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VACCINES

A Prime/Boost PfCS14K^{M}/MVA-sPfCS^{M} Vaccination Protocol Generates Robust CD8^{+} T Cell and Antibody Responses to *Plasmodium falciparum* Circumsporozoite Protein and Protects Mice against Malaria

Aneesh Vijayan,[a]* Ernesto Mejías-Pérez,[a] Diego A. Espinosa,[b] Suresh C. Raman,[a] Carlos Oscar S. Sorzano,[c] Fidel Zavala,[b] Mariano Esteban[a]

Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas (CNB-CSIC), Madrid, Spain[a]; Department of Molecular Microbiology and Immunology, Malaria Research Institute, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, USA[b]; Biocomputing Unit, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas (CNB-CSIC), Madrid, Spain[c]

ABSTRACT Vaccines against the preerythrocytic stages of malaria are appealing because the parasite can be eliminated before disease onset and because they offer the unique possibility of targeting the parasite with both antibodies and T cells. Although the role of CD8^{+} T cells in preerythrocytic malaria stages is well documented, a highly effective T cell-inducing vaccine remains to be advanced. Here we report the development of a prime-boost immunization regimen with the *Plasmodium falciparum* circumsporozoite protein (PfCS) fused to the oligomer-forming vaccinia virus A27 protein and a modified vaccinia virus Ankara (MVA) vector expressing PfCS. This protocol induced polyfunctional CD8^{+} T cells with an effector memory phenotype and high PfCS antibody levels. These immune responses correlated with inhibition of liver-stage parasitemia in 80% and sterile protection in 40% of mice challenged with a transgenic *P. berghei* parasite line that expressed PfCS. Our findings underscore the potential of T and B cell immunization strategies for improving protective effectiveness against malaria.

KEYWORDS *P. falciparum* circumsporozoite, oligomeric PfCS, MVA-PfCS, polyfunctional CD8^{+} T cells, antibodies, transgenic *P. berghei*, malaria vaccine

Malaria continues to be a global killer, with annual mortalities of as high as 438,000 for all age groups and 306,000 for children under the age of 5 years (http://www.who.int/malaria/media/world-malaria-report-2015/en/). The circumsporozoite (CS) protein, the major surface component of the malaria sporozoite, has been studied extensively and is a leading preerythrocytic vaccine candidate (1–3). Many studies highlight the importance of CS protein-specific antibodies and T cells in mediating protection in both animals and human subjects (3–8). Although RTS,S, currently the only malaria vaccine being considered for licensing, is based on the CS protein, its protective efficacy is only 50% in children for the first year and tends to decline rapidly (9–11). RTS,S-mediated protection is attributed mainly to the high titer of anti-CS protein antibodies (9–12). An ideal preerythrocytic vaccine should also generate polyfunctional cytotoxic T cell responses that target liver stages of the parasite. The next generation of preerythrocytic malaria vaccines should thus aim at inducing both humoral and cell-mediated immunity. Studies with murine malaria models have shown that to achieve protection, large numbers of CS protein-specific memory CD8^{+} T cells are needed (13).

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Address correspondence to Mariano Esteban, mesteban@cnb.csic.es.

* Present address: Aneesh Vijayan, Malaria Parasite Biology & Vaccines Unit, Department of Parasites & Insect Vectors, Institut Pasteur, Paris, France.

A.V. and E.M.-P. contributed equally to this article.

It is therefore imperative for protection that a successful preerythrocytic malaria vaccine must generate polyfunctional memory $CD8^+$ T cells as well as high antibody levels.

Previous studies showed that multimerization of an antigen enhances its immunogenicity (14, 15). In agreement with these studies, we showed that oligomerization of the CS protein from *Plasmodium yoelii* by fusion with the vaccinia virus 14K protein (A27) enhances its immunogenicity by inducing a type I interferon response in macrophages (8). A recent study reported that a similar type I interferon response, mediated via IRF-3 and STAT-1, is essential for clearing liver-stage parasitemia (16). These studies highlight the importance of the innate immune system in controlling the liver stage of malaria and indicate that formulations able to generate similar responses may aid in the development of next-generation malaria vaccines.

In this study, we describe an immunization regimen that generates *P. falciparum* CS protein (PfCS)-specific polyfunctional memory $CD8^+$ T cells and high antibody titers able to inhibit infection by a transgenic rodent *P. berghei* parasite expressing PfCS. The strategy is based on priming with oligomeric PfCS fused to the vaccinia virus 14K protein and boosting with a recombinant modified vaccinia virus Ankara (MVA) strain expressing PfCS. Our findings underscore the importance of an immunization protocol that controls the preerythrocytic liver stage of the parasite and may warrant development as a next-generation candidate for a malaria vaccine.

RESULTS

Expression of the oligomeric CS protein from *P. falciparum*. A truncated version of the *PfCS* gene from the *Plasmodium* 3D7 isolate (NCBI reference sequence XM_001351086), excluding the native signal peptide and the C-terminal glycosylphosphatidylinositol (GPI) motif, was amplified from the plasmid pIC-CSPfFL. A point mutation was introduced by site-directed mutagenesis to generate a $CD8^+$ T cell epitope that is absent in the *P. falciparum* 3D7 strain but is found in other *P. falciparum* isolates, such as 7G8 and T4. In the region containing the sequence $_{308}$DYANDIEKKI$_{317}$, the A residue at position 310 was thus replaced by E to give $_{308}$DYENDIEKKI$_{317}$ (Fig. 1A), which is the only H-2^K-restricted *P. falciparum* $CD8^+$ T cell epitope recognized in mice and also partially overlaps the human epitope recognized by HLA-B35 (17). The amplified fragment was fused to a vaccinia virus A27L gene lacking the codons for the first 20 amino acids and then cloned into the pGEX-6P bacterial expression vector as described previously (8). Protein purity was analyzed by SDS-PAGE followed by Coomassie blue staining (Fig. 1B). Immunoblots using monoclonal antibody (MAb) 2A10, a neutralizing antibody against the PfCS repeat region, showed that under nonreducing conditions, the protein existed mainly as high-molecular-mass oligomers (Fig. 1C); under reducing conditions, the protein was present as ~55-kDa monomers. The recognition profile for a polyclonal antibody to the vaccinia virus 14K protein was similar to that for MAb 2A10 (not shown). These results corroborated our previous study showing that 14K fusion with the CS protein in a disulfide-dependent manner (18, 19) aids in the formation of high-molecular-mass oligomers (8).

Generation and characterization of recombinant vaccinia virus MVA-sPfCS^M. The construction and characterization of an MVA construct expressing the full-length *P. falciparum* CS protein were described previously (20). Here we generated a recombinant virus encoding a mutated (A_{310} to E_{310}) CS protein lacking the GPI motif, in which the native signal sequence was replaced by a synthetic signal peptide from human immunodeficiency virus type 1 (HIV-1) glycoprotein 120 (gp120) to enable secretion of the protein (Fig. 2A). We used PCR and DNA sequencing of purified viral stocks to evaluate the integrity and stability of the recombinant virus (Fig. 2B). Immunoblots using MAb 2A10 showed that the recombinant MVA-sPfCS^M strain expressed mutated PfCS as a secreted product, in contrast to recombinant MVA-PfCS, which expressed the native PfCS sequence (Fig. 2C).

To determine whether expression of the heterologous proteins affected viral replication under permissive conditions, we compared the growth kinetics of wild-type virus to those of the recombinant viruses MVA-PfCS and MVA-sPfCS^M. Expression of the

A.

