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Evaluation of the Impact of Codon Optimization and N-Linked Glycosylation on Functional Immunogenicity of Pfs25 DNA Vaccines Delivered by *In Vivo* Electroporation in Preclinical Studies in Mice

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***Plasmodium falciparum* sexual stage surface antigen Pfs25 is a well-established candidate for malaria transmission-blocking vaccine development. Immunization with DNA vaccines encoding Pfs25 has been shown to elicit potent antibody responses in mice and nonhuman primates. Studies aimed at further optimization have revealed improved immunogenicity through the application of *in vivo* electroporation and by using a heterologous prime-boost approach. The goal of the studies reported here was to systematically evaluate the impact of codon optimization, *in vivo* electroporation, and N-linked glycosylation on the immunogenicity of Pfs25 encoded by DNA vaccines. The results from this study demonstrate that while codon optimization and *in vivo* electroporation greatly improved functional immunogenicity of Pfs25 DNA vaccines, the presence or absence of N-linked glycosylation did not significantly impact vaccine efficacy. These findings suggest that N-glycosylation of Pfs25 encoded by DNA vaccines is not detrimental to overall transmission-blocking efficacy.**

Malaria caused by *Plasmodium* species is still endemic in 97 countries, and WHO estimated that 3.3 billion people are at risk of disease, with ~584,000 deaths reported in 2013 (1). *Plasmodium falciparum* is responsible for the most morbidity and mortality and is thus the major focus of current vaccine development efforts (2). The malaria eradication research agenda (malERA) initiative of 2011 underscored the need for a multipronged approach for malaria control and elimination that includes vaccines targeting infection (3) and transmission along with various control measures currently in use such as indoor residual spraying and insecticide-treated mosquito nets (4).

Malaria transmission-blocking vaccines (TBVs) target the sexual life cycle stages of the parasite that develop in the mosquito vector with the goal of interrupting transmission and further spread of the infection (5). The primary mode of action of TBVs is via induction of antibodies that target surface antigens expressed in the sexual stages of the parasite. *P. falciparum* TBV candidates include prefertilization antigens Pfs230 and Pfs48/45 and postfertilization antigens Pfs25 and Pfs28 (6, 7). So far, vaccine approaches based on recombinant protein-adjuvant formulations have met with limited success due to the complex conformational nature of these antigens, often resulting in improperly folded, unstable, and aggregated proteins (6). DNA vaccines encoding specific *P. falciparum* TBV target antigens offer alternatives to traditional platforms as seen in murine (8) and nonhuman primate (9) models. Additional benefits for use of DNA vaccines include ease of design and sequence modification, stability, and transportability (10). Studies in mice with Pfs25 DNA plasmids showed high TBV activity with >95% oocyst reduction in the mosquitoes (8). Similar studies in rhesus macaques, however, revealed only modest immunogenicity even after four immunization doses and required heterologous boosting with recombinant protein for improved immunogenicity (9). In lieu of the low immune responses seen in larger animals, *in vivo* electroporation (EP)-based DNA delivery, which has demonstrated up to a 1,000-fold increase in DNA delivery potential over traditional mechanisms (11), was used as an immunogenicity enhancement tool. EP-based DNA delivery leads to a reversible and short-lived increase in cell membrane permeability and an influx of antigen-presenting cells to the site of vaccine delivery that result in increased uptake of DNA plasmid and efficient processing and presentation of encoded antigen (12). DNA vaccine delivery using EP improved outcomes, and studies in mice revealed that a 2 log lower dose of plasmid was capable of eliciting anti-Pfs25 antibodies comparable to immunization without EP in mice (13). EP combined with a heterologous prime-boost regimen in a nonhuman primate model (olive baboons, *Papio anubis*) also suggested a dose-dependent enhancement in antibody titers and improved functional blocking (14).

Pfs25 and Pfs28 are highly conserved proteins present on the surface of zygotes and ookinetes (15, 16). Pfs25 contains four epidermal growth factor domains, a secretory N-terminal signal sequence, and a C-terminal glycosylphosphatidylinositol (GPI) anchor sequence (17). In addition, the amino acid sequence of Pfs25 contains three putative asparagine N-linked glycosylation sites. Posttranslational modifications such as N-glycosylation have been known to play a crucial role in the folding, stability, and

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functional integrity of proteins (18, 19). Glycosylation of proteins in *Plasmodium* has remained highly controversial (20), and a recent study has suggested formation of severely truncated N-glycan side chains due to the absence of glycosyltransferases required for precursor side chain generation (21). The impact of N-linked glycosylation has been studied with respect to viral virulence and immune evasion (reviewed in reference 22) as well as in limited vaccine studies with DNA plasmids encoding viral antigens (23). In the case of *Plasmodium*, only a few studies have explored the impact of N-glycosylation on immunogenicity. The aim of this study was to investigate whether the protein product of a Pfs25 DNA vaccine is N-glycosylated in mammalian cells, and if so, whether such unnatural glycosylation of Pfs25 has any impact on the stability and functional immunogenicity parameters. Combined with codon optimization and *in vivo* EP, the N-glycosylation status of Pfs25 may suggest ways to further improve the effectiveness of DNA vaccines for further development.

