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Enhanced Immunity to *Plasmodium falciparum* Circumsporozoite Protein (PfCSP) by Using *Salmonella enterica* Serovar Typhi Expressing PfCSP and a PfCSP-Encoding DNA Vaccine in a Heterologous Prime-Boost Strategy▽†

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Two *Salmonella enterica* serovar Typhi strains that express and export a truncated version of *Plasmodium falciparum* circumsporozoite surface protein (tCSP) fused to *Salmonella* serovar Typhi *cytolysin A* (ClyA) were constructed as a first step in the development of a preerythrocytic malaria vaccine. Synthetic codon-optimized genes (t-*csp1* and t-*csp2*), containing immunodominant B- and T-cell epitopes present in native *P. falciparum* circumsporozoite surface protein (PfCSP), were fused in frame to the carboxyl terminus of the ClyA gene (*clyA*::t-*csp*) in genetically stabilized expression plasmids. Expression and export of ClyA-tCSP1 and ClyA-tCSP2 by *Salmonella* serovar Typhi vaccine strain CVD 908-*htrA* were demonstrated by immunoblotting of whole-cell lysates and culture supernatants. The immunogenicity of these constructs was evaluated using a "heterologous prime-boost" approach consisting of mucosal priming with *Salmonella* serovar Typhi expressing ClyA-tCSP1 and ClyA-tCSP2, followed by parenteral boosting with PfCSP DNA vaccines pVR2510 and pVR2571. Mice primed intranasally on days 0 and 28 with CVD 908-*htrA*(pSEC10*tcsp2*) and boosted intradermally on day 56 with PfCSP DNA vaccine pVR2571 induced high titers of serum NANP immunoglobulin G (IgG) (predominantly IgG2a); no serological responses to DNA vaccination were observed in the absence of *Salmonella* serovar Typhi-PfCSP priming. Mice primed with *Salmonella* serovar Typhi expressing tCSP2 and boosted with PfCSP DNA also developed high frequencies of gamma interferon-secreting cells, which surpassed those produced by PfCSP DNA in the absence of priming. A prime-boost regimen consisting of mucosal delivery of PfCSP exported from a *Salmonella*-based live-vector vaccine followed by a parenteral PfCSP DNA boosting is a promising strategy for the development of a live-vector-based malaria vaccine.

Effector immune responses directed against *Plasmodium falciparum* preerythrocytic stages may provide the first line of defense to protect against malaria (54). The *P. falciparum* circumsporozoite protein (PfCSP) is the most abundant antigen expressed on the surface of *P. falciparum* sporozoites, and it has been the main target antigen in the development of preerythrocytic malaria vaccines (47), including subunit proteins with adjuvants or attenuated viral platforms (32, 51, 56). High titers of antibodies directed to PfCSP are believed to interrupt the invasion of hepatocytes by blood-borne sporozoites, thereby abrogating the development of blood-stage malaria (47, 60). Immunization with irradiated sporozoites is known to induce sterile immunity against homologous challenge with *Plasmodium* spp. sporozoites in humans (10). A recombinant PfCSP-based vaccine, RTS,S/AS02A, conferred protection against malaria in humans that was significant, albeit of limited duration (5–7, 51).

We are pursuing the development of *Salmonella enterica* serovar Typhi live-vector vaccines that can efficiently express protective antigens from the various stages in the life cycle of *P. falciparum*, with the ultimate goal of stimulating relevant immune responses in humans (21, 22). The feasibility of using *Salmonella* serovar Typhi expressing PfCSP as an oral live-vector vaccine was demonstrated in an early clinical trial in which serovar Typhi vaccine strain CVD 908 carrying the PfCSP gene integrated into the chromosome stimulated circumsporozoite protein (CSP)-specific serum antibodies and cytotoxic lymphocytes (22). However, these responses were modest and limited to only a fraction of the vaccinated subjects.

