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The *Plasmodium falciparum* circumsporozoite (CS) protein (CSP) is a major vaccine target for preventing malaria infection. Thus, developing strong and durable antibody and T cell responses against CSP with novel immunogens and potent adjuvants may improve upon the success of current approaches. Here, we compare four distinct full-length *P. falciparum* CS proteins expressed in *Escherichia coli* or *Pichia pastoris* for their ability to induce immunity and protection in mice when administered with long-chain poly(I·C) [poly(I·C)LC] as an adjuvant. CS proteins expressed in *E. coli* induced high-titer antibody responses against the NANP repeat region and potent CSP-specific CD4$^+$ T cell responses. Moreover, *E. coli*-derived CS proteins in combination with poly(I·C)LC induced potent multifunctional (interleukin 2-positive [IL-2$^+$], tumor necrosis factor alpha-positive [TNF-α^+], gamma interferon-positive [IFN-γ^+]) CD4$^+$ effector T cell responses in blood, in spleen, and particularly in liver. Using transgenic *Plasmodium berghei* expressing the repeat region of *P. falciparum* CSP [*Pb*-CS(*Pf*)], we showed that there was a 1- to 4-log decrease in malaria rRNA in the liver following a high-dose challenge and ~50% sterilizing protection with a low-dose challenge compared to control levels. Protection was directly correlated with high-level antibody titers but not CD4$^+$ T cell responses. Finally, protective immunity was also induced using the Toll-like receptor 4 agonist glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) as the adjuvant, which also correlated with high antibody titers yet CD4$^+$ T cell immunity that was significantly less potent than that with poly(I·C)LC. Overall, these data suggest that full-length CS proteins and poly(I·C)LC or GLA-SE offer a simple vaccine formulation to be used alone or in combination with other vaccines for preventing malaria infection.

Malaria infection with *Plasmodium falciparum* causes more than 600,000 deaths annually as well as significant morbidity worldwide (1). A range of efforts to control and treat malaria include public health measures such as insecticide-treated bed nets, indoor residual spraying, and widespread usage of antimalarial drugs. Despite the impact of these approaches, the most cost-effective solution to prevent infection and to ultimately control the malaria endemic is to develop a vaccine.

Currently, the most advanced vaccine tested in humans against *P. falciparum* infection is RTS,S, which targets the circumsporozoite (CS) protein (CSP), the major and most abundant antigen expressed on the surfaces of infectious sporozoites. RTS,S given with the AS01 adjuvant (RTS,S/AS01) shows ~30% protection against clinical disease and severe malaria (2, 3). Thus, while these first results in phase III trials with RTS,S/AS01 are encouraging, there may be additional approaches for further optimizing the breadth, potency, and duration of immunity against the CSP using different immunogens or more-potent adjuvants. In terms of antigen design, RTS,S is comprised of a truncated form of CSP containing the central repeat region, NANP, which is a target for antibody-mediated neutralization, as well as CD8$^+$ and CD4$^+$ T cell epitopes at the C-terminal end. This truncated CS protein is then fused to the hepatitis B virus surface antigen, creating an immunogenic particle. Therefore, using a more-full-length CSP,

including the N-terminal end and the R1 region of CSP as well as the minor repeat region (NVDP), might favor broader antibody responses than against the NANP repeat region alone (4–8). Moreover, a full-length CSP may provide additional T cell epitopes, leading to increased breadth of cellular immunity, which could also enhance protection. Another approach is to enhance the humoral and cellular immune responses by altering the type of adjuvant given with the full-length-CSP-based protein vaccine.

Early studies in mice showed that protection was associated with high antibody titers (9–11). The next generation of malaria vaccines combined CSP with more-potent adjuvants, like *Pseu-*

domonas aeruginosa exotoxin A, monophospholipid A (MPL), mycobacterial cell wall skeleton, or squalene (Detox; Ribi Immunochem) (12–14), which resulted in high antibody titers; however, they failed to confer sufficient protective efficacy (15–17). In contrast, studies using irradiated sporozoites for vaccination have shown a critical requirement for gamma interferon (IFN-γ) and cellular immunity in mediating protection against malaria (18, 19). Accordingly, optimizing $CD4^+$ T cell-derived IFN-γ production after RTS,S vaccination by altering the adjuvant formulation enhanced protection (20–22). Using the Toll-like receptor 4 (TLR4) ligand MPL and saponin (QS-21) in an oil-water emulsion (AS02) or liposome (AS01B or -E) formulation led to a strong antibody response and increased $CD4^+$ T cell immunity compared to levels induced with older formulations with alum and MPL (21, 23, 24). Collectively, these data highlight the importance of adjuvant formulations in optimizing immunity and protection.

In this study, long-chain poly(I·C) [poly(I·C)LC] and the TLR4 agonist glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) were compared as adjuvants when they were administered with a number of CS proteins expressed in the yeast *Pichia pastoris* or in *Escherichia coli*, to which we refer as full length but which lack the glycosylphosphatidylinositol (GPI) signal region and contain various numbers of the NANP repeat region. Poly(I·C)LC is a unique formulation of long-chain double-stranded RNA, polyinosinic-poly(C), and carboxy methylcellulose that has extended *in vivo* activity compared to that of poly(I·C). Poly(I·C)LC mediates innate signaling through TLR3 and melanoma differentiation-associated protein 5 (MDA-5), leading to activation of dendritic cells and induction of interleukin 12 (IL-12) and type I interferons (IFNs) (25–27). In addition, poly(I·C) has been shown to promote T cell survival and enhance germinal-center formation through the generation of $CD4^+$ T follicular helper (Tfh) cells (28). As an immune adjuvant, poly(I·C)LC has been shown to elicit strong humoral and cellular immunity when administered with a variety of protein- or dendritic cell-targeting vaccines in a number of mouse and nonhuman primate (NHP) studies (29–35).

GLA is a synthetic and therefore homogeneous variant of the TLR4 agonist lipid A, formulated in a stable oil-in-water emulsion (SE) (36). Strong Th1 immune responses induced by protein antigens in combination with GLA-SE have been observed in mouse models of tuberculosis (37), leishmaniasis (38), and influenza (39). Additionally, GLA-SE showed an adjuvant activity similar to or enhanced relative to that of MPL-SE (one of the adjuvant components in AS01) in various animal models, such as mice, guinea pigs, and nonhuman primates (40, 41).

Here, we compare four full-length *P. falciparum* CS proteins expressed in *E. coli* or in the yeast *P. pastoris* given with poly(I·C)LC or GLA-SE as an adjuvant and determine their relative immunogenicities and levels of protection using an *in vivo* mouse challenge model. Furthermore, one of the CS proteins and poly(I·C)LC was tested in NHPs. Together, these data provide a potential simple vaccine formulation for inducing potent and protective *P. falciparum* CSP responses.

MATERIALS AND METHODS

Animals. Six- to 8-week-old C57BL/6 mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and maintained in the Vaccine Research Center Animal Care Facility (Bethesda, MD) under pathogen-free conditions. Male, Indian rhesus macaques were stratified into comparable groups on the basis of age and weight. The animals were housed at the School of Medicine, Comparative Medicine and Veterinary Resources, University of Maryland. All experiments were conducted according to the guidelines of the National Research Council under protocols approved by the Institutional Animal Care and Use Committee at the National Institutes of Health.

Reagents. Four *P. falciparum* CS proteins were provided by the PATH Malaria Vaccine Initiative (MVI; Washington, DC) and provided to us in a blind manner (they were designated CSP1, CSP2, CSP3, and CSP4). These four proteins are called full length in the text, but they lack the GPI signal region and contain various numbers of the repeat region. After the immune studies were completed, it was revealed to us that two proteins were expressed in *E. coli* (CSP1 and CSP2) and two were expressed in *P. pastoris* (CSP3 and CSP4). CSP1 and CSP2 were provided to the MVI from Gennova (Pune, India) and WRAIR (Silver Spring, MD), respectively. Protein concentration was measured using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Endotoxin contaminants were determined using the *Limulus* amebocyte lysate (LAL) assay (Lonza, Walkersville, MD). If endotoxin levels exceeded 2 endotoxin units (EU)/ml, endotoxin was removed by two-phase extraction with Triton X-114. Small amounts of endotoxin was detected in the *E. coli*-derived proteins. Thus, all four proteins, including the two expressed in yeast, which had no detectable endotoxin, were treated with Triton X-114. All proteins used for immunization had final endotoxin levels below 1 EU/ml. After removal of endotoxin, protein concentration was determined to confirm that all groups of mice received the same amount of protein. *P. falciparum* CSP15-mer peptides overlapping by 11 amino acids (aa) and spanning the entire length of the protein (*P. falciparum* 3D7 strain) were synthesized by GenScript. Each batch of CSP was tested for accurate size using SDS-PAGE.