MATERIALS AND METHODS

DNA plasmids. DNA vaccine vector VR1020 (Vical Inc., San Diego, CA) was used to prepare three different plasmid constructs, each encoding Pfs25 lacking N-terminal signal and C-terminal anchor sequences (8). The first contained a wild-type (WT) coding sequence (Pfs25WT), the second contained a Pfs25 codon optimized for optimum expression in mammalian cells (Pfs25SYN), and the third contained codon-optimized Pfs25 wherein all 3 putative N-linked glycosylation sites in Pfs25 were mutated from asparagine to glutamine (Pfs25MUT). Pfs25SYN and Pfs25MUT were produced as synthetic genes by GenScript (Piscataway, NJ). Plasmid DNA (endotoxin, <30 endotoxin units [EU]/mg), purified by Aldevron (Fargo, ND), was used for all immunizations.

Immunization dose and scheme. Five- to seven-week-old female BALB/c mice (NCI, Bethesda, MD), divided into 4 groups per vaccine construct, received 3 intramuscular doses of DNA with or without EP at 4-week intervals. EP was administered using an Ichor pulse generator and TriGrid electrode array (Ichor Medical Systems Inc., CA) (24). DNA was injected using 0.3-ml U-100 insulin syringes (BD Biosciences, NJ) and followed ~10 s later by electrical pulse at an amplitude of 250 V/cm of electrode spacing (2.5-mm spacing used). Mice were anesthetized using isoflurane, USP (Baxter, IL), and DNA doses were administered in 20 μl phosphate-buffered saline (PBS) in the anterior-tibialis muscle. Group 1 received 25 μg DNA/mouse without EP (no-EP). Groups 2, 3, and 4 were immunized with EP at 25 μg, 2.5 μg, and 0.25 μg dose/mouse, respectively. Mice were bled prior to each dose and 1 month after each immunization, and immunization studies were conducted in three replicates.

Mammalian cell transfection studies. DNA plasmids (Pfs25WT, Pfs25SYN, and Pfs25MUT) were transfected into mammalian HEK293T cells using MegaTran 1.0 transfection reagent (OriGene Technologies, MD) at 2 different concentrations (0.5 and 1.0 μg/ml). Culture medium was changed 4 to 5 h posttransfection, and cells were maintained with or without tunicamycin (TN; 5 μg/ml) (Sigma-Aldrich, MO), an N-glycosylation blocking inhibitor, for 48 h. Culture media and cells were tested for protein expression by Western blotting.

Recognition of recombinant Pfs25 protein by Western blotting. Recombinant Pfs25 (rPfs25) (25) was run on a 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% milk-PBST (PBS + 0.1% Tween 20), and individual strips were incubated for 1 h with pooled immune sera at 1:4,000 dilution and processed using enhanced chemiluminescence (ECL) (Amersham Biosciences, NJ) (26).

Antibody titer, isotype, and avidity analysis by ELISA. Immulon-2 plates (Thermo Scientific, MA) coated with 100 μl/well of 1.5 μg/ml rPfs25 in carbonate buffer, pH 9.6, were used, and the assays were performed as described previously (9). To determine avidity of antibodies, plates were incubated for 15 min with NaSCN (0, 1, 2, 4, 8 M) after primary antibody incubation and washed prior to incubation with secondary antibody and remaining enzyme-linked immunosorbent assay (ELISA) steps. Antigen bound antibody after NaSCN treatment was expressed as a percentage of total binding in the absence of NaSCN. For antibody isotype analysis, mouse sera were tested at 1:500 (no-EP group) and 1:4,000 (EP groups) dilutions. The various secondary antibodies used were peroxidase-conjugated goat anti-mouse IgM, IgG1, IgG2a, IgG2b, and IgG3 (Life Technologies, CA).