One approach to improving the immunogenicity of malaria live-vector vaccines involves the use of genes optimized for prokaryotic codon usage, expressed from genetically stabilized plasmids, to improve the levels of foreign-antigen expression (18, 19). Antigen export systems provide yet another mechanism for enhancing immune responses to foreign antigens (20, 23, 28). Such improvements to foreign-antigen expression in the live vector can also be combined with additional refinements in the immunization strategy, including "heterologous

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prime-boost" approaches. Heterologous prime-boost strategies, which involve sequential administration of the same antigen in two different vaccine formulations by the same or different routes, have also been shown to improve the immunogenicity of foreign antigens expressed by bacterial live vectors. Mucosal priming with *Salmonella* serovar Typhi CVD 908 expressing *Helicobacter pylori* urease followed by parenteral boosting with urease was shown by Londono-Arcila et al. (30) to confer partial protection following challenge with a mouse-adapted strain of *H. pylori*. Further success was reported by Vindurampulle et al. (55), using *Salmonella* serovar Typhi expressing nontoxic fragment C of tetanus toxin. Mice primed intranasally (i.n.) with CVD 908-*htrA* expressing fragment C and boosted intramuscularly (i.m.) with tetanus toxoid mounted enhanced and more rapid antitoxin responses compared to the responses of mice primed and boosted with either the live vector or tetanus toxoid alone.

The success of the heterologous prime-boost strategy has also been illustrated in preerythrocytic-stage falciparum malaria vaccine trials (15, 33, 58). Volunteers immunized parenterally with a DNA vaccine encoding PfCSP followed by parenteral boosting with the PfCSP-derived RTS,S/AS02A vaccine exhibited PfCSP-specific antibodies and gamma interferon (IFN-γ) production and had $CD8^+$-mediated cytotoxic lymphocytes (CTLs) (15, 58). This broad and balanced humoral and cellular immune response was not seen in volunteers immunized either with DNA vaccine alone or with RTS,S/AS02A alone (6, 51, 57).

Here, we describe the development of *Salmonella* serovar Typhi-based preerythrocytic malaria vaccine constructs expressing a truncated version of PfCSP (tCSP). In these constructs, tCSP is exported via fusion to the ClyA cryptic hemolysin of *Salmonella* serovar Typhi (20, 49). Two synthetic codon-optimized genes (t-*csp1* and t-*csp2*) encoding tCSP genetically fused in frame to the carboxyl terminus of ClyA (*clyA*::t-*csp*) were engineered to export ClyA-tCSP from CVD 908-*htrA* to the extracellular milieu. The immunogenicity of these constructs was evaluated using a heterologous prime-boost approach consisting of mucosal priming with *Salmonella* serovar Typhi exporting ClyA-tCSP followed by parenteral boosting with PfCSP-encoding DNA vaccine.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Plasmid constructs were maintained in and recovered from *Escherichia coli* strain DH5α (Invitrogen Life Technologies, Carlsbad, CA). The live vector *Salmonella* serovar Typhi CVD 908-*htrA* is an auxotrophic derivative of wild-type strain Ty2 with deletions in *aroC*, *aroD*, and *htrA* (52). Strains used in this study were grown in Luria-Bertani (LB) medium alone or supplemented with 2,3-dihydroxybenzoic acid (Sigma, St. Louis, MO), as previously described (17, 25). When grown on solid medium, plasmid-bearing derivatives of CVD 908-*htrA* were streaked from frozen (−70°C) master stocks onto 2× LB agar containing 20 g Bacto tryptone, 10 g Bacto yeast extract, and 50 mM NaCl (2×LB50 agar) plus kanamycin at a concentration of 10 μg/ml or carbenicillin at a concentration of 50 μg/ml, as appropriate. Plates were incubated at 30°C for 24 to 36 h to obtain isolated colonies ~2 mm in diameter to minimize any toxicity of heterologous antigen expression in CVD 908-*htrA*.

Molecular genetic techniques. Standard techniques were used to construct the plasmids (44). Unless otherwise noted, *Taq* DNA polymerase (Invitrogen) or Vent DNA polymerase (New England BioLabs, Beverly, MA) was used in PCRs. *Salmonella* serovar Typhi strains were electroporated with recombinant plasmids using standard techniques (17). Isolated transformants were swabbed onto supplemented 2×LB50 agar and incubated at 30°C for 16 h. Frozen master stocks were prepared by harvesting bacteria into LB medium with 30% glycerol without further supplementation and freezing at −70°C.

