs25-vaccinated mice when stimulated with rPfMSP8 and vice versa (Fig 1A and 1B) may be due to a shared epitope(s) between the two antigens associated with a common leader and linker sequence. As expected, cells harvested from all antigen-immunized mice demonstrated high proliferative responses when stimulated with chimeric rPfs25/8 (S1 Fig). These were generally additive of the domain-specific responses. Collectively, these data demonstrate that T cells from CB6F1/J immunized mice recognize epitopes present in both Pfs25 and PfMSP8 domains.

Antigen-specific T cells induced by Alum- and GLA-SE-based formulations are skewed toward Th2 and Th1 profiles, respectively, in a PfMSP8-dependent manner

The effect of adjuvant on the type of antigen-specific T helper cells (Th1 vs. Th2) induced by immunization was evaluated based on cytokine production following antigen re-exposure. Similar to the T cell proliferation studies, splenocytes were collected and stimulated in vitro with rPfs25, rPfs25/8, rPfMSP8 or cultured in media alone. Stimulation with Con A served as a positive control. Culture supernatants were collected and analyzed for production of IL-5, TNFα, IFNγ, IL-2 and IL-4 via a multiplex assay (Luminex). As expected, stimulation with Con A elicited detectible and similar responses in cells from all groups (S1 Table). As shown in Fig 2, cells from most antigen-immunized groups secreted detectible levels of IL-5, IFNγ, IL-2 and IL-4 via a multiplex assay (Luminex). As expected, stimulation with Con A elicited detectible and similar responses in cells from all groups (S1 Table). As shown in Fig 2, cells from most antigen-immunized groups secreted detectible levels of IL-5, IFNγ and TNFα when stimulated with rPfs25/8. However, there was a clear effect of adjuvant on the relative level of each of these cytokines.

Irrespective of antigen dose, T cells from groups immunized with Alum-based formulations produced primarily IL-5 (Fig 2A). These levels were similar for all formulations containing
Pf MSP8 and significantly higher compared to levels produced by T cells from Alum control mice. Of interest, cells from groups immunized with rPfs25 formulated in Alum produced only low levels of IL-5, not significantly different than adjuvant control mice.

The response of T cells from mice immunized with vaccines formulated with GLA-SE was robust at both antigen doses and marked by a more diversified cytokine profile (Fig 2). Cells from mice immunized with PfMSP8-containing vaccines with GLA-SE as adjuvant produced IL-5 at significantly higher levels relative to the control group (Fig 2A). Of note, cells from
mice immunized with unfused rPfs25 formulated with GLA-SE produced low levels of IL-5 that were not statistically above background controls. In pairwise comparisons, the IL-5 levels elicited by GLA-SE-based formulations were similar to those produced by the corresponding Alum-based formulations at both doses. In contrast, groups immunized with PfMSP8-containing vaccines formulated with GLA-SE also produced both IFNγ (Fig 2B) and TNFα (Fig 2C) at levels significantly higher than the corresponding Alum-based antigen groups and GLA-SE controls. This was not true for cells from mice immunized with unfused rPfs25 in GLA-SE, which did not produce significant quantities of either IFNγ or TNFα.

The production of IL-2 and IL-4 by antigen-specific T cells was also assessed following stimulation with rPfs25/8. Production of both analytes was low in all vaccine formulations, with no significant differences observed as a function of immunizing antigen, dose and/or adjuvant (S2 Table). The observation that cytokine production is adjuvant and carrier-dependent was further confirmed by results of domain-specific stimulation with rPfMSP8 alone (S2 Fig) which yielded results similar to stimulation with rPfs25/8. Therefore, in some contrast to the proliferative responses observed upon stimulation with both rPfs25 and rPfMSP8 domains, cytokine production was driven primarily by epitopes present in PfMSP8 domain with choice of adjuvant influencing the profile.
Fusion of rPfs25 to the PfMSP8 carrier elicits strong anti-Pfs25 antibody responses and alleviates antigenic competition

It is well established that transmission-reducing activity of Pfs25-based vaccines is primarily antibody mediated. As such, the magnitude and specificity of the antibody responses induced by each vaccine formulation over time was evaluated. Sera were collected following each of three s.c. immunizations and domain-specific IgG titers determined. As depicted in Fig 3A, immunization with 2.5 μg dose of rPfs25, rPfs25 + rPfMSP8, or rPfs25/8 adjuvanted with Alum elicited strong and comparable anti-Pfs25 IgG titers that were significantly boosted over time. At the 0.5 μg dose in Alum (Fig 3B), high anti-Pfs25 IgG were also generated in response to immunization with rPfs25. However, the anti-Pfs25 IgG response was impaired in mice

