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The C-terminal 19-kDa domain of *Plasmodium falciparum* merozoite surface protein 1 (*Pf*MSP1$_{19}$) is an established target of protective antibodies. However, clinical trials of *Pf*MSP1$_{42}$, a leading blood-stage vaccine candidate which contains the protective epitopes of *Pf*MSP1$_{19}$, revealed suboptimal immunogenicity and efficacy. Based on proof-of-concept studies in the *Plasmodium yoelii* murine model, we produced a chimeric vaccine antigen containing recombinant *Pf*MSP1$_{19}$ (r*Pf*MSP1$_{19}$) fused to the N terminus of *P. falciparum* merozoite surface protein 8 that lacked its low-complexity Asn/Asp-rich domain, r*Pf*MSP8 (ΔAsn/Asp). Immunization of mice with the chimeric r*Pf*MSP1/8 vaccine elicited strong T cell responses to conserved epitopes associated with the r*Pf*MSP8 (ΔAsn/Asp) fusion partner. While specific for *Pf*MSP8, this T cell response was adequate to provide help for the production of high titers of antibodies to both *Pf*MSP1$_{19}$ and r*Pf*MSP8 (ΔAsn/Asp) components. This occurred with formulations adjuvanted with either Quil A or with Montanide ISA 720 plus CpG oligodeoxynucleotide (ODN) and was observed in both inbred and outbred strains of mice. *Pf*MSP1/8-induced antibodies were highly reactive with two major alleles of *Pf*MSP1$_{19}$ (FVO and 3D7). Of particular interest, immunization with *Pf*MSP1/8 elicited higher titers of *Pf*MSP1$_{19}$-specific antibodies than a combined formulation of r*Pf*MSP1$_{42}$ and r*Pf*MSP8 (ΔAsn/Asp). As a measure of functionality, *Pf*MSP1/8-specific rabbit IgG was shown to potently inhibit the *in vitro* growth of blood-stage parasites of the FVO and 3D7 strains of *P. falciparum*. These data support the further testing and evaluation of this chimeric *Pf*MSP1/8 antigen as a component of a multivalent vaccine for *P. falciparum* malaria.

In recent years, there has been an increased international effort to reduce the burden of malaria through the implementation and integration of multiple control programs to provide high coverage of affected populations. These efforts have included the use of insecticide-treated bednets, indoor residual spraying, intermittent preventative treatment, rapid diagnostic tests, and artemisinin-based combination therapies. Some reduction in malaria morbidity and mortality has been achieved, such that in 2010, the number of clinical cases of malaria worldwide was estimated to be 219 million, with approximately 660,000 deaths (1). The development of an effective malaria vaccine could bolster these efforts further, but success has been limited. There has been increased interest and emphasis on development and testing of preerythrocytic and transmission-blocking malaria vaccines (2, 3). RTS,S, the most advanced preerythrocytic vaccine for *Plasmodium falciparum*, is currently being tested in a large, multicenter phase 3 clinical trial in Africa. Initial reports suggest that vaccine efficacy is only around 30% in the most vulnerable target population of infants (4), with a higher efficacy of approximately 50% in young children (5).

The development and testing of blood-stage malaria vaccines have been challenging, and only a limited number of candidate antigens have progressed to evaluation in phase 1 and 2 clinical trials (reviewed in reference 6). Such vaccines are unlikely to prevent infection but may be able to reduce parasite burden, the duration of clinical disease, and/or the severe consequences of malaria associated with mortality. *P. falciparum* merozoite surface protein-1 (*Pf*MSP1) and apical membrane antigen-1 (*Pf*AMA1) are two of the leading blood-stage vaccine candidates (6). In clinical trials in human subjects, the efficacy of *Pf*MSP1- and *Pf*AMA1-based vaccines has been low, with problems associated with inadequate immunogenicity, the polymorphism of both B and T cell epitopes, and the short duration of protection (6–10). As the ability to evaluate the protective efficacy of blood-stage vaccines requires large and expensive field trials, pursuit of such vaccines appears to be declining in recent years. Nevertheless, an effective blood-stage malaria vaccine could have a very significant impact on reducing the burden of malaria in settings in which it is endemic, even if such a vaccine does not completely prevent infection.

Merozoite surface protein 8 (MSP8) is a target of protective, vaccine-induced antibody responses in the *Plasmodium yoelii* rodent model of malaria (11–13). Proof-of-concept studies showed that a high level of vaccine efficacy against challenge infection with lethal *P. yoelii* 17XL could be achieved by immunization of mice with a multicomponent vaccine including both *P. yoelii* MSP1 (*Py*MSP1) and *Py*MSP8 (14, 15). Of interest, this potency was not achieved by immunization with an adjuvanted mixture of recom-

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binant *Py*MSP1 and *Py*MSP8 vaccines. To achieve maximum protection, it was necessary to fuse *Py*MSP1$_{19}$, a target of neutralizing antibodies, to the N terminus of full-length *Py*MSP8. Upon immunization, a strong T cell response to epitopes within the *Py*MSP8 portion of the chimeric antigen was elicited. This T cell response provided help for the production of high and sustained levels of IgG that recognized conformational, protective B cell epitopes of both *Py*MSP1$_{19}$ and *Py*MSP8 (15).

MSP8 is well conserved among the species of plasmodial parasites which infect a range of hosts, including rodents, nonhuman primates, and human subjects (16, 17). Full-length *P. falciparum* MSP8 contains ~600 amino acids, slightly larger than its orthologs in other plasmodial species due to the presence of an asparagine and aspartic acid (Asn/Asp)-rich domain of ~170 amino acids near its N terminus. Sequence conservation of *Pf*MSP8 among diverse strains of *P. falciparum* is very high, with variability mainly restricted to small insertions and/or deletions in the Asn/Asp-rich domain (16). In assessing the immunogenicity of full-length recombinant *Pf*MSP8 (r*Pf*MSP8) and r*Pf*MSP8 (ΔAsn/Asp), we mapped immunogenic T cell epitopes to highly conserved regions of the protein outside the Asn/Asp-rich domain (18). The Asn/Asp-rich domain was somewhat immunogenic for B cells, but the majority of immunization-induced antibodies recognized conformational epitopes of *Pf*MSP8 located outside the Asn/Asp-rich region. These antibodies did not exhibit significant functional activity *in vitro* as measured in a standard parasite growth inhibition assay. The functional activity of these antibodies against *P. falciparum* blood-stage parasites *in vivo* has not been evaluated.

Based on the *P. yoelii* MSP1/8 studies in mice and information gained from immunogenicity studies of r*Pf*MSP8 vaccines in mice and rabbits, we have now produced a chimeric *Pf*MSP1/8 vaccine antigen. In this study, we evaluated the immunogenicity of r*Pf*MSP1/8 for both T cells and B cells, assessed the specificity of the responses for *Pf*MSP1 and *Pf*MSP8 epitopes, and determined the ability of *Pf*MSP1/8-specific IgG to inhibit the *in vitro* growth of homologous and heterologous strains of *P. falciparum* blood-stage parasites. In mice and rabbits, we also compared *Pf*MSP1/8 to *Pf*MSP1$_{42}$ vaccines to assess differences in the immunogenicity of *Pf*MSP$_{19}$ and the induction of cross-strain growth inhibitory antibodies. The results indicate that the chimeric r*Pf*MSP1/8 vaccine antigen has clear vaccine potential, and its further development and testing should be pursued.

MATERIALS AND METHODS

Production and purification of chimeric r*Pf*MSP1/8. To generate the expression construct for the chimeric *Pf*MSP1/8 vaccine, the codon-harmonized gene sequence of *Pf*MSP1$_{19}$ (19) was fused to the 5′ end of the codon-harmonized gene sequence of r*Pf*MSP8 (ΔAsn/Asp) (18) via a glycine-serine linker (GGSGSG). The sequences of *Pf*MSP1$_{19}$ and *Pf*MSP8 from the FVO strain of *P. falciparum* were utilized. The algorithm for codon harmonization for recombinant antigen expression in *Escherichia coli* has been previously described (19) and was used effectively to enhance production of full-length r*Pf*MSP8 and r*Pf*MSP8 (ΔAsn/Asp) (18), r*Pf*MSP1$_{42}$ (19), r*Pf*LSA1 (20), and r*Pf*s48/45 (21). A leader sequence that includes a histidine tag, MAHHHHHHPGGSGSGT, was incorporated at the N terminus, and two stop codons (TGA and TAA) were added at the 3′ end of the chimeric gene. The codon-harmonized synthetic gene was commercially prepared by Blue Heron Biotechnology, Inc. (Bothell, WA), and the DNA was sequence verified. The gene insert was then subcloned into the NcoI and NotI sites of the pET-28 expression vector (EMD Biosciences, San Diego, CA). The *Pf*MSP1/8 expression plasmid was transformed into Shuffle T7 express *lysY* competent *E. coli* cells (New England BioLabs, Ipswich, MA). This strain was previously used successfully for the production of full-length, properly folded r*Pf*MSP8 (18).