Construction of *clyA*::t-*csp* expression plasmids and expression analysis. Primers used for construction of the t-*csp* alleles are described in Table S1 in the supplemental material. Both t-*csp* alleles were inserted into the unique NheI site of pSEC10, a low-copy-number expression plasmid containing the origin of replication from pSC101, which normally exists at a level of five copies per chromosomal equivalent (49). The sequence encoding the *csp* allele used for codon optimization was taken from the published sequence of *csp* determined for a recent field isolate from an area of Thailand where the organism is endemic (27). The central part of wild-type *csp* encodes repeating NANP and NVDP tetrapeptides, which comprise crucial immunodominant B-cell epitopes (11). This central repeat region is flanked at the 5′ and 3′ termini by nonrepeat regions containing two strongly conserved regions, regions I and II-plus (11, 46), implicated in the invasion of hepatocytes (16, 39, 46, 47). To minimize genetic instability due to homologous recombination and to facilitate PCR amplification, the central repeat region was truncated from 44 NANP repeats to 11 and from three NVDP repeats to two, while regions I and II-plus were preserved. The N-terminal and C-terminal regions that contain hydrophobic sequences were also eliminated. Since multiple helper and cytotoxic T-cell epitopes are found in the highly polymorphic carboxyl terminus of PfCSP in various *P. falciparum* strains, we elected to synthesize two alleles of t-*csp*, one closely resembling the parent Thailand allele (t-*csp1*) and the other incorporating most of the epitopes thought to be involved in immunity to the 3D7 allele (t-*csp2*) (Fig. 1A and Table 1) (4, 8, 12, 31, 60). To increase expression, the open reading frames of both alleles were codon optimized using the codon preferences for *Salmonella* serovar Typhi available at the Codon Usage Database of the Kazusa DNA Research Institute (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=Salmonella+typhi+[gbbct]). ClyA-tCSP fusions were detected using anti-NANP monoclonal antibody (MAb) 2A10, kindly provided by Anthony W. Stowers (Malaria Vaccine Development Branch, NIAID, NIH, Rockville, MD) (50, 60). Immunoblotting was performed as previously described (20) using the ECL+Plus detection system (Amersham Biosciences, Piscataway, NJ) and Kodak X-OMAT XAR-2 film.

(i) t-*csp1*. t-*csp1* was constructed in a two-step PCR using seven overlapping oligonucleotides (primers 1 to 7 [see Table S1 in the supplemental material]) with 21-base regions of complementarity in an initial assembly reaction and two shorter primers, primers 8 and 9, in an amplification reaction to generate sufficient full-length synthetic t-*csp1* for subcloning. The assembly reaction was carried out in a 50-μl mixture using 2.5 U *Taq* DNA polymerase (Invitrogen) in the presence of primers 1 to 7, 1 mM $MgCl_2$, and 250 μM deoxynucleoside triphosphates in reaction buffer, as recommended by the manufacturer. The PCR parameters were as follows: one cycle of 94°C for 4 min, 30 cycles of 92°C for 1.5 min, 45°C for 1 min, and 72°C for 1 min, and one cycle of 72°C for 5 min. The amplification reaction was carried out in a 100-μl mixture using 4 μl of the assembly reaction mixture as the template in the presence of primers 8 and 9 plus 5 U of *Taq* and 1 U of Deep Vent DNA polymerase (New England BioLabs); the other reaction and cycle parameters were the same as those used for the assembly reaction. Since preliminary immunoblot analysis of t-*csp1* indicated that there was poor expression of unfused tCSP1, we reengineered this optimized synthetic gene as a t-*csp1*–*tetA* cassette that could be inserted in frame into the NheI fusion insertion site at the 3′ terminus of *clyA*; this was accomplished by overlapping PCR using primers 10 and 11 with synthetic t-*csp1* template DNA and primers 12 and 13 with a template derived from pBR322. The resulting t-*csp1*–*tetA* product was ultimately inserted as an 1,812-bp SpeI fragment into pSEC10 cleaved with NheI to generate the desired *clyA*::t-*csp1* gene fusion in pSEC10*tcsp1* (Fig. 1B).