***In vivo* transmission-blocking analysis.** Groups of BALB/c mice immunized with 25 μg DNA (Pfs25SYN and Pfs25MUT groups, EP and no-EP) after three DNA doses were divided into test and control groups. Test mice were infected with 10^6 transgenic *Plasmodium berghei* parasites expressing Pfs25 (TrPfs25Pb) (27), and control mice received wild-type *P. berghei* parasites. WT *P. berghei* mice do not express Pfs25, and antibodies to Pfs25 will not target transmission of WT *P. berghei* unlike transmission of TrPfs25Pb, which express Pfs25. Five days after infection, mice were used to infect *Anopheles stephensi* mosquitoes (25 to 30 per mouse). Fed mosquitoes were maintained at 19°C and 80% to 90% relative humidity, and midguts were dissected (9 to 10 days after blood feeding) to enumerate oocysts (13).

Total IgG purification, parasite culture, and standard membrane feeding assays. Total IgG was purified from pooled mouse sera using protein A-Sepharose beads (25). Mature, stage V gametocytes of *P. falciparum* (NF54) produced *in vitro* (28) were used in standard membrane feeding assays (SMFAs). Gametocytes were mixed with IgG (50 to 1,000 μg/ml) and human red blood cells (RBCs) to a final 50% hematocrit and 0.3% gametocytemia and fed to female *Anopheles gambiae* (Keele strain) mosquitoes (4 to 5 days old) (14). All SMFAs included negative controls of IgG from unimmunized mice sera and normal human sera. Transmission-blocking activity was determined by calculating reduction in the percentage of infected mosquitoes and also reduction in the number of oocysts per midgut as described previously (13).

Statistical analysis. Antibody endpoint titers were defined as serum dilution with absorbance higher than that of the average absorbance of preimmune sera plus 3 standard deviations (SD). Percent inhibition of oocyst development and mosquito infectivity differences were analyzed as described previously (13). Statistical analysis was performed using the Prism (GraphPad, CA) software package.

RESULTS

Evidence for N-linked glycosylation of Pfs25 in mammalian cells. Mammalian cells (HEK293T) transfected with Pfs25WT, Pfs25SYN, or Pfs25MUT DNA were analyzed by Western blotting to determine protein expression and N-linked glycosylation (Fig. 1). Expressed Pfs25 protein was detected in supernatant and cell lysates, indicating partial secretion of protein out of the cytoplasm. On a per cell basis, Pfs25SYN DNA revealed higher levels of protein expression than Pfs25WT and Pfs25MUT DNA. ImageJ (http://rsbweb.nih.gov/ij/) analysis of band intensity in the supernatants gave a ratio of 1:1.77:1 for Pfs25WT, Pfs25SYN, and Pfs25MUT groups (before TN treatment), respectively. Similar ImageJ analysis of band intensity in the cell lysates revealed a ratio of 1:1.17:0.57 for Pfs25WT, Pfs25SYN, and Pfs25MUT groups before TN treatment, respectively. The molecular mass of Pfs25 expressed using the Pfs25MUT plasmid (19 kDa) was smaller than the 25 kDa from Pfs25WT- and 23 kDa from Pfs25SYN-transfected cells. The size of the expressed protein from both Pfs25WT and Pfs25SYN plasmids after tunicamycin treatment was smaller than that before tunicamycin treatment and comparable to that from the Pfs25MUT plasmid. As expected, tunicamycin treatment had no such effect on proteins expressed from the Pfs25MUT plasmid (Fig. 1).

Antibody analysis. Immune sera were analyzed by ELISA, and

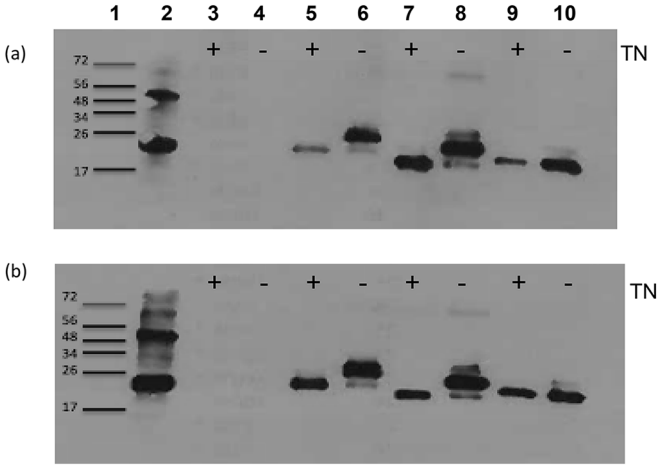

FIG 1 Evidence for N-linked glycosylation of Pfs25 by *in vitro* mammalian cell transfections. HEK293T cells were transfected with DNA plasmids and cultured in the presence or absence of tunicamycin (TN) followed by Western blotting. Cell supernatant (a) and lysate (b) were analyzed by SDS-PAGE and Western blotting using anti-rPfs25 antisera under nonreducing conditions. Lane 1, standard protein markers. Lane 2, rPfs25 protein control. Lanes 3 and 4, no DNA negative control. Lanes 5 through 10, Pfs25WT, Pfs25SYN, or Pfs25MUT DNA-transfected cells cultured in the presence (+) or absence (−) of TN.