Production of r*Pf*MSP1/8 was accomplished in 3-liter bacterial cultures using a BioFLo115 benchtop bioreactor (New Brunswick Scientific, Edison, NJ) as previously described (18). r*Pf*MSP1/8 expression was induced by the addition of β-D-1-thiogalactopyranoside (IPTG; Fisher Scientific, Pittsburg, PA) to a final concentration of 1 mM. Three hours postinduction, cells were harvested by centrifugation at 8,000 × *g* for 20 min at 4°C, and cell paste was stored frozen at −80°C. Purification of r*Pf*MSP1/8 followed protocols previously established for both r*Pf*MSP8 and r*Pf*MSP8 (ΔAsn/Asp). Briefly, bacterial cells were lysed using the BugBuster HT protein extraction reagent (EMD Biosciences) in the presence of benzonase nuclease (EMD Biosciences) and recombinant lysozyme (EMD Biosciences). The lysate was clarified by centrifugation, and the resulting pellet was solubilized in column binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 0.5 M NaCl) containing 0.2% *N*-lauroylsarcosine sodium salt (Sarkosyl; Sigma-Aldrich, St. Louis, MO). r*Pf*MSP1/8 was purified from the detergent-soluble fractions by nickel-chelate affinity chromatography on nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Valencia, CA) under nondenaturing conditions as described previously (18). The eluted r*Pf*MSP1/8 was dialyzed overnight at 4°C against 4 liters of binding buffer containing 0.2% Sarkosyl. The final protein concentration was determined by bicinchoninic acid protein assay (BCA; Thermo Scientific, Rockford, IL). Protein purity and conformation were assessed by Coomassie blue staining following SDS-PAGE on 10% gels, run under both reduced and nonreduced conditions. Corresponding immunoblots were probed with a *Pf*MSP1$_{19}$ (FVO)-specific monoclonal antibody (MAb 5.2) (MRA-94; Malaria Research and Reference Reagent Resource Center, MR4, Manassas, VA), rabbit anti-r*Pf*MSP1$_{19}$ (FVO) (MR4 and MRA-33), rabbit anti-r*Pf*MSP8 (18), or rabbit anti-r*Pf*MSP8 (ΔAsn/Asp) (18). Additional antigens used in this study, including r*Pf*MSP8 (ΔAsn/Asp), r*Pf*MSP1$_{42}$, recombinant glutathione *S*-transferase-*Pf*MSP1$_{19}$ (rGST-*Pf*MSP1$_{19}$) (FVO), and rGST-*Pf*MSP1$_{19}$ (3D7), were expressed and purified as previously reported (14, 18, 19, 22–24).

Mice and immunization protocols. Male CB6F1/J (BALB/cJ × C57BL/6J) mice or outbred CD1 mice, 5 to 6 weeks of age, were purchased from The Jackson Laboratory and housed in the Animal Care Facility of Drexel University College of Medicine under specific-pathogen-free conditions. All animal studies were reviewed, approved, and conducted in compliance with the Institutional Animal Care and Use Committee (IACUC) of Drexel University College of Medicine. Three immunogenicity experiments were completed. In each experiment, serum and/or splenocytes were recovered from each animal approximately 2 weeks following the last immunization.

Experiment 1. Groups of CB6F1/J or outbred CD1 mice ($n = 10$) were immunized subcutaneously with 10 μg/dose of purified r*Pf*MSP1/8 formulated with either (i) 25 μg/dose of Quil A as the adjuvant (Accurate Chemical and Scientific Corporation, Westbury, NY) or (ii) combined with 25 μg/dose of CpG oligodeoxynucleotide (ODN) 1826 (Eurofins MWG Operon, Huntsville, AL) emulsified in Montanide ISA 720 VG (Seppic Inc., Paris, France) at a ratio of 70:30 (vol/vol) (termed M plus CpG). For all mouse immunizations, r*Pf*MSP1/8 was diluted in saline, resulting in a final Sarkosyl concentration of ~0.004%, which did not interfere with emulsification. Control mice were immunized with the corresponding adjuvants alone. All mice were boosted twice at 3-week intervals with the same antigen/adjuvant formulation as that used in the priming immunization.

Experiment 2. Groups of CB6F1/J mice ($n = 5$) were immunized and boosted 3 weeks later with the following prime-boost antigen combinations: (i) *Pf*MSP1/8 (primary immunization [1°])-*Pf*MSP1/8 (secondary immunization [2°]), (ii) *Pf*MSP1/8 (1°)-*Pf*RBC lysate (2°), (iii) adjuvant (1°)-*Pf*RBC lysate (2°), or (iv) adjuvant (1°)-adjuvant (2°). *Pf*MSP1/8 (10

μg/dose) and *P. falciparum* red blood cell (*Pf*RBC) lysate (100 μg/dose) were administered as subcutaneous formulations with 25 μg/dose Quil A as the adjuvant (Accurate Chemical and Scientific). *P. falciparum* FVO strain (ATCC, Manassas, VA) blood-stage parasites were grown *in vitro* as asynchronous cultures in O^+ human RBCs as previously described (25). *P. falciparum* parasites were recovered by centrifugation following treatment of infected RBCs with 0.15% saponin, and the *Pf*RBC lysate was prepared following 10 freeze/thaw cycles.

Experiment 3. Groups of CB6F1/J mice ($n = 5$) were immunized subcutaneously with (i) r*Pf*MSP1/8, (ii) r*Pf*MSP8 (ΔAsn/Asp), (iii) r*Pf*MSP1$_{42}$, (iv) r*Pf*MSP8 (ΔAsn/Asp) plus r*Pf*MSP1$_{42}$, or (v) adjuvant alone. Antigens (10 μg/dose) were formulated with either 25 μg/dose Quil A as the adjuvant (Accurate Chemical and Scientific Corporation) or combined with 25 μg/dose CpG ODN 1826 (Eurofins MWG Operon) and emulsified in Montanide ISA 720 VG (Seppic Inc.) at a ratio of 70:30 (vol/vol). For the r*Pf*MSP8 (ΔAsn/Asp) plus r*Pf*MSP1$_{42}$ group, recombinant antigens were combined, formulated with adjuvant, and injected at the same site. Control mice were immunized with the corresponding adjuvants alone. All mice were boosted twice at 3-week intervals with the same antigen/adjuvant formulation as that used in the priming immunization.

T cell proliferation assay. Fifty-one peptides (see Table S1 in the supplemental material) spanning the length of the chimeric r*Pf*MSP1/8 sequence were custom synthesized and purified (GenScript USA Inc., Piscataway, NJ). These peptides overlapped by 9 amino acids and were >90% pure. Peptides 1 to 11 were based on the *Pf*MSP1$_{19}$ (FVO) sequence; peptides 12 and 13 were based on the linker and flanking sequence where *Pf*MSP1$_{19}$ and r*Pf*MSP8 (ΔAsn/Asp) were joined; peptides 23 to 60 were based on the r*Pf*MSP8 (ΔAsn/Asp) sequence with peptide numbering consistent with that used in the original publication (18). The lyophilized peptides were reconstituted to a working concentration of 1 mg/ml as recommended by the manufacturer. T cell proliferation induced by recombinant antigens (5 μg/ml) or peptides (15 μg/ml) was measured by [^{3}H]thymidine incorporation as previously described (15, 18). An additional set of cells was stimulated with 1 μg/ml of concanavalin A (ConA; Sigma-Aldrich) or left unstimulated to serve as positive and negative controls. The stimulation index was calculated as the mean counts per minute of stimulated wells divided by the mean counts per minute of unstimulated wells.