Antibodies. The following anti-mouse antibodies for flow cytometry were purchased from BD Pharmingen: purified anti-CD28 (37.51), PerCP–Cy55–anti-CD3 (145-2C11), and Alexa 700–anti-CD4 (RM4-5). The following antibodies were purchased from BioLegend: allophycocyanin (APC)–Cy7–anti-CD8 (53-6.7). LIVE/DEAD fixable violet dead cell stain (ViViD) were purchased from Molecular Probes, and staining was performed as described by Perfetto et al. (42). Intracellular staining was performed according to the BD Cytofix/Cytoperm kit instructions using APC–anti-IFN-γ (XMG1.2), phycoerythrin (PE)–Cy7–anti-tumor necrosis factor alpha (anti-TNF-α) (MP6-XT22), and PE–anti-IL-2 (MQ1-17H12), which were purchased from BD Biosciences, and Alexa Fluor 488–anti-IL-10 (JES5-16E3), which was purchased from eBioscience.

For staining of nonhuman primate (NHP) cells, the following anti-human antibodies were purchased from BD Pharmingen: purified anti-CD49d (9F10), APC–Cy7–anti-CD3 (SP34-2), PE–Cy7–anti-TNF-α (MAb11), PE–anti-IL-2 (MQ1-17H12), PE–Cy5–anti-CD95 (DX2), and fluorescein isothiocyanate (FITC)–anti-IFN-γ (B27). PacificBlue–anti-CCR7 (TG8/CCR7) was purchased from BioLegend. Anti-CD45RA–R phycoerythrin-Texas Red (ECD) (clone 2H4LDH11LDB9) was purchased from Beckman Coulter, and Qdot605–anti-CD4 (S3.5) was purchased from Invitrogen. The conjugates Alexa 680–anti-CD28 (CD28.2) and Qdot655–anti-CD8 (RPA-T8) were produced in-house by the laboratory of Mario Roederer (NIAID, NIH, Bethesda, MD).

Immunizations. C57BL/6 mice were immunized with 2 μg or 20 μg of CSP, with or without 50 μg poly(I·C)LC (Oncovir, Inc., Washington, DC) or 5 μg GLA-SE (Infectious Disease Research Institute, Seattle, WA) which are the optimal doses for these adjuvants. Naive mice or mice immunized with the adjuvant alone served as controls. The vaccines were administered subcutaneously (s.c.) in both hind footpads in a total volume of 50 μl per foot. Animals were immunized at weeks 0 and 3 or 0, 3, and 6.

Rhesus macaques were immunized with 100 μg of *P. falciparum* CSP mixed with 1 mg of poly(I·C)LC. A total volume of 1 ml was injected s.c. into the deltoid area of the upper arm. For boosting, the opposite arm was used. Animals were immunized at weeks 0, 5, and 16.
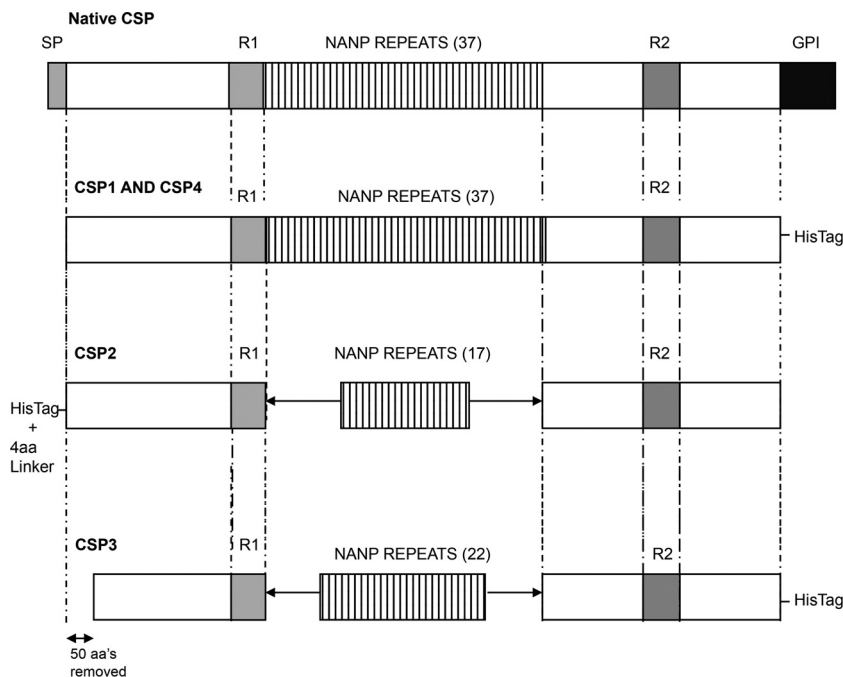
FIG 1 Graphic diagrams of the characteristics of four different CSPs. Full-length CSP consists of the signal peptide (SP), two highly conserved motifs at the N- and C-terminal ends of the protein, region 1 and region 2 plus (R1 and R2), tandem repeats (37 NANP repeats), and the GPI anchor signal (GPI). CSP1 and CSP2 were produced by expression in *Escherichia coli*, CSP3 and CSP4 by expression in *P. pastoris*.

Analysis of CD4$^+$ T cell responses. Cells from mice were harvested from spleens at various times postvaccination, and single-cell suspensions (2×10^6 cells/well) from individual mice were incubated for 5 h with anti-CD28, 10 μg/ml Brefeldin A, and 2 μg/ml CSP peptide pool. Cells were stained with the viability dye LIVE/DEAD fixable violet dead cell stain (ViViD), CD4, and CD8, followed by intracellular staining for CD3, IFN-γ, IL-2, IL-10, and TNF-α using the BD Cytofix/Cytoperm kit according to the manufacturer's instructions. For intrahepatic-lymphocyte isolation, the liver was perfused with phosphate-buffered saline (PBS) before being processed, and lymphocytes were isolated by Percoll density centrifugation. Stimulation and staining were performed as described for splenocytes in this section.

For analysis of NHP peripheral blood mononuclear cells (PBMCs), cryopreserved cells were thawed and rested overnight at 37°C. The next day, cells (2×10^6 cells/well) were stimulated for 5 h with anti-CD49d and anti-CD28, 10 μg/ml Brefeldin A, and 2 μg/ml CSP peptide pool. Cells were stained in warm medium with CCR7, followed by surface staining with a LIVE/DEAD fixable aqua dead cell stain kit (Invitrogen), CD45RA, CD95, CD4, and CD8 in PBS. Intracellular cytokine staining was performed using IFN-γ, IL-2, CD3, and TNF-α with the BD Cytofix/Cytoperm kit according to the manufacturer's instructions. Cells were resuspended in 1% paraformaldehyde, acquired on a modified BD LSR II flow cytometer, and analyzed using FlowJo software (Tree Star), Pestle, and SPICE (Mario Roederer, NIAID, NIH).

Detection of CSP-specific antibodies. Serum samples from immunized mice and NHPs were analyzed for IgG antibodies specific for the repeat region ([NANP]$_6$ peptide) of *P. falciparum* CSP by the Malaria Serology Laboratory at WRAIR. Plates were read at 414 nm, and endpoint titers were calculated at an optical density (OD) of 1.0. For analysis of total CSP-specific IgG1 and IgG2a titers, enzyme-linked immunosorbent assay (ELISA) plates were coated with CSP1 protein at a concentration of 1 μg/ml and washed. Serially diluted serum samples from immunized or control mice were added in duplicate for 2 h. After being washed, samples were incubated with either anti-mouse IgG1-horseradish peroxidase (HRP) or anti-mouse IgG2a-HRP. Plates were then developed with

3,3′,5,5′-tetramethylbenzidine substrate-chromogen (Dako) and read at 450 nm. Midpoint 50% effective concentration (EC$_{50}$) titers were calculated using 4PL curve fitting.