Fig 3. Antigen-specific IgG titers elicited by Alum-based formulations. CB6F1/J sera collected 3 weeks following each subcutaneous immunization were analyzed for antigen-specific antibodies by ELISA using plates coated with rPfs25 (A and B) or PfMSP8 (C and D) (0.25 μg/well). Graphs depict the mean IgG titers +/- standard deviation. Asterisks over horizontal lines within an immunization group indicate significant boosting of antigen-specific IgG titers over time (Friedman Test; P < 0.05 considered significant). Asterisks over horizontal lines comparing different immunization groups indicate significant differences between final titers achieved by those groups (Kruskal Wallis Test, P < 0.05 considered significant; ns, not significant).

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immunized with the admixture of rPfs25 + rPfMSP8 relative to animals immunized with rPfs25 alone, indicative of competition between rPfs25 and rPfMSP8 when administered as separate, non-fused components. Importantly, this competition was eliminated upon immunization with 0.5 μg of the chimeric rPfs25/8 in Alum with a strong anti-Pfs25 IgG response comparable to that observed in the rPfs25 group. Immunization with either dose of rPfs25/8, the rPfs25 + rPfMSP8 admixture or PfMSP8 antigens formulated in Alum induced strong and similar titers against the highly immunogenic rPfMSP8 carrier, which were increased significantly over time (Fig 3C and 3D). Of note, antibodies induced by immunization with rPfs25 alone exhibited some reactivity with rPfMSP8 (Fig 3C and 3D). This reactivity is associated with a shared epitope(s) present within the His-tag and linker that are common to both antigens. This reactivity is relatively low, representing only 1–2% of the overall anti-Pfs25 titer induced by immunization with unfused rPfs25 (Fig 3A and 3B).

Likewise, the anti-Pfs25 response induced by immunization with 2.5 μg of rPfs25, rPfs25 + rPfMSP8 admixture or rPfs25/8 formulated in GLA-SE was assessed (Fig 4A). Responses were detected in all groups that were significantly boosted over time. However, there was a 10-fold reduction in final anti-Pfs25 titer in mice immunized with the rPfs25 + rPfMSP8 admixture relative to the group immunized with rPfs25 alone, highlighting competition between antigens. Importantly, this response was restored in mice immunized with the chimeric rPfs25/8, resulting in a higher final titer relative to the rPfs25 immunized mice group. Antibody responses elicited by immunization with these antigens formulated at 0.5 μg also increased significantly over time (Fig 4B). However, the anti-Pfs25 response elicited by rPfs25 at the 0.5 μg dose was more than 10-fold lower than that induced at the 2.5 μg dose. Again, anti-Pfs25 titers were further decreased in the admixture group. Similar to the 2.5 μg dose group, immunization with chimeric rPfs25/8 was able restore this response with final anti-Pfs25 titers even greater than those observed in mice immunized with unfused rPfs25. Despite divergent responses against Pfs25, the anti-PfMSP8 responses elicited in mice immunized with rPfs25 + rPfMSP8, rPfs25/8 or PfMSP8 carrier alone formulated with GLA-SE were strong and comparable irrespective of dose and was significantly boosted over time (Fig 4C and 4D). Antibody titers measured against rPfs25/8 coated wells were generally additive of the two individual domain-specific responses (S3 Fig).

Finally, the effectiveness of Alum vs GLA-SE as adjuvant in mice immunized with rPfs25 or the chimeric rPfs25/8 was considered. As shown in Fig 5, both rPf25-containing vaccines induced high and comparable titers against Pfs25 when formulated with Alum irrespective of dose. However, there were significant differences in final titer induced by the two antigens when GLA-SE was used as an adjuvant. Here, the final anti-Pfs25 titer induced in the rPfs25-immunized group was 10-fold lower than the corresponding group formulated with Alum. Importantly, chimeric rPfs25/8 formulated with GLA-SE elicited significantly higher final titers of anti-Pfs25 IgG relative to immunization with unfused rPfs25 at the same dose; these titers were comparable to those induced by Alum-based formulations. Together, these data indicate that Alum was an equally potent adjuvant for both vaccine antigens, while maximal anti-Pfs25 responses elicited by GLA-SE based formulations depended on genetic fusion of rPfs25 to the rPfMSP8 carrier protein.