Measurement of antigen-specific antibody responses by ELISA. The antigen-specific antibody responses induced by immunization with r*Pf*MSP1/8, r*Pf*MSP8 (ΔAsn/Asp), or r*Pf*MSP1$_{42}$ were measured by direct binding enzyme-linked immunosorbent assay (ELISA) as previously described (18). Briefly, ELISA plates were coated with 0.25 μg per well of purified r*Pf*MSP1/8, r*Pf*MSP8 (ΔAsn/Asp), r*Pf*MSP1$_{42}$, rGST-*Pf*MSP1$_{19}$ (FVO), or rGST-*Pf*MSP1$_{19}$ (3D7). Plates were incubated with 2-fold serial dilutions of mouse or rabbit sera starting at 1:2,500. Corresponding adjuvant control sera were used as negative controls and subtracted as background. Bound antibodies were detected by horseradish peroxidase-conjugated rabbit anti-mouse IgG (Thermo Scientific) or goat anti-rabbit IgG (Invitrogen) with ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] as the substrate. A_{405} values between 1.0 and 0.1 were plotted, and titers were calculated as the reciprocal of the dilution that yielded an A_{405} of 0.5. A high-titer pool of serum obtained from r*Pf*MSP1/8-immunized mice ($n = 5$) was included on each assay plate as an internal reference to normalize the data between assays.

Production of polyclonal rabbit antisera. Polyclonal rabbit antisera were generated by Lampire Biological Laboratories (Pipersville, PA) by following their "classic-line basic" protocol. Briefly, adult New Zealand White rabbits (three/antigen) were immunized once with 200 μg of either r*Pf*MSP1/8 or r*Pf*MSP1$_{42}$ formulated with complete Freund's adjuvant (CFA), followed by 4 booster immunizations (200 μg each) with the same antigen formulated in incomplete Freund's adjuvant (IFA). For r*Pf*MSP1/8, a final concentration of ~0.01% Sarkosyl did not interfere with emulsification. Approximately 2 weeks after the final immunization, antisera were recovered.

***P. falciparum* growth inhibition assays.** The growth inhibitory activity (GIA) of purified rabbit anti-r*Pf*MSP1/8 IgG and anti-r*Pf*MSP1$_{42}$ IgG was assessed *in vitro* by the measurement of parasite lactate dehydrogenase activity (26) using standard protocols. Prebleed and adjuvant control IgG served as negative controls. Each rabbit IgG was tested at final concentrations ranging between 1.25 and 5 mg/ml as indicated. Growth inhibitory activity was calculated relative to *P. falciparum* blood-stage parasites growing in complete media in the absence of any added rabbit IgG.

Statistical analysis. When comparing data from two groups, the statistical significance of the differences in antigen-specific IgG titers and T cell proliferation stimulation indices was determined by the Mann-Whitney test. The statistical significance of increases in antigen-specific titers between paired primary and secondary immunization sera was determined using the Wilcoxon signed-rank test. Nonparametric tests were utilized considering the limited ability to ensure normality of the data sets due to sample size. Probability (P) values of ≤0.05 were considered significant.

RESULTS

Expression and purification of chimeric r*Pf*MSP1/8. Based on earlier supporting studies in the *P. yoelii* rodent model (14, 15), an effort was initiated to produce and characterize a chimeric MSP1/8-based vaccine for *P. falciparum*. Comparative immunogenicity studies with full-length r*Pf*MSP8 and r*Pf*MSP8 (ΔAsn/Asp) identified the shorter r*Pf*MSP8 (ΔAsn/Asp) as the appropriate fusion partner for *Pf*MSP1$_{19}$ (18). The Asn/Asp-rich domain lacked T cell epitopes and was only weakly immunogenic for B cells, and deletion of the domain did not alter the display of conformational B cell epitopes of *Pf*MSP8. A schematic for the chimeric *Pf*MSP1/8 vaccine is shown in Fig. 1A with the double-EGF-like domains of *Pf*MSP1$_{19}$ and *Pf*MSP8 indicated at the N and C termini, respectively. A codon-harmonized gene sequence for the chimeric *Pf*MSP1/8 was synthesized and cloned into the pET28 plasmid. Shuffle T7 express *lysY E. coli* cells were used as the expression host. r*Pf*MSP1/8 was successfully purified under nondenaturing conditions by nickel-chelate affinity chromatography with a final yield of ~2.4 mg/g (wet weight) cells. The quality of the purified product was assessed by SDS-PAGE. In the absence of reducing agent, purified r*Pf*MSP1/8 migrates as a prominent doublet of ~48 to 50 kDa with a second higher-molecular-mass band migrating at ~100 kDa (Fig. 1B). This pattern is consistent with that observed previously with r*Pf*MSP8 (ΔAsn/Asp), which contained a single intrachain disulfide bond resulting in a 1:1 mixture of monomers and dimers. Under reducing conditions, r*Pf*MSP1/8 migrated as a single prominent band of ~58 kDa, close to its predicted molecular mass. As shown in Fig. 1C, immunoblot analysis performed under nonreducing conditions revealed strong seroreactivity of the 48- to 50-kDa band with MAb 5.2, rabbit anti-*Pf*MSP1$_{19}$, and rabbit anti-*Pf*MSP8 antibodies. MAb 5.2 recognizes a conformation-dependent epitope within *Pf*MSP1$_{19}$ (27). The results indicate that the chimeric r*Pf*MSP1/8, displaying correct disulfide-dependent epitopes, could be produced in *E. coli* and purified in reasonable quantities.

Immunization with r*Pf*MSP1/8 elicits robust T cell responses toward *Pf*MSP8-associated epitopes. Previously, immunization with full-length r*Pf*MSP8 was shown to elicit strong T cell responses to epitopes also present within the shorter r*Pf*MSP8 (ΔAsn/Asp) (18). *Pf*MSP1$_{19}$ has been reported to be poorly immunogenic for T cells due to the difficulty in processing this highly disulfide bond-constrained domain (28–30). CB6F1/J mice were

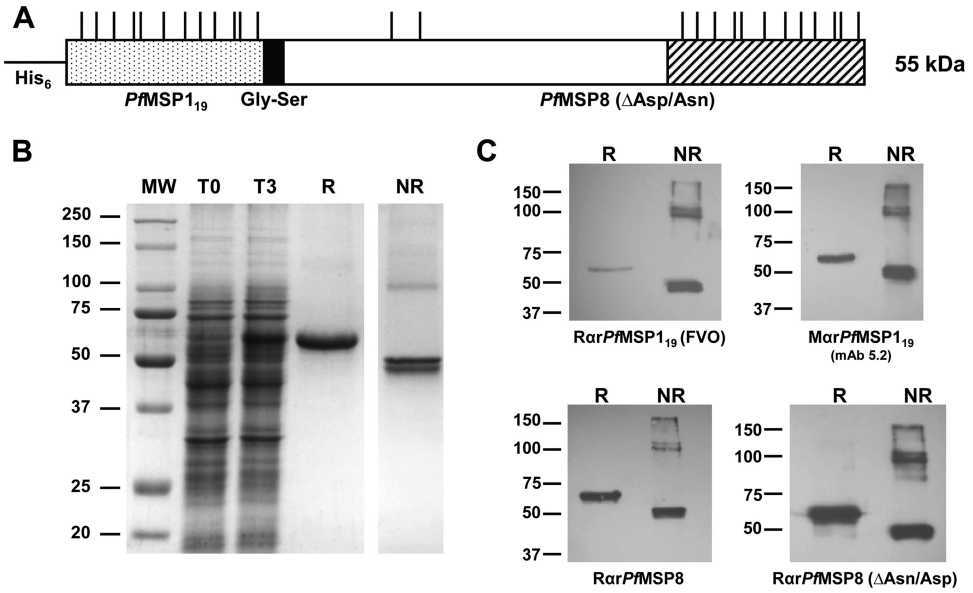

FIG 1 Design, production, and analysis of a chimeric r*Pf*MSP1/8 (FVO) vaccine antigen. (A) Cartoon of the chimeric *Pf*MSP1/8 expression construct with sequences encoding the double EGF-like domains of *Pf*MSP1$_{19}$ (stippled box), followed by a Gly-Ser spacer (filled box) and *Pf*MSP8 (ΔAsn/Asp) including the C-terminal EGF-like domains (open and hatched boxes). Cysteine residues are indicated by vertical lines. (B) Coomassie blue-stained 10% SDS-polyacrylamide gel containing lysates of *E. coli* (reduced) expressing r*Pf*MSP1/8 at the time of induction (T0) or 3 h postinduction (T3) and nickel-chelate affinity-purified r*Pf*MSP1/8 (3 μg per lane) under reducing (R) and nonreducing (NR) conditions. (C) Immunoblot analysis of purified r*Pf*MSP1/8 (0.1 μg per lane) under reducing (R) and nonreducing (NR) conditions probed with rabbit anti-r*Pf*MSP1$_{19}$ (FVO), *Pf*MSP1$_{19}$ (FVO)-specific monoclonal antibody (MAb 5.2), rabbit anti-r*Pf*MSP8, or rabbit anti-r*Pf*MSP8 (ΔAsn/Asp). Molecular weight markers (MW; in thousands) are indicated.

immunized three times with r*Pf*MSP1/8 formulated with Quil A as the adjuvant. After a period of rest following the third immunization, splenocytes were harvested and stimulated *in vitro* with r*Pf*MSP1/8, r*Pf*MSP8 (ΔAsn/Asp), rGST-*Pf*MSP1$_{19}$, or GST alone. Antigen-specific proliferation of T cells was measured in a standard [^{3}H]thymidine incorporation assay. As shown in Fig. 2A, stimulation with r*Pf*MSP1/8 or r*Pf*MSP8 (ΔAsn/Asp) elicited high proliferative responses of cells from r*Pf*MSP1/8-immunized mice that were significantly greater than the responses of splenocytes from adjuvant control mice ($P < 0.01$). In contrast, no significant proliferation of cells from r*Pf*MSP1/8-immunized or adjuvant control mice was noted upon stimulation with rGST-*Pf*MSP1$_{19}$ or GST alone ($P > 0.1$).