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PFCSM   1    MMNYYGKQENWYSLKKNSRSLGENDDGNNEDNEKLRKPKHKKLKQPADGNPDPNANPNVD   60
PFCS    1    MMNYYGKQENWYSLKKNSRSLGENDDGNNEDNEKLRKPKHKKLKQPADGNPDPNANPNVD   60

PFCSM   61   PNANPNVDPNANPNVDPNANPNANPNANPNANPNANPNANPNANPNANPHANPNAN   120
PFCS    61   PNANPNVDPNANPNVDPNANPNANPNANPNANPNANPNANPNANPNANPHANPNAN   120

PFCSM   121  PNANPNANPNANPNANPNANPNANPNVDPNANPNANPNANPNANPNANPNANPNAN   180
PFCS    121  PNANPNANPNANPNANPNANPNANPNVDPNANPNANPNANPNANPNANPNANPNAN   180

PFCSM   181  PNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNKNNQGNGQGHNMPNDPNR   240
PFCS    181  PNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNKNNQGNGQGHNMPNDPNR   240

PFCSM   241  NVDENANANSAVKNNNNEEPSDKHIKEYLNKIQNSLSTEWSPCSVTCGNGIQVRIKPGSA   300
PFCS    241  NVDENANANSAVKNNNNEEPSDKHIKEYLNKIQNSLSTEWSPCSVTCGNGIQVRIKPGSA   300

PFCSM   301  NKPKDELDYENDIEKKICKMEKCSSVFN   328
PFCS    301  NKPKDELDYANDIEKKICKMEKCSSVFN   328
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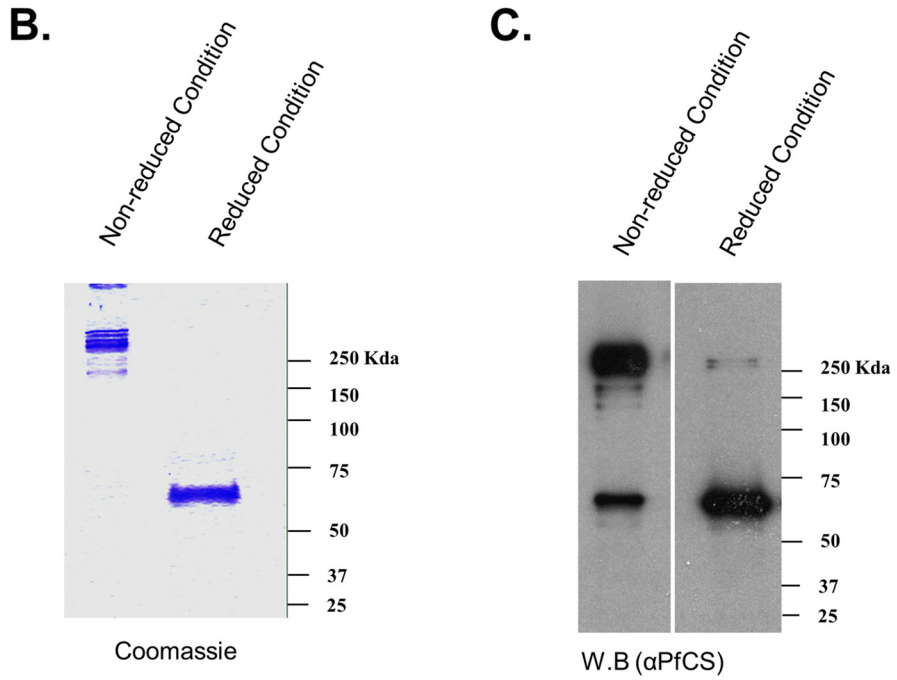

FIG 1 Characterization of PfCS14K^{M} recombinant protein. (A) Sequence of the *Plasmodium falciparum* (strain 3D7) CS protein used in this study, lacking the signal sequence and the GPI motif at the C terminus. A mutant form of the CS protein (PfCS^{M}) was generated by introducing an $A_{310} \rightarrow E_{310}$ mutation to generate the CD8^{+} T cell epitope DYENDIEKKI. The purity of the recombinant PfCS14K protein from *Escherichia coli* was evaluated under reducing and nonreducing conditions by Coomassie blue staining (B) and by immunoblotting (W.B) using MAb 2A10 (anti-PfCS) (C).

heterologous antigen did not influence viral replication in permissive chick DF-1 cells (not shown). We thus successfully generated a recombinant MVA strain that is able to express and secrete mutated PfCS from infected cells.

Generation and characterization of transgenic *P. berghei-P. falciparum* chimeric parasites. We generated a *P. berghei* transgenic parasite expressing the full-length *P. falciparum* CS protein (3D7). In this transgenic line, a single amino acid in the CS protein region containing the sequence DYANDIEKKI was modified to incorporate the cytotoxic CD8^{+} T cell epitope DYENDIEKKI. This epitope is not found in the 3D7 strain but is present in other *P. falciparum* isolates (such as 7G8 and T4). Full-length PfCS with the

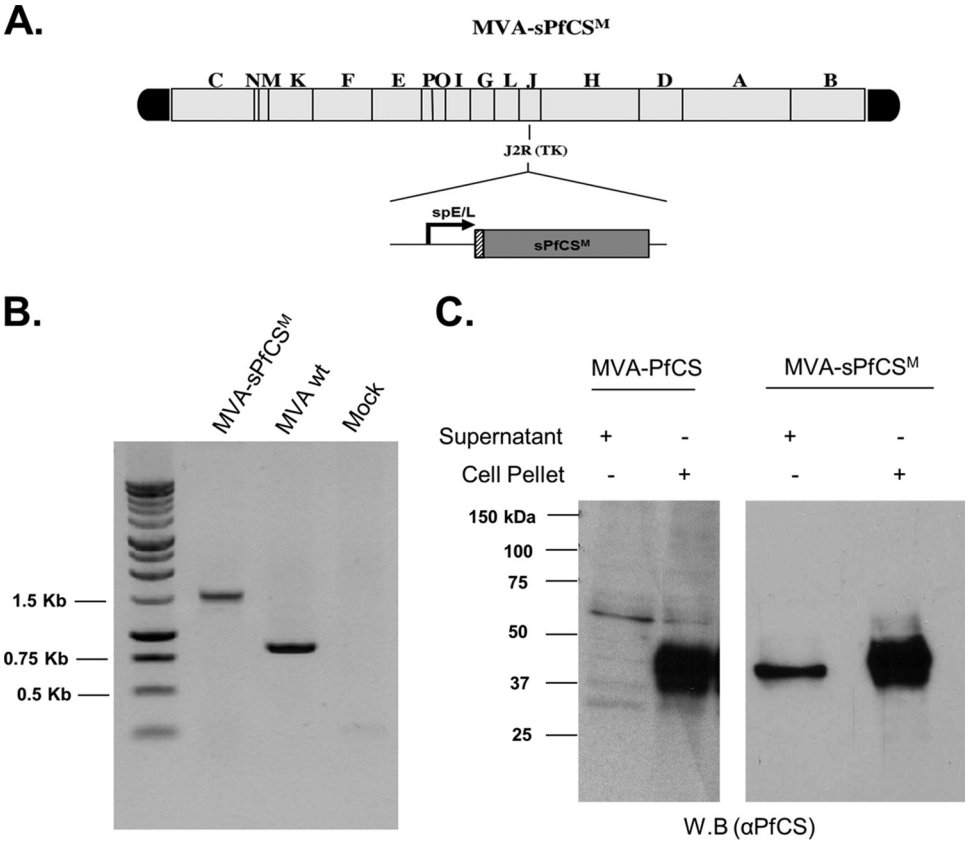

FIG 2 Construction and characterization of MVA-sPfCS^{M}. (A) Scheme of the MVA-sPfCS^{M} genome map. The *CS* gene with a synthetic HIV-1 gp120 signal peptide was inserted into the vaccinia virus TK locus (J2R), whose expression was driven by a synthetic early/late viral promoter (spE/L). (B) PCR analysis of the TK locus in recombinant viral vectors. Viral DNA was purified from infected DF-1 cells and probed with primers flanking the TK locus. DNA from the wild-type virus (wt) was used as a control for heterologous gene insertion (band sizes: MVA-sPfCS^{M}, 1,600 bp; and MVA wt, 800 bp). (C) PfCS expression by recombinant MVA. DF-1 cells were infected with MVA-PfCS or MVA-sPfCS^{M} at a multiplicity of infection (MOI) of 1. After 24 h, cell pellets and supernatants were collected and resolved by 10% SDS-PAGE. Protein was detected using the 2A10 antibody.

P. berghei native signal sequence was cloned into the CS locus of green fluorescent protein (GFP)-luciferase-expressing *P. berghei* ANKA (Fig. 3A). Transfected parasites were selected by pyrimethamine treatment of infected Swiss Webster mice. The transgenic parasites were cloned by limiting dilution of total parasites obtained from the mice. PCR analysis confirmed recombination at the 5′ and 3′ ends of the replaced gene (Fig. 3B) and was followed by sequencing of the insert from the cloned isolate (Fig. 3C).

To verify the infectiousness of the cloned isolate of the *P. berghei-P. falciparum* transgenic parasite, we compared the chimeric parasite with the wild-type strain. *Aedes stephensi* mosquitoes were allowed to feed on parasite-infected mice. Oocyst counts in mosquito midguts were analyzed after 12 days and were ~55 to 60 for both groups. The development pattern of the transgenic parasite was similar to that of the wild-type parasite (Table 1).

