2013

Transgenic Parasites Stably Expressing Full-Length *Plasmodium falciparum* Circumsporozoite Protein as a Model for Vaccine Down- Selection in Mice Using Sterile Protection as an Endpoint

Michael D. Porter
Walter Reed Army Institute of Research

Jennifer Nicki
Walter Reed Army Institute of Research

Christopher D. Pool
Walter Reed Army Institute of Research

Margot DeBot
Walter Reed Army Institute of Research

Ratish M. Illam
Walter Reed Army Institute of Research

See next page for additional authors

CVI
Journals.ASM.org

Transgenic Parasites Stably Expressing Full-Length *Plasmodium falciparum* Circumsporozoite Protein as a Model for Vaccine Down-Selection in Mice Using Sterile Protection as an Endpoint

Michael D. Porter,[a] Jennifer Nicki,[a] Christopher D. Pool,[a] Margot DeBot,[a] Ratish M. Illam,[a] Clara Brando,[b] Brooke Bozick,[b] Patricia De La Vega,[a] Divya Angra,[a] Roberta Spaccapelo,[c] Andrea Crisanti,[d] Jittawadee R. Murphy,[b] Jason W. Bennett,[a] Robert J. Schwenk,[a] Christian F. Ockenhouse,[a] Sheetij Dutta[a]

Malaria Vaccine Development Branch[a] and Entomology Branch,[b] Walter Reed Army Institute of Research, Silver Spring, Maryland, USA; Università degli Studi di Perugia, Perugia, Italy[c]; Imperial College London, London, United Kingdom[d]

Circumsporozoite protein (CSP) of *Plasmodium falciparum* is a protective human malaria vaccine candidate. There is an urgent need for models that can rapidly down-select novel CSP-based vaccine candidates. In the present study, the mouse-mosquito transmission cycle of a transgenic *Plasmodium berghei* malaria parasite stably expressing a functional full-length *P. falciparum* CSP was optimized to consistently produce infective sporozoites for protection studies. A minimal sporozoite challenge dose was established, and protection was defined as the absence of blood-stage parasites 14 days after intravenous challenge. The specificity of protection was confirmed by vaccinating mice with multiple CSP constructs of differing lengths and compositions. Constructs that induced high NANP repeat-specific antibody titers in enzyme-linked immunosorbent assays were protective, and the degree of protection was dependent on the antigen dose. There was a positive correlation between antibody avidity and protection. The antibodies in the protected mice recognized the native CSP on the parasites and showed sporozoite invasion inhibitory activity. Passive transfer of anti-CSP antibodies into naive mice also induced protection. Thus, we have demonstrated the utility of a mouse efficacy model to down-select human CSP-based vaccine formulations.

A malaria parasite-infected mosquito injects approximately 10 to 200 sporozoites into a vertebrate host's skin during a blood meal (1). These sporozoites travel to the liver, where each successful invasion of a liver hepatocyte yields approximately 30,000 blood-stage merozoites (2). Hence, immune interventions that block sporozoite invasion are thought currently to be the most effective way to protect against malaria. The most abundant *Plasmodium falciparum* sporozoite surface protein is the 397-amino acid circumsporozoite protein (CSP). Genetic analysis of CSP from multiple *Plasmodium* species reveals a highly conserved structure (3). The central region of CSP is composed of species-specific repeats that are flanked by an amino-terminal region, region I, containing a conserved five-amino-acid sequence and a carboxyl-terminal region, region II, containing a conserved cell adhesion motif similar to that observed in the mammalian thrombospondin protein (4).

Malaria still causes extensive morbidity and mortality, and the development of a vaccine against this parasite is an urgent research priority. Because of its abundance and exposed location on the sporozoite surface, CSP has been widely investigated as a candidate malaria vaccine antigen. RTS,S, the most advanced human malaria vaccine candidate to date, contains the central repeats and the cysteine-rich C-terminal region of *Plasmodium falciparum* CSP (*Pf*CSP). Vaccination with RTS,S induces sterile protection against experimental sporozoite challenge in about 50% of vaccinees (5); this vaccine is now undergoing advanced phase III trials at multiple centers in Africa. Although RTS,S marked a critical point as a proof-of-concept recombinant protein vaccine against malaria, the efficacy and duration of RTS,S-based protection are not sufficient to eradicate the disease. It is hypothesized that a full-length CSP-based vaccine might confer improved protection by eliciting immune responses to the N-terminal region of CSP, antibodies against which have been associated with protection from disease (6).

Although nonhuman primates are considered the best model to predict human protection against malaria, these models are costly and often require splenectomy of the monkeys. Moreover, the few *P. falciparum* strains that have been shown to infect New World monkeys do not match the commonly used vaccine strain 3D7 (7–9). Although transgenic monkey parasites expressing a full-length *P. falciparum* CSP would be ideal, none is currently available for use. Several reports suggest that transgenic rodent parasites expressing the *P. falciparum* CSP gene are viable and infective in mice. One such parasite, in which the central repeat region of *Plasmodium berghei* CSP was exchanged with that of *P. falciparum*, has been used recently to evaluate the protective efficacy of *P. falciparum* CSP vaccines in mice (10, 11). However, such parasites provide no information on the protective role of the N- and C-terminal epitopes of CSP. Furthermore, the outcomes of these murine challenge studies might be clouded by measurement of the reduction of parasite-specific RNA, rather than direct assessment of protective efficacy. To address these issues, we have optimized a vaccine evaluation model based on a previously described transgenic parasite in which the full-length *P. berghei* CSP gene was replaced with *P. falciparum* CSP (4). This parasite line

Received 5 February 2013 Returned for modification 11 March 2013
Accepted 19 March 2013
Published ahead of print 27 March 2013
Address correspondence to Sheetij Dutta, sheetij.dutta@us.army.mil.

