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Comparative functional potency of DNA vaccines encoding *Plasmodium falciparum* transmission blocking target antigens Pfs48/45 and Pfs25 administered alone or in combination by *in vivo* electroporation in rhesus macaques

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ABSTRACT

Antibodies recognizing conformational epitopes in Pfs48/45, an antigen expressed on the surface of *Plasmodium falciparum* gametes and zygotes, have firmly established Pfs48/45 as a promising transmission blocking vaccine (TBV) candidate. However, it has been difficult to reproducibly express Pfs48/45 in a variety of recombinant expression systems. The goal of our studies was to evaluate functional immunogenicity of Pfs48/45 using DNA vaccine format in rhesus macaques. An additional goal was to ensure that when used in combination with another malarial antigen, specific immunity to both antigens was not compromised. For testing combination vaccines, we employed Pfs25 DNA plasmids that have previously undergone evaluations in rodents and nonhuman primates. Pfs25 is expressed on the surface of parasites after fertilization and is also a lead TBV candidate. DNA plasmids based on codon-optimized sequences of Pfs48/45 and Pfs25 were administered by *in vivo* electroporation, followed by a final recombinant protein boost. Our studies demonstrate that Pfs48/45 encoded by DNA plasmids is capable of inducing potent transmission blocking antibody responses, and such transmission blocking immune potency of Pfs48/45 was not compromised when tested in combination with Pfs25, These findings provide the evidence in favor of further studies on Pfs48/45 and Pfs25, either alone or in combination with other known malaria vaccine candidates for developing effective vaccines capable of interrupting malaria transmission.

1. Introduction

Vaccines have been crucial in the control and eradication of several infectious diseases and represent one of the most effective public health tools available. Development of vaccines for malaria has focused on antigens expressed during various stages of the parasite, and malaria transmission blocking vaccines (TBVs) target antigens in sexual and mosquito midgut stage parasites. In *Plasmodium falciparum* these TBV target antigens include Pfs230 and Pfs48/45 expressed on circulating intra-erythrocytic male and female gametocytes and gametes, as well as Pfs25 expressed during mosquito midgut stage development (zygote to ookinete) [1]. Pfs25 has undergone extensive pre-clinical evaluation and a few phase I clinical trials as adjuvant formulated recombinant protein with mixed and varying outcomes [2–5]. Advancements with Pfs48/45 and Pfs230 have lagged, largely because of difficulties in reproducibly expressing recombinant forms of these antigens after initial success [6–8]. Moreover, the paucity of adjuvants for adequate vaccine formulations also hampers overall vaccine development efforts [9].

DNA vaccines, however, provide a single step approach for expressing the antigens in the immunized host cells and simultaneously presenting antigens to the immune system [10]. DNA vaccines encoding Pfs25 and Pvs25 (a *P. vivax* ortholog) have revealed

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highly potent immunogenicity, especially when administered using *in vivo* electroporation in mice and nonhuman primates [11–16]. We have recently also reported on induction of transmission-blocking antibodies in mice by DNA vaccine encoding Pfs48/45 [17]. DNA vaccines were first described in the early 1990s and generated much interest due to their simple design, manufacturability, and the ability to induce both cellular and humoral immune responses [18–23]. DNA vaccines also offer a convenient platform wherein either a single plasmid or a mixture of plasmids each encoding different antigens can be combined to develop a combination vaccine to target multiple stages or multiple species of the malaria parasite [24,25]. Despite initial promising results, clinical development of DNA plasmid based vaccine development has been hampered, largely due to the relatively low potency seen in nonhuman primates and a few phase I clinical trials, particularly when the DNA is administered by conventional injection [26]. Exact mechanisms of poor immunogenicity of DNA vaccines in *Homo sapiens* are not known and few studies have systematically evaluated several approaches to enhance immune responses, including *in vivo* electroporation based DNA delivery, the use of genetic adjuvants, and sequence optimization for improved protein expression [25,27–29]. Additional studies evaluating these approaches individually, as well as in various combinations, are warranted, especially in nonhuman primate owing to their phylogenetic closeness to humans [30] and their presumed ability to mimic the outcomes expected in humans [26].

The primary objective of the study reported here was to investigate TBV potential of Pfs48/45 encoded by DNA plasmids in rhesus macaques. Additionally, we were able to conduct comparative immunogenicity outcome studies for two *P. falciparum* TBV antigens (Pfs25 and Pfs48/45, individually and in combination) in nonhuman primates, and assess relative contributions of (i) codon optimization [29], (ii) *in vivo* electroporation [31], (iii) DNA prime – protein boost regimen [32], and (iv) the role of N-linked glycosylation [33]. The underlying goal was to delineate factors that may catalyze further studies to facilitate development of vaccines capable of interrupting malaria transmission.