Switch from Alum to GLA-SE as adjuvant for Pfs25-based vaccines shifts the B cell response to the production of cytophilic IgG in a PfMSP8-dependent manner

In addition to titer, the functionality of vaccine-induced IgG may be influenced by heavy chain subclass depending on adjuvant selection. The profile of IgG subclasses in the final sera
from each vaccine group was measured by ELISA using plates coated with rPfs25/8 and secondary antibodies specific for IgG1, IgG2a/c, IgG2b and IgG3. As shown in Fig 6, vaccines formulated with Alum, regardless of antigen or dose, elicited antibodies primarily of the IgG1 subclass, with low but detectible IgG2a/c, IgG2b and IgG3. In the same way, immunization with rPfMSP8-containing vaccines formulated with GLA-SE, irrespective of dose, produced high and similar levels of IgG1 compared to the Alum-formulated counterparts. One exception was noted in the rPfs25 + PfMSP8 admixture group that showed a modest but statistically significant reduction in IgG1. In stark contrast to the IgG profiles induced by Alum-based formulations, mice immunized with GLA-SE-based formulations also produced significantly higher

Fig 4. Antigen-specific IgG titers elicited by GLA-SE-based formulations. CB6F1/J sera collected 3 weeks following each subcutaneous immunization were analyzed for antigen-specific antibodies by ELISA using plates coated with rPfs25 (A and B) or PfMSP8 (C and D) (0.25 μg/well). Graphs depict the mean IgG titers +/- standard deviation. Asterisks over horizontal lines within an immunization group indicate significant boosting of antigen-specific IgG titers over time (Friedman Test; \( P < 0.05 \) considered significant). Asterisks over horizontal lines comparing different immunization groups indicate significant differences between final titers achieved by those groups (Kruskal Wallis Test, \( P < 0.05 \) considered significant).

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levels of antigen-specific IgG2a/c, IgG2b and IgG3 relative to the Alum-formulated counterparts. This was not true for rPfs25-immunized animals, which primarily produced antigen-specific IgG1 when formulated with either Alum or GLA-SE. These results demonstrate a strong influence of adjuvant on IgG subtype profile generated in response to vaccination, with IgG class-switching in the GLA-SE formulations dependent on the presence of the rPfMSP8 carrier.

Transmission-reducing activity (TRA) of IgG induced by immunization with rPfs25-based vaccines formulated with Alum vs GLA-SE

In addition to the magnitude and profile of antibody responses, demonstrating the ability of Pfs25-containing vaccines to induce IgG that inhibits development of sexual-stage parasites in the mosquito vector is important. To test the relative functionality of IgG induced by rPfs25 and rPfs25/8 vaccines, total IgG was purified from pools of sera derived from each vaccine group and tested in the SMFA at a concentration of 750 μg/ml. As depicted in Table 1, all Alum-based formulations elicited potent and comparable TRA relative to the IgG derived from adjuvant-immunized control groups. Similarly, IgG from rPfs25/8 + GLA-SE immunized groups also demonstrated potent TRA relative to control IgG. As predicted based on analysis of anti-Pfs25 antibody responses, groups immunized with rPfs25 formulated with GLA-SE had much lower TRA that were not statistically different from controls.
Bivalent formulations containing rPfs25/8 and rPfMSP1/8 elicit strong antibody titers against both fusion partners in outbred mice, with no indication of antigen competition.

Based on the above immunogenicity and functionality data, chimeric rPfs25/8 was evaluated in combination with rPfMSP1/8, which elicits potent merozoite invasion inhibitory antibodies. The immunogenicity of rPfs25/8 + rPfMSP1/8 (2.5 μg each antigen/dose) was compared to

Table 1. Transmission-reducing activity of vaccine-induced IgG is a function of antigen and adjuvant.