doi:10.1128/CVI.00066-13

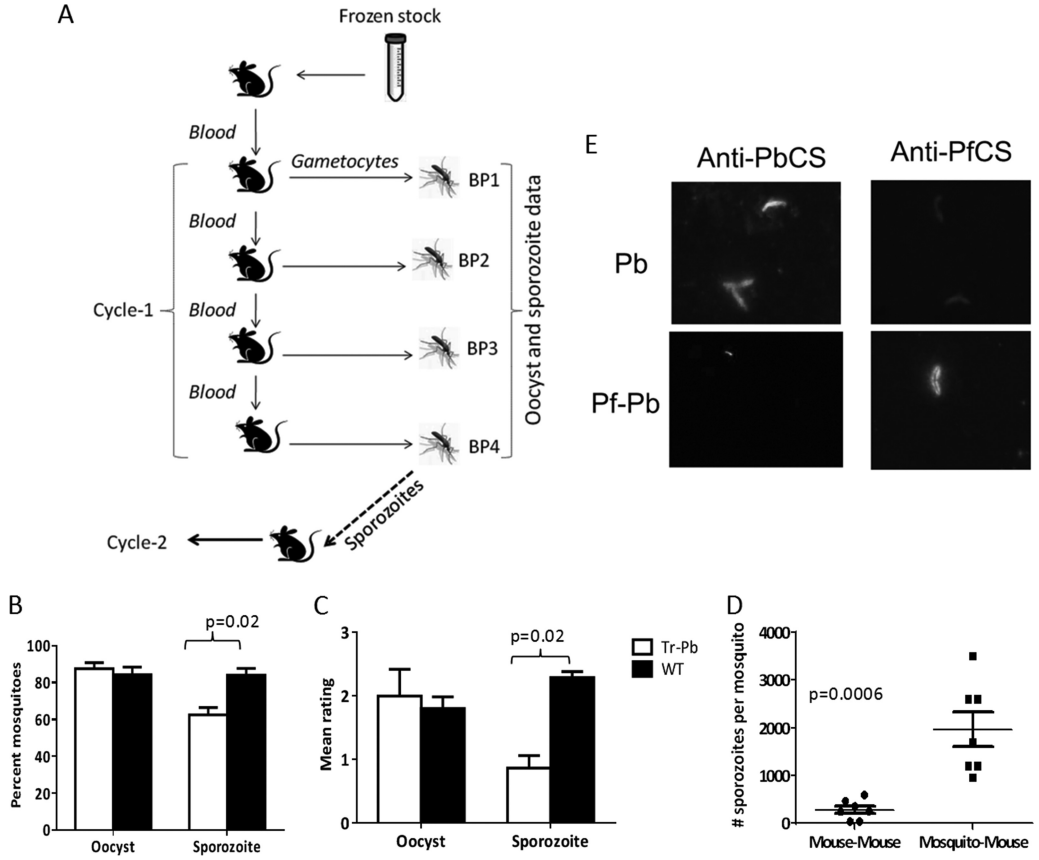

FIG 1 Transgenic *P. berghei* optimization in the mosquito-mouse model. (A) Passage cycles of the transgenic *P. berghei* parasite (Tr-Pb) from mouse to mosquito. Data from 10 mosquito-mouse cycles, each consisting of 4 blood passages, were collected. (B) Percentage of mosquitoes infected with oocysts and sporozoites of transgenic or wild-type *P. berghei* parasites. Means + standard errors of the mean (SEM) and Mann-Whitney *P* values are shown. (C) Oocyst and sporozoite ratings (means + SEM). Oocyst ratings were defined as follows: 0, 0 oocysts; 1, 1 to 25 oocysts per midgut; 2, 26 to 50 oocysts per midgut; 3, 51 to 100 oocysts per midgut; and 4, >100 oocysts per midgut. Sporozoite load ratings were defined as follows: 0, 0 sporozoites; 1, 1 to 100 sporozoites per mosquito; 2, 100 to 1,000 sporozoites per mosquito; 3, 1,000 to 10,000 sporozoites per mosquito; and 4, >10,000 sporozoites per mosquito. (D) Sporozoite yield per mosquito using mouse-mouse blood transmission or mouse-mosquito (vector-borne) transmission. (E) Reactivity of anti-*P. berghei* CSP MAb 4B10 (anti-*Pb*CS) and anti-*P. falciparum* CSP MAb 49-1B2 (anti-*Pf*CS) against Tr *P. berghei* (Pf-Pb) and WT *P. berghei* (Pb) sporozoites.

was originally produced to study the structure-function relationship of CSP during salivary gland and hepatocyte invasion. While the full-length CSP-transgenic sporozoites showed reduced salivary gland invasion, the mouse infectivity of these parasites was similar to that of wild-type *P. berghei* sporozoites (4). Our data indicate that this full-length transgenic parasite is usable for rapid down-selection of recombinant *P. falciparum* CSP-based vaccines, with sterile protection as an endpoint.

MATERIALS AND METHODS

Recombinant CSP vaccination. The genes for the CSP constructs used to immunize mice were codon optimized for high-level expression in *Escherichia coli* using the 3D7 strain CSP sequence (GenBank accession number XM_001351086.1). The histidine-tagged proteins from the soluble fraction were purified to homogeneity using chromatography columns (purification process to be presented elsewhere). The endotoxin content of the vaccine proteins was less than 5 endotoxin units/μg, as measured with the *Limulus* amebocyte lysate endotoxin assay (Associates of Cape Cod, East Falmouth, MA). The antigens were mixed with Montanide ISA720 adjuvant (Seppic Inc., Paris, France) in a 3:7 (vol/vol) antigen/adjuvant ratio, and the formulation was emulsified by vigorous vortex mixing for 10 to 15 min. One hundred microliters of vaccine was administered to C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) intraperitoneally, and three immunizations were given at 2-week intervals. Mice were bled 2 weeks after each vaccination. IgG used in the passive transfer experiment was pooled from three rabbits that had been vaccinated three times with 100 μg of N-$(NANP)_{19}$-C protein emulsified with Montanide ISA720 adjuvant, at 4-week intervals.

Adaptation of transgenic *P. berghei* in the rodent and mosquito hosts. Transgenic (Tr) *P. berghei* parasites, previously described by Tewari et al. (4), were obtained and injected intraperitoneally into three outbred mice. Six days following inoculation, the presence of blood-stage parasitemia was confirmed by Giemsa-stained blood smear examination. Blood from one infected mouse was collected and diluted 1:1 with phosphate-buffered saline (PBS), and 0.2 ml was injected into three naive mice to start blood passage cycle 1 (BP-1) (Fig. 1A). Three days postinfection, 200 to 300 female *Anopheles stephensi* mosquitoes were allowed to feed on two of the three BP-1-infected mice, while the third mouse was used to generate a cohort of BP-2-infected mice via infected blood transfer. This process was repeated until the fourth blood passage, at which point three naive mice were inoculated with infectious sporozoites via mosquito bites, thus generating a new set for which the cycle of four blood passages was repeated.

Oocyst and sporozoite ratings. The oocyst and sporozoite data from 10 cycles, each consisting of four blood passages, were collected and statistically analyzed to determine infectivity differences between the wild-

type and transgenic parasites. At 8 to 11 days after feeding on parasitemic mice, 10 mosquitoes were randomly sampled from each carton, and their midguts were removed and stained with methylene blue. Oocyst ratings were defined as follows: 0, 0 oocysts; 1, 1 to 25 oocysts; 2, 26 to 50 oocysts; 3, 51 to 100 oocysts; 4, >100 oocysts. Eighteen days after the infectious blood meal, salivary glands dissected from 10 mosquitoes were examined for the presence and estimated quantity of sporozoites. Sporozoite ratings were defined as follows: 0, 0 sporozoites; 1, 1 to 100 sporozoites per mosquito; 2, 100 to 1,000 sporozoites per mosquito; 3, 1,000 to 10,000 sporozoites per mosquito; and 4, >10,000 sporozoites per mosquito.