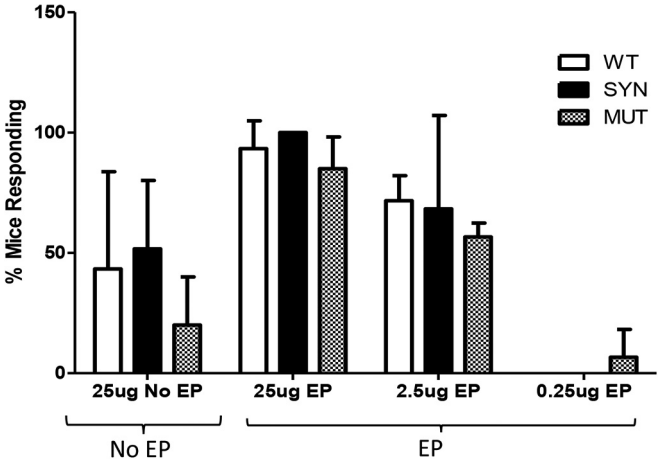

FIG 3 Percent mice responding after primary immunization. Immune sera obtained from all immunization groups after primary immunization were analyzed at 1:100 dilution. Percent responding mice were determined based on positive ELISA absorbance reading over preimmune negative control + 3 SD. Data shown are averages from three experimental replicates ($n = 15$ mice per vaccine group); error bars indicate SD.

the average endpoint titers after 3 DNA doses are shown (Fig. 2). EP groups showed significantly higher titers than no-EP groups. Even at a 10-fold lower immunization dose, Pfs25WT DNA and Pfs25SYN DNA (2.5-μg DNA dose with EP) outperformed no-EP groups. Additionally, we were interested in investigating the impact of N-linked glycosylation on Pfs25-specific antibody responses. Our findings from three independent replicates show a similar trend, and the antibody titers with Pfs25SYN DNA were consistently higher than those with Pfs25MUT DNA. Moreover, the antibody titers with Pfs25SYN DNA were also higher than those of Pfs25WT DNA, suggesting the benefits of codon optimization. Further analysis of sera after the priming DNA dose revealed the value of EP in giving higher percentages of seroconversion. As seen in Fig. 3, the percentage of mice responding after a single immunization with 25 μg DNA EP (Pfs25WT, Pfs25SYN, and Pfs25MUT) ranged between 90% and 100%. Even at a 10-fold lower dose of 2.5 μg DNA, EP groups had 60% to 80% mice responding. In contrast, no-EP groups immunized with 25 μg DNA had only 20% to 50% of the mice responding after the priming dose.

Antibody avidity was compared in sera from mice immunized with a 25-μg DNA dose, without or with EP. NaSCN concentrations resulting in 50% loss of bound antibodies were not significantly different among Pfs25WT, Pfs25SYN, and Pfs25MUT DNA groups and when comparing EP to no-EP groups. Concentrations of NaSCN required for 50% dissociation of bound antibodies ranged from 1 to 1.5 M for no-EP groups and from 2.6 to 3.0 M for EP groups and were not statistically significantly different between the groups (data not shown). In addition, we investigated whether