Immunofluorescent antibody (IFA) assay. To determine whether serum bound to sporozoites, slides (Tekdon Inc.; poly-L-lysine coated) were coated with a 10-μl suspension of sporozoites of *P. berghei* expressing the repeat region of CSP from *P. falciparum* [*Pb*-CS(*Pf*)] at a concentration of 4×10^5 to 6×10^5 sporozoites/ml and air dried. Serum samples from immunized mice or NHPs were diluted in PBS-1% bovine serum albumin (BSA) prior to their addition to the slides (10 μl of sample) and then incubated for 30 min at room temperature. Slides were then washed with PBS-1% BSA, and 10 μl of a secondary-antibody solution [Alexa Fluor 488 F(ab′)$_2$ fragment of goat anti-mouse IgG(H+L) (2 μg/ml; Invitrogen) and FITC-labeled goat anti-monkey IgG(H+L) (KPL) for mice and NHPs, respectively] was added for 30 min at room temperature; then slides were washed with PBS-1% BSA. Fluorescent sporozoites were visualized using an upright fluorescence microscope (Nikon Eclipse 90i). The lowest titer at which sporozoites were visualized was scored as positive. Binding of serum antibodies to sporozoites was scored as +++ (very good), ++ (good), + (weak), or − (no) binding.

Sporozoite challenge. Mice were challenged intravenously (i.v.) with 1.5×10^4 transgenic *Pb*-CS(*Pf*) sporozoites, kindly provided by E. Nardin (10). Approximately 40 h later, mice were euthanized to assess parasite burden in livers. Parasite loads were determined by quantitative PCR (qPCR) for *P. berghei* 18S rRNA (43). For the assessment of blood-stage parasitemia, mice were challenged i.v. with 1×10^3 of the transgenic *Pb*-CS(*Pf*) sporozoites. Starting on day 4, blood smears were taken and observed under a microscope. Smears were fixed with methanol (for 30 s) before being stained with a 10% Giemsa stain solution (Sigma-Aldrich) for 15 min.

Serum transfer. Serum was collected from individual mice 2 weeks after three immunizations with 20 μg CSP1 and poly(I·C)LC, and 0.5 ml was transferred i.v. immediately prior to challenge.

Isolation of PBMCs from NHPs. PBMCs were isolated from fresh blood by Ficoll density centrifugation using LeucoSep tubes (Greiner Bio
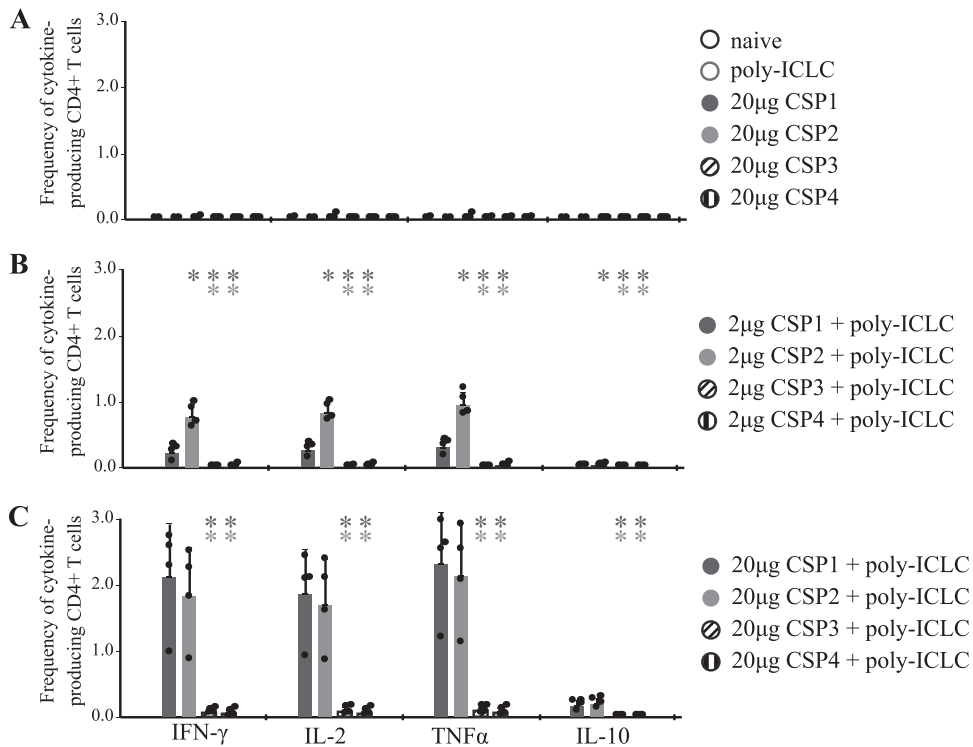
FIG 2 C57BL/6 mice ($n = 4$) were immunized twice, 3 weeks apart, with either 2 μg or 20 μg of various CSPs and 50 μg poly(I·C)LC. Naive mice and mice that received poly(I·C)LC alone or 20 μg of each protein alone served as controls. Four weeks later, T cells in spleens were analyzed for cytokine production by flow cytometry. Frequencies of CSP-specific IFN-γ-, IL-2-, TNF-α-, or IL-10-producing CD4$^+$ T cells are shown for protein-only and control mice (A) and mice that received either 2 μg (B) or 20 μg (C) of CSP with poly(I·C)LC. Data are expressed as means + SD and are representative of two independent experiments. *, $P < 0.05$. The color of the asterisk indicates the group to which the value is being compared.

One) according to the manufacturer's instructions. After several washes, cells were cryopreserved.

Statistics. The majority of the data and statistical analysis were created using Prism software (GraphPad) and a Mann-Whitney test. Differences were found to be significant when P was less than 0.05 or 0.01. Data are represented as means + standard deviations (SD) or as geometrical means, as indicated in the figure legends. Bar and pie charts of cytokine production were created using FlowJo software (Tree Star), Pestle, and SPICE (Mario Roederer, NIAID, NIH).

RESULTS

E. coli-derived CSP induces robust CD4$^+$ T cell cytokine responses. The goal of this blinded study was to compare the immunogenicities of four full-length CSPs (CSP1 to -4) in combination with different adjuvants. CSP1 and CSP2 were produced in *E. coli*, while CSP3 and CSP4 were expressed in the yeast *P. pastoris*. The amino acid sequences of CSP2 and CSP3, derived from the 3D7 strain of the malaria parasite, share strong homology at the amino- and carboxy-terminal regions but differ in their numbers of NANP repeats (Fig. 1). CSP1 and CSP4 are derived from the India strain IND637HDD1, contain all 37 NANP repeats, and share strong amino acid sequence homology. The 3D7 strain has a deletion in the N-terminal region (aa 91 to 97), whereas this region is intact in the India strain. We first selected poly(I·C)LC as an adjuvant based on its potency for generating T cell and antibody responses shown in prior studies by our lab and others (34, 35). C57BL/6 mice were immunized twice, 3 weeks apart, with 2 μg or 20 μg of CSP with or without 50 μg poly(I·C)LC. Four weeks after the second immunization, CD4$^+$ T cell responses in
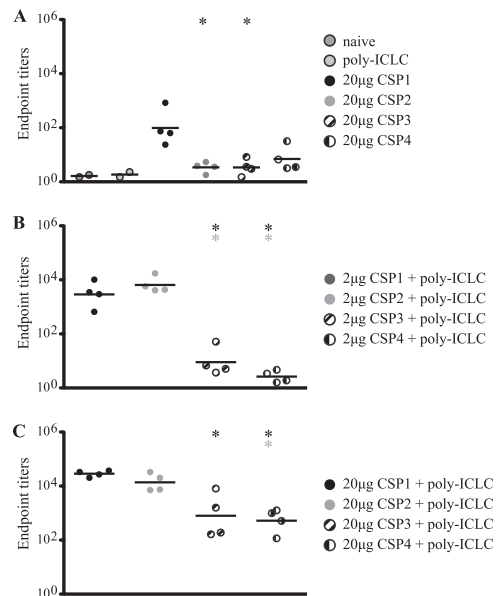
FIG 3 C57BL/6 mice ($n = 4$) were immunized as described in the legend of Fig. 2. At the time of tissue harvest, sera were collected and analyzed for CSP-specific IgG antibodies in protein-only and control mice (A) and mice that received either 2 μg (B) or 20 μg (C) of CSP with poly(I·C)LC. Antibodies were measured against part of the repeat region ([NANP]$_6$). Data points are graphed as geometrical means of endpoint titers and are representative of two independent experiments. *, $P < 0.05$. The color of the asterisk indicates the group to which the value is being compared.