The restriction of the r*Pf*MSP1/8-elicited T cell response to epitopes present within the *Pf*MSP8 (ΔAsn/Asp) domain was confirmed. Splenocytes from r*Pf*MSP1/8-immunized and adjuvant control mice were stimulated with a panel of overlapping peptides covering the complete sequence of the chimeric r*Pf*MSP1/8 antigen. Relative to adjuvant controls, T cells from r*Pf*MSP1/8-immunized mice showed significantly higher proliferation in response to 4 dominant peptides ($P \leq 0.05$) (Fig. 2B) that mapped to the *Pf*MSP8 (ΔAsn/Asp) region of the chimeric vaccine antigen. Neither *Pf*MSP1$_{19}$-associated epitopes (peptides 1 to 11) nor epitopes present at the linker/junction of the *Pf*MSP1$_{19}$ and *Pf*MSP8 (ΔAsn/Asp) sequences (peptides 12 and 13) were immunogenic.

Immunization with r*Pf*MSP1/8 elicits high-titer antibodies to both *Pf*MSP1$_{19}$ and *Pf*MSP8. The ability of the *Pf*MSP8-restricted T cell response to provide help for production of antibodies specific for *Pf*MSP1$_{19}$ and *Pf*MSP8 B cell epitopes was evaluated. CB6F1/J mice were immunized three times with r*Pf*MSP1/8 formulated with either Quil A adjuvant or with a combination of Montanide ISA 720 and CpG ODN (M plus CpG) as the adjuvant. Sera were collected 2 weeks following the last immunization, and antibody titers to r*Pf*MSP1/8, r*Pf*MSP8 (ΔAsn/Asp), rGST-*Pf*MSP1$_{19}$ (FVO), and rGST-*Pf*MSP1$_{19}$ (3D7) were determined by ELISA. As shown in Fig. 3A, immunization with the chimeric r*Pf*MSP1/8 elicited high titers of IgG that recognized r*Pf*MSP1/8 as well as epitopes present within the r*Pf*MSP8 (ΔAsn/Asp) domain. Just as important, immunization with r*Pf*MSP1/8 also elicited a very strong antibody response to the homologous r*Pf*MSP1$_{19}$ (FVO) domain, which was highly cross-reactive with the heterologous r*Pf*MSP1$_{19}$ (3D7) antigen. The use of Quil A as the adjuvant induced somewhat higher titers of antibodies to r*Pf*MSP8 (ΔAsn/Asp) epitopes than formulations with M plus CpG ($P = 0.03$). However, titers of antibodies to *Pf*MSP1$_{19}$-specific epitopes were comparable to Quil A and M plus CpG formulations ($P > 0.2$).

To determine if the broad specificity of the antibody responses elicited by r*Pf*MSP1/8 was generalizable to genetically diverse populations, outbred CD-1 mice were immunized as described above with r*Pf*MSP1/8 formulated with either Quil A or M plus CpG as the adjuvant. Sera collected 2 weeks following the last immunizations were analyzed by ELISA as described above to determine the response to *Pf*MSP1$_{19}$ and *Pf*MSP8 domains. As with inbred CB6F1/J mice, immunization of outbred CD-1 mice with the chimeric r*Pf*MSP1/8 elicited high titers of antigen-specific IgG, and r*Pf*MSP8 (ΔAsn/Asp) epitopes were highly immunogenic (Fig. 3B). Most importantly, immunization with r*Pf*MSP1/8 also elicited a very strong antibody response to the r*Pf*MSP1$_{19}$ (FVO) domain, which again was cross-reactive with the heterologous r*Pf*MSP1$_{19}$ (3D7) allele. In contrast to the data from immunization of CB6F1/J mice, differences in mean antibody titers to r*Pf*MSP8 (ΔAsn/Asp) epitopes elicited by immunization of out-

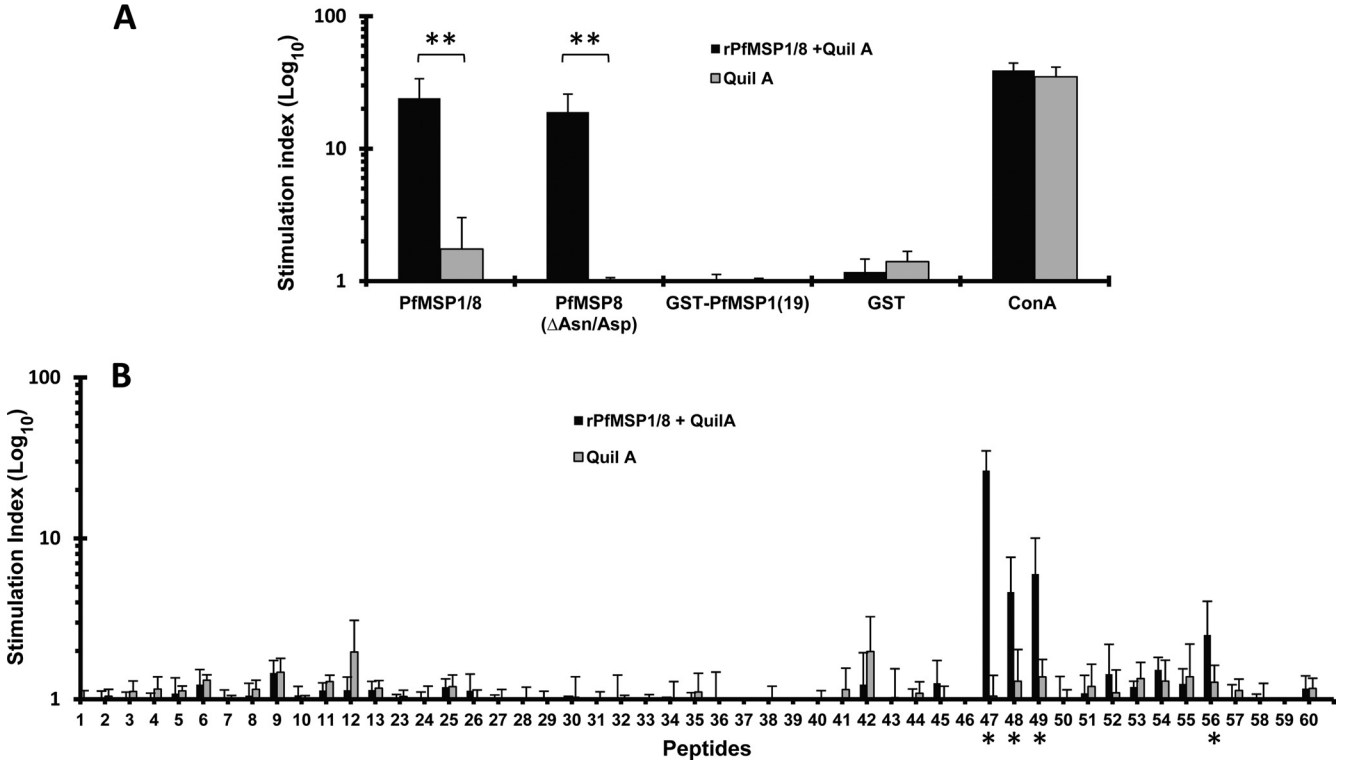

FIG 2 T cells induced by immunization with r*Pf*MSP1/8 recognize epitopes within r*Pf*MSP8 (ΔAsn/Asp). CB6F1/J mice (5 mice/group) were immunized three times with r*Pf*MSP1/8 with Quil A as the adjuvant (black bars) or with Quil A alone (gray bars). Following the third immunization, splenocytes were harvested and stimulated with r*Pf*MSP1/8, r*Pf*MSP8 (ΔAsn/Asp), GST-*Pf*MSP1$_{19}$ (FVO), GST, or ConA (A) or with overlapping synthetic peptides spanning the entire length of r*Pf*MSP1/8 (B), as indicated on the *x* axes. See Table S1 in the supplemental material for sequence of individual peptides. After 4 days of culture, proliferation was quantitated by [^{3}H]thymidine incorporation. The stimulation index was calculated as mean counts per minute in stimulated cultures/mean counts per minute in unstimulated cultures. Mean values ± standard deviations (SD) are shown with significant responses elicited by 4 peptides marked by an asterisk on the *y* axis (*, $P < 0.05$; **, $P < 0.01$).

bred CD1 mice with r*Pf*MSP1/8 formulated in Quil A versus M plus CpG were not statistically significant ($P > 0.3$).