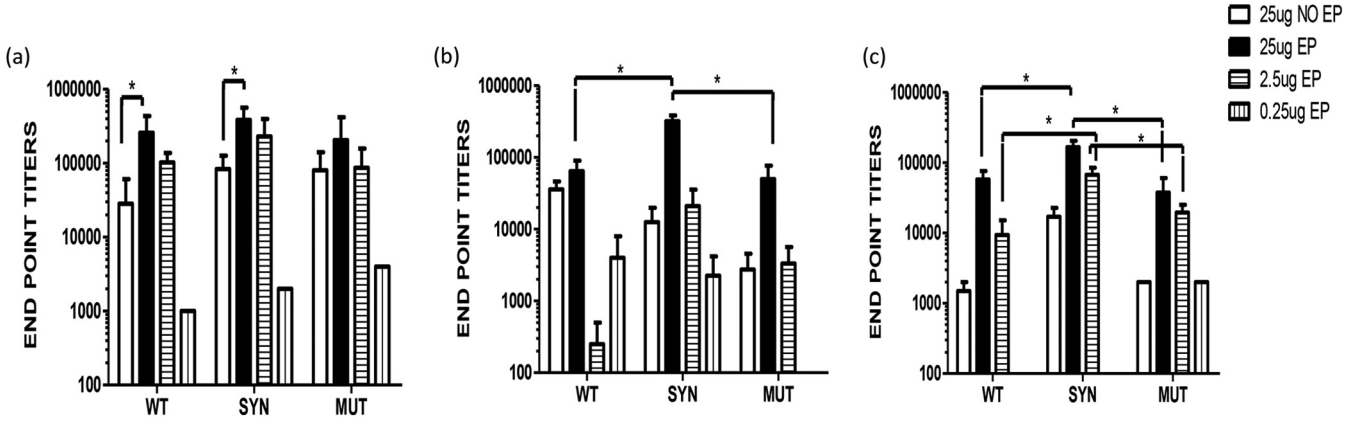

FIG 2 Analysis of final antibody titers after third immunization by ELISA from mice ($n = 5$) immunized with different DNA vaccine constructs. Data shown are from three independent experimental replicates. Sera collected after DNA immunizations (intramuscular) were evaluated to determine average antibody endpoint titers, defined as serum dilutions giving an absorbance (405 nm) higher than that with preimmune sera + 3 SD. The antibody titers after the second DNA immunization are shown in Table S1 in the supplemental material. Statistically significant differences in immune responses between groups were determined by Student *t* tests at a *P* of <0.003 to 0.05, indicated by *. The error bars indicate SD.