FIG 4 C57BL/6 mice ($n = 6$) were immunized two or three times, 3 weeks apart, with 2 or 20 μg of CSP1 and 50 μg poly(I·C)LC. Naive mice and mice that received poly(I·C)LC alone or 20 μg of CSP1 alone served as controls. Four weeks later, mice were challenged with 1.5×10^4 Pb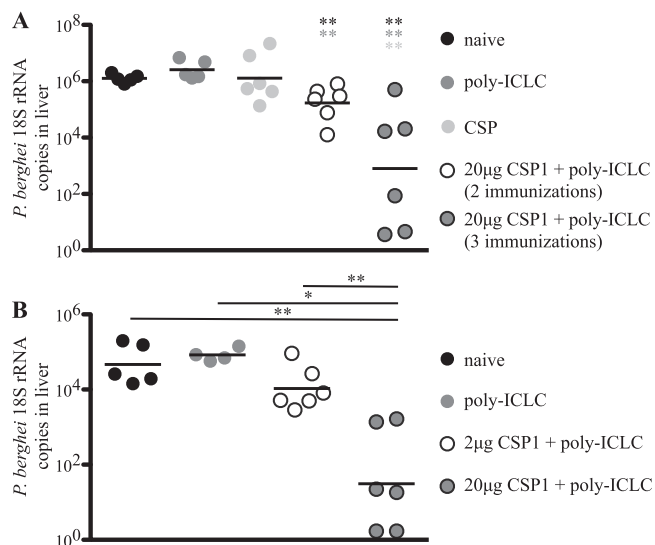-CS(Pf) sporozoites. Parasite burden was determined 40 h postchallenge by counting the copies of 18S rRNA in the livers by RT-PCR. Shown are comparisons of levels of protective immunity in mice that received either two or three immunizations of 20 μg CSP1 and poly(I·C)LC (A) or three immunizations with 2 μg or 20 μg CSP1 and poly(I·C)LC (B). Data points are graphed as geometrical means and are representative of two independent experiments*, $P < 0.05$; **, $P < 0.01$. The color of the asterisk (A) or the length of the bar (B) indicates the group to which the value is being compared.
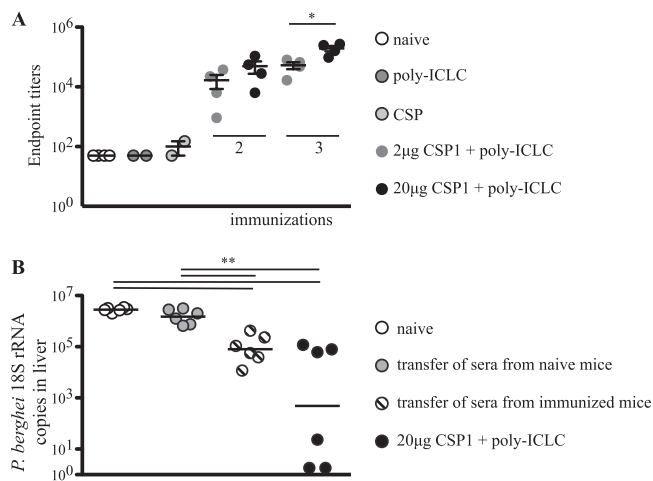

FIG 5 (A) C57BL/6 mice ($n = 4$ to 6) were immunized two or three times, 3 weeks apart, with 2 or 20 μg of CSP1 and 50 μg poly(I·C)LC. Naive mice and mice that received poly(I·C)LC alone or 20 μg of CSP1 alone served as controls. Two weeks later, sera were collected and CSP-specific IgG antibodies were determined. Data points are graphed as means + SD. *, $P < 0.05$. The values for all control groups are significantly lower than those for vaccine groups. (B) Serum from mice immunized three times with CSP1 and poly(I·C)LC or serum from naive mice was transferred into naive mice at the time of challenge with 1.5×10^4 Pb-CS(Pf) sporozoites. Vaccinated mice (CSP1 and poly(I·C)LC) served as a positive control. Forty hours postchallenge, parasite burden was determined by counting the copies of 18S rRNA in the livers by RT-PCR. Black bars show the geometrical means. **, $P < 0.01$. Results are representative of at least two independent experiments.

the spleens were assessed by multiparameter flow cytometry. Mice immunized with poly(I·C)LC or any of the CSPs without adjuvant had no measurable CSP-specific CD4$^+$ T cell cytokine responses (Fig. 2A). In contrast, 2 μg of CSP1 and CSP2 administered with poly(I·C)LC induced robust CD4$^+$ T cell cytokine responses, producing IFN-γ, IL-2, and TNF-α (Fig. 2B). Such responses were increased ~2- to 3-fold by using 20 μg of CSP with poly(I·C)LC (Fig. 2C). Remarkably, both CSPs produced in yeast (CSP3 and CSP4) failed to induce significant antigen-specific CD4$^+$ T cell responses with poly(I·C)LC, even at the higher dose (20 μg).

E. coli-derived CSP induces antibodies against the NANP repeat region. Since antibodies against CSP are critical for protection, we assessed responses against the NANP repeat region following immunization of all vaccine groups. Consistent with the data in Fig. 2, CSP1 and CSP2 vaccines produced in E. coli elicited significantly more potent antibody responses than did CSP3 and CSP4 expressed in yeast (Fig. 3A to C). Higher antibody titers were observed in mice that received the higher dose (20 μg) of CSP and poly(I·C)LC. Taken together, the E. coli-derived proteins were more potent for CSP-specific antibody and CD4$^+$ T cell immunity in this study with poly(I·C)LC or with CpG as an adjuvant (data not shown). Therefore, CSP1 was selected for further testing.

Protection requires three immunizations with CSP and poly(I·C)LC. We next sought to establish the optimal antigen dose and number of immunizations required to induce protection against a challenge. Because mice are not susceptible to P. falciparum, we used a recombinant P. berghei parasite expressing the P. falciparum CSP repeat region [Pb-CS(Pf)] (10) as a challenge for our P. falciparum CSP-vaccinated mice. Mice were immunized two or three times at 3-week intervals with 20 μg of CSP1 and

poly(I·C)LC. Four weeks after the last immunization, mice were challenged i.v. with 1.5×10^4 Pb-CS(Pf) parasites. Forty hours later, livers of infected mice were harvested and parasite-derived 18S rRNA was measured by real-time qPCR (RT-qPCR). Mice that received two immunizations with 20 μg of CSP1 and poly(I·C)LC showed a modest but significant (~0.5 log) reduction ($P < 0.01$) of parasite-derived 18S rRNA levels compared to naive mice or mice that received poly(I·C)LC or CSP1 alone (Fig. 4A). Moreover, three immunizations with 20 μg CSP1 and poly(I·C)LC resulted in an ~2- to 4-log reduction in 18S rRNA (Fig. 4A) compared to levels in mice immunized with protein alone, poly(I·C)LC alone, or CSP1 protein and poly(I·C)LC (two immunizations). A separate series of experiments sought to determine whether the dose of CSP1 influenced protection. We compared three immunizations with 2 or 20 μg of CSP1 and poly(I·C)LC. As shown in Fig. 4B, the higher dose of CSP1 provided a significant ($P = 0.005$) (~2-log) reduction in parasite load in the livers of mice compared to those with the lower dose of CSP1. Overall, these data show that three immunizations with the higher dose (20 μg) of CSP1 and poly(I·C)LC provided significant reduction of parasite load against a high-dose i.v. challenge with sporozoites.