Native *P. falciparum* blood-stage antigens boost r*Pf*MSP1/8-primed responses. The restriction of the T cell response to *Pf*MSP8 epitopes raised the question of whether or not antibody responses to both *Pf*MSP1$_{19}$ and *Pf*MSP8 would be boosted upon exposure to native, noncoupled antigens during natural infection. To test this, CB6F1/J mice were immunized once with r*Pf*MSP1/8 formulated with Quil A as the adjuvant. Three weeks later, mice were boosted with either r*Pf*MSP1/8 or a total antigen lysate prepared from *in vitro*-cultured *P. falciparum* (FVO) blood-stage parasites. Antibody titers specific for r*Pf*MSP1/8, r*Pf*MSP8 (ΔAsn/

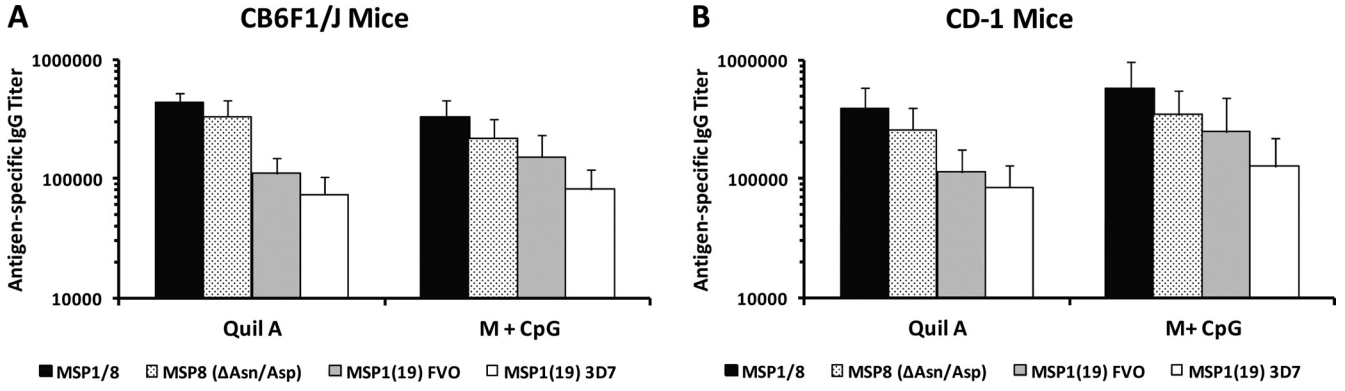

FIG 3 Immunization of inbred CB6F1/J mice and outbred CD1 mice with r*Pf*MSP1/8 elicits high-titer antibodies to both *Pf*MSP1$_{19}$ and *Pf*MSP8 (ΔAsn/Asp). Antigen-specific IgG titers (means ± standard deviations) in sera collected from CB6F1/J (A) and CD-1 (B) mice (10 mice/group) immunized with r*Pf*MSP1/8 formulated with Quil A or M plus CpG were determined by ELISA on plates coated with either r*Pf*MSP1/8, r*Pf*MSP8 (ΔAsn/Asp), rGST-*Pf*MSP1$_{19}$ (FVO), or rGST-*Pf*MSP1$_{19}$ (3D7) as indicated. For each dilution, the mean absorbance values at A_{405} of the pooled sera from adjuvant control mice ($n = 5$) was subtracted as the background.

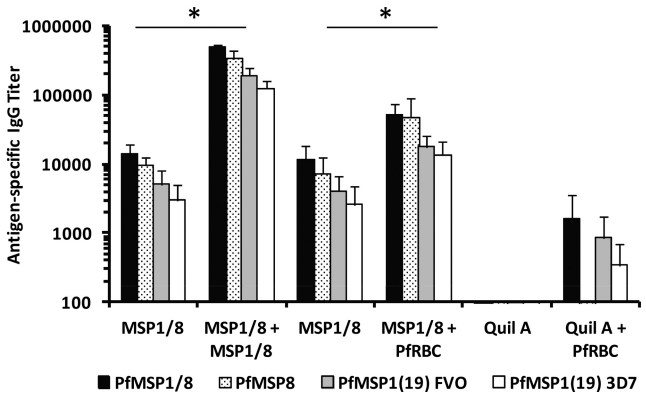

FIG 4 Native *P. falciparum* blood-stage antigens boost *Pf*MSP1$_{19}$- and *Pf*MSP8 (ΔAsn/Asp)-specific antibody responses primed by r*Pf*MSP1/8 immunization. CB6F1/J mice (5 mice per group) received a primary immunization with r*Pf*MSP1/8 or adjuvant and then were boosted with either r*Pf*MSP1/8 or a total antigen lysate (*Pf*RBC) prepared from *in vitro*-cultured *P. falciparum* (FVO) blood-stage parasites. Antigens were formulated with Quil A as the adjuvant. Antibody titers specific for r*Pf*MSP1/8, r*Pf*MSP8 (ΔAsn/Asp), rGST-*Pf*MSP1$_{19}$ (FVO), and rGST-*Pf*MSP1$_{19}$ (3D7) were measured by ELISA following primary and secondary immunizations. Immunization groups are indicated on the *x* axis. *, $P < 0.05$.

Asp), rGST-*Pf*MSP1$_{19}$ (FVO), and rGST-*Pf*MSP1$_{19}$ (3D7) were measured by ELISA following primary and secondary immunizations. As expected, boosting with r*Pf*MSP1/8 led to a sharp and significant increase (40 to 50-fold) ($P < 0.05$) in antibody titers to both *Pf*MSP1$_{19}$ and *Pf*MSP8 epitopes (Fig. 4). A single immunization with *Pf*RBC lysate formulated with Quil A as the adjuvant elicited low titers of antibodies to *Pf*MSP1$_{19}$ with little or no response to r*Pf*MSP8 (ΔAsn/Asp)-associated epitopes. Of importance, boosting of r*Pf*MSP1/8-primed mice with *Pf*RBC lysate also led to a significant increase in antibody titers (6- to 10-fold; $P < 0.05$) to both *Pf*MSP1$_{19}$ and *Pf*MSP8 epitopes (Fig. 4). Not unexpectedly, the antibody titers to both r*Pf*MSP8 (ΔAsn/Asp) and *Pf*MSP1$_{19}$ epitopes following secondary immunization with a heterogeneous mixture of native *P. falciparum* blood-stage antigens were significantly lower than that observed upon a secondary immunization with r*Pf*MSP1/8 ($P < 0.01$). Nonetheless, the data suggest that upon exposure to native, parasite-associated antigen during natural infection, there is the potential for boosting of both *Pf*MSP1$_{19}$- and *Pf*MSP8-specific antibody responses primed by immunization with the chimeric r*Pf*MSP1/8 vaccine.