An immunization regimen based on recombinant protein priming and virus boosting enhances PfCS-specific CD8^{+} T cell numbers. The rationale for developing mutated versions of a PfCS-based recombinant protein (PfCS14K^{M}) and a recombinant virus (MVA-sPfCS^{M}) was that our initial studies with unmutated PfCS14K and MVA-PfCS immunization strategies did not elicit T cell responses in BALB/c mice (not shown). Because previous studies described the generation of H-2^{K}-restricted CD8^{+} T cells specific for the *P. falciparum* CS protein epitope DYENDIEKKI in C3H mice (20), we developed an immunization strategy with PfCS and MVA vectors in which the *P.*

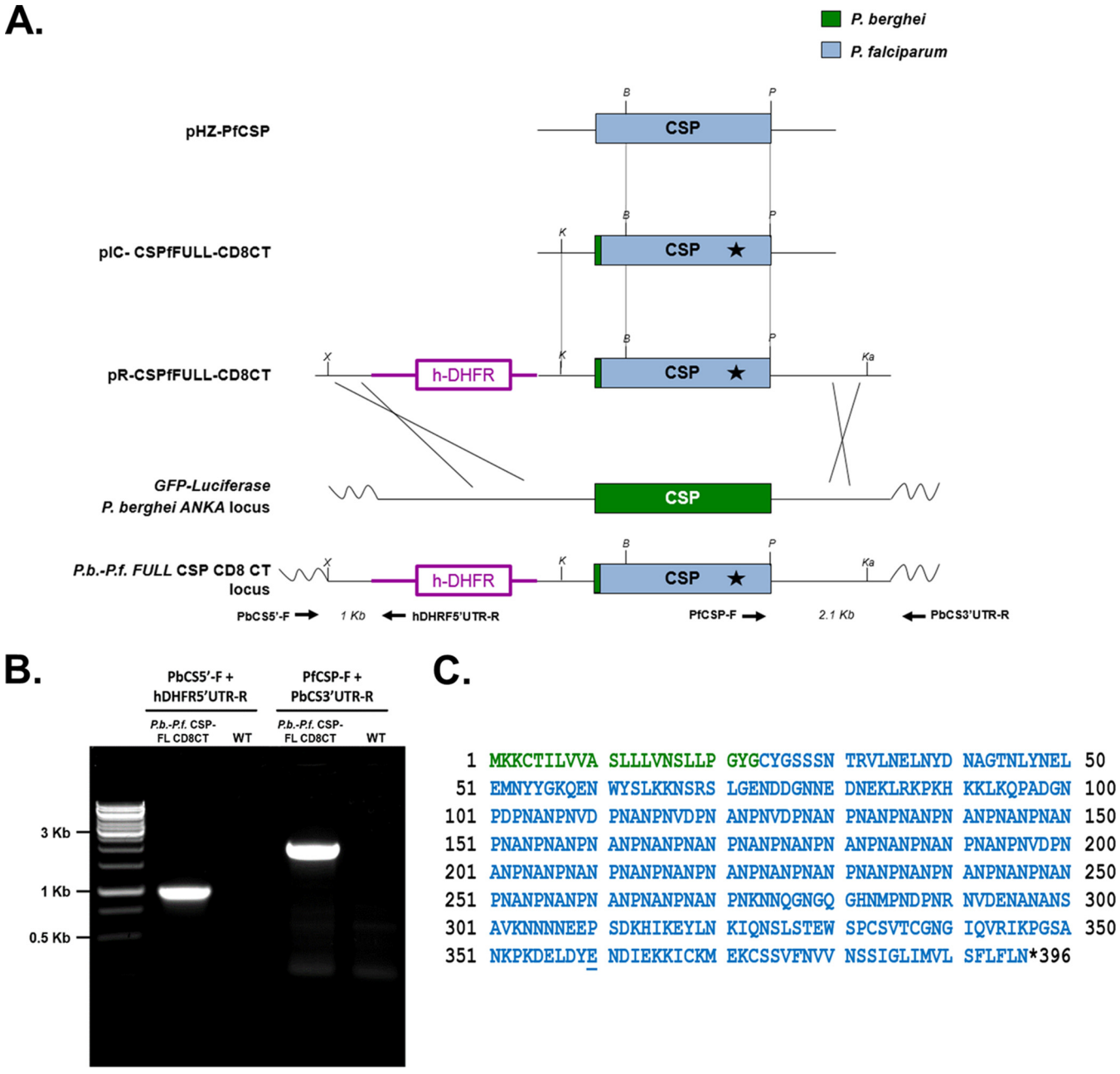

FIG 3 Development of *P. berghei* transgenic parasites expressing a mutant form of PfCS. (A) Scheme of the strategy to replace the *CSP* gene of GFP-luciferase-expressing *P. berghei* (ANKA) with *P. falciparum* (3D7) *CSP* incorporating the *P. berghei* signal sequence. A star indicates the location of the nucleotide change introduced to generate the DYENDIEKKI $CD8^+$ T cell epitope. The annealing sites of the PCR primers used to verify recombination at the *CSP* locus are indicated at the bottom. Restriction sites are indicated as follows: B, BbsI; K, KpnI; Ka, KasI; P, PacI; and X, XhoI. (B) Using genomic DNA from *P. berghei-P. falciparum* (P.b.-P.f.) CSP-FL CD8CT parasites, 5′ and 3′ integration at the *CSP* locus was verified by PCR. Primers PbCS5′-F and hDHFR5′UTR-R yielded a 1,000-bp product, and primers PfCSP-F and PbCS3′UTR-R yielded a 2,100-bp product. (C) Amino acid sequence of the CS protein in the *P. berghei-P. falciparum* CSP-FL CD8CT parasite. The *P. berghei* signal sequence is shown in green, and *P. falciparum* residues are shown in blue. The Ala (A)-to-Glu (E) change introduced to generate the cytotoxic epitope is underlined.

falciparum 3D7 CS protein was modified to incorporate the cytotoxic epitope (Fig. 1A), which we used to define the profile of immune responses in mice (Fig. 4A).

Stimulation of splenocytes from mice immunized with the peptide showed a robust expansion of PfCS-specific $CD8^+$ T cells during the adaptive and memory phases (Fig. 4B); CD4-specific responses were low for all mouse groups given that the peptide used for stimulation is CD8 specific (Fig. 4C). Mice primed with the oligomeric protein and boosted with the recombinant virus had the highest $CD8^+$ T cell levels. To determine

TABLE 1 Developmental characteristics and infectivity of *P. berghei-P. falciparum* CSP-FL CD8CT transgenic parasites in *A. stephensi* mosquitoes[a]

Parasite	% of midguts infected	No. of oocysts/midgut	% of salivary glands infected	No. of sporozoites/salivary gland (10^3)	Prepatent period (days) (SD)[b]
P. berghei-P. falciparum CSP-FL CD8CT	87.50	60.33	80	9.5	4.4 (0.502)
P. berghei-P. falciparum CSP-FL	70.58	83.25	85	10	4.6 (0.767)
P. berghei ANKA	80.20	55.25	75	10	4.7 (0.788)

[a]The data are means for 3 experiments, with at least 20 mosquitoes examined in each experiment.
[b]Three infected mosquitoes were allowed to feed on C57BL/6 mice ($n = 5$) for 3 min. Blood-stage parasitemia was assessed 5 days later, and 100% of mice were infected for all groups. SD, standard deviation.

whether PfCS-specific $CD4^+$ or $CD8^+$ T cells targeted other protein epitopes, we stimulated splenocytes from vaccinated mice with sonicated unmutated recombinant PfCS. Using this approach, we did not detect other H-2^K-restricted PfCS-specific $CD4^+$ or $CD8^+$ T cells, which supported our hypothesis that the cellular response generated by the vaccine regimen was restricted to the $CD8^+$ T cell epitope (not shown). Mice primed with protein and boosted with recombinant virus maintained similar levels of CS peptide-specific $CD8^+$ T cells at 2 weeks and 2 months postboost (Fig. 4B).

These immunizations generated high titers of PfCS-specific antibodies in the memory phase. The PfCS14K^M/PfCS14K^M-immunized mouse group produced high levels of PfCS antibodies similar to those for the heterologous PfCS14K^M/MVA-sPfCS^M prime-boost group (Fig. 4D). Since the vaccination protocols led to antibody production, the low $CD4^+$ T cell responses after splenocyte stimulation with whole PfCS might be linked to the use of C3H mice, which are low responders to immunization using synthetic peptides with PfCS $CD4^+$ T cell epitopes (21).

These data suggest that PfCS14K^M priming/MVA-sPfCS^M boosting increased H-2^K-restricted CS protein-specific splenic $CD8^+$ T cells during the adaptive and memory phases, in contrast to the results of homologous immunogen combination. This strategy also produced high anti-PfCS titers comparable to those induced by homologous protein PfCS14K^M priming and boosting.