Antibody against CSP correlates with protection. In the next series of experiments, we sought to determine the immunological correlates of protection following immunization with CSP1 and poly(I·C)LC. Prior studies in mice and humans showed that antibodies and CD4$^+$ T cells play a role in protection (20, 22, 44–48). In Fig. 3, we show that two immunizations with 2 or 20 μg of CSP1 induced potent antibody responses against the NANP repeat region. To extend this analysis, we assessed CSP-specific antibody
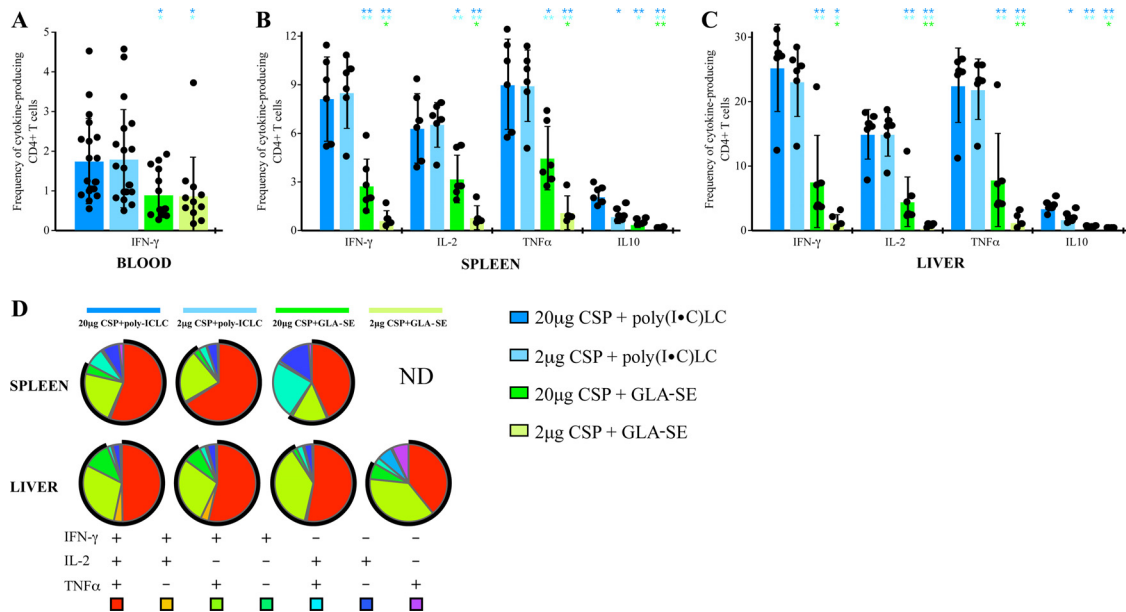
FIG 6 C57BL/6 mice (n = 6 to 18) were immunized three times, 3 weeks apart, with the indicated doses of CSP1 with either 50 μg poly(I·C)LC or 5 μg GLA-SE as the adjuvant. Mice that received poly(I·C)LC, GLA-SE, or CSP1 alone, mice that received CSP1 and SE, or naive mice served as controls (not shown). CD4$^+$ T cells in blood, spleen, and liver were analyzed for cytokine production by flow cytometry. (A) Frequencies of CSP-specific IFN-γ-producing CD4$^+$ T cells in the blood 7 days after the third immunization; (B, C) frequencies of CSP-specific IFN-γ-, IL-2-, TNF-α-, or IL-10-producing CD4$^+$ T cells in the spleen (B) and liver (C) 2 weeks after three immunizations; (D) relative proportions of each individual combination of IFN-γ-, IL-2-, or TNF-α-producing cells in the spleen and liver 2 weeks after three immunizations. ND, not determined (because the frequency was too low). The black circle represents the percentage of CD4$^+$ T cells that make IFN-γ. Bars show the means ± SD. *, $P < 0.05$; **, $P < 0.01$. All control groups had values below the limit of detection. The color of the asterisk indicates the group to which the value is being compared. Results are representative of two independent experiments.

titers at both doses of CSP after two or three immunizations. As shown in Fig. 5A, the greatest antibody response was detected with the higher dose of CSP1 given three times, consistent with improved protection in this group (Fig. 4). To substantiate a direct role of antibodies in mediating protection, pooled serum from mice immunized three times with 20 μg CSP1 and poly(I·C)LC was transferred into naive recipients, and then the mice were challenged. As a negative control, serum from naive mice was transferred to naive mice. As a positive control, mice immunized three times with 20 μg of CSP1 and poly(I·C)LC were challenged at the same time. Naive mice that received serum from CSP1- and poly(I·C)LC-vaccinated animals showed an ~1-log reduction in parasite burden compared to mice that received control serum or naive mice (Fig. 5B). However, this protection was not as robust as that seen in animals immunized with CSP1 and poly(I·C)LC. This could be due to dilution of transferred antibodies in the blood of recipient animals or to a contribution of vaccine-elicited CD4$^+$ T cells absent in passively transferred animals. Nevertheless, these data show that humoral immunity is sufficient to confer some protection against this high-dose challenge.

Poly(I·C)LC induces higher CD4$^+$ Th1 cell immunity than GLA-SE. The RTS,S vaccine has been formulated with the TLR4 ligand MPL and QS-21 in either a liposomal solution (AS01) or oil-in-water emulsion (AS02). Thus, to compare poly(I·C)LC (which signals through TLR3 and MDA-5) to an adjuvant with some commonality to AS02, we choose GLA-SE. This formulation consists of a synthetic form of the TLR4 agonist lipid A, which has been shown to provide adjuvant activity that is similar to, if not better than, that of MPL-SE (49).

Mice were immunized three times with 2 μg or 20 μg of CSP1

formulated with either poly(I·C)LC or GLA-SE, and the CD4$^+$ T cell responses were analyzed in the blood, spleen, and liver 7 to 14 days after the third vaccination. At this peak time point, the frequencies of IFN-γ-producing CD4$^+$ T cells in the blood (Fig. 6A) were similar when we compared the 2-μg and 20-μg doses of CSP1 protein using either poly(I·C)LC or GLA-SE as an adjuvant. However, CD4$^+$ IFN-γ responses were significantly higher when poly(I·C)LC was used than when GLA-SE was used for both doses of CSP1 (Fig. 6A). Moreover, in spleen (Fig. 6B) and liver (Fig. 6C), CD4$^+$ T cell responses were also significantly higher using poly(I·C)LC than using GLA-SE. Mice immunized with either adjuvant alone or CSP1 alone showed no detectable CD4$^+$ T cell responses (data not shown). Lastly, we did not detect any CD8$^+$ T cell responses (data not shown), consistent with the absence of a *P. falciparum* CSP-specific CD8$^+$ T cell epitope in C57BL/6 mice.

To extend this analysis, we assessed the quality of the CD4$^+$ T cell cytokine response based on the relative proportions of cells producing IL-2, IFN-γ, and TNF-α, either alone or simultaneously. The quality of the CD4$^+$ T cell response may be an important metric because of evidence that multifunctional CD4$^+$ T cells correlate with protection (20, 23, 50). As shown in Fig. 6D, ~50% of the total cytokine-producing responses were multifunctional, producing IL-2, TNF-α, and IFN-γ, with another ~25% making IFN-γ and TNF-α without IL-2. Thus, both adjuvants elicit the induction of CD4$^+$ T cells that secrete two critical effector cytokines that may mediate parasite killing (51–53).

Poly(I·C)LC and GLA-SE confer comparable levels of protection correlating with antibody titers. We next compared antibody responses induced with 2 or 20 μg of CSP1 and GLA-SE to poly(I·C)LC following three immunizations. In contrast to the
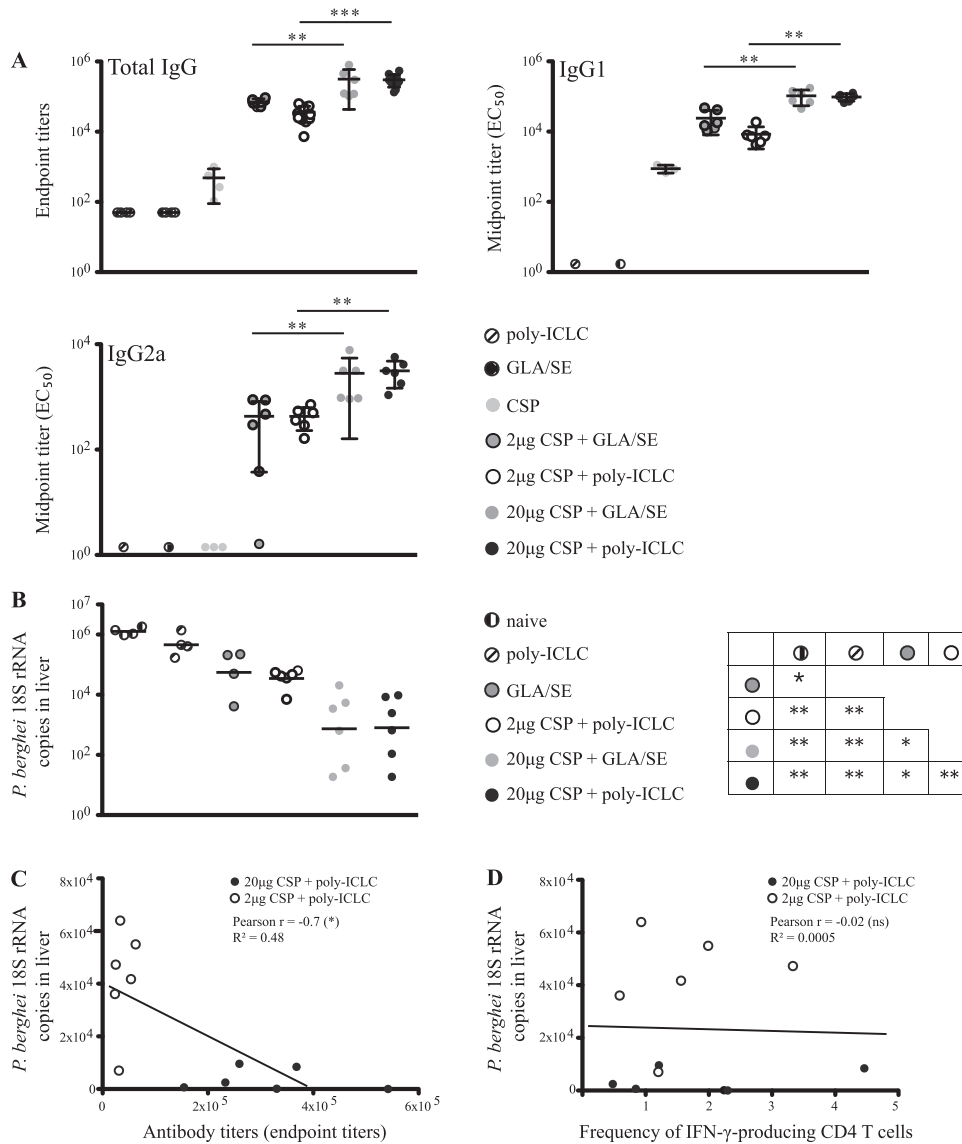
FIG 7 C57BL/6 mice ($n = 6$) were immunized three times, 3 weeks apart, with the indicated doses of CSP1 together with either 50 μg poly(I·C)LC or 5 μg GLA-SE. Mice that received poly(I·C)LC, GLA-SE, or CSP1 (20 μg) alone or naive mice served as controls. (A) Two weeks after the third immunization, sera were collected and CSP-specific IgG, IgG1, and IgG2a antibodies were measured. Bars are graphed as means ± SD. All control groups showed significantly lower antibody titers than the vaccine groups. (B) Four weeks after the third immunization, mice were challenged with 1.5×10^4 *Pb*-CS(*Pf*) sporozoites. Forty hours postchallenge, parasite burden was determined by counting 18S rRNA copies in the livers by RT-PCR. Black bars show the geometrical means. The matrix (B) represents significances among various immunization groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) Correlation between antibody titers and parasite burden in the liver. (D) Correlation between frequency of IFN-γ-producing CD4^{+} T cells in the blood (day 7) and parasite burden in the liver. (C and D) As indicated, Pearson's *r* was calculated. Results are representative of at least two independent experiments.