Isolation of sporozoites for challenge and passive transfer. Fresh naive mouse serum was isolated from whole blood using serum separator tubes (Becton, Dickinson and Co., Franklin Lakes, NJ) and was used to supplement RPMI 1640 medium (BioWhittaker, Walkersfield, MD) to a final concentration of 5% (vol/vol) (RPMI/serum). Sporozoites were collected using the method described by Ozaki et al. (12). Briefly, a hole was punctured at the bottom of a 0.5-ml siliconized Eppendorf tube and plugged with a small amount of glass wool, and the tube was placed inside a larger, 1.5-ml, siliconized Eppendorf collection tube. Mosquitoes were killed by immersion in 70% ethanol for approximately 1 min, rinsed with tap water, and bathed in a small volume of RPMI/serum. Dissections were performed in a drop of RPMI/serum under a dissecting microscope. Heads plus salivary glands were gently separated from the thoraxes, and up to 50 heads were added per Ozaki tube. One hundred microliters RPMI/serum was added, followed by centrifugation at 9,000 × *g* for 2 min. The sporozoite pellet was resuspended in the eluate and transferred to another Eppendorf tube. Another 100 μl of RPMI/serum was added to the original tube, which was respun at 9,000 × *g* for 2 min, and the pellet was combined with the initial recovery. The sporozoites were stored on ice after isolation. Sporozoites were counted with a hemocytometer and diluted to 25,000 per ml in RPMI/serum, and 100 μl of this suspension was injected intravenously into the lateral tail vein of each mouse. In the passive transfer experiment, mice were injected intraperitoneally with 1 mg of protein G (GE Healthcare, Pittsburgh, PA)-purified rabbit anti-*Pf*CSP IgG or 1 mg of rabbit anti-*P. falciparum* apical membrane antigen 1 control IgG. A second dose of IgG was given the next morning, and mice were challenged with 2,500 sporozoites 5 h later.

Monitoring of mouse infection. Levels of parasitemia in the mice were monitored daily using thin blood smears, from day 5 to day 14 postchallenge. Blood smears were fixed with methanol and stained with Giemsa stain. Positive infection in mice was defined as the appearance of two parasites in 25 high-power fields (100× magnification). Mice found to be infected with blood stages of the parasite (not protected) were sacrificed, and mice that did not develop blood-stage parasitemia by day 14 were reported as being protected.

ELISA. Immulon 2HB plates (Thermo Scientific, Rochester, NY) were coated overnight at 4°C with either 50 ng/well recombinant CSP [N-$(NANP)_{19}$-C] or 20 ng/well $(NANP)_6$ peptide. Plates were washed with PBS containing 0.05% Tween 20 (PBS/T) and blocked for 1 h with PBS containing 1% casein. Fifty microliters of diluted primary antibody was added to the wells in duplicate for 2 h at 22°C, plates were washed 3 times with PBS/T, and 50 μl/well of a 1:15,000 dilution of peroxidase-conjugated anti-mouse IgG (Southern Biotech, Birmingham, AL) was added. After 1 h of incubation, plates were washed 4 times with PBS/T and developed using 50 μl/well ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] peroxidase substrate system (KPL, Gaithersburg, MD) for 1 h. The optical density at 415 nm (OD_{415}) was measured using a Synergy4 microplate reader (BioTek, Highland Park, VT) and the endpoint titer, defined as the serum dilution that resulted in an OD_{415} value of 1.0, was calculated using Gen5 software (BioTek). The avidity enzyme-linked immunosorbent assay (ELISA) was performed similarly, with 75 μl of a 1:1,000 serum dilution added to the blocked wells for 1 h. After washing, 100 μl of either 6 M urea or PBS was incubated in the wells for 10 min. Plates were washed, and the rest of the ELISA procedure was performed as described above. The avidity index was defined as the ratio of OD_{415} values obtained in the presence and absence of 6 M urea.

Indirect immunofluorescence assay. Sporozoites were placed on an immunofluorescence assay (IFA) slide, air dried, and fixed with chilled methanol for 1 min. Wells were blocked with PBS containing 5% bovine serum albumin, and serial dilutions of the test antibodies were added and incubated for 2 h. Slides were washed 3 times with PBS and incubated with a 1:100 dilution of fluorescein-labeled anti-mouse IgG (Southern Biotech) for 1 h. The slides were washed again, and anti-fade solution (Molecular Probes, Eugene, OR) was applied. Fluorescence was observed with a UV microscope (200× magnification). An adjuvant control serum pool diluted 1:100 was used as a negative control. The mouse *P. berghei* CSP (*Pb*CSP)-specific monoclonal antibody (MAb) 4B10 and the *Pf*CSP-specific MAb 49-1B2 were used in the study.

Sporozoite invasion inhibition assay. Mouse serum was tested for its ability to inhibit sporozoite entry into hepatocytes (13). Briefly, the wells of a Lab-Tek glass chamber slide were coated with entactin-collagen IV-laminin (ECL) attachment matrix (Millipore, Billerica, MA), 45,000 HepG2-A16 cells were added, and the slide was incubated overnight at 37°C in 5% CO_2. Fifty microliters of the appropriate serum dilution, along with 50 μl of *P. falciparum* NF54 sporozoite suspension (25,000 sporozoites), was added per well. The chamber slides were then incubated for 3 h at 37°C in 5% CO_2. Slides were washed with PBS and fixed with chilled methanol. Sporozoites that had invaded hepatocytes were visualized by staining with a *P. falciparum* CSP-specific MAb (49-1B2), followed by the addition of a 1:200 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (KPL, Gaithersburg, MD). Color was developed using 3,3′-diaminobenzidine reagent (KPL), and slides were mounted with Permount mounting medium (Fisher Scientific, Hampton, NH). The number of intracellular sporozoites per well was counted with a phase-contrast microscope at ×200 magnification. The percent inhibition of sporozoite invasion was calculated as (invasion events in the medium control − invasion events in the test serum)/invasion events in the medium control × 100.

Native CSP Western blotting. Proteins from 50,000 sporozoites per well were separated by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane, and blocked for 1 h with PBS with casein. The blot was incubated with a 1:1,000 dilution of immune serum for 1 h, washed with PBS/T, and incubated for 1 h with a 1:5,000 dilution of alkaline phosphatase-conjugated anti-mouse IgG secondary antibody (Southern Biotech). The blots were washed again and developed with nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate substrate tablets (Roche, Nutley, NJ).