PfCS14K^M/MVA-sPfCS^M immunization triggers polyfunctional PfCS-specific $CD8^+$ T cell responses. Having established that the PfCS14K^M/MVA-sPfCS^M prime/boost immunization protocol generated PfCS-specific $CD8^+$ T cells, we characterized these T cells based on their polyfunctional responses. Polyfunctionality was defined by the ability of the antigen-specific T cells to simultaneously produce two or more cytokines (gamma interferon [IFN-γ], tumor necrosis factor alpha [TNF-α], and/or interleukin-2 [IL-2]) and/or perforin (CD107a) after peptide stimulation of splenocytes from vaccinated mice. A strongly polyfunctional T cell secretes a large number of cytokines (quadruple-positive population). Intracellular cytokine staining (ICS) analysis of adaptive and memory immune responses clearly showed a shift in $CD8^+$ T cell polyfunctionality in PfCS14K^M-primed/MVA-sPfCS^M-boosted mice (Fig. 5). Although the size of the CS protein-specific H-2^K-restricted $CD8^+$ T cell population remained unchanged between the adaptive and memory phases, there was a significant shift in polyfunctionality. Most responses during the adaptive phase in protein-primed/recombinant virus-boosted mice were monofunctional ($CD107a^+$ or IFN-γ^+), bifunctional ($CD107a^+$ IFN-γ^+), or trifunctional ($CD107^+$ IFN-γ^+ TNF-α^+) (Fig. 5A). We found a 6-fold increase in $CD107^+$ IFN-γ^+ TNF-α^+ IL-2^+ $CD8^+$ T cells ($P < 0.005$) in this group during the memory compared to the adaptive phase (Fig. 5). The proportion of $CD107^+$ IFN-γ^+ TNF-α^+ IL-2^+ $CD8^+$ T cells (quadruple positive) increased 31% during the memory phase ($P < 0.005$), while the triple-positive population doubled, to 40% of total $CD8^+$ T cells ($P < 0.05$), compared to the levels of the same populations in the adaptive phase (Fig. 5A and B, pie charts).

PfCS14K^M/MVA-sPfCS^M immunization enhances effector memory T cell levels. The correlation between induction of memory $CD8^+$ T cells specific to liver-stage

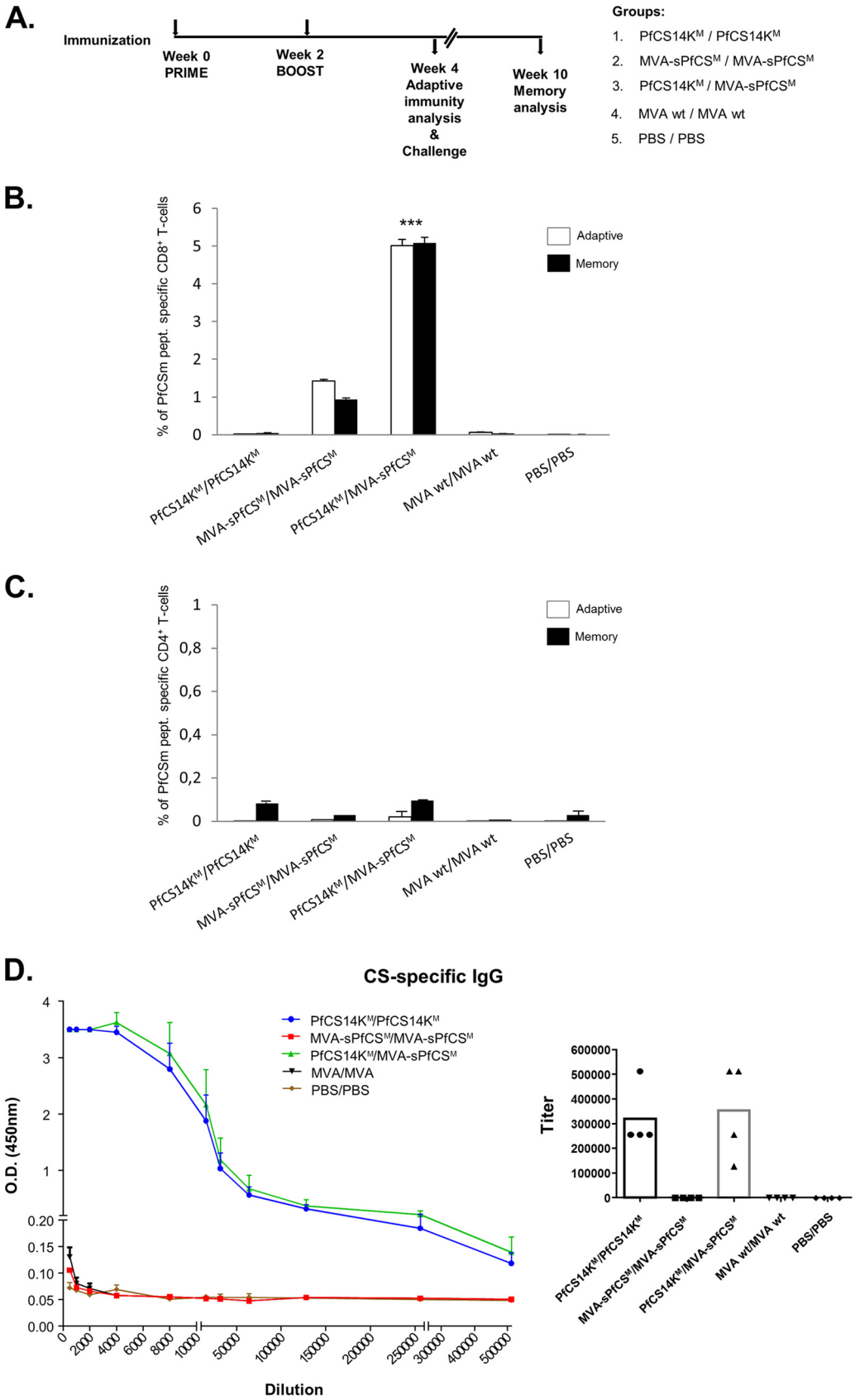

FIG 4 Levels of PfCS (peptide)-specific $CD8^+$ and $CD4^+$ T cells and antibodies generated by different immunization protocols. (A) Immunization schedule. C3H (H-2^K) mice received distinct combinations of immunogens in a prime/boost approach. For each dose, each mouse received 20 μg protein by the intradermal (i.d.) route or 2×10^7 PFU of MVA-sPfCS^M

(Continued on next page)

antigens and malaria protection is well established for small mammals as well as humans (6, 7, 22). To characterize the phenotype of CS protein-specific memory $CD8^+$ T cells generated in response to vaccination, we used the CD127 and CD62L surface markers. These markers differentiate memory cells into central memory (T_{CM}; $CD127^{Hi}$ $CD62L^{Hi}$), effector memory (T_{EM}; $CD127^{Hi}$ $CD62L^{Lo}$), and effector (T_E; $CD127^{Lo}$ $CD62L^{Lo}$) T cell subsets (23, 24). Our data showed that the majority of $CD8^+$ T cells generated in response to vaccine had an effector memory phenotype (Fig. 6). Furthermore, we found a 3.5-fold increase in T_{EM} $CD8^+$ T cell levels in the protein/MVA protocol group compared to those in the other groups ($P < 0.005$). This confirmed that the $PfCS14K^M$/MVA-$sPfCS^M$ immunization regimen significantly enhanced development of PfCS-specific $CD8^+$ T cells with an effector memory phenotype.

Parasite protection in mice immunized with $PfCS14K^M$/MVA-$sPfCS^M$. Since the protein prime/recombinant virus boost regimen induced larger numbers of polyfunctional $CD8^+$ T cells in mice than those induced by homologous combinations of immunogens, we tested whether the former protocol protected mice after challenge with transgenic *P. berghei* sporozoites expressing the mutated *P. falciparum* CS protein (*P. berghei-P. falciparum* CSP-FL CD8CT). Mice primed with $PfCS14K^M$ and boosted with MVA-$sPfCS^M$ showed an 80% inhibition of parasite load in the liver compared to the loads of the other groups (Fig. 7A, left half).