magnitudes of CD4^{+} T cell cytokine responses, which were significantly different, the total titers of IgG against the NANP repeats measured with the two adjuvants were not significantly different (Fig. 7A, upper left). Of note, the lower dose (2 μg) of CSP1 consistently induced ~2- to 5-fold-lower total antibody titers than the higher dose (20 μg) for both adjuvants. Similar results were seen after analyzing IgG1 and IgG2a antibody titers (Fig. 7A). In terms of protection, there was a 2- to 3-log reduction of 18S rRNA after challenge with *Pb*-CS(*Pf*) in mice immunized with 20 μg of CSP1 with poly(I·C)LC compared to that in naive mice, consistent with the results of previous experiments (Fig. 4). Immunization with 20 μg of CSP1 and

GLA-SE resulted in similar levels of protection (Fig. 7B). Thus, in a setting of equivalent antibody titers, there was no significant difference in protection using poly(I·C)LC or GLA-SE as an adjuvant.

To determine whether humoral or cellular immune responses correlated with protection, prechallenge antibody titers (Fig. 7C) and CD4^{+} T cell cytokine responses (Fig. 7D) were plotted against the level of protection achieved upon consecutive challenge. There was a highly significant correlation between CSP-specific antibody titers and the level of 18S rRNA measured in livers after parasite challenge (Pearson $r = -0.7$) (Fig. 7C). In contrast, there was no correlation between the frequency of IFN-γ-producing CD4^{+} T

TABLE 1 Antibodies in sera from mice that were immunized with a high dose of CSP1 and adjuvant showed a strong binding capacity to sporozoites *in vitro*

Vaccine	Binding of serum antibodies to sporozoites as determined by fluorescence intensity at an antibody dilution of[a]:					
	1:300	1:900	1:2,700	1:8,100	1:24,300	1:72,900
Poly(I·C)LC	−	−	−	−	−	−
GLA-SE	−	−	−	−	−	−
CSP	+	+/−	−	−	−	−
2 µg CSP + GLA-SE	++	++	+	+	−	−
2 µg CSP + poly(I·C)LC	++	+	+/−	+/−	−	−
20 µg CSP + GLA-SE	+++	++	++	+	+	+/−
20 µg CSP + poly(I·C)LC	+++	+++	++	+	+	+/−

[a] Serum samples that were collected 2 weeks after the third immunization (as described in the legend of Fig. 6) were pooled by group. The serum pools at different dilutions were incubated on slides coated with fixed sporozoites. The slides were washed and incubated with a secondary fluorescently labeled antibody. Finally, slides were analyzed under a microscope. +++, very good binding; ++, good binding; +, weak binding; −, no binding. Results are representative of two independent experiments.

cells in the blood and the level protection after parasite challenge (Pearson $r = -0.02$) (Fig. 7D). Finally, to confirm that our vaccine-elicited CSP-specific antibodies had binding activity against the intact parasite, we used an IFA assay to visualize direct antibody binding to sporozoites (Table 1). Consistent with the CSP-specific antibody titers, poly(I·C)LC and GLA-SE induced strong and comparable IFA titers when they were administered with 20 µg of CSP1 protein. Taken together, these data show that poly(I·C)LC and GLA-SE differ in their capacities to induce $CD4^+$/Th1 immune responses after immunization with CSP; however, they induce comparable antibody responses that correlate best with protection.

While reduction in parasite load in the liver following a high-dose i.v. challenge may have important clinical consequences by reducing the prepatent period and time to infection (20, 54, 55), the most important and clearest clinical outcome is sterilizing immunity. Thus, mice vaccinated with CSP1 and poly(I·C)LC were i.v. challenged with a low dose of 1×10^3 *Pb*-CS(*Pf*) sporozoites and monitored daily by blood smear to establish the onset of infection. As shown in Fig. 8, all naive mice and mice immunized with poly(I·C)LC alone developed parasitemia within 5 days. In contrast, 5 out of 10 mice immunized with CSP1 and poly(I·C)LC did not develop parasitemia during the 15 days of follow-up. Also, the prepatent period was significantly delayed ($P < 0.05$) in mice that received CSP1 and poly(I·C)LC (5.8 days) compared to mice that received poly(I·C)LC alone or naive animals (4.6 days and 4.8 days, respectively) (Fig. 8A; Table 2). Analysis of prechallenge sera from mice vaccinated with CSP1 and poly(I·C)LC revealed that protected mice from this group displayed significantly higher antibody titers than the nonprotected mice from this same vaccine group (Fig. 8B). These data are consistent with human trials showing that a very high level of antibody is required for sterilizing immunity (20, 56).

CSP and poly(I·C)LC induce humoral and cellular responses in NHPs. Based on the results from the mouse studies, we determined whether CSP1 and poly(I·C)LC could induce potent antibody and $CD4^+$ T cell responses in NHPs. NHPs are a more useful animal model than mice for testing vaccine adjuvants because of greater similarities to humans, with respect to innate immune pathways and TLR expression. Rhesus macaques received either two or three immunizations of 100 µg CSP1 and poly(I·C)LC, and $CD4^+$ T cell and antibody responses were assessed at different time points after immunization. For $CD4^+$ T cell cytokine responses, a batch analysis was done on frozen PBMCs from various

time points. As shown in Fig. S1 in the supplemental material, as with the mouse data in Fig. 2, the frequency of CSP-specific cytokine-producing $CD4^+$ T cells is increased in NHPs that received three immunizations rather than only two immunizations (see Fig. S1A and B in the supplemental material), with a substantial portion of multifunctional cells secreting IFN-γ, IL-2, and TNF-α or IFN-γ and TNF-α (Fig. S1C and D). There were also robust CSP antibody titers that were maximal after two immunizations with CSP1 and poly(I·C)LC. Waning antibody titers could be boosted with a third immunization but did not exceed the levels that were reached after two immunizations (Fig. S1E and F). Moreover, in the IFA assay, sera from animals that received three immunizations contained antibodies with a stronger binding ca-

FIG 8 C57BL/6 mice ($n = 10$ per group) were immunized three times, 3 weeks apart, with 20 µg of CSP1 together with 50 µg poly(I·C)LC as the adjuvant. Mice that received poly(I·C)LC or naive mice served as controls. (A) Five weeks later, mice were challenged i.v. with 1×10^3 *Pb*-CS(*Pf*) sporozoites. Starting on day 4, daily blood smears were taken and analyzed under a microscope. Kaplan-Meier plots show the time to detection of parasites in the blood for each vaccine group after the challenge. (B) Sera collected 3 weeks before challenge were analyzed for CSP-specific IgG antibodies. Endpoint titers are grouped by whether mice went on to be protected from challenge. *, $P < 0.05$. Results are representative of two independent experiments.