2. Materials and methods

2.1. DNA plasmids

DNA vector VR1020 (Vical Inc. San Diego, CA) encoding codon-optimized *Pfs48/45* or *Pfs25*, lacking signal and anchor sequences were constructed (SYN *Pfs48/45* or SYN *Pfs25*). Additionally, an N-glycosylation mutant form of the codon-optimized *Pfs48/45* sequence was developed where all seven putative N-glycosylation sites were mutated (MUT *Pfs48/45*). Sequence modifications to block N-linked glycosylation included $N_{50} \rightarrow D$, $N_{131} \rightarrow D$, $T_{192} \rightarrow A$, $N_{204} \rightarrow T$, $N_{254} \rightarrow K$, $S_{301} \rightarrow A$, $N_{303} \rightarrow D$ in all NXS/T sites. Plasmid DNA (<30 EU/mg) was purified by Aldevron (Fargo, ND) and supplied at a 2.5 mg/mL concentration.

2.2. Animals

Twenty adult male and female rhesus macaques (*Macaca mulatta*), aged 4–13 years and weighing 4–10 kg, were utilized in this study. The macaques were housed at the Tulane National Primate Research Center (TNPRC; Covington, LA), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC000594), and were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee (OLAW Assurance A4499-01). All procedures complied with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and TNPRC standards for minimizing animal distress.

2.3. Immunizations

Animals were assigned randomly to one of five immunization groups (4 per group) (Table 1). Groups 1 and 2 were immunized with SYN *Pfs25* without or with electroporation (EP), respectively. Animals in groups 3 and 4 were immunized with SYN *Pfs48/45* and MUT *Pfs48/45*, respectively, both with EP. Group 5 animals were immunized with a combination of SYN *Pfs48/45* and SYN *Pfs25* with EP. Plasmids (2.5 mg of each plasmid) were administered in 1.0 mL of PBS divided in equal volumes in quadriceps muscle of both legs for groups 1 to 4. Group 5 animals received a mixture of 2.5 mg of each SYN *Pfs25* and SYN *Pfs48/45* plasmid in 2.0 mL total volume equally divided between both legs. The animals received three repeat immunizations of respective DNA vaccines followed by a final protein boost with recombinant Pfs25 [5] (groups 1, 2), Pfs48/45 [8] (groups 3, 4), and a mixture of both rPfs25 and Pfs48/45 (group 5). Proteins were adsorbed with Alhydrogel (Brenntag Biosector) at a ratio of 1:3.2 (w/w) in PBS, pH 7.4, and each animal received 50 μg protein in a total volume of 1.0 mL. Animals were bled prior to immunization and at one month after each dose of vaccine.

2.4. Antibody analysis

Sera were analyzed for antibody titers by ELISA using 96-well Immulon 4HBX plates coated with 1.5 μg/mL rPfs25 [5] or 1.5 μg/mL rPfs48/45 proteins [13]. HRP-conjugated goat anti-monkey IgG (KPL, Inc., MD) was used at 1:10,000 dilution and the plates were developed using ABTS substrate (KPL, Inc., MD) and absorbance was read at 405 nm using an ELISA reader (VersaMax, Molecular Devices, CA).

Antibody avidity was determined using varying concentrations (0, 1, 2, 4, 8 M) of sodium thiocyanate (NaSCN) during an incubation (15 minutes) included in the standard ELISA protocol after primary antibody incubation, followed by the remaining ELISA steps [15]. Binding of antibody to antigen at 0 M NaSCN was considered as 100% (total) binding and avidity of antibody was expressed as NaSCN concentration giving 50% dissociation of bound antibody.

2.5. Standard membrane feeding assay (SMFA)

Mature gametocytes of *P. falciparum* NF54 strain were produced *in vitro* as described earlier [34]. Whole IgG was purified using Protein A - Sepharose (Sigma-Aldrich, MO) beads from sera of individual animals in each group. SMFAs were conducted by mixing different concentrations of purified IgG with mature *P. falciparum* gametocytes (0.3% gametocytemia) and human erythrocytes (50% hematocrit), and feeding the final mix to 4–5 day old *Anopheles gambiae* (Keele strain) mosquitoes that were starved for 4–6 hours. Blood fed mosquitoes were then maintained for 8–10 days at 26 °C and 80–90% relative humidity. Mosquitoes were dissected and midguts were stained with 0.1% mercurochrome for oocyst enumeration by microscopy. Percent transmission reduction activity (TRA) is defined as percent reduction in the number of oocysts and was calculated using the following formula: percentage oocyst reduction = 100 − [(geometric mean number of oocysts with test sera/geometric mean number of oocysts with NMS) × 100].

2.6. Statistical analysis

All statistical tests were conducted using GraphPad Prism software (GraphPad Software Inc., CA). Antibody end point titers were defined as serum dilutions giving an absorbance higher than the