Statistical analysis. Data were plotted using Excel (Microsoft, Redmond, WA) and GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Unpaired groups of data were compared for significant differences utilizing Mann-Whitney nonparametric tests. *P* values of <0.05 were used to determine statistical significance.

RESULTS

Adaptation of the transgenic *P. berghei* parasite in the mouse and mosquito hosts. Data from 10 independent mosquito-mouse transmission cycles showed no significant differences in the percentages of mosquitoes infected with oocysts and in the oocyst ratings between transgenic *P. berghei*-infected and wild-type (WT) *P. berghei*-infected mosquitoes (Fig. 1B and C). However, the percentage of mosquitoes infected with salivary gland sporozoites and the mean sporozoite ratings (on a scale of 0 to 4) were significantly lower for Tr *P. berghei*-infected mosquitoes (Mann-Whitney test, $P < 0.05$ for both comparisons). Initial batches obtained by feeding mosquitoes on mice that were infected by blood stages typically yielded <500 sporozoites per mosquito, compared with >10,000 sporozoites for the WT *P. berghei*-infected mosquitoes. The transmission cycle was then modified, eliminating the

TABLE 1 Optimization of the challenge dose in C57BL/6 mice[a]

Expt	Sporozoite dose (no. of sporozoites/mouse)	Route	No. of mice that were:		Mean day of patency
			Challenged	Infected	
I	5,000	s.c.	7	1	8
	7,500	s.c.	7	1	5
II	10,000	s.c.	7	5	6.3
	2,500	i.v.	7	7	5.5
III	2,500	i.v.	7	7	5
	1,000	i.v.	7	7	5
IV	2,500	i.v.	10	10	5.2
	1,000	i.v.	10	9	5.4

[a] The sporozoite dose required for 100% infectivity in mice was established with subcutaneous (s.c.) or intravenous (i.v.) injection of sporozoites. Shown are the number of challenged mice, the number of infected mice (determined by patent parasitemia in the blood), and the mean day of patency for each group.

blood-stage transmission, and mosquitoes were fed only on mice that had been infected directly by mosquito bites. This modification selected for a line that was better adapted to produce salivary gland sporozoites, increasing the average yield to ~2,000 sporozoites per mosquito (Fig. 1D). However, no change in the rodent infectivity of the adapted parasites was notable. The genetic replacement of the *Pb*CSP gene with *Pf*CSP was confirmed by IFA using a *P. falciparum*-specific monoclonal antibody (MAb), 49-1B2. WT *P. berghei* sporozoites reacted with a *Pb*CSP-specific MAb, 4B10, but only weak reactivity was observed with the *Pf*CSP-specific MAb 49-1B2 (Fig. 1E). In contrast, the Tr *P. berghei* sporozoites reacted only with the *Pf*CSP-specific MAb.

Challenge route and dose optimization. To reproduce the natural mode of transmission used for experimental human challenges, either 5 or 10 Tr *P. berghei* sporozoite-infected mosquitoes were fed on individual C57BL/6 mice. This challenge route yielded variable infectivity data that were strongly dependent on the sporozoite loads of individual mosquitoes in the carton. A subcutaneous challenge using isolated sporozoites also showed low infectivity, despite a high sporozoite inoculum (Table 1, experiments I and II). The challenge route was then modified to intravenous administration of defined numbers of isolated sporozoites. Intravenous injection of 2,500 sporozoites successfully infected all inoculated mice (Table 1, experiment II), with patency typically being observed by 6 days postchallenge (Table 1, experiments III and IV). The BALB/c strain was more resistant to Tr *P. berghei* parasites than were C57BL/6 mice and required a 4 to 5 times greater sporozoite dose than did the C57BL/6 strain. Based on these data, all subsequent challenge experiments were performed with C57BL/6 mice, using an intravenous dose of 2,500 Tr *P. berghei* sporozoites.

Utility and validity of the Tr *P. berghei* model. A major goal of this study was to establish the effectiveness of the Tr *P. berghei* sporozoite model for the down-selection of different CSP-based vaccine antigens. To accomplish this, the following four closely related *Pf*CSP constructs were prepared and tested: construct N-$(NANP)_{19}$-C contained the N-terminal region, 19 repeats, and the C-terminal cysteine-rich region; construct N-$(NANP)_5$-C contained the N-terminal region, 5 repeats, and the C-terminal region; construct $(NANP)_{18}$-C contained 18 repeats and the C-terminal region; and construct Cterm contained only the C-terminal region of *Pf*CSP (Fig. 2A and B). Groups of nine mice received three intraperitoneal vaccinations of 2.5 μg antigen formulated with Montanide ISA720, at 2-week intervals. At 2 weeks after the third vaccination, mice were challenged with 2,500 Tr *P. berghei* sporozoites. Antibody titers were measured against the repeat peptide $(NANP)_6$ or the nearly full-length construct N-$(NANP)_{19}$-C protein coated onto ELISA plates.

As expected, the 5-repeat-containing construct induced lower repeat-specific antibody titers than did constructs that contained either 19 or 18 repeats (Fig. 2C and D). Interestingly, the $(NANP)_{18}$-C construct lacking the N terminus exhibited faster kinetics of repeat-specific antibody acquisition after the second vaccination than did the constructs that contained the N-terminal region [N-$(NANP)_{19}$-C and N-$(NANP)_5$-C] (Fig. 2C). Similarly, after the third vaccination, the magnitude of repeat-specific antibody levels in the $(NANP)_{18}$-C-immunized group was greater than that observed for the N-$(NANP)_{19}$-C- and N-$(NANP)_5$-C-immunized groups ($P = 0.02$ and 0.0001, respectively) (Fig. 2D). The presence of the N-terminal region, however, did not globally reduce the immunogenicity of the N-$(NANP)_{19}$-C and N-$(NANP)_5$-C constructs, as their group titers were similar to the $(NANP)_{18}$-C-induced titers when measured against the nearly full-length CSP and Cterm plate antigens ($P > 0.2$ for all comparisons) (Fig. 2D). Upon challenge with Tr *P. berghei* parasites, none of the adjuvant control-, N-$(NANP)_5$-C-, or Cterm-vaccinated mice were protected (Fig. 2D, open triangles). In contrast, 4 of 9 N-$(NANP)_{19}$-C-vaccinated mice and 4 of 9 $(NANP)_{18}$-C-vaccinated mice were protected.