Comparative immunogenicity of r*Pf*MSP1/8 and constituent single-antigen vaccines. Data from studies of the chimeric MSP1/8 vaccine in the *P. yoelii* model indicated superiority of r*Py*MSP1/8 in eliciting strong *Py*MSP1$_{19}$-specific antibody responses compared to an admixture of two recombinant antigens, r*Py*MSP1$_{42}$ and r*Py*MSP8 (14). To evaluate this with the chimeric *P. falciparum* vaccine, CB6F1/J mice were immunized with r*Pf*MSP1/8, r*Pf*MSP8 (ΔAsn/Asp), r*Pf*MSP1$_{42}$, or a combination of r*Pf*MSP1$_{42}$ and r*Pf*MSP8 (ΔAsn/Asp) formulated with Quil A or M plus CpG as the adjuvant. Two weeks following the last immunization, sera were collected and the specificity and titer of elicited antibodies analyzed by ELISA. The antibody responses to r*Pf*MSP1/8 (Fig. 5A) and r*Pf*MSP8 (ΔAsn/Asp) (Fig. 5B) elicited by immunization with r*Pf*MSP1/8 or r*Pf*MSP8 (ΔAsn/Asp) alone or the combination of r*Pf*MSP1$_{42}$ and r*Pf*MSP8 (ΔAsn/Asp) were consistently strong. Immunization with r*Pf*MSP1/8 elicited high titers of antibodies to *Pf*MSP1$_{19}$, which were highly cross-reactive with FVO and 3D7 alleles of *Pf*MSP1$_{19}$ (Fig. 5C to E), confirming the data presented in Fig. 3. The titers of *Pf*MSP1$_{19}$-specific antibodies elicited by immunization with r*Pf*MSP1$_{42}$ plus Quil A were also comparable to those elicited by immunization with r*Pf*MSP1/8 plus Quil A ($P > 0.30$) (Fig. 5D and E). Most importantly, immunization with a mixture of r*Pf*MSP1$_{42}$ and r*Pf*MSP8 (ΔAsn/Asp) resulted in production of high titers of antibodies specific for r*Pf*MSP8 (ΔAsn/Asp) epitopes (Fig. 5B) but markedly inhibited the response to *Pf*MSP1$_{19}$ epitopes with the use of Quil A ($P < 0.01$) or M plus CpG ($P < 0.01$) as the adjuvant relative to that observed with the r*Pf*MSP1/8 vaccine (Fig. 5D and E). Consistent with the previous *P. yoelii* studies, the data indicate that immunization with the r*Pf*MSP1/8 chimeric vaccine promotes superior antibody responses to *Pf*MSP1$_{19}$ epitopes relative to immunization with a combined formulation of r*Pf*MSP1$_{42}$ and r*Pf*MSP8 (ΔAsn/Asp).

Immunization with r*Pf*MSP1/8 elicits potent, growth inhibitory antibodies against homologous and heterologous strains of *P. falciparum*. High-titer polyclonal rabbit sera were raised against r*Pf*MSP1/8 and r*Pf*MSP1$_{42}$. As with inbred and outbred strains of mice, immunization of rabbits with r*Pf*MSP1/8 elicited high titers of IgG specific for both *Pf*MSP8 and *Pf*MSP1$_{19}$ epitopes (Table 1). Elicited antibodies were highly cross-reactive between the two major alleles (FVO and 3D7) of *Pf*MSP1$_{19}$. Consistent with previous reports (22, 31, 32), immunization with r*Pf*MSP1$_{42}$ also elicited high titers of antibodies to *Pf*MSP1$_{19}$ epitopes, and these were comparable in magnitude to those elicited by r*Pf*MSP1/8 immunization. The functionality of these antibodies was measured by the ability to inhibit the *in vitro* growth of *P. falciparum* blood-stage parasites. As shown in Table 2, r*Pf*MSP1/8-specific antibodies inhibited the growth of homologous (FVO) and heterologous (3D7) strains of *P. falciparum* by >90 and >70%, respectively, when tested at a final concentration of 2.5 mg/ml of IgG. While rabbit sera elicited by immunization with r*Pf*MSP1$_{42}$ contained equally high titers of r*Pf*MSP1$_{19}$-specific antibodies, their ability to inhibit the *in vitro* growth of *P. falciparum* blood-stage parasites was somewhat less effective and variable, with growth inhibition ranging from 30 to 80% when tested at a final concentration of 2.5 mg/ml IgG (Table 2). Testing of higher concentrations of rabbit anti-r*Pf*MSP1/8 and anti-r*Pf*MSP1$_{42}$ IgG (5 mg/ml) resulted in modest increases in growth inhibitory activity (Table 2). Background inhibition with prebleed pools and adjuvant control IgG were generally below 10%, with the exception of one sample. Previous studies indicated that r*Pf*MSP8 (ΔAsn/Asp) antibodies do not inhibit the *in vitro* growth of *P. falciparum* blood-stage parasites. This was confirmed. As shown in Fig. 6, the growth inhibitory activity of r*Pf*MSP1/8-specific IgG could be reversed by preincubation of r*Pf*MSP1/8-specific IgG with either r*Pf*MSP1/8 or with GST-*Pf*MSP1$_{19}$ (FVO) but not with r*Pf*MSP8 (ΔAsn/Asp).

DISCUSSION

The ability of antibodies to conformational epitopes of MSP1$_{19}$ to suppress the growth of blood-stage parasites *in vitro* and *in vivo* is well established (24, 33–40). This has laid the foundation for the effort to include MSP1$_{19}$ as a key component of a blood-stage malaria vaccine. The leading MSP1-based vaccine candidate that has progressed to testing in clinical trials is *Pf*MSP1$_{42}$. This is comprised of the C-terminal *Pf*MSP1$_{33}$ and *Pf*MSP1$_{19}$ domains of the