(ii) t-*csp2*. DNA sequence analysis of t-*csp1* revealed that point mutations within several intended T-cell epitopes had arisen during construction. Rather than simply site specifically correcting these mutations, we elected to engineer a second t-*csp* allele containing six additional epitopes not contained in t-*csp1* (Table 1).

t-*csp2* was constructed through ligation of three smaller fragments (t-*csp2A*, t-*csp2B*, and t-*csp2C*), synthesized using a total of six primers (primers 14 to 19 [see Table S1 in the supplemental material]). To facilitate sequential assembly of t-*csp2*, individual fragments were generated as genetic fusions with *tetA* (again using primers 12 and 13 with a pBR322-derived template), and the desired cassettes were recovered using pBluescript II SK(+) (Stratagene Corp., La Jolla, CA). PCRs were carried out in 25-μl mixtures using 0.5 U of Vent DNA polymerase (New England BioLabs) in the presence of primers and template, as recommended by the manufacturer. The PCR parameters were as follows: one cycle of 94°C for 3 min, 30 cycles of 94°C for 1.0 min, 55°C for 1.5 min, and 72°C for 2 min, and one cycle of 72°C for 3 min. Primers 14 and 15 were used in reactions with the t-*csp1* template to synthesize the first 348 bp of t-*csp2*, which