Titration of vaccine-induced protection. Groups of mice received, at 2-week intervals, three intraperitoneal injections of incremental doses of N-$(NANP)_{19}$-C protein formulated with Montanide ISA720. The geometric mean antibody titers against all three plate antigens at 2 weeks after the third vaccination increased with increasing antigen dose (Fig. 3A). Mice were challenged with 2,500 sporozoites 2 weeks after the third vaccination. While no protection was observed in the adjuvant control group, 1 of 6 mice in the 1-μg-dose group, 5 of 7 mice in the 2.5-μg group, 3 of 7 mice in the 5-μg group, and 7 of 7 mice in the 10-μg group were protected. Hence, the degree of protection increased with escalating antigen dose.

Association between antibody titers and protection against Tr *P. berghei*. IgG subclasses and antibody avidity were measured using the sera of individual mice from the various immunization regimens. The protection status of the mice correlated with antibody titers against the nearly full-length protein and the repeat peptide. As seen in Fig. 3B, 70% of the mice with ELISA titers of >10,000 against both the full-length and repeat antigens were protected, in contrast to only 28% protection for mice with low repeat-specific antibody titers and 21% protection for mice with low antibody titers to both full-length and repeat antigens. Mice in the three highest-dose groups (10, 5, and 2.5 μg CSP) were tested for antibody avidity. The avidity index was associated with protection, as 100% of mice with avidity index values of >80% and full-length antigen ELISA titers of >10,000 were protected (Fig. 3C). Low levels of IgG2 induced by Montanide ISA720 formulations precluded any correlation of subclasses with protection.

Biological activity of CSP-specific antibodies. Antibodies against the N-$(NANP)_{19}$-C protein showed positive reactivity with fixed sporozoites by indirect immunofluorescence assay (IFA), and pooled sera showed increasing IFA titers with escalating antigen dose (Fig. 4A and B). Antibodies after N-$(NANP)_{19}$-C immunization showed positive reactivity with an ~60-kDa band

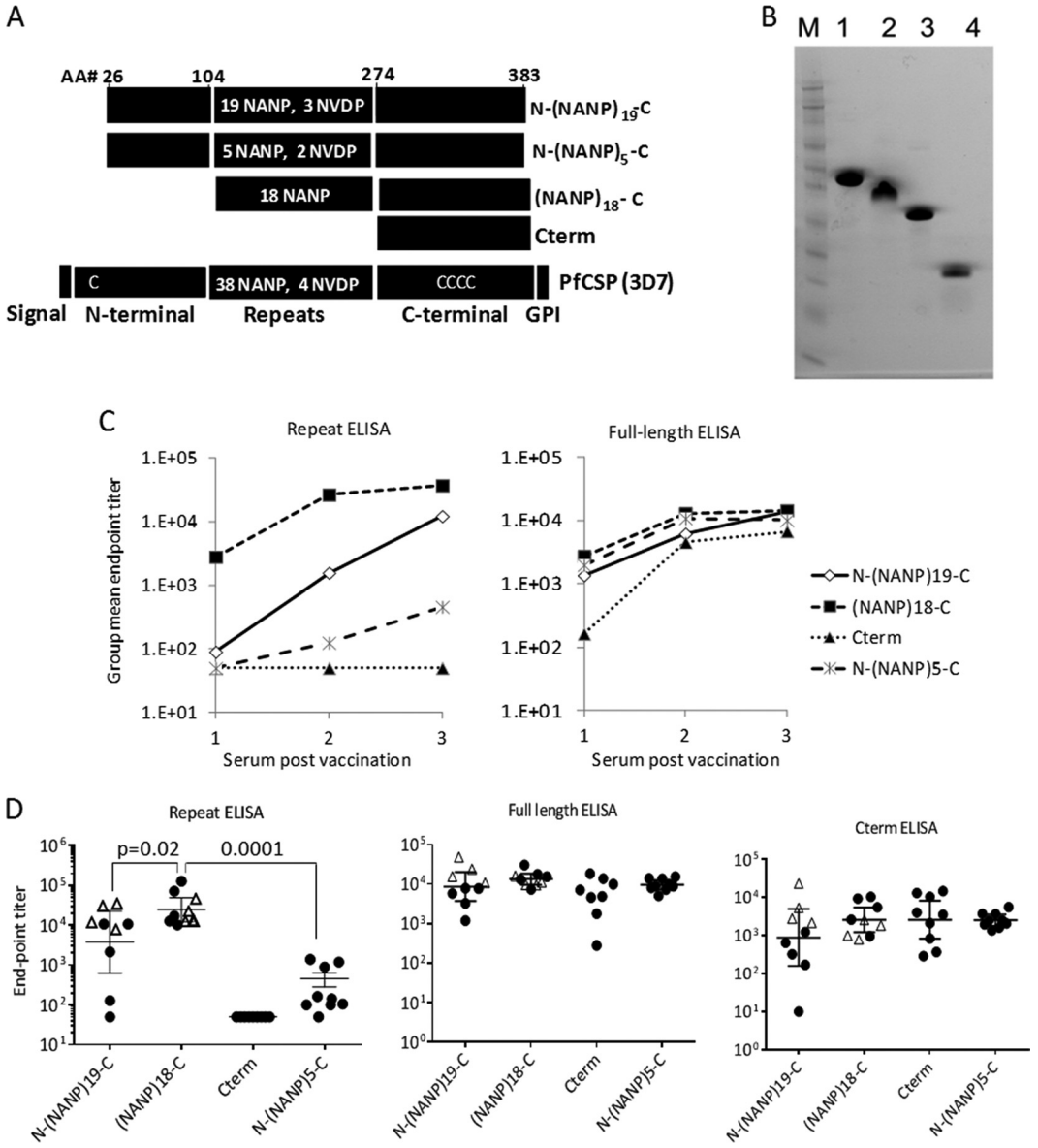

FIG 2 Protection data on various *Pf*CSP constructs. (A) Four constructs were expressed in *E. coli*, two of which included the N- and C-terminal regions and either 19 or 5 of the 38 NANP repeats of 3D7 *P. falciparum* CSP. A third construct contained 18 NANP repeats and the C-terminal region, and a fourth construct (Cterm) contained only the C-terminal region. AA, amino acid; GPI, glycosylphosphatidylinisotol. (B) Coomassie blue-stained gel of purified proteins separated through nonreduced SDS-PAGE. Lane 1, N-$(NANP)_{19}$-C; lane 2, $(NANP)_{18}$-C; lane 3, N-$(NANP)_5$-C; lane 4, Cterm; M, markers. (C) Mean ELISA endpoint titers against the repeat and nearly full-length constructs, measured 2 weeks after each vaccination. (D) Titers of individual mice in each group measured against the $(NANP)_6$ peptide, the nearly full-length protein, or the Cterm protein coated on plates. Geometric mean titers ± 95% confidence intervals are shown. △, mice protected against Tr *P. berghei* challenge; ●, nonprotected mice.

on a Western blot against an extract of sporozoite-infected mosquito salivary glands (Fig. 4C). In a sporozoite invasion inhibition assay, anti-N-$(NANP)_{19}$-C serum (1:100 dilution) caused ~90% inhibition of sporozoite invasion of HepG2-A16 cells, compared with ~20% inhibition by normal mouse serum (Fig. 4D). Inhibition of sporozoite invasion activity increased with the antigen dose, and ~90% inhibition was observed using a serum pool from the 10-μg N-$(NANP)_{19}$-C group, in which all of the mice were protected (Fig. 4E).