To further determine if liver-stage inhibition would be observed after immunization with the unmutated PfCS antigen, which did not generate T cell responses, we tested a prime-boost regimen by using unmutated versions of the recombinant protein (PfCS14K) and virus (MVA-PfCS) (without the $A_{310} \rightarrow E_{310}$ substitution). In immunized mice challenged with transgenic *P. berghei* sporozoites expressing the unmutated full-length *P. falciparum* 3D7 CS protein (*P. berghei-P. falciparum* CSP-FL), liver parasite burdens were comparable to those in controls (Fig. 7A, right half). It would be anticipated that mice immunized with $PfCS14K^M$/MVA-$sPfCS^M$ and challenged with transgenic *P. berghei* parasites expressing PfCSP lacking the $H\text{-}2^K$-restricted $CD8^+$ T cell epitope would not be protected and thus would have higher parasite loads, which would further support a protective role for $CD8^+$ T cells. Nonetheless, given the large number of controls needed to allow accurate measurement and interpretation of the relative protective efficacies of the individual immune mechanisms involved, this experiment was not performed.

Finally, we tested whether the reduction in liver-stage parasite burden translates into sterile protection. We observed that 40% of $PfCS14K^M$/MVA-$sPfCS^M$-immunized mice did not develop blood-stage parasitemia after challenge by infectious mosquito bites harboring transgenic *P. berghei* sporozoites expressing the mutated *P. falciparum* CS protein (*P. berghei-P. falciparum* CSP-FL CD8CT) (Fig. 7B). The $PfCS14K^M$ prime/MVA-$sPfCS^M$ boost regimen is thus an effective vaccine protocol for controlling parasite loads in the liver and achieving sterile protection.

DISCUSSION

The aims of this study were to demonstrate the potency of a novel malaria vaccine candidate generated by fusion of the *P. falciparum* CS protein with the vaccinia virus

FIG 4 Legend (Continued)
by the intraperitoneal (i.p.) route. The groups received the following immunogens: group 1, protein $PfCS14K^M$/protein $PfCS14K^M$ (i.d.); group 2, recombinant MVA-$sPfCS^M$/MVA-$sPfCS^M$ (i.p.); group 3, $PfCS14K^M$ (i.d.)/MVA-$sPfCS^M$ (i.p.); group 4, parental MVA/MVA (i.p.); and group 5, PBS/PBS (i.p.). (B and C) Comparisons of total $CD8^+$ (B) and $CD4^+$ (C) T cell responses generated by different vaccination schedules during the adaptive and memory phases. Splenocytes from vaccinated mice were obtained at 2 weeks (adaptive) or 10 weeks (memory) postboost. Splenocytes were stimulated *in vitro* by use of 10 μg/ml CS protein-specific $CD8^+$ T cell peptide. Data are representative of two independent experiments ($n = 4$ mice/group). (D) Sera from mice (vaccinated as described for panel A) at the memory phase were analyzed by ELISA for the levels of PfCS-specific IgG antibodies. (Left) Data are presented as OD_{450} values versus serum dilutions; the maximum OD is represented by a value of 3.5 (data correspond to the means for 4 animals per group and 3 repeats per animal). (Right) CS protein-specific IgG titers. The titer was defined as the inverse of the highest serum dilution that exhibited at least 3 times the mean OD for the naive control (the presented data correspond to individual mouse titers for 4 animals/group and are means for 3 repetitions per mouse). Statistical analysis was used to compare the $PfCS14K^M$ prime/MVA-$sPfCS^M$ boost group with all other groups. ***, $P < 0.001$.

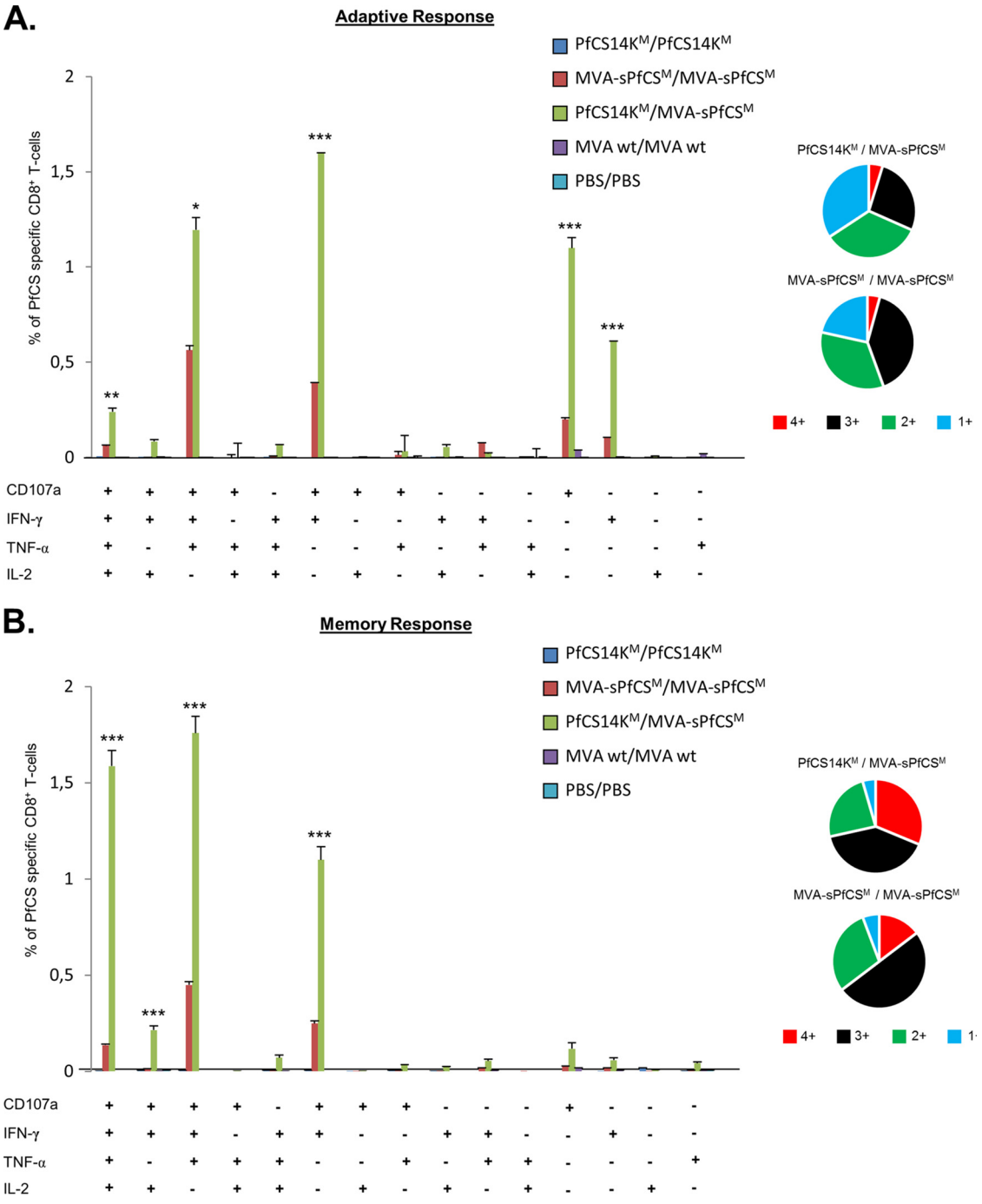

FIG 5 PfCS-specific CD8$^+$ T cell polyfunctionality in mice. Splenocytes from vaccinated mice were stimulated with 10 μg/ml CS protein-specific peptide. Live lymphocytes were gated on CD3 and then gated on CD8. CD8$^+$ T cells were further gated on the memory markers CD127 and CD62L (for samples collected at the memory phase). Gated CD8$^+$ T cells were probed for polyfunctionality based on secretion of different combinations of IFN-γ, TNF-α, IL-2, and CD107a. (A) Polyfunctional CS protein-specific CD8$^+$ T cells during the adaptive phase. (B) Polyfunctional CS protein-specific CD8$^+$ T cells during the memory phase. Data are representative of two independent experiments ($n = 4$ mice/group). Pie charts compare polyfunctional CD8$^+$ T cell populations induced by the distinct immunization schedules during the adaptive and memory phases. Peptide-specific T cells that secreted one, two, three, or all four factors (IFN-γ, TNF-α, IL-2, and CD107a) were classified based on intracellular cytokine staining (ICS). Statistical significance between the PBS prime/PBS boost control group and the PfCS14K^M prime/MVA-sPfCS^M boost test group is indicated. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$.