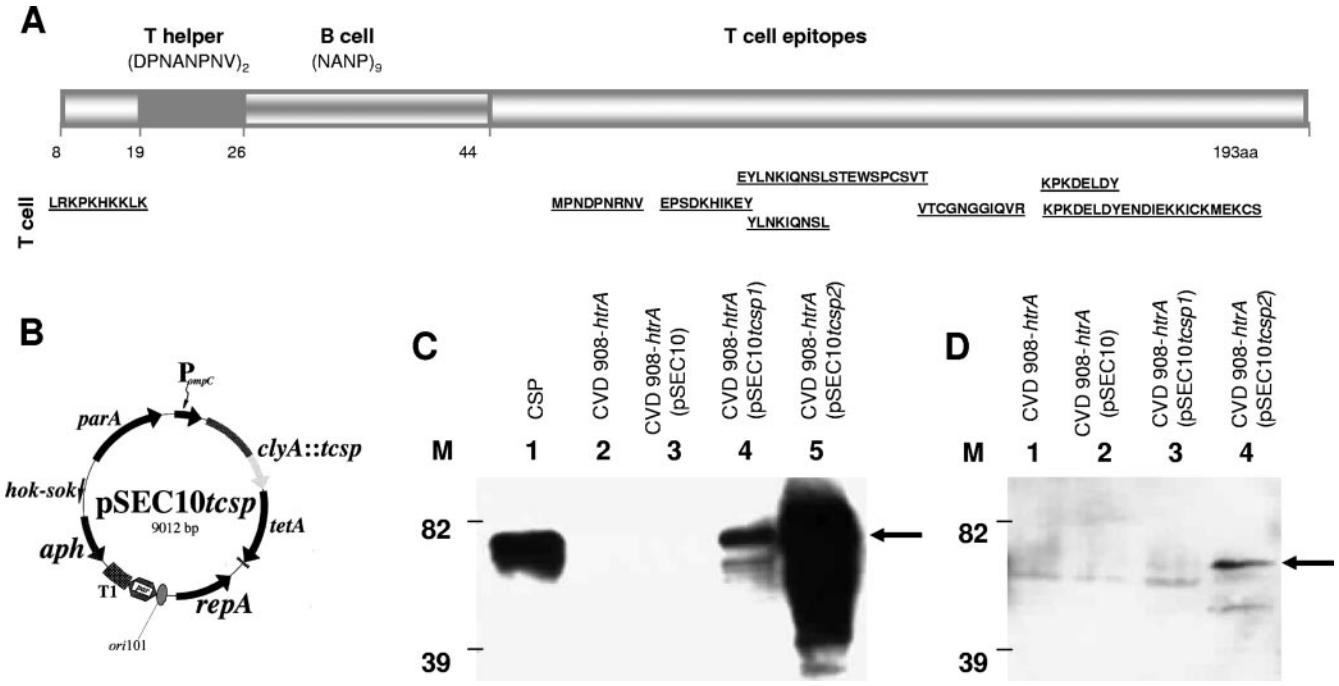

FIG. 1. (A) Schematic diagram showing B- and T-cell epitopes in tCPS2. aa, amino acids. (B) Genetic map of pSEC10*tcsp1* or pSEC10*tcsp2*. Alleles 1 and 2 are not specified as the two expression plasmids were constructed in similar ways. P_{ompC} is a modified osmotically controlled *ompC* promoter from *E. coli*; *clyA* encodes cytolysin A from *Salmonella* serovar Typhi; *clyA*::*tcsp* encodes prokaryotic codon-optimized truncated CSP fused to the carboxyl terminus of ClyA; *tetA* encodes a promoterless tetracycline efflux protein from pBR322; *aph* encodes the aminoglycoside 3′-phosphotransferase conferring resistance to kanamycin; *ori*101 is the origin of replication from pSC101 providing an expected expression plasmid copy number of ~5 copies per chromosomal equivalent; *repA* encodes the replication protein essential for replication of *ori*101; *par* is the passive partitioning locus from pSC101; T1 is the transcriptional terminator from the *rrnB* rRNA operon of *E. coli*; *hok-sok* is the postsegregational killing locus from the multiple-antibiotic-resistance R-plasmid pR1; *parA* encodes the active partitioning system from pR1. (C) Western immunoblot analysis of whole bacterial lysates of CVD 908-*htrA* (lane 2), CVD 908-*htrA*(pSEC10) (lane 3), CVD 908-*htrA*(pSEC10*tcsp1*) (lane 4), and CVD 908-*htrA*(pSEC10*tcsp2*) (lane 5). *P. falciparum* sporozoite crude lysate (7.5 ng) was included in lane 1 as a positive control. The membrane was probed with MAb 2A10 specific for the NANP repeat region of CSP. M, molecular mass (in kilodaltons). (D) Western immunoblot analysis of filtered culture supernatants from CVD 908-*htrA* (lane 1), CVD 908-*htrA*(pSEC10) (lane 2), CVD 908-*htrA*(pSEC10*tcsp1*) (lane 3), and CVD 908-*htrA*(pSEC10*tcsp2*) (lane 4). The membrane was probed with MAb 2A10. The arrows indicate the ClyA-tCSP fusion protein; although the expected position was 54 kDa, a band at a slightly higher molecular mass was observed both in whole bacterial lysates and in culture supernatants.

was in turn combined with *tetA* in overlapping PCRs to generate a 1,585-bp t-*csp2A*–*tetA* cassette. Primers 16 and 17 and primers 18 and 19 were used in reactions with the t-*csp1* template to synthesize 210- and 179-bp fragments, respectively, which were also combined with *tetA* in overlapping PCRs to generate the desired 1,393-bp t-*csp2B*–*tetA* and 1,362-bp t-*csp2C*–*tetA* cassettes. t-*csp2A* was then ligated with t-*csp2B* to create a 480-bp t-*csp2AB* SpeI-NheI cassette, into which t-*csp2C* was inserted at the NheI site to create the final t-*csp2* allele. The integrity of t-*csp2* was confirmed by DNA sequence analysis. Similar

TABLE 1. Recognized human lymphocyte epitopes of CSP included in the engineering of t-*csp1* and t-*csp2*

Allele	B-cell epitope	T-cell epitope (MHC restriction)	Position[a]	Reference(s)
t-*csp1*	NANP (11 repeats)			15, 35, 47
		DPNANPNVDPNANPNV ("T1"; HLA-DQ/DR)	103–118, 111–126	8
		MPNDPNRNV (HLA-B7)	285–293	56
		VTCGNGIQVR (HLA-A3 supertype)	336–345	13
t-*csp2*	NANP (11 repeats)			15, 34, 35, 47
		DPNANPNVDPNANPNV ("T1"; HLA-DQ/DR)	103–118, 111–126	8
		LRKPKHKKLK (HLA-B8)	86–95	3
		MPNDPNRNV (HLA-B7)	285–293	56
		EPSDKHIKEY (HLA-A1)	310–319	56
		YLNKIQNSL (HLA-A2)	319–327	13
		EYLNKIQNSLSTEWSPCSVT (T* universal CD4)	318–337	8
		VTCGNGIQVR (HLA-A3 supertype)	336–345	13
		KPKDELDY (HLA-B35)	353–360	24
		KPKDELDYENDIEKKICKMEKCS (defined only as an MHC class I-restricted epitope)	353–375	31

[a] Position relative to the amino acid sequence of the *P. falciparum* 3D7 isolate.

to t-*csp1*, t-*csp2* was inserted as an 1,815-bp t-*csp2*–*tetA* SpeI fragment into pSEC10 cleaved with NheI to generate the desired *clyA*::t-*csp2* gene fusion in pSEC10*tcsp2* (Fig. 1B).