TABLE 2 Vaccination with CSP1 and poly(I·C)LC prevents 50% of mice from developing parasitemia after a low-dose sporozoite challenge[a]

Immunization	No. of infected/no. of challenged mice	Prepatent period (days)	Protection (%)
20 μg CSP + poly(I·C)LC	5/10	5.8	50
Poly(I·C)LC	10/10	4.6	0
None (naive mice)	10/10	4.8	0

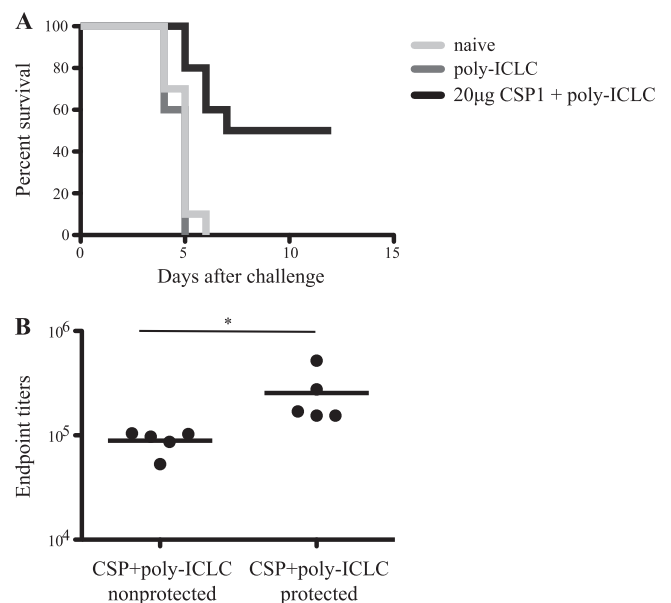[a] C57BL/6 mice (n = 10/group) were immunized three times, 3 weeks apart, with 20 μg CSP1 together with 50 μg poly(I·C)LC as the adjuvant. Mice that received poly(I·C)LC only or naive mice served as controls. Five weeks after the last immunization, mice were challenged i.v. with 1×10^3 Pb-CS(Pf) sporozoites. Starting at day 4, daily blood smears were taken and observed under a microscope to look for the onset of parasitemia. Results are representative of two independent experiments.

pacity than those from animals that received two immunizations (see Table S1 in the supplemental material). Therefore, we conclude that the CSP1 protein and poly(I·C)LC are strongly immunogenic in NHPs.

DISCUSSION

Currently, the RTS,S vaccine administered with AS01 is the most advanced malaria vaccine, providing ~30 to 50% protection in humans (2, 3). The recent results of the pivotal phase III clinical efficacy trial were a major breakthrough in establishing that a protein/particle-based vaccine with a well-formulated adjuvant system could achieve some protective efficacy against a parasite infection and confirms this approach as a first step toward developing a successful malaria vaccine. In the study presented here, we compared various full-length CS proteins for their ability to induce strong immunity and protection. Such proteins were provided by four partners, in partnership with MVI, to enable direct comparison and down-selection of one or more proteins for further development, thereby providing a potentially simpler formulation than RTS,S/AS01 with comparable or improved efficacy. The data presented here using poly(I·C)LC as an adjuvant show a striking difference between the immunogenicities elicited by *E. coli*-derived CSP and yeast-derived CSP. The relatively low responses to the yeast-derived protein were surprising, since RTS,S (as well as other successful protein-based vaccines) is produced in yeast (57) and one of the yeast-derived proteins used in this study when given with Montanide did elicit potent antibody titers in mice (data not shown), showing that at least for antibody responses, the yeast-derived protein was immunogenic. We speculate that differences in the amino acid sequences or mannosylation sites of CSPs expressed in yeast and *E. coli* may have accounted for the limited immunogenicity. Indeed, one of the yeast-derived proteins induced higher antibody and CD4^{+} T cell responses when the same amino acid sequence was expressed in *E. coli* (data not shown). However, modifications in the mannosylation sites did not significantly alter the immunogenicity of yeast-expressed CSP (data not shown). Therefore, the mechanistic basis for the differences in immunogenicity of yeast- and *E. coli*-expressed CSPs used in this experiment is not entirely clear. In view of these results, CSP1 was used with the two adjuvants, poly(I·C)LC and GLA-SE, for the remainder of the studies.

Both poly(I·C)LC and GLA-SE elicited strong and comparable levels of CSP-specific IgG antibody titers, which conferred similar degrees of protection against high-dose sporozoite challenge. However, poly(I·C)LC induced a substantially higher frequency of CSP-specific CD4^{+} T cell cytokine responses than GLA-SE. Moreover, such responses were comprised of a large percentage of multifunctional cells secreting IFN-γ, IL-2, and TNF-α, which have been proposed to contribute to antimalarial protection after RTS,S/AS01 vaccination (20, 23). Of note, while vaccination with CSP (*P. vivax*) and GLA-SE in mice has been shown to induce a robust CD4^{+} T cell effector response (58), the same vaccine induced monofunctional (IL-2 only) CD4^{+} T cell responses in NHPs (49). In contrast, we show that CSP and poly(I·C)LC induced robust multifunctional Th1 CD4^{+} T cell responses in both mice and NHPs. These data highlight potential differences in the magnitudes and quality of CD4^{+} T cell responses induced in mice and NHPs and are consistent with the results of other studies showing poly(I·C) or poly(I·C)LC to be an especially potent adjuvant in NHPs for such responses (34, 59). Finally, it is notable that despite the dramatic differences in the CD4^{+}/Th1 responses between poly(I·C)LC and GLA-SE in mice, the antibody titers were similar. These data highlight the possibility that adjuvants can differentially mediate effects on antibody and Th1 immunity, which is currently being investigated.

In terms of correlates of protection, both antibody and CD4^{+} T cells have been described to occur in mice or humans with CSP vaccines. Here, we show that the antibody titers using poly(I·C)LC or GLA-SE strongly correlate with protection (Fig. 7C). Moreover, serum transfer from CSP-immunized animals reduced the parasite burden after high-dose i.v. challenge. Thus, antibodies are necessary and sufficient in this model. Of note, protection was best with the higher dose of CSP (20 μg), which induced an ~1-log increase in antibody titer compared to that induced by the lower dose of protein (2 μg). It is notable that, in humans, 25 μg or 50 μg of RTS,S induced antibody titers better than and comparable to those induced by 10 μg, suggesting some dose effect similar to what we observed in this study (60). This suggests that there may be a threshold for the amount of CSP-specific antibody required to provide or increase protection. Indeed, when we analyzed antibody titers from protected and nonprotected animals (Fig. 7C and 8B), there was a threshold for the amount of antibody required to mediate protection after low- or high-dose challenge. This is consistent with data from humans in which anti-CSP antibody titers needed to be above 40 EU/ml (56) to induce protection. In addition, we show that nonprotected mice had a significantly delayed prepatent period compared to the control animals (Table 2). Accordingly, studies using RTS,S and different adjuvants in humans also show that the prepatent period can be delayed. At present, it still is not clear whether this delay might play a role in improving the clinical outcome of a malaria infection (20, 54, 55).

Regarding the role of cellular immunity in mediating protection, previous studies have shown that CD4^{+} T cells specific for *P. yoelii* CSP eliminate infected hepatocytes *in vitro* and, when adoptively transferred, mediate protection *in vivo* (61, 62). The protective role of CD4^{+} effector cells against malaria was further substantiated in other murine and human malaria models (22, 63, 64). In data not shown, antibody depletion of CD4^{+} T cells in mice immunized with CSP1 and poly(I·C)LC at the time of sporozoite challenge resulted in only modestly reduced protection. Hence, in our model, the data support a far more critical role for CSP antibodies in mediating protection than for CD4^{+} effector T

cells. Nevertheless, human studies using the AS02 or AS01 adjuvant showing that improved CD4$^+$ T cell responses were associated with improved protection after challenge with *P. falciparum* (20, 22) provide evidence for the importance of these T cells. Therefore, the ability of poly(I·C)LC to elicit such potent CD4$^+$ T cells in blood, liver, and spleen may be of importance in humans through a variety of mechanisms. Indeed, IFN-γ has been shown to inhibit the development of liver-stage malaria in various animal models (52, 65). That would provide a potential advantageous role for poly(I·C)LC, based on the high frequency of those cells detected in the livers of immunized mice.