| Immunization Group | IgG level (μg/ml) | Transmission-reducing activity |
|--------------------|-------------------|--------------------------------|
|                    |                   | % inhibition | 95% CI (low) | 95% CI (high) | P value (vs. control pool) |
| Adjuvant Control Sera | N/A | 750 | 0 | |
| Alum               | rPfs25 2.5 μg | 750 | 100.0 | 98.7 | 100.0 | 0.001 |
|                   | rPfs25/8 2.5 μg | 750 | 99.3 | 97.5 | 100.0 | 0.001 |
|                   | rPfs25 0.5 μg | 750 | 99.3 | 97.6 | 100.0 | 0.001 |
|                   | rPfs25/8 0.5 μg | 750 | 98.0 | 95.4 | 99.5 | 0.001 |
| GLA-SE             | rPfs25 2.5 μg | 750 | 46.5 | -23.1 | 75.5 | 0.128 |
|                   | rPfs25/8 2.5 μg | 750 | 97.4 | 90.1 | 100.0 | 0.001 |
|                   | rPfs25 0.5 μg | 750 | 46.5 | -25.8 | 79.5 | 0.135 |
|                   | rPfs25/8 0.5 μg | 750 | 89.4 | 75.4 | 95.9 | 0.001 |

Total IgG from pools of tertiary sera collected from C66F1/J mice immunized with rPfs25 or rPfs25/8 formulated as indicated was purified and tested in a standard membrane feeding assay at a concentration of 750 μg/ml. Each IgG sample was fed to 20 mosquitoes. The best estimates and 95% confidence intervals (CIs) of percent inhibitions and P values were calculated for each pool compared to IgG derived from adjuvant-immunized control mice.

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corresponding monovalent vaccines (2.5 μg/dose) when adjuvanted with Alum or GLA-SE. Immunizations were conducted in male (n = 5) and female (n = 5) outbred (CD1) mice in order to i) assess the consistency of vaccine-induced responses in a genetically diverse population, ii) evaluate sex as a variable with potential to influence immune responses, and iii) determine the impact of concurrent immunization with two subunit vaccines fused to the same PfMSP8 carrier. Mice were immunized three times and sera collected following each immunization. These samples were then analyzed for antigen-specific titers against both PfMSP1
19 and PfMSP8 carrier.

As shown in Fig 7A–7C, all antigen-immunized groups mounted strong antigen-specific antibody response against component domains contained in the formulation (PfMSP1
19, Pf25, and/or PfMSP8). These antigen-specific antibody responses were significantly boosted over time in all groups. Importantly, final domain-specific IgG titers elicited by immunization with the bivalent formulations were comparable to those induced by the corresponding monovalent vaccines, regardless of adjuvant or antigen (Fig 7). Following three immunizations, the anti-PfMSP1
19 and anti-Pf25 titers in male and female mice in each immunization group were comparable, indicating that host sex did not influence vaccine immunogenicity (S4 Fig). Overall, these data indicate that concurrent responses against B cell determinants within two
different PfMSP8 fusion partners can be effectively induced by a bivalent vaccine without antigenic competition.

The effect of adjuvant on the domain-specific IgG titers induced by each antigen formulation was compared. As shown in Fig 8, the magnitude of the anti-Pf$_{s25}$ response was high and comparable in monovalent vs. bivalent vaccines, and similar between Alum- and GLA-SE-based formulations. These findings indicate that immunization with rPf$_{s25}$/8 induces strong anti-Pf$_{s25}$ responses that are independent of adjuvant selection and are not inhibited by the presence of rPfMSP1/8 in the formulation. In contrast, anti-PfMSP$_{19}$ titers induced by the bivalent vaccine were significantly higher when formulated with GLA-SE vs. Alum. In addition, responses to the PfMSP8 carrier were significantly higher when formulated with GLA-SE vs. Alum across all immunization groups. As such, the strength of anti-PfMSP$_{19}$ titers generated by immunization with rPfMSP1/8 was dependent upon adjuvant, with GLA-SE-based formulations inducing superior responses.

As above, the profile of anti-PfMSP8 IgG induced by each formulation was evaluated to determine the relative levels of IgG1, IgG2a/c, IgG2b and IgG3 subtypes. As shown in Fig 9, strong and similar levels of IgG1 were detected in all groups regardless of antigen formulation or adjuvant. All Alum-based formulations induced detectible but very low levels of IgG2a/c and IgG2b, consistent with the expected Th$_2$ associated response. In contrast, strong and significant production of IgG2a/c, IgG2b and IgG3 was observed in all GLA-SE-based
formulations. These data confirm results from studies in inbred mice with vaccines utilizing PfMSP8 as a carrier (Fig 6), showing that the production of cytophilic IgG occurs in the context of the Th1-baising adjuvant, GLA-SE, and leads to a more diverse IgG subclass profile.