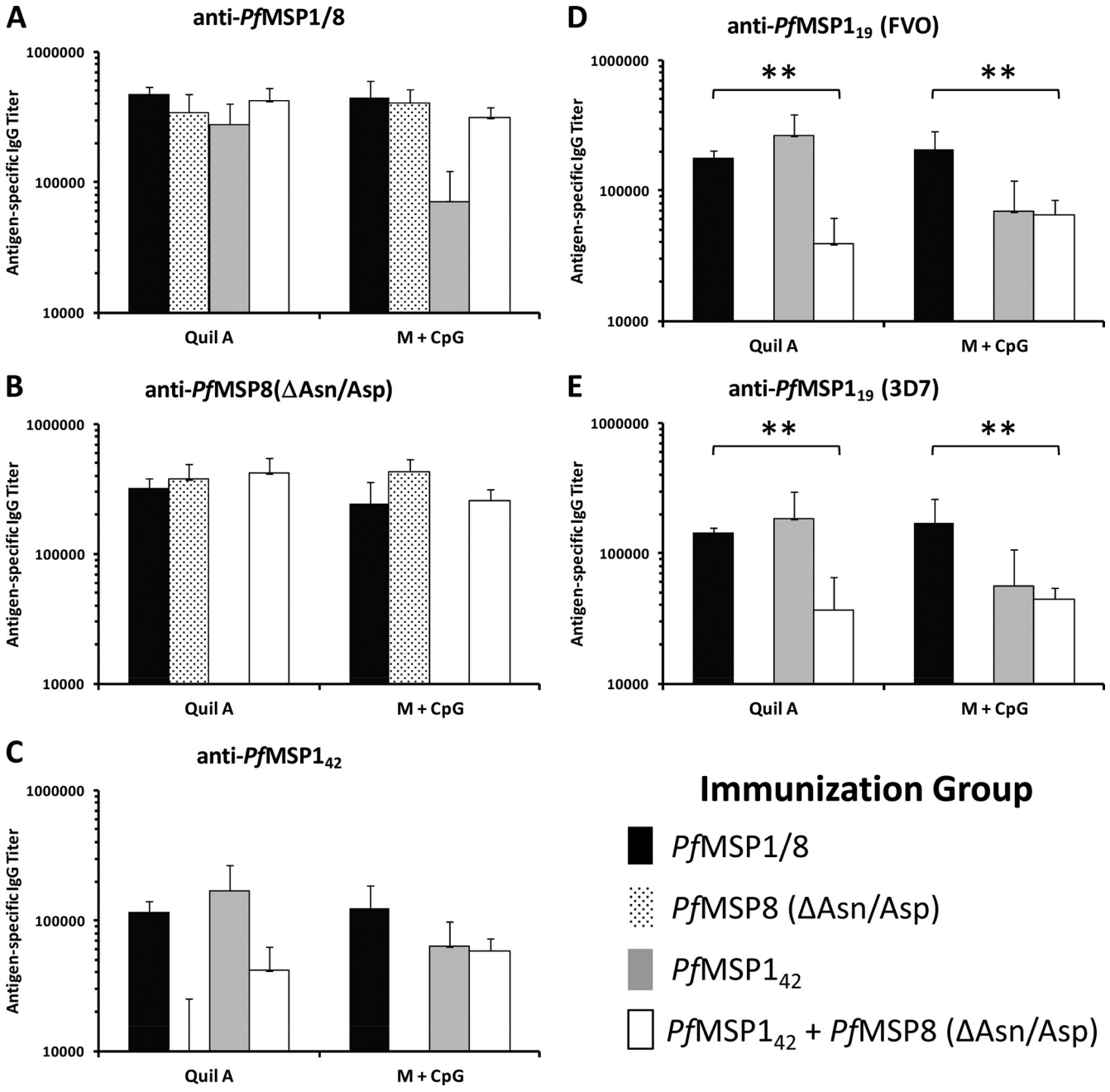

FIG 5 Enhanced immunogenicity of the chimeric r*Pf*MSP1/8 vaccine relative to r*Pf*MSP1$_{42}$ vaccine formulations. CB6F1/J mice (5 mice per group) were immunized with r*Pf*MSP1/8, *Pf*MSP8 (ΔAsn/Asp), r*Pf*MSP1$_{42}$, or a mixture of *Pf*MSP8 (ΔAsn/Asp) and r*Pf*MSP1$_{42}$, formulated with either Quil A or M plus CpG as the adjuvant. Antigen-specific IgG titers (means ± standard deviations) in sera collected following the third immunization were determined by ELISA on plates coated with either r*Pf*MSP1/8, r*Pf*MSP8 (ΔAsn/Asp), rGST-*Pf*MSP1$_{19}$ (FVO), or rGST-*Pf*MSP1$_{19}$ (3D7) as indicated. For each dilution, the mean absorbance values at A_{405} of the pooled sera from adjuvant control mice ($n = 5$) was subtracted as the background. **, $P < 0.01$.

larger 200-kDa merozoite protein. Two challenges facing the development of this subunit vaccine remain to be addressed. The first relates to the immunogenicity of *Pf*MSP1$_{42}$ and the induction of growth inhibitory antibodies which have been suboptimal in phase 1 and 2 trials that utilized either AS01, AS02A, or Alhydrogel (with or without CpG ODN) as the adjuvant (41–46). The second relates to the polymorphism of *Pf*MSP1$_{42}$ (47, 48). In one phase 2 trial completed in Kenyan children, the overall efficacy of an r*Pf*MSP1$_{42}$ (3D7) vaccine was limited (10). In this trial, the possibility that some level of allele-specific protection was elicited is being considered. One approach being considered to address this issue of polymorphism involves immunization with a combined formulation containing multiple alleles of *Pf*MSP1$_{42}$ (42, 49).

Many successful *P. yoelii* MSP-1 efficacy studies utilized *Py*MSP-1$_{19}$ fused to the glutathione *S*-transferase of *Schistosoma japonicum* formulated with a variety of adjuvants (24, 50, 51). Use

TABLE 1 Rabbit MSP-specific antibody response induced by immunization with *Pf*MSP1/8 and *Pf*MSP1$_{42}$ vaccines

Rabbit antiserum (animal no.)	Antigen-specific IgG titer ($\times 10^6$) induced by vaccine:				
	r*Pf*MSP1/8	r*Pf*MSP8	r*Pf*MSP1$_{42}$	GST-*Pf*MSP1$_{19}$ (FVO)	GST-*Pf*MSP1$_{19}$ (3D7)
*Pf*MSP1/8 (1)	3.19	1.37	1.39	1.95	1.30
*Pf*MSP1/8 (2)	10.72	4.64	4.85	6.46	5.94
*Pf*MSP1/8 (3)	6.50	2.87	2.57	3.96	2.32
*Pf*MSP1$_{42}$ (1)	3.28	0.01	3.83	3.60	3.22
*Pf*MSP1$_{42}$ (2)	1.66	0.01	1.88	1.71	1.52
*Pf*MSP1$_{42}$ (3)	3.79	0.01	4.46	4.11	2.95

of GST as a heterologous carrier could be eliminated by inclusion of other heterologous T cell epitopes (52, 53) or by 4 to 5 immunizations with nonfused *Py*MSP-1$_{19}$ emulsified in potent adjuvants, including CFA/IFA (37, 54) or M plus CpG ODN (55–57). We are unaware of any studies where immunization with nonfused, recombinant *Py*MSP-1$_{42}$ demonstrated comparable protective efficacy. Our efficacy studies with r*Py*MSP1$_{42}$ formulated with Quil A as the adjuvant revealed only partial protection against *P. yoelii* 17XL malaria (14). Working with the *P. yoelii* model, however, we produced and tested a chimeric MSP-based vaccine containing *Py*MSP1$_{19}$ fused to the N terminus of full-length *Py*MSP8 (14, 15). Fusion of *Py*MSP1$_{19}$ to *Py*MSP8 significantly increased the immunogenicity of *Py*MSP1$_{19}$ over that observed with *Py*MSP1$_{42}$, with r*Py*MSP1/8 immunization resulting in the production of high and sustained levels of *Py*MSP1$_{19}$-specific antibodies. Most importantly, immunization with the chimeric r*Py*MSP1/8 vaccine formulated with Quil A as the adjuvant elicited nearly complete protection against lethal *P. yoelii* 17XL. r*Py*MSP1/8-induced protection was markedly improved over that of vaccines based on single or combined antigen formulations of *Py*MSP1$_{42}$ and *Py*MSP8. Enhanced protection was attributed to an improved antibody response to *Py*MSP1$_{19}$ and the concurrent targeting of protective, conformation-dependent epitopes present on both *Py*MSP1$_{19}$ and *Py*MSP8.

TABLE 2 *In vitro* inhibition of *P. falciparum* growth by rabbit MSP-specific antibodies[a]

Assay no. and rabbit antiserum (animal no.)	% growth inhibition of:			
	P. falciparum (FVO)		*P. falciparum* (3D7)	
	5 mg/ml IgG	2.5 mg/ml IgG	5 mg/ml IgG	2.5 mg/ml IgG
1				
*Pf*MSP1/8 (1)	93	91	87	74
*Pf*MSP1/8 (2)	95	94	90	79
*Pf*MSP1/8 (3)	94	92	88	75
Prebleed pool	ND	25	ND	ND
Adjuvant control	ND	9	ND	ND
2				
*Pf*MSP1$_{42}$ (1)	58	33	62	30
*Pf*MSP1$_{42}$ (2)	63	47	59	35
*Pf*MSP1$_{42}$ (3)	91	75	92	82
Prebleed pool	2	ND	-4	ND

[a] *In vitro* growth inhibitory activity of rabbit anti-MSP IgG for *P. falciparum* FVO blood-stage parasites was based on measurement of parasite lactate dehydrogenase levels. Percent growth inhibition in the presence or rabbit IgG relative to controls in the absence of IgG is shown. ND, not determined.

We applied our findings in the *P. yoelii* model to the design and testing of a comparable MSP-based vaccine for *P. falciparum*. We successfully produced a chimeric recombinant vaccine by fusing r*Pf*MSP1$_{19}$ (FVO) to the N terminus of r*Pf*MSP8 (ΔAsn/Asp). Specific selection of r*Pf*MSP8 (ΔAsn/Asp) as the fusion partner was based on comparative T and B cell immunogenicity studies of full-length r*Pf*MSP8 and of r*Pf*MSP8 (ΔAsn/Asp) (18). We chose two very distinct adjuvants for comparative immunogenicity studies to reduce the possibility that any enhancement in the quantity and/or quality of *Pf*MSP1/8 vaccine-induced responses was unique to a specific formulation. Quil A is a nontoxic, aqueous adjuvant, and its derivative, QS21, has been tested in vaccine trials in human subjects (3, 6). Montanide is a very different oil-based adjuvant combined with a CpG DNA-based adjuvant designed to promote a Th1-biased response. Our immunogenicity studies with the chimeric r*Pf*MSP1/8 showed remarkable concordance with the earlier data on the *P. yoelii* MSP1/8 vaccine. Immunization with r*Pf*MSP1/8 induced high titers of antibodies to both *Pf*MSP1 and *Pf*MSP8 epitopes. As anticipated, the T cell response was restricted to epitopes within the *Pf*MSP8 domain but clearly provided adequate help for the activation and differentiation of both MSP1- and MSP8-specific B cells. Immunization with r*Pf*MSP1/8 was superior to immunization with a mixture of r*Pf*MSP1$_{42}$ and r*Pf*MSP8 (ΔAsn/Asp) with respect to the production of high titers of *Pf*MSP1$_{19}$-specific antibodies. As a measure of functionality, the r*Pf*MSP1/8-elicited antibodies showed a high level of *in vitro* growth inhibitory activity against both homologous (FVO) and heterologous (3D7) strains of *P. falciparum* blood-stage parasites.

