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

Dibyadyuti Datta, Geetha P. Bansal, Rajesh Kumar, Barry Ellefsen, Drew Hannaman, Nirbhay Kumar

Department of Tropical Medicine, School of Public Health and Tropical Medicine and Vector Borne Infectious Diseases Research Center, Tulane University, New Orleans, Louisiana, USA; Ichor Medical Systems, Inc., San Diego, California, USA

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 (malarIA) 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 approaches 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 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).

DNA vaccines encoding specific Plasmodium falciparum 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
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). Mammalian cell transfection studies.

DNA plasmids (Pfs25WT, Pfs25SYN, and Pfs25MUT) were transfected into mammalian HEK293T cells using enhanced chemiluminescence (ECL) (Amersham Biosciences, NJ) and DNA doses were administered in 20 µl phosphate-buffered saline (PBS) in the anterior-tibialis muscle. Group 1 received 25 µg 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 *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).

**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
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
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 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% to 100%). 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).

**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 (10⁶, 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.
Membrane feeding assays to determine transmission-blocking activity of purified IgG from pooled immune sera of Pfs25WT, Pfs25SYN, and Pfs25MUT DNA vaccines in mice, revealed a need for heterologous protein delivery [8,13]. However, similar issues exist in trials after EP-mediated immunization with DNA vaccines in baboons [14].

Findings from SMFAs using different concentrations of purified IgG from pooled immune sera of Pfs25WT, Pfs25SYN, and Pfs25MUT DNA vaccines in mice, especially after EP-mediated immunization with DNA vaccines in baboons [14]. However, similar issues exist in trials after EP-mediated immunization with DNA vaccines in baboons [14].
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)
PfAMA-1 modifications (32). However, unlike the results of another, which showed that immunogenicity and 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) (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 falci grooming 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.

ACKNOWLEDGMENTS

These studies were supported by NIH grants AI47089 and AI101427.
21. Bushkin GG, Ratner DM, Banerjee S, Duraisingh MT, Jennings CV, Dvorin JD, Gubbels M-J, Robertson SD, Steffen M, O’Keefe BR, Robbins PW, Samuelson J. 2010. Suggestive evidence for Darwinian selection against asparagine-linked glycans of Plasmodium falciparum and Toxoplasma gondii. Eukaryot Cell 9:228–241. http://dx.doi.org/10.1128/EC.00197-09.

22. Vigerust DJ, Shepherd VL. 2007. Virus glycosylation: role in virulence and immune interactions. Trends Microbiol 15:211–218. http://dx.doi.org/10.1016/j.tim.2007.03.003.

23. Zhang Y, Chen P, Cao R, Gu J. 2011. Mutation of putative N-linked glycosylation sites in Japanese encephalitis virus premembrane and envelope proteins enhances humoral immunity in BALB/c mice after DNA vaccination. Virol J 8:138. http://dx.doi.org/10.1186/1743-422X-8-138.

24. Luxembourg A, Hannaman D, Ellefsen B, Nakamura G, Bernard R. 2006. Enhancement of immune responses to an HBV DNA vaccine by electroporation. Vaccine 24:4490–4493. http://dx.doi.org/10.1016/j.vaccine.2005.08.014.

25. Kumar R, Angov E, Kumar N. 2014. Potent malaria transmission-blocking antibody responses elicited by Plasmodium falciparum Pf625 expressed in Escherichia coli after successful protein refolding. Infect Immun 82:1453–1459. http://dx.doi.org/10.1128/IAI.01438-13.

26. Kongkasuriyachai D, Bartels-Andrews L, Stowers A, Collins WE, Sullivan J, Sattabongkot J, Torii M, Tsuboi T, Kumar N. 2004. Potent immunogenicity of DNA vaccines encoding Plasmodium vivax transmission-blocking vaccine candidates Pvs25 and Pvs28 — evaluation of homologous and heterologous antigen–delivery prime-boost strategy. Vaccine 22:3205–3213. http://dx.doi.org/10.1016/j.vaccine.2003.11.060.

27. Mlambo G, Maciel J, Kumar N. 2008. Murine model for assessment of Plasmodium falciparum transmission-blocking vaccine using transgenic Plasmodium berghei parasites expressing the target antigen Pf625. Infect Immun 76:2018–2024. http://dx.doi.org/10.1128/IAI.01409-07.

28. Ponnudurai T, Meuwissen JH, Leeuwenberg AD, Verhave JP, Lenssen AH. 1982. The production of mature gametocytes of Plasmodium falciparum in continuous cultures of different isolates infective to mosquitoes. Trans R Soc Trop Med Hyg 76:242–250. http://dx.doi.org/10.1016/0035-9203(82)90289-9.

29. González-Valdez J, Aguilar-Yáñez JM, Benavides J, Rito-Palomares M. 2013. DNA based vaccines offer improved vaccination supply for the developing world. J Chem Technol Biotechnol 88:979–982. http://dx.doi.org/10.1002/jctb.4046.

30. Stowers AW, Chen LH, Zhang Y, Kennedy MC, Zou L, Lambert L, Rice TJ, Kaslow DC, Saul A, Long CA, Meade H, Miller LH. 2002. A recombinant vaccine expressed in the milk of transgenic mice protects aotus monkeys from a lethal challenge with Plasmodium falciparum. Proc Natl Acad Sci U S A 99:339–344.

31. Mamedov T, Ghosh A, Jones RM, Mett V, Farrance CE, Musiychuk K, Horsey A, Yusibov V. 2012. Production of non-glycosylated recombinant proteins in Nicotiana benthamiana plants by co-expressing bacterial PNGase F. Plant Biotechnol J 10:979–982. http://dx.doi.org/10.1111/j.1467-7652.2012.00694.x.

32. Giersing B, Miura K, Shrimp R, Wang J, Zhou H, Orcutt A, Stowers A, Saul A, Miller LH, Long C, Singh S. 2005. Posttranslational modification of recombinant Plasmodium falciparum apical membrane antigen 1: impact on functional immune responses to a malaria vaccine candidate. Infect Immun 73:3963–3970. http://dx.doi.org/10.1128/IAI.73.7.3963-3970.2005.

33. Boes A, Spiegel H, Edgue G, Kapelski S, Scheuermayer M, Fendel R, Remarque E, Altmann F, Maresch D, Reimann A, Pradel G, Schillberg S, Fischer R. 2014. Detailed functional characterization of glycosylated and nonglycosylated variants of malaria vaccine candidate PfAMA1 produced in Nicotiana benthamiana and analysis of growth inhibitory responses in rabbits. Plant Biotechnol Jul 13:222–234.