**Discussion**

Though conceptually simple, the rational production of a multivalent, multistage subunit malaria vaccine is a challenge. The first requirement is that recombinant forms of each component be produced so that they bear proper conformation and induce antibodies that demonstrate functionality against the targeted stage(s) of plasmodial parasites. The production of recombinant antigens with routine heterologous expressions systems has often resulted in low yields and/or inconsistent, misfolded products incapable of producing protective antibodies to
neutralizing, conformational B cell epitopes. [43–46]. This is true for PbS25, but transmission blocking antibodies have been induced by rPbS25 produced in scalable yeast [43, 47, 48] and baculovirus [49] systems and recently with a non-glycosylated, folded product of an E. coli expression system [50].

An equally important impediment to overcome is the inherently poor immunogenicity of many malaria vaccine candidates. This is often attributed to the small size of the antigen or, more often, the inability to elicit effective CD4+ T cell responses capable of providing adequate help to B cells for the production of protective antibodies. In some cases, this deficit may be due to a lack of immunogenic CD4+ T cell epitopes. However, it may also be due to the presence of complex tertiary structures that inhibit antigen processing and presentation, such as highly constrained EGF-like domains. This appears to be the case for PbMSP119 [51, 52]. Further complicating the task is the need to induce protective and durable responses against each component that can be maintained when formulated in a multiantigen combination. Antigenic competition between co-administered components has been noted in several studies [4–6]. In the recent Phase III RTS,S trial, one of several contributing factors to lower responses in children compared to adults may relate to the administration of RTS,S concurrently with other childhood vaccines through the Expanded Program for Immunization (EPI) [53, 54].

Common strategies to address issues relating to immunogenicity, include i) use of heterologous carrier proteins, ii) formulation with potent adjuvants, and iii) optimization of vaccine dose, route of administration and/or timing of vaccinations. These considerations, as well as the nature of the target itself, are likely to impact overall immunogenicity of a given formulation. Variations of these parameters were evaluated in an effort to improve the efficacy and duration of responses generated by immunization with the PbS25-Exoprotein A conjugate [55], the leading PbS25-based clinical candidate. Results of those studies indicated that anti-PbS25 responses were influenced by both adjuvant and specific carrier selected [55]. Similarly, our comparative immunogenicity studies indicate that the induction of potent anti-PbS25 responses is significantly influenced by the presence of the PbMSP8 carrier as well as by adjuvant formulation.

We began a systematic evaluation of our candidate antigens by assessing how these parameters affected cellular responses. Using a [3H]-thymidine incorporation assay to determine the specificity of vaccine-induced cellular responses, we showed that immunization with any of the PbMSP8-containing vaccines induced significant and similar levels of antigen-specific T cell proliferation against PbMSP8. This was expected, as previous studies conducted in both inbred and outbred mice demonstrated the presence of potent CD4+ T cell epitopes within the PbMSP8 carrier [24, 25]. Interestingly, proliferative responses against PbS25 were also detected following immunization with all three rPbS25-containing vaccines, though at a lower magnitude relative to PbMSP8. Nevertheless, this indicated that PbS25 possesses one or more MHC II epitopes capable of inducing PbS25-specific CD4+ T cell responses in mice, even in the absence of a carrier. Indeed, PbS25 contains at least one epitope predicted to bind MHC II. Unlike PbMSP119, the CD4+ epitope(s) within PbS25 appear(s) to be available for processing and presentation despite the highly constrained nature of this antigen. The specificity of CD4+ T cells induced by these formulations was not affected by adjuvant selection.

The phenotype of T cells generated in response to PbS25-based vaccines was influenced by adjuvant. Alum-based formulations containing rPbS25/8, regardless of dose, elicited significant levels of Th2-associated cytokine, IL-5, and very low levels of Th1-associated cytokines, IFNγ and TNFα. This was expected, as Alum is a known Th2-biasing adjuvant. Downstream analysis of the IgG subclasses induced by vaccines adjuvanted with Alum reflected this Th2-biasing effect, as the vast majority of antigen-specific antibodies were IgG1. Vaccines formulated with GLA-SE, an adjuvant known to drive responses to a Th1 phenotype, elicited T cells that
produced significantly elevated levels of Th1-associated IFNγ and TNFα in comparison to Alum-based formulations, in a PfMSP8-dependent manner. These T cells also produced IL-5 at similar levels to those achieved by Alum-based formulations. However, the elevated levels of Th1-associated cytokines in GLA-SE based formulations influenced downstream class switching, leading to a more diversified IgG profile of antigen-specific antibodies that featured significant increases in cytophilic IgG2a/c, as well as IgG2b and IgG3.