Both *P. yoelii* and *P. falciparum* MSP8 sequences are highly immunogenic. Our studies show that this is true in both inbred and outbred strains of mice and is not adjuvant dependent. This makes the use of r*Pf*MSP8 (ΔAsn/Asp) as a carrier protein for MSP1$_{19}$ very attractive. With the r*Pf*MSP1/8 vaccine, the *Pf*MSP1-specific antibody response is directly focused on the most protective epitopes of the *Pf*MSP1$_{19}$ domain. In contrast, immunization with *Pf*MSP1$_{42}$ elicits competing antibody responses directed against *Pf*MSP1$_{33}$-associated epitopes which are not clearly linked with protection (54, 58). T cell responses to epitopes within the *Pf*MSP1$_{33}$ domain can provide help for the production of *Pf*MSP1$_{19}$-specific antibodies (29, 59, 60), but this region of MSP1 is only semiconserved among *P. falciparum* isolates (47) and can lead to allele-specific responses. Furthermore, there is some evidence that the presence of certain T cell epitopes within *Pf*MSP1$_{33}$ impedes the development of memory T cells (61) and/or protective antibody responses to *Pf*MSP1$_{19}$ (62). In the *P. yoelii* model, improved vaccine efficacy has been demonstrated by replacement of *Py*MSP1$_{33}$ with promiscuous CD4$^+$ T cell epitopes from the

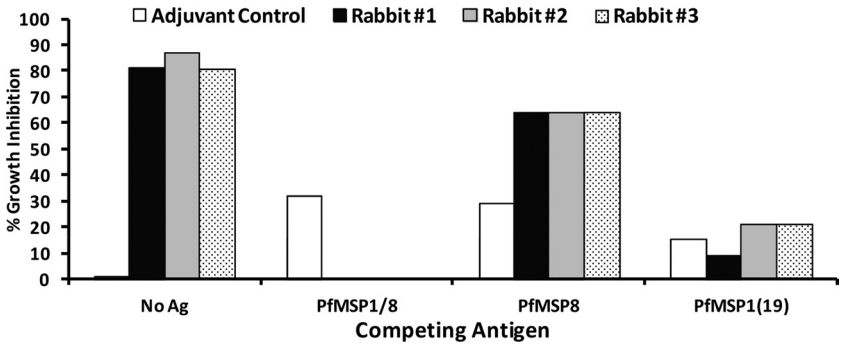

FIG 6 Rabbit anti-*Pf*MSP1/8-dependent inhibition of *P. falciparum* growth is reversed in the presence of rGST-*Pf*MSP1$_{19}$ (FVO) but not r*Pf*MSP8 (ΔAsn/Asp). Inhibition of the *in vitro* growth of *P. falciparum* FVO blood-stage parasites was assessed by measuring parasite lactate dehydrogenase levels in the presence of rabbit anti-r*Pf*MSP1/8 IgG (rabbits 1 to 3) or adjuvant control IgG. All purified rabbit IgG samples were tested at a constant concentration of 1.25 mg/ml. To test the specificity of growth inhibitory antibodies, anti-r*Pf*MSP1/8 IgG was preincubated with r*Pf*MSP1/8, r*Pf*MSP8 (ΔAsn/Asp), or rGST-*Pf*MSP1$_{19}$ (FVO) at an antigen concentration of 100 μg/ml. Residual growth inhibitory activity was then measured. No Ag, no antigen.

N-terminal regions of *Py*MSP1 (63). In addition to its high immunogenicity for T cells, the sequence of r*Pf*MSP8 (ΔAsn/Asp) is highly conserved among *P. falciparum* isolates, effectively eliminating the problem associated with the strain specificity of T cell responses. While our efforts have focused on improving immunogenicity through vaccine design, other efforts in the field have enjoyed recent success in developing and testing new, potent adjuvants for use in human subjects (64, 65). We are excited about the possibility of further enhancing the immunogenicity of r*Pf*MSP1/8 with the use of adjuvants other than Quil A.

Protective antibodies to MSP1 are believed to function by several mechanisms, including blocking merozoite invasion, inhibiting the processing of MSP1$_{42}$, and impairing the intracellular growth of parasites that were opsonized prior to erythrocyte invasion (26, 66–70). *In vivo*, contributing effector mechanisms may also involve other cells and soluble mediators. *In vitro*, antibodies elicited by immunization with r*Pf*MSP1/8 are highly neutralizing, as measured by GIA against *P. falciparum*. The r*Pf*MSP1/8 vaccine-elicited antibodies that are functional in the inhibition of *P. falciparum* growth appear to be specific for *Pf*MSP1$_{19}$ exclusively. High titers of *Pf*MSP1$_{19}$-specific antibodies can also be elicited in rabbits by immunization with *Pf*MSP1$_{42}$. However, *Pf*MSP1$_{42}$-elicited antibodies are less effective in inhibiting *P. falciparum* growth than those elicited by immunization with r*Pf*MSP1/8 when tested at comparable concentrations. This suggests that the fine specificity of *Pf*MSP1$_{19}$-specific antibodies is important for parasite neutralization. Alternatively, immunization with the larger *Pf*MSP1$_{42}$ may also elicit the production of blocking antibodies which have been recognized for their ability to hinder the activity of otherwise neutralizing antibodies (66–68). As a first step to test this, it will be informative to determine the 50% growth inhibitory concentration of *Pf*MSP1$_{19}$-specific antibodies affinity purified from sera from *Pf*MSP1$_{42}$- versus *Pf*MSP1/8-immunized animals when tested individually and in combination.

The present study indicates that *Pf*MSP8-specific antibodies are not active in the GIA, and this is consistent with our prior report (18). Previously we showed that antibodies induced by r*Pf*MSP8 (ΔAsn/Asp) immunization predominantly recognized conformation epitopes of the full-length and processed products of native *Pf*MSP8 by immunoblotting (nonreducing conditions) and yielded strong binding in immunofluorescence assays to native *Pf*MSP8 expressed by ring-, trophozoite-, and schizont-stage parasites. However, these *Pf*MSP8-specific antibodies do not inhibit the growth of *P. falciparum* blood-stage parasites *in vitro*. As such, we feel it unlikely that the lack of *Pf*MSP8-dependent GIA activity in the present study is due to altered conformation of *Pf*MSP8 in the chimeric *Pf*MSP1/8 antigen. Importantly, we do not know the *in vivo* mechanism of action of protective MSP8-specific antibodies in the *P. yoelii* model system. As with MSP1, it is possible that *in vivo*, anti-MSP8 antibodies could function in cooperation with other components of the immune system, such as complement or FcR-bearing cells. However, it also appears that *P. falciparum* MSP8 differs to some degree from *P. yoelii* MSP8 with respect to the timing and extent of proteolytic processing and subcellular localization (18, 71, 72). The low and/or transient expression of MSP8 epitopes on the surface of extracellular *P. falciparum* merozoites may limit the ability of *Pf*MSP8-specific antibodies to inhibit the *in vitro* growth of blood-stage parasites.

Supporting data clearly demonstrate the ability of r*Pf*MSP8 (ΔAsn/Asp) to function as an effective carrier for *Pf*MSP1$_{19}$, leading to the production of high titers of antibodies that effectively inhibit the growth of *P. falciparum* blood-stage parasites. Upon immunization of human subjects, we anticipate similarly strong CD4$^+$ T cell responses for B cell help, as r*Pf*MSP8 (ΔAsn/Asp) is predicted to contain epitopes that will bind with high affinity to multiple HLA-DR, HLA-DP, and HLA-DQ alleles (73). From a broader perspective then, it may be worthwhile to consider the use of r*Pf*MSP8 (ΔAsn/Asp) as a carrier for domains of other vaccine candidates that are targets of neutralizing antibodies but are poorly immunogenic. The results presented here also extend our work to specifically develop a chimeric r*Pf*MSP1/8 vaccine. Based on the encouraging data obtained, further testing of this vaccine in immunization and challenge studies in an *Aotus* monkey model of *P. falciparum* malaria is being pursued. From such studies, we will also be able to determine if immunization with r*Pf*MSP8 (ΔAsn/Asp) alone contributes to protection against challenge infection *in vivo*. These nonhuman primate studies will be important for establishing clear correlates of r*Pf*MSP1/8-induced protection and a foundation to guide further testing.

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J.M.B. is an inventor listed on U.S. patent no. 7,931,908, entitled "Chimeric MSP-based malaria vaccine."

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