Passive transfer of *Pf*CSP-specific antibodies confers protection in the Tr *P. berghei* challenge model. Mice were administered purified rabbit IgG against the N-$(NANP)_{19}$-C vaccine and were challenged with 2,500 Tr *P. berghei* sporozoites. All five mice that received anti-apical membrane antigen 1 IgG developed parasitemia by day 5, while 3 of 5 mice that received anti-CSP IgG were protected through day 14 postchallenge. In another experiment, Tr *P. berghei* sporozoites were preincubated with a 1:4 dilution of either mouse serum against N-$(NANP)_{19}$-C or control normal mouse serum. After a 5-min incubation at 37°C, 3,000 sporozoites were injected intravenously into each of three naive C57BL/6 mice. Two of three mice in the anti-CSP group were protected through day 14,

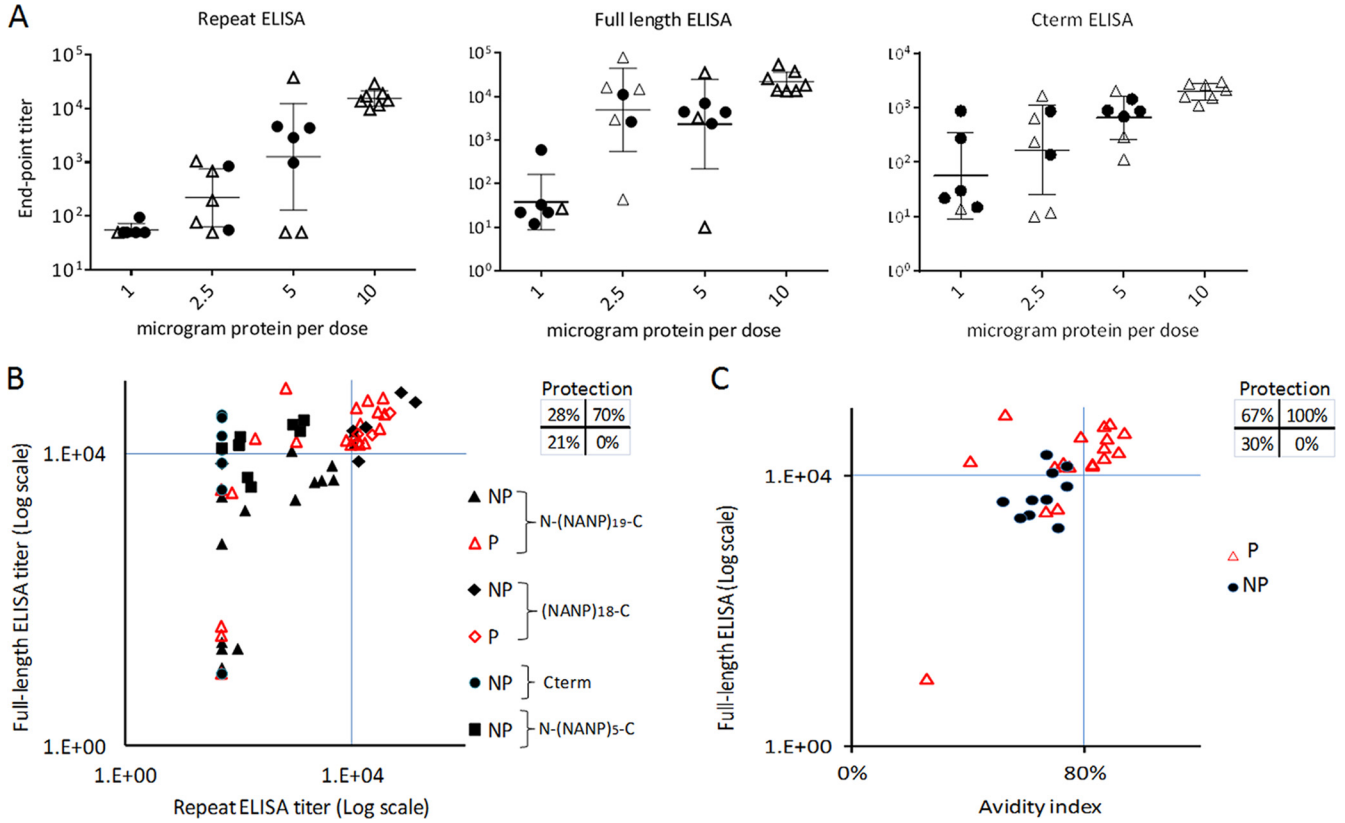

FIG 3 Protection data with incremental doses of *Pf*CSP. (A) Endpoint titers for mice vaccinated with increasing doses of N-$(NANP)_{19}$-C protein, measured 2 weeks after the third dose, against the $(NANP)_6$ repeat peptide, the nearly full-length N-$(NANP)_{19}$-C protein, or the Cterm protein coated on ELISA plates. Geometric means ± 95% confidence intervals are shown. △, protected mice; ●, nonprotected mice. (B) Endpoint titers for mice from the construct and dose-titration experiments, 2 weeks after the third vaccination. Full-length CSP ELISA titers were plotted against the repeat peptide ELISA titers. P, protected mice; NP, nonprotected mice. Inset, percentage of mice protected in each quadrant. (C) Nearly full-length ELISA titers for N-$(NANP)_{19}$-C-immunized mice, from the construct and dose-titration studies, plotted against the respective avidity index values.

while all three control mice tested positive by day 6 postchallenge.

DISCUSSION

There is substantial evidence that antibodies and T-cell-mediated immune responses to CSP can protect against the pre-erythrocytic stage of malaria (14). Owing to the escalating costs and regulatory constraints associated with human vaccine trials and due to the limitations of primate models of *P. falciparum* sporozoite challenge (7–9), there is an urgent need to develop rodent models for the routine down-selection of second-generation vaccine formulations of *Pf*CSP vaccines. Here we present studies that led to the optimization of one such rodent challenge model, based on transgenic *P. berghei* parasites that express full-length *Pf*CSP (4). Our data showed that this model can distinguish between the protective efficacies of several closely related CSP antigens, using a highly stringent endpoint of sterile protection following intravenous injection of sporozoites.