We assessed the domain-specific responses of total IgG induced by the various vaccine formulations as an indicator of potential efficacy. All formulations containing rPfMSP8 resulted in high and comparable titers of anti-PfMSP8 IgG irrespective of adjuvant or dose. However, the induction of optimal anti-PfS25 humoral immunity was dependent on both carrier and adjuvant. Consistent with the proliferation data, immunization with rPfS25 elicited high anti-PfS25 antibody titers when formulated with Alum at both antigen doses. In contrast, only modest titers of anti-PfS25 IgG were elicited by unfused rPfS25 when formulated with GLA-SE, despite the detection of proliferative T cell responses in these groups. These results differ from a previous study in which Chlamydomonas reinhardtii-produced rPfS25 formulated with GLA-SE effectively induced anti-PfS25 antibodies that exhibited transmission-reducing activity in the SMFA [56]. However, this discrepancy may be a result of significant differences in the total amount of rPfS25 administered in the two studies (50 μg vs 7.5 μg). In three of the four groups immunized with the admixture of rPfS25 and PfMSP8, we observed a notable reduction in anti-PfS25 titers. These data indicate that antigenic competition is a potential problem and responses to rPfS25 may be impaired by the presence of additional immunogenic vaccine components.

In agreement with our previous rPfMSP1/8 studies, immunization with rPfS25/8 elicited strong humoral responses against both the carrier and PfS25 domains, effectively rescuing the anti-PfS25 response. This was true when rPfS25/8 was formulated with Alum, where the anti-PfS25 responses were restored to levels similar to those achieved by unfused rPfS25. The improvement was even more pronounced when rPfS25/8 was formulated with GLA-SE. Here, anti-PfS25 IgG titers were enhanced relative to the modest titers achieved by rPfS25 formulated with GLA-SE. In fact, the 2.5 μg dose of rPfS25/8 formulated with GLA-SE elicited anti-PfS25 responses comparable to those induced by either PfS25-based antigen when formulated with Alum. Importantly, the anti-PfS25 IgG induced by either PfS25-based vaccine demonstrated potent transmission-reducing activity, irrespective of notable differences in IgG subclass profile. Consistent with previous reports [57], the magnitude of the anti-PfS25 response primarily influenced transmission-reducing activity. Mice immunized with rPfS25 formulated with GLA-SE displayed only modest anti-PfS25 IgG responses with little or no functional activity. Together, these results showed that the genetic fusion of PfS25 to the PfMSP8 carrier was required for i) induction of anti-PfS25 responses in the presence of additional immunogenic targets in a multivalent formulation and ii) induction of anti-PfS25 IgG with functional activity in the context of a GLA-SE-based vaccine formulation. Furthermore, we observed potent transmission-reducing activity of vaccine-induced IgG at a concentration of 750 μg/ml, a value 1- to 3-fold lower than the normal level of IgG in mouse serum. These data increase the likelihood that immunization of human subjects with PfS25/8 formulated with GLA-SE can induce functional antibodies that significantly impact parasite transmission if comparable vaccine immunogenicity is achieved.

Initial testing of a bivalent vaccine containing rPfS25/8 and rPfMSP1/8 in outbred mice demonstrated the induction of strong B cell responses against both PfS25 and PfMSP1,9 that were comparable to those induced by corresponding monovalent vaccines. Anti-PfS25 responses induced by rPfS25/8 were strong and similar regardless of adjuvant selection or the presence of rPfMSP1/8. Interestingly, anti-PfMSP1,9 responses induced by immunization with
the bivalent vaccine were adjuvant dependent, with the GLA-SE-based formulations eliciting superior responses relative to the Alum-based formulations. This is reflective of several clinical trials in which rPfMSP142 formulated with Alum resulted in suboptimal anti-PfMSP1 responses [19, 58]. In addition to the lack of antigenic interference with either adjuvant formulation, these studies also showed that PfMSP8 can be effectively used as a carrier for two distinct vaccine components when administered in the same formulation to genetically heterogeneous, male and female mice.