PfCSP DNA vaccines. Three DNA vaccine plasmids (kindly provided by Stephen L. Hoffman, Malaria Program, Naval Medical Research Center, Silver Spring, MD) were used: (i) pVR2510, which includes the full-length *csp* allele of the 3D7 strain of *P. falciparum* (56); (ii) pVR2571, which includes a full-length mammalian codon-optimized *csp* allele from 3D7 (45); and (iii) control plasmid pVR2576, which encodes *P. falciparum* sporozoite surface protein SSP-2 (45). Plasmids used for immunization were purified using endo-free QIAGEN columns as indicated by the manufacturer (QIAGEN Inc, Valencia, CA) and were resuspended in sterile phosphate-buffered saline (PBS).

Immunization of mice. Female BALB/c mice that were 6 to 8 weeks old (Charles River, Wilmington, MA) were inoculated i.n. on days 0 and 28 with *Salmonella* serovar Typhi vaccine suspensions (1×10^9 to 3×10^9 CFU in 10 μl) as previously described (17). Mice were boosted with PfCSP DNA vaccine plasmids pVR2510 and pVR2571 at doses of 100 μg and 20 μg in 100 μl of PBS, respectively. Plasmids were injected i.m. into the tibialis anterioris with a needle and syringe or intradermally (i.d.) into a shaved thigh using a Biojector 2000 needle-free jet injector (Bioject Medical Technologies, Portland, OR) (2). In experiments to establish optimal conditions for boosting, mice were immunized with increasing doses of pVR2571 (1 to 100 μg) i.m. or i.d., as described above. Blood samples were collected on days 0, 28, 56, 70, and 85 after primary immunization; sera were stored at −70°C until they were tested.

Measurement of antibodies to NANP and *Salmonella* serovar Typhi LPS. Levels of serum immunoglobulin G (IgG) antibodies against NANP and *Salmonella* serovar Typhi lipopolysaccharide (LPS) were measured by an enzyme-linked immunosorbent assay (ELISA) (37). Briefly, 96-well plates were coated with 100 μl of $(NANP)_{50}$ synthetic repeat polymer (Hoffman-La Roche, Basel, Switzerland) at 5 μg/ml or with *Salmonella* serovar Typhi LPS (Sigma) at 10 μg/ml in carbonate buffer (pH 9) and blocked with 10% dry milk (Nestle USA Inc., Glendale, CA) in PBS. Sera were tested in serial dilutions in PBS containing Tween 20 and milk. Specific antibodies were detected with goat anti-mouse IgG-, IgG1-, or IgG2a-horseradish peroxidase (HRP) conjugates (Roche Diagnostics Corporation, Indianapolis, IN), followed by the 3,3,5′,5′-tetramethylbenzidine microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). After 15 min of incubation, the reaction was stopped by addition of 100 μl of 1 M H_2PO_3, and the absorbance at 450 nm was measured. Negative and positive controls were included in each assay. NANP antibody titers were calculated by interpolation of regression-corrected absorbance values into a standard curve with known concentrations of anti-NANP MAb 2A10 and were expressed in ng/ml. NANP IgG subclass and LPS IgG endpoint titers were calculated through a linear regression equation as the reciprocal of the serum dilution that produced an absorbance value that was 0.2 U above the blank and were expressed in ELISA units/ml. Seroconversion was defined as a fourfold increase in the antibody titer postvaccination.