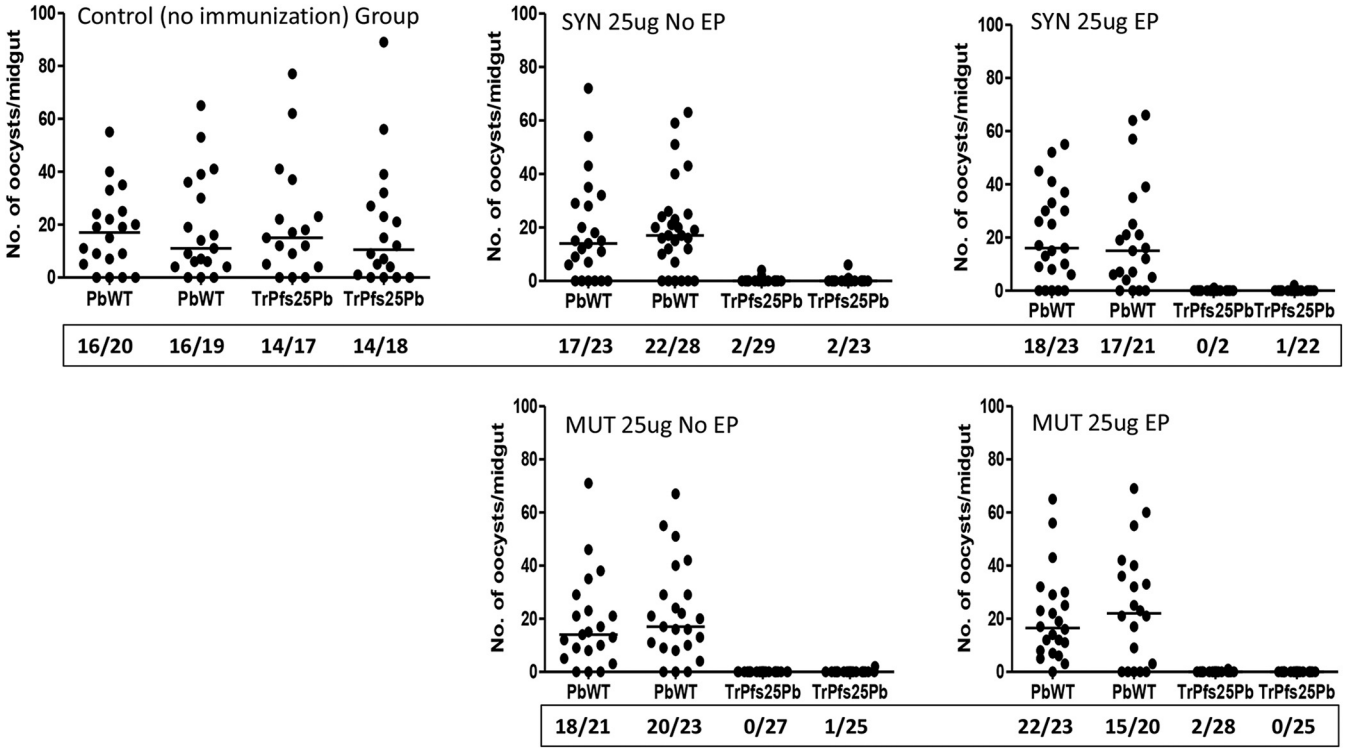

FIG 4 Evaluation of *in vivo* transmission-blocking activity using a TrPfs25Pb-murine model. Pfs25SYN and Pfs25MUT DNA immunized mice (three DNA doses of 25 μg each, no-EP and EP) were divided into control and test animals ($n = 2$ per group). Control mice were infected with *P. berghei* WT (PbWT) parasites, and test mice were infected with TrPfs25Pb parasites (10^6, intraperitoneal route). Five days after infection, starved *A. stephensi* mosquitoes ($n = 25$ to 30) were allowed to feed on control and test mice. Eight to ten days later, mosquito midguts were dissected to assess infectivity and transmission blocking activity. Nonimmunized mice served as controls for infection and transmission with *P. berghei* WT and TrPfs25Pb parasites. Numbers in the boxes indicate total number of infected/total number of mosquitoes dissected for each group.

mutating N-glycosylation sites would skew isotypes of elicited antibody responses. Sera from mice in all groups revealed balanced IgG1 and IgG2a isotypes (see Fig. S1 in the supplemental material). To further confirm antigen specificity of induced antibodies, sera from all groups with EP were evaluated by Western blotting using rPfs25. Sera from mice immunized with Pfs25WT, Pfs25SYN, or Pfs25MUT plasmids recognized the nonreduced and reduced forms of Pfs25 expressed in *Escherichia coli* (nonglycosylated), and the recognition by antibodies generated against the antigen potentially modified *in vivo* by N-linked glycosylation was not impaired (not shown).

***In vivo* transmission-blocking activity of immune sera after DNA immunization.** Mice immunized with Pfs25SYN and Pfs25MUT DNA (25 μg, no-EP and EP) were challenged using transgenic *P. berghei* parasites expressing Pfs25 (TrPfs25Pb) followed by *in vivo* transmission to *A. stephensi* mosquitoes (Fig. 4). The Pfs25SYN and Pfs25MUT test group mosquitoes showed a drastically reduced number of oocysts and percentage of infected mosquitoes with transgenic parasites in the test groups (0% to 8% infectivity) compared with those of the control groups infected with wild-type *P. berghei* parasites (74% to 95% infectivity). Mice immunized with Pfs25SYN and Pfs25MUT DNA with EP or no-EP revealed potent transmission-blocking activity, suggesting that a three-dose immunization is sufficient to elicit potent transmission-blocking antibody responses. As expected, transmission of transgenic and wild-type *P. berghei* parasites was not affected when nonvaccinated mice were challenged likewise.

Standard membrane feeding assays with purified IgGs. In order to evaluate transmission-blocking differences among various DNA groups, SMFAs were conducted using purified IgG from pooled immune sera. In the two experimental replicates, IgG from all EP groups tested at 1,000 and 500 μg/ml showed potent transmission blocking (96% to100%). Even at 250 μg/ml, all the groups revealed significant transmission blocking (80% to 99%). In contrast, IgG from the no-EP groups in one experimental replicate showed significant blocking (88%) only at 1,000 μg/ml and 94% to 98% blocking at 1,000 and 500 μg/ml for Pfs25SYN DNA. These replicate variations are possibly due to differences in the overall endpoint titers. Taken together, the results showed improved blocking for EP groups. Further evidence for antibody dose-dependent transmission-blocking activity is apparent from reduced blocking seen at lower concentrations (100 μg/ml and 50 μg/ml) of IgG used in SMFAs (Table 1).

DISCUSSION

DNA vaccines are still in their nascent stages with respect to research and development, and we still do not entirely understand the mechanisms with which they induce immune responses. Considering the challenges faced by various other subunit vaccine approaches requiring adjuvant formulations and complex production stages, DNA vaccines with relatively simpler design offer a realistic approach (29) and warrant further investigations to understand the mechanisms involved and also to improve the immunogenicity. Previous studies have revealed potent immunoge-

nicity of Pfs25 DNA vaccines in mice, especially after EP-mediated delivery (8, 13). However, similar studies in rhesus without EP (9) and in baboons (14) revealed a need for heterologous protein boost for optimum transmission-blocking effectiveness. In the present study, we undertook a systematic evaluation of the comparative immunogenicity of DNA vaccines based on the native Pfs25 sequence (Pfs25WT), the Pfs25 sequence codon optimized for optimal expression in mammalian cells (Pfs25SYN), and the codon-optimized Pfs25 sequence wherein all three putative N-glycosylation sites were mutated. We wished to determine (i) whether codon optimization for mammalian expression improves immunogenicity of Pfs25 DNA vaccines, (ii) whether Pfs25 undergoes N-linked glycosylation when expressed in mammalian cells, and (iii) whether the presence of N-glycosylation impacts the functional immunogenicity outcome.