A notable area of concern with parasites that are transgenic for full-length CSP is reduced sporozoite salivary gland invasion. Rodent parasites expressing either *Plasmodium falciparum* or avian *Plasmodium gallinaceum* CSP develop normally in the midgut yet show significantly reduced salivary gland burdens in mosquitoes. In such studies, the N-terminal and repeat regions of CSP were specifically implicated in migration to the salivary glands (15, 16). We found in our studies that switching from blood-stage passage to more-natural, vector-borne, mosquito-to-mouse passage yielded an adapted line of Tr *P. berghei* that routinely produced sufficient (although still relatively low) numbers of infective sporozoites for challenge experiments. Other criteria for successful challenge included sporozoite yields of >1,000 per mosquito, addition of 5% freshly collected mouse serum to the dissection medium, minimization of the dissection time to under 1 h, and storage of dissected sporozoites on ice until injection.

The observation that constructs containing the N terminus induced lower levels of repeat-specific antibodies than did those lacking the N terminus requires further investigation, as it may reflect the shielding of CSP domains by the N-terminal region that is known to occur during sporozoite passage from the mosquito to the liver (17). Although the relative immunogenicity of different CSP regions may be different in humans and mice, it is notable that the only successful CSP-based malaria vaccine, RTS,S, also lacks the N-terminal region. Thus, these observations in mice might have implications for improving CSP vaccine design (18). Although the C-terminal region of *P. berghei* CSP has been associated with protection in mice (19, 20), our C-terminal construct of *Pf*CSP did not confer protection against a Tr *P. berghei* challenge. This result was reminiscent of a human trial in which a

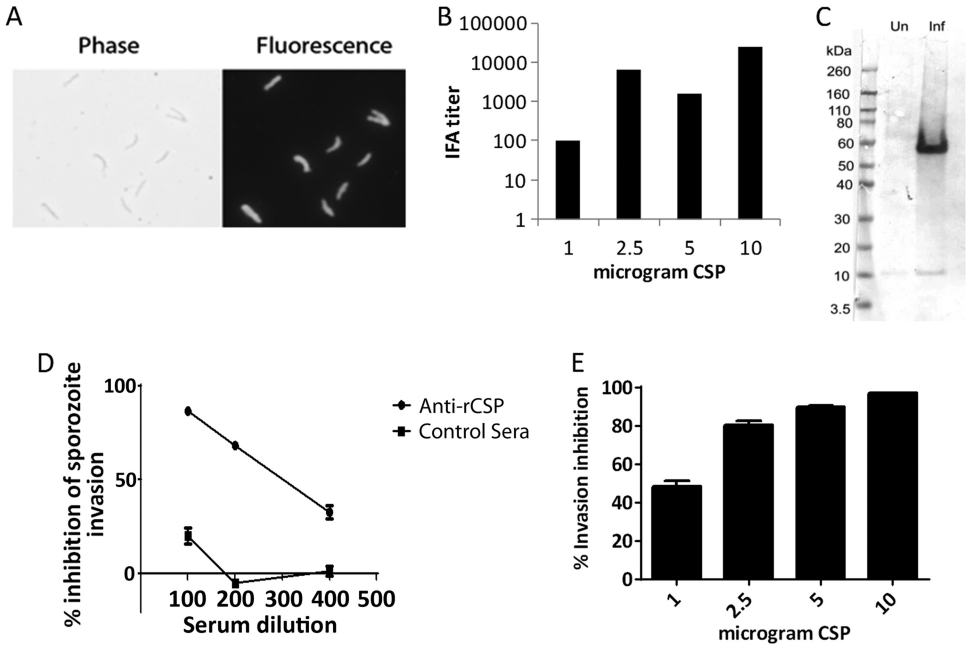

FIG 4 Biological activity of mouse antibodies against the N-$(NANP)_{19}$-C construct. (A) Recognition of CSP on the surface of fixed sporozoites. Typical phase-contrast and fluorescence images are shown. (B) IFA titers from a single experiment using pooled sera from N-$(NANP)_{19}$-C-vaccinated mice from the dose-titration study. (C) Western blot reactivity, at 1:1,000 serum dilution, against salivary gland preparations of *P. falciparum* sporozoite-infected (Inf) or uninfected (Un) mosquitoes. (D) Dose-response assay, showing the inhibition of sporozoite invasion using sera from N-$(NANP)_{19}$-C vaccinated mice. (E) Pooled sera from the N-$(NANP)_{19}$-C dose-titration experiment, at 1:100 dilution, tested in an assay of the inhibition of sporozoite invasion. Mean + SEM values for three replicate wells are shown.

repeatless CSP construct did not protect the vaccinees (21). Doud et al. have recently shown that key epitopes in the C-terminal region involve noncontiguous residues of the molecule (22). It is possible that the isolated C-terminal protein used for immunization in our study did not adopt the same conformation as the C-terminal region in the intact CSP molecule. Additionally, it is noteworthy that several mice with low repeat-specific antibody titers were protected in the present study, as is often observed with RTS,S-immunized humans. Although cellular immune responses to CSP were not measured, T-cell epitopes in the N- and C-terminal regions of CSP might have contributed to protection (6, 19), as observed by others using CSP-transgenic parasite models (11, 23).

In conclusion, we report sterile protection by recombinant *Pf*CSP vaccines against a full-length *Pf*CSP-transgenic mouse parasite challenge. The protection was dependent on the antigen dose and the nature of the CSP immunogen. In support of our observations, it is noteworthy that, during the course of our study, Kaba et al. reported using the same Tr *P. berghei* parasite line to monitor protection induced in mice following immunization with a nanoparticle vaccine expressing B-cell and $CD8^+$ T-cell epitopes from the *Pf*CSP (23). Although the biological relevance of this mouse protection model with respect to human vaccine development remains to be confirmed, sterile protection as the efficacy endpoint provides a means to evaluate *Pf*CSP-based vaccines rapidly for future human trials.

ACKNOWLEDGMENTS

The funding for this work was provided by the USAID Malaria Vaccine Development Program.

We thank Lorraine Soisson and Carter Diggs for support and advice.

The opinions expressed in this publication are those of the authors and are not to be construed as the official position of the United States Department of the Army or the Department of Defense. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animal experiments and was adherent to the principals stated in the *Guide for the Care and Use of Laboratory Animals* (24).