A27 protein in oligomer form and to evaluate its effectiveness in a mouse preclinical setting, alone or combined with an MVA vector expressing PfCS. We generated PfCS immunogens based on a recombinant chimeric protein (PfCS14K) and recombinant MVA (MVA-PfCS). As mice would not produce PfCS-specific T cells in response to this

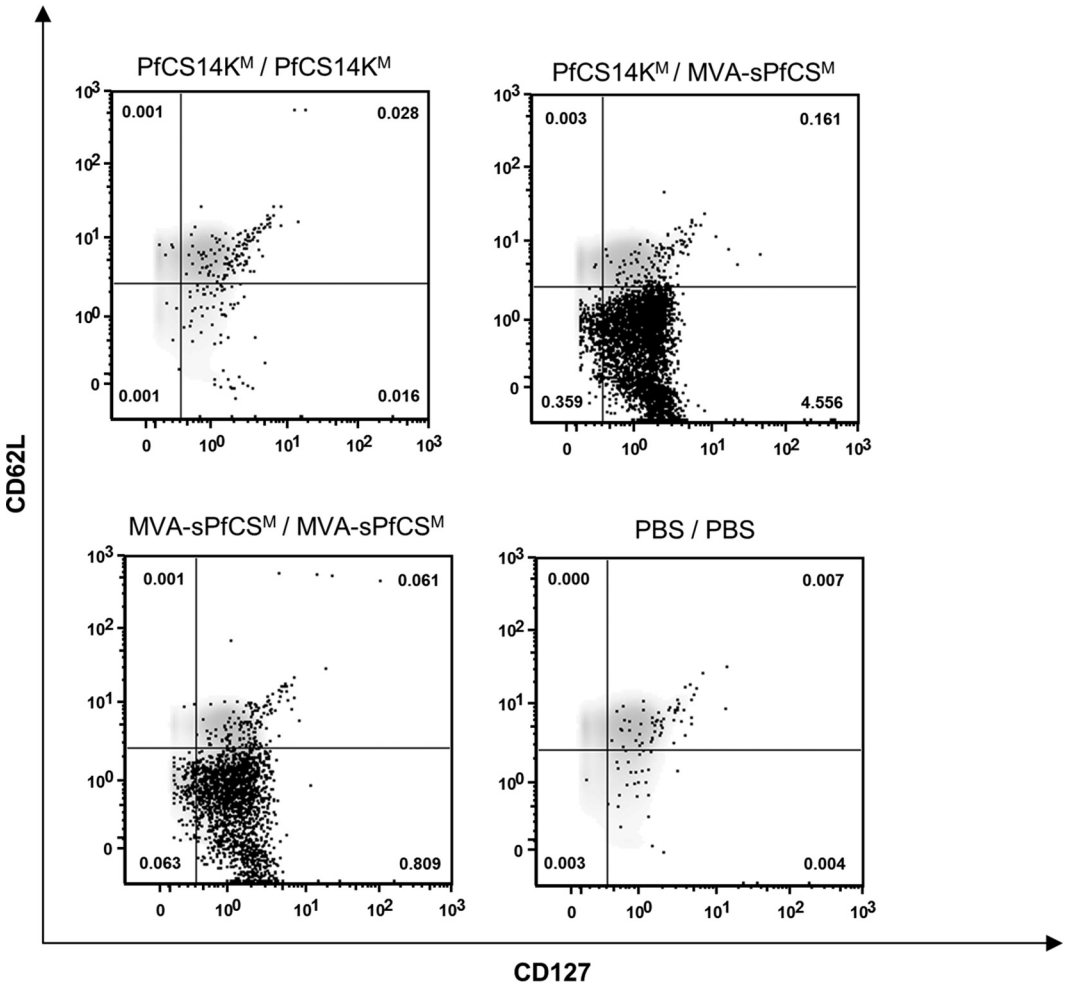

FIG 6 Phenotypic characterization of PfCS-specific memory $CD8^+$ T cells generated by vaccination. Lymphocytes gated on CD3 and CD8 were further selected, based on differential expression of the memory markers CD127 and CD62L, as naive ($CD127^-$ $CD62L^+$), central memory (T_{CM}; $CD127^+$ $CD62L^+$), effector (T_E; $CD127^-$ $CD62L^-$), or effector memory (T_{EM}; $CD127^+$ $CD62L^-$) cells. Values in the quadrants indicate the contributions of the memory phenotype subpopulations to the total antigen-specific response. Data are representative of two independent experiments ($n = 4$ mice/group).

antigen, we generated a mutated PfCS-based recombinant protein ($PfCS14K^M$) and viral immunogen ($MVA\text{-}sPfCS^M$) with an $H\text{-}2^K$-restricted $CD8^+$ T cell epitope. For challenge in the mouse model, we used transgenic *P. berghei* sporozoites expressing full-length PfCS with the $CD8^+$ T cell epitope. We found that the combined $PfCS14K^M$/$MVA\text{-}sPfCS^M$ immunization protocol offered protection against malaria that correlated with induction of PfCS-specific $CD8^+$ T cell and antibody responses.

RTS,S vaccine trials showed that protection against malaria is mediated by high anti-CS protein antibody titers that wane rapidly (9, 25), and current vaccination strategies do not maintain high levels of circulating anti-CS protein antibodies. A recent study showed that the protection offered by RTS,S appears to be strain specific (for strain 3D7) (26). Human studies using irradiated sporozoites or chloroquine prophylaxis showed that maintenance of IFN-γ-secreting T cells can be used as a surrogate protection marker during preerythrocytic stages (22, 27). A cytotoxic T lymphocyte (CTL)-based malaria vaccine expressing the thrombospondin-related adhesive protein (TRAP) antigen in combination with several T cell epitopes from preerythrocytic antigens also provided sterile protection of 21% of individuals in an adenovirus prime/MVA vector boost regimen; although the protection levels were modest, the study highlighted the importance and feasibility of developing a $CD8^+$ T cell malaria vaccine (28). The same group nonetheless also found higher levels of vaccine efficacy with an

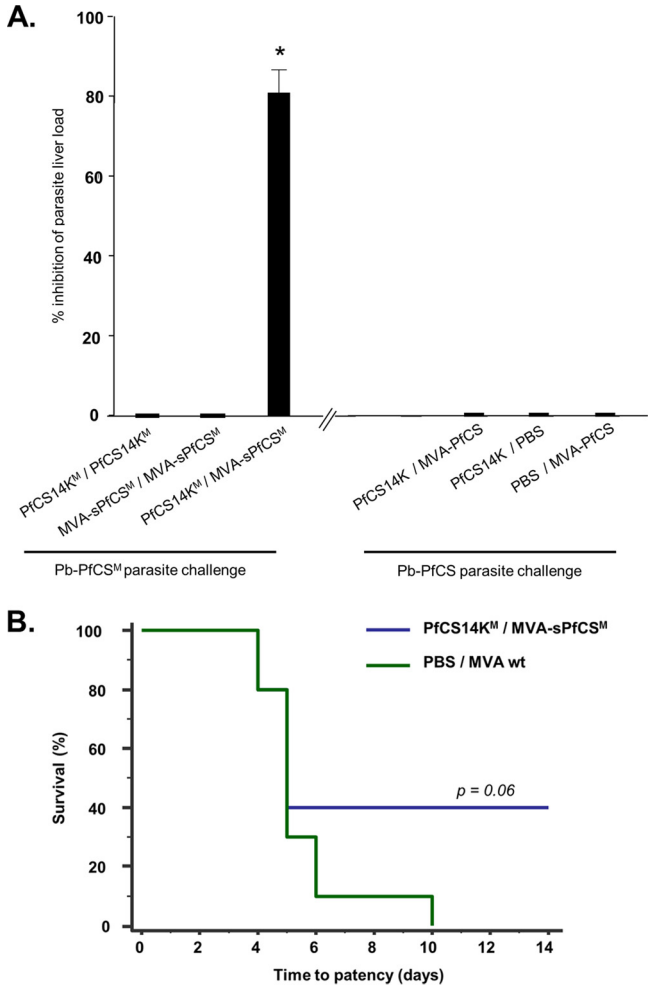

FIG 7 PfCS14K^{M} prime/MVA-sPfCS^{M} boost immunization protects mice against chimeric *P. berghei* transgenic parasites. (A) At 2 weeks postboost, mice ($n = 5$/group) were challenged intravenously with 2,000 *P. berghei* sporozoites expressing mutated PfCS bearing the CD8^{+} T cell epitope DYENDIEKKI (Pb-PfCS^{M}) (left) or unmodified full-length PfCS (Pb-PfCS) (right). After 40 h, the liver parasitemia level was evaluated by RT-qPCR. The percent reduction in liver-stage transgenic parasite development compared to the level for the control groups was determined. (B) Kaplan-Meier survival curves for mice ($n = 10$/group) vaccinated with PfCS14K^{M}/MVA-sPfCS^{M} or with PBS/wild-type MVA after challenge with mosquitoes harboring *P. berghei* PfCS^{M}-transgenic parasites. Statistical significance was determined for differences between the PBS/MVA controls and the protected group. *, $P < 0.05$.

adenovirus-MVA combination expressing ME-TRAP (multiepitope string containing malaria antigens fused to thrombospondin-related adhesion protein), with no apparent protective correlation with CD4^{+} or CD8^{+} T cell responses (29). Other human studies with an irradiated sporozoite vaccine showed a significant, dose-dependent increase in sporozoite-specific T cells in protected individuals (30, 31). The differences in these immunization protocols might account for the distinct induction of activated T cells for vaccine effectiveness. A CD8^{+} T cell malaria vaccine would appear to be more efficient for targeting the preerythrocytic parasite stages.