There is also strong evidence for cytotoxic CD8$^+$ T cells in mediating protective immunity against liver-stage malaria (18, 46, 66, 67). As our study used a strain of mice in which there was no major histocompatibility complex (MHC) class I recognition of *P. falciparum* CSP peptides, we did not detect any CD8$^+$ T cell responses. However, we and others have previously shown that type I IFN is essential for mediating cross-priming of protein in mice (68, 69), highlighting the potential advantage of poly(I·C)LC over other adjuvants (25). Nevertheless, while adjuvants that induce type I IFN should be preferred to induce CD8$^+$ T cell responses upon protein vaccination, virus-based vaccines seem preferable if robust CD8$^+$ T cell immunity is required. Improving how the protein is formulated and targeting it to specific dendritic cell subsets may ultimately improve the capacity of such vaccines to consistently and efficiently induce CD8$^+$ T cells.

In conclusion, we show that a full-length *P. falciparum* CS protein when combined with poly(I·C)LC or GLA-SE induces potent immunity and protection in mice. Moreover, poly(I·C)LC is an effective adjuvant for eliciting such responses in NHPs, providing a predictive model for what might occur in humans (20, 21, 70, 71). It is possible that the increased breadth of CSP responses with a full-length CSP (compared to the CSP responses with truncated RTS,S) and/or the enhanced CD4$^+$ Th1 immunity that would potentially be elicited by poly(I·C)LC would enhance protection and/or durability. Whether a full-length protein vaccine with an adjuvant would substantially improve the outcome over the current platforms of RTS,S/AS01 and RTS,S/AS02, which have the advantages of being particles with well-formulated adjuvants and of being safe and scalable, remains a question for further development. Our study shows that a protein platform can provide effective protection against malaria. The key question for future studies is whether this protection will also provide a prolonged durability compared to that of the RTS,S vaccine.
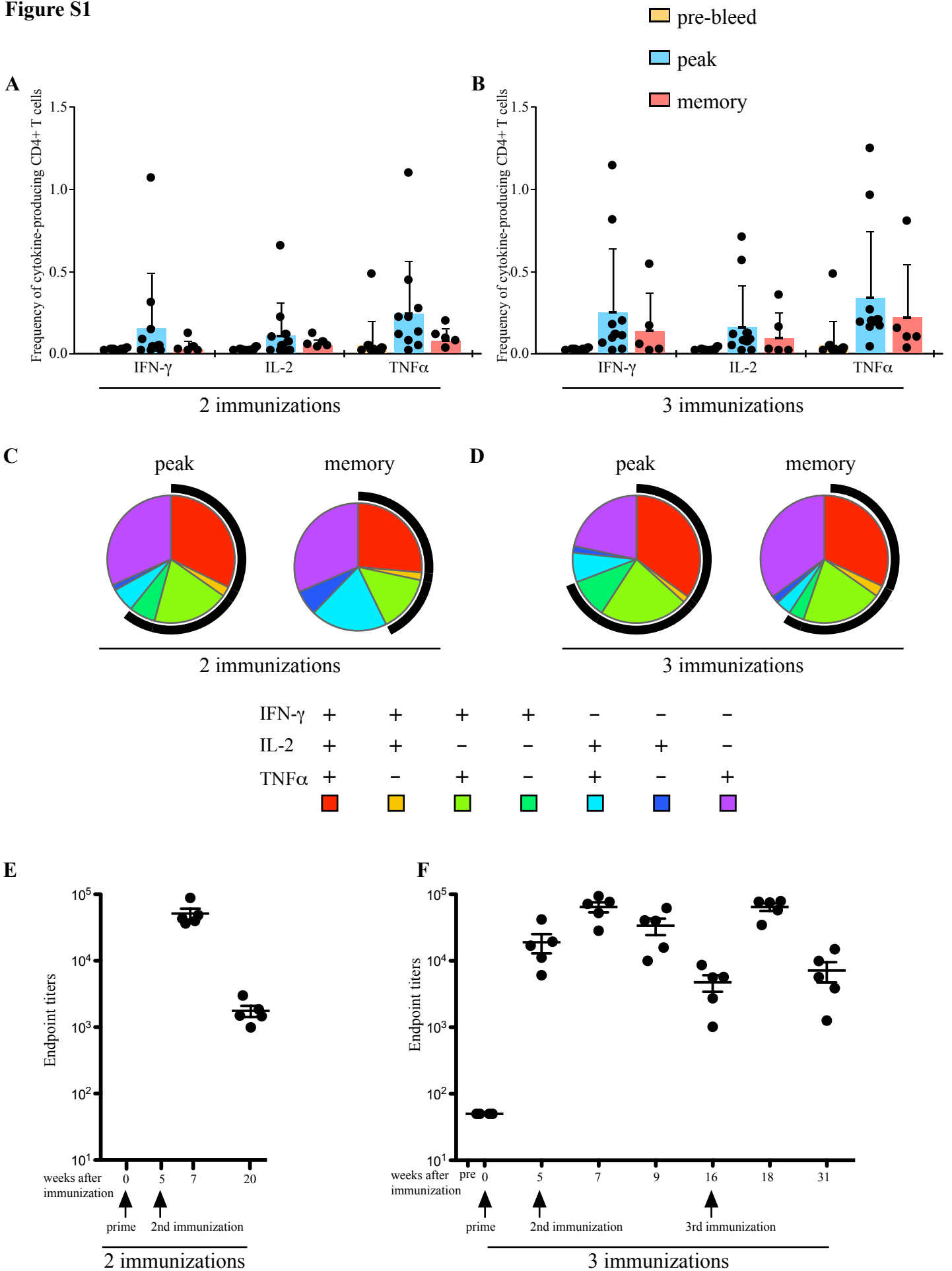
ACKNOWLEDGMENTS

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Figure S1

A

Frequency of cytokine-producing CD4+ T cells

pre-bleed
peak
memory

IFN-γ   IL-2   TNFα

2 immunizations

B

Frequency of cytokine-producing CD4+ T cells

IFN-γ   IL-2   TNFα

3 immunizations

C

peak   memory

2 immunizations

D

peak   memory

3 immunizations

IFN-γ	+	+	+	+	−	−	−
IL-2	+	+	−	−	+	+	−
TNFα	+	−	+	−	+	−	+

E

Endpoint titers

10^5
10^4
10^3
10^2
10^1

weeks after immunization   0   5   7   20

prime   2nd immunization

2 immunizations

F

Endpoint titers

10^5
10^4
10^3
10^2
10^1

weeks after immunization   pre   0   5   7   9   16   18   31

prime   2nd immunization   3rd immunization

3 immunizations

Supplementary Figure 1:

Rhesus macaques were immunized with 100μg of CSP1 protein mixed with 1mg of poly-ICLC. A total volume of 1ml was injected s.c. in the deltoid area of the upper arm. For boosting, the opposite arm was used. NHPs were immunized at weeks 0, 5 and 16. At indicated time-points after immunizations blood was collected and PBMCs were used for stimulation for ICS. Serum was also collected at indicated time-points to determine antibody titers. Frequency of CSP-specific IFN-γ-, IL-2- or TNFα-producing CD4+ T cells in PBMCs collected at peak (two weeks) and memory (15 weeks) after two (A) or three (B) immunizations. Relative proportion of each individual combination of IFN-γ-, IL-2-, or TNFα-producing cells in PBMCs collected at peak (two weeks) and memory (15 weeks) after two (C) or three (D) immunizations. (E and F) CSP-specific IgG antibody titers.

Supplementary Table 1:

TIME-POINT	FLUORESCENT INTENSITY BY DILUTION					
	1:100	1:300	1:900	1:2700	1:8100	1:24300
PRE	-	-	-	-	-	-
2 weeks after 2 immunizations	++	++	++	+	-	-
2 weeks after 3 immunizations	+++	+++	++	+	+/-	+/-
15 weeks after 2 immunizations	+	+	+/-	-	-	-
15 weeks after 3 immunizations	++	+	+/-	+/-	-	-

Supplementary Table 1: Antibodies in serum from monkeys that received three immunizations with CSP1 and poly-ICLC adjuvant showed a strong binding capacity to sporozoites *in vitro*. Serum was collected at indicated time-points and IFA was performed as described in Table 1. +++ very good binding, ++ good binding, + weak binding, - no binding.