In vitro transfection studies demonstrated that Pfs25 encoded by DNA vaccines is posttranslationally modified in mammalian cells, resulting in the addition of N-glycan side chains at putative N-glycosylation sites, which is unlike the native form of the protein in the parasite. Mutating putative N-glycosylation sites blocked the addition of N-glycan side chains to the polypeptide backbone. Treatment of cells transfected with Pfs25WT and Pfs25SYN plasmids with tunicamycin also confirmed that Pfs25 expressed in mammalian cells undergoes N-linked glycosylation in HEK293 cells. It is not clear that mature myofibers share the glycosylation characteristics of HEK293 cells. This is an area of interest for future studies using *in situ* immunostaining of immunized skeletal muscles to investigate *in vivo* glycosylation of expressed proteins. Analysis of protein band intensity demonstrated an increased yield of protein by Pfs25SYN DNA-transfected cells compared with those of Pfs25WT and Pfs25MUT, suggesting that while codon optimization is likely to aid in increased expression and immunogenicity, mutating glycosylation sites does not confer an advantage in the case of Pfs25 DNA immunization.

Furthermore, higher immunogenicity of the codon-optimized Pfs25 plasmid with N-glycosylation sites intact (Pfs25SYN) than that of the native Pfs25 sequence or that of a codon-optimized N-glycosylation site mutant sequence (Pfs25MUT) as seen by ELISA-based antibody analysis also suggests that mutations to block N-glycosylation did not result in benefits for Pfs25-specific antibody responses. We do not know if these *in vivo* differences are due to enhanced expression of Pfs25 from Pfs25SYN DNA or to reduced expression and/or stability or altered conformation of Pfs25 encoded by Pfs25MUT DNA. Regardless, the polyclonal antibodies elicited by various DNA vaccines were capable of recognizing nonglycosylated rPfs25 expressed in *E. coli*. Despite differences in antibody titers between Pfs25SYN- and Pfs25MUT-immunized mice, *in vivo* transmission blocking studies using transgenic *P. berghei* parasites in a mouse model showed similar blocking (~95% to 99%) by antibodies in the two groups. Because of the nature of this *in vivo* assay, which does not allow various antibody concentrations, we further evaluated functional activity using an *ex vivo* SMFA.

Findings from SMFAs using different concentrations of purified IgGs showed no significant differences in the transmission blocking among the various DNA groups, with blocking potential plateauing at approximately 250 μg/ml total serum IgG. A significant difference between groups was seen with enhanced immunogenicity when comparing EP. Comparing ELISA endpoint titers and percent responder mice, our results suggest at least a

TABLE 1 Membrane feeding assays to determine transmission-blocking activity of purified IgG from pooled immune sera of Pfs25WT, Pfs25SYN, and Pfs25MUT DNA

Expt no.	IgG (μg/ml)	NMS[a] control (no. infected/total) median (geometric mean) (range of oocysts)	EP use	% TBA[b] (no. infected/total) median (geometric mean) (range of oocysts) with:		
				Pfs25WT	Pfs25SYN	Pfs25MUT
1	500	(18/21) 6.5 (4.9) (0–25)	No-EP	97.2 (2/21) 0 (0.14) (0–9)*[c]	97.6 (2/20) 0 (0.12) (0–3)*	99.43 (1/19) 0 (0.04) (0–1)*
			EP	98.6 (2/19) 0 (0.07) (0–1)*	99 (1/21) 0 (0.05) (0–2)*	99.4 (1/22) 0 (0.03) (0–1)*
	250	(14/17) 9 (5.8) (0–21)	No-EP	99.15 (1/18) 0 (0.05) (0–2)*	98.75 (3/25) 0 (0.08) (0–1)*	99.57 (1/23) 0 (0.05) (0–1)*
			EP	100 (0/22) 0 (0.01) (0–0)*	100 (0/20) 0 (0.01) (0–0)*	99.15 (1/20) 0 (0.03) (0–2)*
2	1,000	(16/19) 16 (11.4) (0–120)	No-EP	59.7 (11/19) 5.5 (4.6) (0–79)	89.1 (12/24) 0 (1.25) (0–12)*	36.9 (18/21) 7 (7.2) (0–61)
			EP	96.7 (5/21) 0 (0.3) (0–4)*	99.5 (2/22) 0 (0.06) (0–1)*	96.9 (9/24) 0 (0.3) (0–2)*
	500	(18/20) 111 (70.8) (0–235)	No-EP	NB[d] (20/20) 113 (114.7) (61–251)	NB (17/18) 96 (89.5) (0–220)	NB (19/20) 105 (80.02) (0–243)
			EP	97.6 (10/19) 2 (1.76) (0–19)*	98.2 (11/21) 2 (1.2) (0–7)*	99.5 (7/20) 0 (0.4) (0–3)*
	250	(19/21) 82.5 (60.8) (0–234)	No-EP	NB (19/19) 85 (82.8) (29–192)	NB (18/18) 89 (88.1) (31–208)	NB (19/20) 88 (68.5) (0–171)
			EP	93 (14/21) 8.5 (4.2) (0–31)*	96.3 (10/23) 8 (2.28) (0–25)*	97.7 (10/21) 2 (1.35) (0–11)*
	100	(17/20) 28 (18.5) (0–69)	EP	NB (17/19) 24 (20.1) (0–80)	33 (16/20) 22.5 (12.4) (0–35)	54.1 (16/22) 18 (8.5) (0–36)*
	50	(21/24) 24.84 (12) (0–103)	EP	NB (14/18) 28 (14.4) (0–63)	NB (16/20) 24.5 (15) (0–69)	NB (16/19) 23 (15.6) (0–65)