Here we report a prime-boost vaccine protocol based on a chimeric PfCS construct and a recombinant MVA strain that resulted in an 80% reduction in the liver-stage parasite burden in mice and provided 40% sterile protection against infection following parasite challenge with transgenic *P. berghei* expressing the *P. falciparum* CS protein. In a murine malaria model, we previously showed that induction of a large *P. yoelii* CS protein-specific polyfunctional CD8^{+} T cell repertoire after priming with multimeric CS14K and boosting with an MVA vector expressing PyCS can induce sterile protection (8). We also found that the CS14K protein triggers a Th1 signaling cascade via IRF-3 and

STAT-1 activation. Here we used a similar strategy to demonstrate that the *P. yoelii* results can be extended to the human malaria antigen PfCS and that high levels of specific $CD8^+$ T cells and anti-PfCS antibodies can be obtained by this strategy.

To assess the *in vivo* effectiveness of the chimeric immunogen, we generated a new transgenic *P. berghei* strain harboring full-length PfCS with the H-2^K-restricted cytotoxic epitope DYENDIEKKI. This chimeric line was compared to a transgenic *P. berghei* line expressing unmutated full-length PfCS in which the C-terminal region was not modified but retained the original 3D7 sequence (DYANDIEKKI). The cytotoxic DYENDIEKKI epitope is found naturally in certain *P. falciparum* isolates (such as 7G8 and T4) and is recognized by H-2^K C3H mice (20). The availability of these two transgenic lines allowed us to assess protection based on PfCS-specific $CD8^+$ T cells as well as antibodies.

Although the protein/protein- and protein/MVA-immunized mice had similar PfCS-specific antibody levels, liver infection was inhibited only in those that received the mutated chimeric protein and recombinant virus bearing the DYENDIEKKI epitope. The same vaccine regimen with unmutated antigens (PfCS14K/MVA-PfCS) did not protect mice challenged with the *P. berghei* transgenic parasite expressing unmutated PfCS. The protection conferred by the heterologous prime/boost strategy thus suggests an important role for DYENDIEKKI epitope-specific $CD8^+$ T cells. $PfCS14K^M$/$PfCS14K^M$ immunization induced high levels of anti-PfCS antibodies, low levels of $CD8^+$ T cells, and no parasite protection, whereas the $PfCS14K^M$/MVA-$sPfCS^M$ vaccination induced high anti-PfCS titers, high levels of specific $CD8^+$ T cells, and protection from parasite infection. These findings suggest that parasite inhibition in this system is based at least partly on $CD8^+$ T cells. We nonetheless cannot rule out that the combined action of PfCS antibodies and specific $CD8^+$ T cells contributes to parasite inhibition in the protein/MVA-treated group. It would be interesting to compare the biological properties of the PfCS antibodies induced by the $PfCS14K^M$/$PfCS14K^M$ and $PfCS14K^M$/MVA-$sPfCS^M$ protocols. Parasite protection should be more complete when both PfCS $CD8^+$ T cells and anti-PfCS antibodies are produced.

Numerous studies have shown that a strong T cell-mediated response correlates with malaria protection (22, 27, 30). The requirement for high CS protein-specific $CD8^+$ T cell levels for sterile protection is a hurdle that current vaccination strategies must overcome. The high levels of polyfunctional CS protein-specific $CD8^+$ T cells and of PfCS-specific antibodies generated by our heterologous $PfCS14K^M$/MVA-$sPfCS^M$ vaccination regimen suggest its potential as an improved malaria vaccine. Exhaustion of T cell responses, characterized by a loss of polyfunctionality with age, is considered an important factor in *Trypanosoma cruzi* infection in adults compared to that in children (32). The shift in $CD8^+$ T cell polyfunctionality observed in our experiments during the memory phase compared to the adaptive phase might be beneficial. Analysis of the memory $CD8^+$ T cell phenotype showed mainly T_E and T_{EM} cells, which coincides with our previous observations and with studies using an irradiated sporozoite vaccine (8, 33). This spectrum of results indicates the complexity of the CS protein-specific $CD8^+$ T cell functions generated by distinct vaccination protocols.

Considering the evidence that indicates a critical role for PfCS-specific antibodies in protection, our results suggest that vaccine-induced T cell responses can also play a significant role in malaria protection. Other mouse studies using *P. yoelii* showed the importance of CS protein-specific $CD8^+$ T cells in mediating protection against this parasite, but such a role has not been reported for PfCS in a murine model. Our findings with transgenic *P. berghei* parasites and protein/MVA immunogens suggest a role for PfCS-specific $CD8^+$ T cells in malaria protection. The combined $PfCS14K^M$/MVA-$sPfCS^M$ immunization protocol, which generates strong $CD8^+$ T cell and antibody responses to PfCS, together with inhibition of *P. berghei* parasites, may have value in an antimalarial vaccine regimen.

MATERIALS AND METHODS

Cells, plasmid, and viruses. Established chick DF-1 and primary chicken embryo fibroblast (CEF) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-

inactivated fetal calf serum (FCS), as described previously (34–36). Cell cultures were maintained at 37°C (CEF) or 39°C (DF-1) in a humidified incubator (85%) with 5% CO_2.

The pCyA-20/sPfCS^M plasmid was generated to insert the nearly full-length PfCS^M sequence, with the $A_{310} \rightarrow E_{310}$ amino acid substitution, and an additional synthetic secretion signal from the HIV-1 gp120 protein into the thymidine kinase (TK) locus of MVA. The single amino acid substitution was made to incorporate a cytotoxic CD8$^+$ T cell epitope (DYENDIEKKI) not present in the *P. falciparum* 3D7 strain but found in other *P. falciparum* isolates, such as 7G8 and T4. Wild-type MVA and the recombinant virus MVA-sPfCS^M used for *in vivo* studies were grown in CEF cells, purified, and titrated as described previously (34).

Construction and characterization of MVA-sPfCS^M. To generate the recombinant MVA construct, DF-1 cells (cultured in 6-cm plates to 70% confluence) were infected with wild-type MVA at 0.01 PFU/ml (1 h) and transfected with 8 μg pCyA-20/sPfCS^M plasmid by use of Lipofectamine (Invitrogen) to allow insertion of the antigen sequence into the virus TK locus. Cells were harvested at 48 h postinfection, when cytopathic effect was observed. The pCyA-20/sPfCS^M plasmid includes the β-galactosidase marker gene between two identical TK locus-flanking regions within the sequence to be inserted into the viral genome. In the first stage, the recombinant virus was isolated by serial plaque passages, using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (1.2 mg/ml; Sigma-Aldrich) as a substrate for the selection marker. After four passages, plaques negative for β-galactosidase activity were selected and tested for expression of secreted PfCS^M in supernatants. After six continuous passages, a recombinant virus free of wild-type contamination was isolated. Virus stocks were analyzed by PCR and Western blotting for the presence and expression of the antigen sequence. For PCR analysis, we used primers targeting the TK locus (TK_L forward [TGATTAGTTTGATGCGATTC] and TK_R reverse [TGTCCTTGATACGGC AG]). The correct PfCS^M sequence was confirmed by DNA sequencing. Virus stocks were produced in CEF cells and purified after sedimentation in two 37% sucrose cushions, as described previously (34).

Protein purification. The PfCS14K and PfCS14K^M proteins were purified from *Escherichia coli* (BL21) transformed with the pGEX-6P-PfCS14K and pGEX-6P-PfCS14K^M vectors, respectively. Bacteria were grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.7. Following induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), cultures were incubated at 30°C for 5 h before harvesting. Cells were suspended in extraction buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, and protease inhibitor cocktail), lysed using a French press (Thermo Scientific), and centrifuged, and the clarified lysate was incubated with glutathione Sepharose 4B beads (GE Life Sciences) at 4°C overnight. The beads were washed three times with extraction buffer containing 0.5% Triton X-114, followed by three washes with extraction buffer alone. Protein was eluted using 20 mM reduced glutathione in extraction buffer. Protein-containing fractions were pooled and dialyzed against 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl. The glutathione *S*-transferase (GST) tag was cleaved using PreScission protease (GE Life Sciences) according to the manufacturer's instructions. The cleaved protein was further purified to homogeneity by use of HiTrap MonoQ anion columns (GE Life Sciences). The protein was dialyzed against phosphate-buffered saline (PBS) and concentrated using centrifugal concentrators (Millipore). Proteins were tested for endotoxin contamination by use of a *Limulus* amoebocyte lysate (LAL) kit (Lonza), which indicated the presence of $<$10 endotoxin units (EU)/μg protein.