Immunofluorescence. Slides containing air-dried sporozoites (3D7 strain of *P. falciparum*) were kindly provided by John B. Sacci, University of Maryland Baltimore. Pooled serum samples from each group were tested in serial dilutions (starting at 1:40 in 1% bovine serum albumin). Antibodies recognizing the *P. falciparum* sporozoite were detected with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Kirkegaard & Perry Laboratories). To visualize the sporozoite nuclei, slides were subsequently incubated with 1% 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS for 2 min. Prolong Gold (Invitrogen) was used as a mounting solution. Endpoint titers were determined as the inverse of the highest serum dilution producing specific fluorescence. Pictures were obtained using a Nikon Eclipse 2000-E UV fluorescent microscope and MetaVue software, version 6.1 (Universal Imaging Corp.).

Measurement of the frequency of IFN-γ- and IL-5-secreting T cells. PfCSP-specific gamma interferon (IFN-γ)-secreting cells were measured by an enzyme-linked immunospot (ELISPOT) assay (21). Briefly, 96-well nitrocellulose plates (Multiscreen-HA; Millipore, Bedford, MA) were coated with 5 μg/ml of anti-mouse IFN-γ (BD Biosciences/Pharmingen, San Diego, CA) and blocked with RPMI supplemented with 10% fetal calf serum (GIBCO, BRL). Splenocytes (1×10^6 to 1.25×10^5 cells/well) were added to the plates and incubated for 40 h with irradiated major histocompatibility complex (MHC)-matched ($H\text{-}2^d$) P815 cells previously infected with vaccinia virus encoding PfCSP (vP1255; WR-PfCSP) or a control (WR) (Virogenetics, Troy, NY). Vaccinia virus infection was performed the day before the assay using a multiplicity of infection of 5 virus PFU/cell for 2.5 h. The efficiency of infection was assessed by flow cytometry using an FITC-labeled rabbit anti-vaccinia Lister strain antiserum (Virostat, Portland, ME). Effector cells were tested in triplicate wells. Following incubation, plates were washed with PBS containing Tween 20 and incubated with biotin-labeled anti-IFN-γ MAb (BD Biosciences/Pharmingen) for 2 h. Wells were washed and incubated with 100 μl of streptavidin-HRP (Sigma) for 1 h at 37°C and then with TrueBlue peroxidase substrate (Kirkegaard & Perry Laboratories, Inc.). The number of IFN-γ spot-forming cells (SFC) was determined using a stereomicroscope. The results were expressed as the mean number of SFC per 10^6 cells ± standard deviation for replicate wells after subtraction of the number of SFC in control wells. PfCSP-specific interleukin-5 (IL-5) responses were measured as described above by incubating splenocytes with purified PfCSP (kindly provided by David E. Lanar, Division of Malaria Vaccine Development, Walter Reed Army Institute of Research, Silver Spring, MD) or a medium control in nitrocellulose plates coated with anti-IL-5 MAb, followed by biotin-labeled anti-IL-5 (BD Biosciences/Pharmingen) and streptavidin-HRP.

Statistical analysis. Antibody titers and frequencies of IFN-γ SFC were compared using the *t* test and the Mann-Whitney rank sum test (for nonparametric data). The level of significance was set at a P value of <0.05. Statistical analyses were performed using SigmaStat 2.0 software (Systat Software, Inc., Point Richmond, CA).

RESULTS

Construction of ClyA-tCSP1 and ClyA-tCSP2 and analysis of protein expression. To improve expression of a ClyA-CSP fusion protein in *Salmonella*, we engineered synthetic codon-optimized t-*csp* alleles (i.e., t-*csp1* and t-*csp2*). The synthetic *clyA*–t-*csp1* cassette contained an open reading frame encoding a tCSP1 protein consisting of 192 residues and having an expected molecular mass of 20.7 kDa; therefore, the expected mass of the ClyA-tCSP1 protein fusion would be 54.4 kDa. Similarly, *clyA*–t-*csp2* encoded a 54.6-kDa fusion protein that included a 193-residue, 20.9-kDa tCSP2 protein (Fig. 1A).

Repeated attempts to insert an 1,812-bp SpeI t-*csp1*–*tetA* fragment into the higher-copy-number pSEC84 (~60 copies) and pSEC91 (~15 copies) expression plasmids failed. Success inserting the cassette was finally achieved with the low-copy-number pSEC10 plasmid cleaved with NheI, generating the desired *clyA*::t-*csp1* gene fusion plasmid pSEC10*tcsp1* (Fig. 1B).