REFERENCES

1. **Jin Y, Kebaier C, Vanderberg J.** 2007. Direct microscopic quantification of dynamics of *Plasmodium berghei* sporozoite transmission from mosquitoes to mice. Infect. Immun. **75**:5532–5539.
2. **Meis JF, Verhave JP.** 1988. Exoerythrocytic development of malarial parasites. Adv. Parasitol. **27**:1–61.
3. **Dame JB, Williams JL, McCutchan TF, Weber JL, Wirtz RA, Hockmeyer WT, Maloy WL, Haynes JD, Schneider I, Roberts D, Sanders GS, Reddy P, Diggs CL, Miller LH.** 1984. Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. Science **225**:593–599.
4. **Tewari R, Spaccapelo R, Bistoni F, Holder AA, Crisanti A.** 2002. Function of region I and II adhesive motifs of *Plasmodium falciparum* circumsporozoite protein in sporozoite motility and infectivity. J. Biol. Chem. **277**:47613–47618.
5. **Vekemans J, Leach A, Cohen J.** 2009. Development of the RTS,S/AS malaria candidate vaccine. Vaccine **27**(Suppl 6):G67–G71.
6. **Bongfen SE, Ntsama PM, Offner S, Smith T, Felger I, Tanner M, Alonso P, Nebie I, Romero JF, Silvie O, Torgler R, Corradin G.** 2009. The N-terminal domain of *Plasmodium falciparum* circumsporozoite protein represents a target of protective immunity. Vaccine **27**:328–335.
7. **Collins WE, Sullivan JS, Williams A, Galland GG, Nace D, Williams T, Barnwell JW.** 2009. The Santa Lucia strain of *Plasmodium falciparum* in *Aotus* monkeys. Am. J. Trop. Med. Hyg. **80**:536–540.
8. **Hayes DE, Ward RA.** 1977. Sporozoite transmission of falciparum malaria (Burma-Thau. strain) from man to *Aotus* monkey. Am. J. Trop. Med. Hyg. **26**:184–185.
9. **Collins WE, Sullivan JS, Morris CL, Galland GG, Richardson BB,**

Roberts JM. 1997. The Malayan IV strain of *Plasmodium falciparum* in *Aotus* monkeys. Am. J. Trop. Med. Hyg. **56**:49–56.

10. **Persson C, Oliveira GA, Sultan AA, Bhanot P, Nussenzweig V, Nardin E.** 2002. Cutting edge: a new tool to evaluate human pre-erythrocytic malaria vaccines: rodent parasites bearing a hybrid *Plasmodium falciparum* circumsporozoite protein. J. Immunol. **169**:6681–6685.
11. **Kastenmuller K, Espinosa DA, Trager L, Stoyanov C, Salazar AM, Pokalwar S, Singh S, Dutta S, Ockenhouse CF, Zavala F, Seder RA.** 2013. Full-length *Plasmodium falciparum* circumsporozoite protein administered with long-chain poly(I·C) or the Toll-like receptor 4 agonist glucopyranosyl lipid adjuvant-stable emulsion elicits potent antibody and $CD4^+$ T cell immunity and protection in mice. Infect. Immun. **81**:789–800.
12. **Ozaki LS, Gwadz RW, Godson GN.** 1984. Simple centrifugation method for rapid separation of sporozoites from mosquitoes. J. Parasitol. **70**:831–833.
13. **Hollingdale MR, Nardin EH, Tharavanij S, Schwartz AL, Nussenzweig RS.** 1984. Inhibition of entry of *Plasmodium falciparum* and *P. vivax* sporozoites into cultured cells: an in vitro assay of protective antibodies. J. Immunol. **132**:909–913.
14. **Schwenk RJ, Richie TL.** 2011. Protective immunity to pre-erythrocytic stage malaria. Trends Parasitol. **27**:306–314.
15. **Myung JM, Marshall P, Sinnis P.** 2004. The *Plasmodium* circumsporozoite protein is involved in mosquito salivary gland invasion by sporozoites. Mol. Biochem. Parasitol. **133**:53–59.
16. **Aldrich C, Magini A, Emiliani C, Dottorini T, Bistoni F, Crisanti A, Spaccapelo R.** 2012. Roles of the amino terminal region and repeat region of the *Plasmodium berghei* circumsporozoite protein in parasite infectivity. PLoS One **7**:e32524. doi:10.1371/journal.pone.0032524.
17. **Coppi A, Pinzon-Ortiz C, Hutter C, Sinnis P.** 2005. The *Plasmodium* circumsporozoite protein is proteolytically processed during cell invasion. J. Exp. Med. **201**:27–33.
18. **Kester KE, Cummings JF, Ofori-Anyinam O, Ockenhouse CF, Krzych U, Moris P, Schwenk R, Nielsen RA, Debebe Z, Pinelis E, Juompan L, Williams J, Dowler M, Stewart VA, Wirtz RA, Dubois MC, Lievens M, Cohen J, Ballou WR, Heppner DG, Jr, RTS,S Vaccine Evaluation Group.** 2009. Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. J. Infect. Dis. **200**:337–346.
19. **Roggero MA, Meraldi V, Lopez JA, Eberl G, Romero JC, Matile H, Betschart B, Corradin G, Renggli J.** 2000. The synthetic, oxidized C-terminal fragment of the *Plasmodium berghei* circumsporozoite protein elicits a high protective response. Eur. J. Immunol. **30**:2679–2685.
20. **Bergmann-Leitner ES, Scheiblhofer S, Weiss R, Duncan EH, Leitner WW, Chen D, Angov E, Khan F, Williams JL, Winter DB, Thalhamer J, Lyon JA, Tsokos GC.** 2005. C3d binding to the circumsporozoite protein carboxy-terminus deviates immunity against malaria. Int. Immunol. **17**:245–255.
21. **Heppner DG, Gordon DM, Gross M, Wellde B, Leitner W, Krzych U, Schneider I, Wirtz RA, Richards RL, Trofa A, Hall T, Sadoff JC, Boerger P, Alving CR, Sylvester DR, Porter TG, Ballou WR.** 1996. Safety, immunogenicity, and efficacy of *Plasmodium falciparum* repeatless circumsporozoite protein vaccine encapsulated in liposomes. J. Infect. Dis. **174**:361–366.
22. **Doud MB, Koksal AC, Mi LZ, Song G, Lu C, Springer TA.** 2012. Unexpected fold in the circumsporozoite protein target of malaria vaccines. Proc. Natl. Acad. Sci. U. S. A. **109**:7817–7822.
23. **Kaba SA, McCoy ME, Doll TA, Brando C, Guo Q, Dasgupta D, Yang Y, Mittelholzer C, Spaccapelo R, Crisanti A, Burkhard P, Lanar DE.** 2012. Protective antibody and $CD8^+$ T-cell responses to the *Plasmodium falciparum* circumsporozoite protein induced by a nanoparticle vaccine. PLoS One **7**:e48304. doi:10.1371/journal.pone.0048304.
24. **Institute for Laboratory Animal Research.** 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington, DC.