SDS-PAGE analysis of protein. Recombinant proteins were analyzed by SDS-PAGE with Coomassie blue staining or by Western blotting using the anti-*P. falciparum* CS protein MAb 2A10 (8). Briefly, proteins were mixed with 4$\times$ Laemmli buffer and separated by 7% SDS-PAGE alone and with β-mercaptoethanol (nonreducing and reducing conditions, respectively). For immunoblots, proteins transferred to nitrocellulose membranes were detected using MAb 2A10 (1:1,000 dilution; Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas [CNB-CSIC]) followed by a goat anti-mouse antibody (1:2,000; Sigma).

Animals and immunizations. The Ethical Committee of Animal Experimentation at the Centro Nacional de Biotecnología (CEEA-CNB) and the Institutional Animal Care and Use Committee of The Johns Hopkins University approved all animal procedures. Female C3H/HeNHsd mice (H-2^K) aged 5 to 8 weeks were obtained from Harlan Laboratories and used for immunogenicity studies, female C57BL/6 mice aged 5 to 8 weeks were used for parasite characterization, Swiss Webster mice were used for parasite cloning/selection, and female C3H/HeNCr MTV (mammary tumor virus negative; H-2^K) mice aged 5 to 8 weeks (Charles River) were used for challenge studies (34). Several homologous or heterologous prime/boost immunization protocols were used to assay fusion protein immunogenicity (see the text and the figure legends). The PfCS14K^M protein was administered intradermally (i.d.), whereas recombinant MVA-sPfCS^M was administered by the intraperitoneal (i.p.) route. The amount of protein given to each mouse was 20 μg in PBS for each priming dose, and that of virus was 2×10^7 PFU/mouse for each booster dose. Mice were sacrificed at 2 weeks (adaptive response) or 2 months (memory response) postboost for studies of immune responses. Two independent experiments were performed.

Transgenic sporozoite generation and challenge. To develop the chimeric line *P. berghei-P. falciparum* CSP-FL CD8CT, a 784-bp restriction fragment encompassing bp 246 to 1029 of the *P. berghei-P. falciparum* CSP NT *csp* chimeric gene was excised from the plasmid pIC-CSPPfNT (37) by use of the restriction enzymes BbsI and PacI (New England BioLabs). This portion was replaced with a 943-bp fragment which was released by using the same restriction enzymes and the pHZ-PfCSP plasmid. The *csp* gene (1,188 bp) in the resulting plasmid, pIC-CSPfFL-CD8CT, consisted of a full-length *P. falciparum* 3D7 CS protein in which the signal sequence was replaced with that from the *P. berghei* CS protein (bp 1 to 69). In addition, a single base pair in the plasmid pIC-CSPfFL-CD8CT *CS* gene was replaced to incorporate a cytotoxic epitope not present in the *P. falciparum* 3D7 strain. This change was introduced at bp 1079 by site-directed mutagenesis using a QuikChange II XL site-directed mutagenesis kit (Agilent Technol-

ogies). We then excised the *CS* gene from pIC-CSPfFL-CD8CT as a KpnI-PacI fragment and inserted it into the transfection plasmid pR-CSPfFL-CD8CT. XhoI and KasI were used to linearize pR-CSPfFL-CD8CT prior to its transfection into GFP-luciferase-expressing *P. berghei* ANKA parasites (38), as described previously (39).

P. berghei-P. falciparum CSP-FL CD8CT transgenic parasites were selected in Swiss Webster mice by treatment with pyrimethamine (MP Biomedicals, Solon, OH) in drinking water (0.07 mg/ml). Drug-resistant parasites were cloned by limiting dilution. Successful recombination at the 5′ and 3′ ends of the modified locus was verified by PCR. The primers used to confirm 5′ integration were PbCS5′-F (TGCCC TATTCTCATATTTACCAC) and hDHRF5′UTR-R (CACCATTTTTGAAAAGATTAATTTGA), and the primers used to verify integration at the 3′ end were PfCSP-F (TGAATGGTCCCCATGTAGTG) and PbCS3′UTR-R (CGCC TTAATGTGCATTGCT). DNA isolated from chimeric parasites was sequenced to verify the replaced gene.

Challenge studies to determine liver-stage parasitemia were performed as described previously (8, 40). Briefly, 2,000 sporozoites isolated from salivary glands of *Anopheles stephensi* mosquitoes were injected into the tail veins of mice. After 42 h, mice were euthanized, and their liver parasitemia levels were assessed by quantitative reverse transcription-PCR (RT-qPCR) as described previously (40). To evaluate blood-stage parasitemia after vaccination, 5 *P. berghei-P. falciparum* CSP-FL CD8CT-infected mosquitoes were allowed to feed on immunized mice for ~10 min, and the presence of parasites in blood was determined by examination of Giemsa-stained blood smears on days 4 to 14 after infection; the survival probability for challenged mice in this experiment was calculated relative to the time to patency, measured in days.

Multiparameter flow cytometry. The levels, polyfunctionality, and phenotypes of PfCS-specific T cell responses in the adaptive and memory phases were analyzed by flow cytometry and intracellular cytokine staining (ICS) as described previously (41). Briefly, 4×10^6 splenocytes were stimulated with 10 μg/ml $CD8^+$ T cell-specific epitope DYENDIEKKI (based on the sequences of *P. falciparum* 7G8 and T4 isolates [20]; obtained from Peptide 2.0 Inc.), 1 μl/ml GolgiPlug (BD Biosciences), anti-CD107a–Alexa 488 (BD Biosciences), and monensin (1×; eBioscience) in RPMI 1640 medium supplemented with 10% fetal calf serum (6 h) in a 96-well plate. In the case of samples stimulated with PfCS (Pfenex; produced in *Pseudomonas fluorescens*), splenocytes were incubated with 10 μg/ml protein and anti-CD107a–Alexa 488 (2 h) followed by GolgiPlug and monensin as described above (4 h). Following stimulation, cells were washed and their Fc receptors blocked by use of anti-CD16/CD32 (BD Biosciences), and the cells were then stained for surface markers, fixed, permeabilized (Cytofix/Cytoperm kit; BD Biosciences), and stained intracellularly for cytokines with appropriate fluorochromes. Dead cells were excluded by use of a violet LIVE/DEAD staining kit (Invitrogen). Cells were stained with mouse antibodies, including CD3-phycoerythrin (PE)-CF594, CD4-allophycocyanin (APC)-Cy7, CD8-V500, IFN-γ–PE–Cy7, IL-2–APC, and TNF-α–PE (all from BD Biosciences). For differentiation of memory T cells, we used CD62L-Alexa 700 (BD Biosciences) and CD127-peridinin chlorophyll protein (PerCP)-Cy5.5 (eBioscience) antibodies (only the memory PfCS-specific $CD8^+$ T cells that expressed any of the analyzed cytokines were selected and represented). Cells (10^6) were passed through a Gallios flow cytometer (Beckman Coulter), and data were analyzed with FlowJo (Tree Star Inc.). Appropriate controls were used, and values for unstimulated samples were subtracted.

Antibody measurement by ELISA. Antibodies against the CS protein were determined by enzyme-linked immunosorbent assay (ELISA) performed on sera from immunized mice in the memory phase, as previously described (8). Serum samples from individual mice of each naive and immunized group were serially diluted 2-fold in triplicate and incubated with purified recombinant CS protein (2 μg/ml; Pfenex)-coated plates. The CS protein-specific IgG data are depicted as the mean OD_{450} for each dilution assayed for each group. The CS protein-specific IgG antibody titer was defined as the inverse of the highest serum dilution showing at least 3 times the mean OD_{450} for the naive control. The data presented correspond to the serum analysis of each vaccinated mouse (titer graph) and the mean of the values obtained for each mouse (OD graph).

Data analysis. Statistical analysis was performed using Minitab for Windows. One-way analysis of variance (ANOVA) was used to determine the statistical significance of differences between groups. For ICS, statistical analysis was based on previously described methods (8, 36, 41).

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