[a] NMS, normal mouse sera.
[b] % TBA = 100 − (geometric mean of oocysts in test groups/geometric mean of oocysts in normal mouse IgG) × 100.
[c] *, Significant blocking against NMS control (Mann-Whitney Test, $P < 0.0001$ to 0.015).
[d] NB, no blocking (infectivity higher than NMS control).

10-fold vaccine dose-sparing effect by EP compared with that of the no-EP approach. Furthermore, the quality of antigen-antibody binding as determined by avidity assays, Western blotting, and IgG isotypes did not reveal significant differences among various immunization groups.

Collectively, these findings indicate that Pfs25 produced in mammalian cells is N-glycosylated; however, the modifications are not detrimental to the immunogenicity of Pfs25, and immune sera are able to recognize glycosylated and unglycosylated forms of the antigen by ELISA and Western blotting. Furthermore, the functional transmission blocking potential of Pfs25-specific immune sera induced by the N-glycosylated form of the protein was not compromised. Our N-glycosylation mutational studies were not extended to determine which of the 3 putative sites was glycosylated or whether introducing different mutation combinations would result in different outcomes. Our studies, however, do suggest that leaving glycosylation sites intact does not compromise immunogenicity, and additional N-glycosylation mutation studies are unlikely to inform strategies for improving the immunogenicity of Pfs25 DNA vaccines. A few studies have examined the role of N-glycosylation for immunogenicity of *Plasmodium* antigens. An unglycosylated merozoite surface protein 1 (MSP-1) proved more efficient in monkey experiments when challenged with a lethal dose of *P. falciparum* (30). Our findings with Pfs25 are in contrast to those of the Pfs48/45 studies in *Nicotiana benthamiana* that suggest that aberrant N-linked glycosylation of *Plasmodium* proteins by mammalian posttranslational modification machinery is likely to be detrimental to immunological outcomes of the antigen (31). On the other hand, our findings align well with posttranslational modification studies with *Plasmodium falciparum* apical membrane antigen 1 (PfAMA-1) produced in *Pichia pastoris*, which showed that immunogenicity and functional responses to PfAMA-1 were not altered by posttranslational modifications (32). However, unlike the results of another PfAMA-1 study in *N. benthamiana* (33), we did not see any significant enhancement in immunogenicity resulting from N-glycosylation.

Our findings, reviewed in light of previous studies with varied outcomes with different viral and *Plasmodium* antigens, highlight the importance of examining the role of N-glycosylation on the immunogenicity of DNA vaccine candidates on a case-by-case basis. Our studies also highlight the importance of extending these immunogenicity evaluation studies to understand the specific immune correlates of protection underlying individual candidate vaccines so that novel enhancement methods can be utilized for immunogenicity studies. Additionally, our studies lend further support for the EP-based delivery method as an invaluable tool for enhanced immunogenicity. EP allows the possibility of accommodating combinations of multiple plasmid molecules without affecting total DNA dose, a feature that can be exploited for developing a multi-life cycle-stage, multiantigen DNA vaccine (11). The safety of EP technology has also been reproducibly demonstrated in various preclinical studies (13), and EP delivery of DNA vaccines should be investigated further in functional immunogenicity enhancement studies in varied animal models.

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