The systematic evaluation of immune responses generated by the two PfS25-based vaccines as a function of various formulation parameters informed the selection of rPfS25/8 as the more effective candidate. This was most apparent for the induction of transmission-blocking immunity particularly when GLA-SE was selected as adjuvant. In addition, results of the bivalent study suggest that anti-PfMSP19 responses are superior when formulated with GLA-SE, providing some incentive for ultimate selection of this adjuvant. The results of our ongoing comparative immunogenicity studies with inclusion of additional antigens such as PfMSP2 into the multivalent formulation will also impact the choice of adjuvant for advanced testing. With PfMSP2-containing vaccines, we expect that adjuvants such as GLA-SE will be required to effectively induce cytophilic IgG that is needed for opsonization and phagocytosis of merozoites. Overall, these studies further demonstrate the value of PfMSP8 as a carrier protein to help induce effective humoral responses against protective, but poorly immunogenic vaccine components, targeting both blood-stage and sexual stage malaria parasites.

Supporting information

**S1 Fig. Proliferative responses elicited following stimulation with rPfS25/8 antigen.** CB6F1/J splenocytes (2 x 10^5/well) harvested from the indicated immunization groups were stimulated ex vivo in triplicate with rPfS25/8 (2 μg/well) for 96 hrs. [3H]-thymidine (1 μCi/well) was added for the final 18 hours. Average counts of incorporated [3H]-thymidine were measured for rPfS25/8-stimulated wells and converted into a Stimulation Index (SI) that represents the fold change in proliferation of the indicated condition over the corresponding control wells (media alone). Graphs depict mean SI +/- standard deviation. (DOCX)

**S2 Fig. Profile of cytokines elicited following stimulation with rPfMSP8.** CB6F1/J splenocytes (5 x 10^5/well) were harvested from the indicated immunization groups and stimulated ex vivo in triplicate with rPfMSP8 (2 μg/well) or in media alone for 96 hours. Culture supernatants were collected and analyzed for production of IL-5, IFNγ, TNFα, -4 and IL-2 using a multiplex assay (Luminex®). To calculate the final concentration of each analyte, the levels found in the corresponding unstimulated conditions were subtracted out as background. Graphs depict mean concentration of each analyte +/- standard deviation. (DOCX)

**S3 Fig. Anti-rPfS25/8 IgG titers induced by the indicated rPfS25 containing vaccine formulations.** CB6F1/J sera collected 3 weeks following each subcutaneous immunization were analyzed for antigen-specific IgG via ELISA using plates coated with rPfS25/8 (0.25 μg/well). Graphs depict mean IgG titers +/- standard deviation. Asterisks over bars within groups indicate significant boosting of antigen-specific responses over time (Friedman Test; P < 0.05 considered significant). (DOCX)

**S4 Fig. Evaluation of the effect of sex on humoral responses to immunization with combined formulations of rPfS25/8 and rPfMSP1/8 vaccines.** CD1 mice (10/group with 5 male
and 5 female mice) were immunized as indicated and sera were collected following the third immunization. Titers of antigen-specific IgG were measured by ELISA with plates coated with rPfs25 or rGST-PfMSP19 (0.25 ug/well). Graph depicts mean IgG titers +/- standard deviation. Antibody responses in male and female mice within the same immunization group were compared. Statistical significant of differences between sexes were evaluated (Mann-Whitney U Test; P < 0.05 considered significant; ns, not significant).

(1) S1 Table. Cytokine production by cells from mice immunized with Pfs25-based vaccines in response to stimulation with Con A. Splenocytes (5 x 10^5/well) were harvested from groups of CB6F1/J mice (n = 5) immunized as indicated and stimulated ex vivo with Con A (0.2 μg/well) or cultured in media alone for 96 hours. Culture supernatants were collected and analyzed for production of IL-5, IFNγ, TNFα, IL-2 and IL-4 using a multiplex assay (Luminex®). To calculate the final concentration of each analyte, the levels found in the corresponding unstimulated conditions were subtracted as background. (ND = not detected).

(2) S2 Table. Production of IL-2 and IL-4 by cells from mice immunized with Pfs25-based vaccines in response to stimulation with rPfs25/8. Splenocytes (5 x 10^5/well) were harvested from groups of CB6F1/J mice (n = 5) immunized as indicated and stimulated with ex vivo with rPfs25/8 (2 μg/well) or cultured in media alone for 96 hours. Culture supernatants were collected and analyzed for production of IL-2 and IL-4 using a multiplex assay (Luminex®). To calculate the final concentration of each analyte, the levels found in the corresponding unstimulated conditions were subtracted as background. (ND = not detected).

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