Sequence analysis of t-*csp1* confirmed that both conserved region I and conserved region II-plus remained intact. In addition, a number of well-described human helper T-cell epitopes were preserved, including the highly conserved HLA-DQ/DR-restricted human helper T1 epitope (DPNANPNVD PNANPNV) characterized by Calvo-Calle et al. (8). The presence of the human CTL epitope HLA-B7 (MPNDPNRNV [56]) and the presence of the supertype HLA.A3 epitope (VT CGNGIQVR [13]) within region II-plus (46)] were also verified (Table 1).

The t-*csp2*--*tetA* cassette was also inserted as an 1,815-bp SpeI fragment into pSEC10 cleaved with NheI, creating pSEC10*tcsp2*. tCSP2 contains the same 11 NANP and 2 NVDP repeats that are present within tCSP1 and the three T-cell epitopes, but it also contains an additional universal T-helper epitope (T*) (EYLNKIQNSLSTEWSPCSVT [8]) and five additional $CD8^+$-restricted T-cell epitopes (Fig. 1A and Table 1). As such, the 193-residue tCSP2 protein is 89% identical at the amino acid level to residues 67 to 189 of the RTS,S nonrepeat carboxyl terminus.

Following introduction of pSEC10*tcsp1* and pSEC10*tcsp2* into CVD 908-*htrA* by electroporation, we examined the expression and export of the ClyA-tCSP fusion proteins by Western immunoblot analysis, using anti-NANP MAb 2A10. As shown in Fig. 1C, both protein fusions were detected in whole-cell lysates of CVD 908-*htrA*(pSEC10*tcsp1*) and CVD 908-

htrA(pSEC10*tcsp2*). We observed a striking disparity in expression levels, with ClyA-tCSP1 expression being much lower than ClyA-tCSP2 expression (Fig. 1C, lanes 4 and 5). Whereas both fusion proteins were also detected (albeit at much lower levels) in trichloroacetic acid-precipitated supernatants from these cultures, the level of ClyA-tCSP1 expression was negligible (Fig. 1D, lanes 3 and 4). No protein was recognized by MAb 2A10 in whole-cell lysates or supernatants from either CVD 908-*htrA* or CVD 908-*htrA*(pSEC10) negative controls.

Humoral and cellular immune responses to ClyA-tCSP1 exported from *Salmonella* serovar Typhi using a heterologous mucosal prime-parenteral boost approach. A proof-of-principle experiment was performed to evaluate the effectiveness of a heterologous prime-boost regimen that involved mucosal priming immunization with PfCSP exported from *Salmonella* serovar Typhi live-vector strain CVD 908-*htrA*(pSEC10*tcsp1*), followed by parenteral boosting with a PfCSP-encoding DNA vaccine. Mice were primed i.n. with two doses of CVD 908-*htrA* alone or CVD 908-*htrA*(pSEC10*tcsp1*) on days 0 and 28 and boosted i.m. on day 62 with 100 μg of PfCSP-encoding pVR2510. A positive control group received three doses of pVR2510 i.m., and a negative control group received PBS at the same time points. The results are shown in Fig. 2. Mice primed with two doses of CVD 908-*htrA* (or CVD 908-*htrA* carrying empty plasmid pSEC10 [data not shown]) and boosted with pVR2510 failed to develop NANP-specific serum antibody responses. Notably, significant NANP serum IgG levels (>4-fold rise over preimmunization titers) were observed in 5 of 10 mice primed with CVD 908-*htrA*(pSEC10*tcsp1*) and boosted with pVR2510 (geometric mean titer [GMT], 6 ng/ml; $P = 0.025$ for a comparison with the CVD 908-*htrA*-primed group) on day 85. The highest antibody titers were observed in 8 of 12 mice that received three doses of pVR2510 [peak GMT, 84 ng/ml; $P = 0.038$ for a comparison with mice primed with CVD 908-*htrA*(pSEC10*tcsp1*) and boosted with pVR2510] on day 85 after immunization.

We also examined the IgG subclass distribution as an indicator of the T-helper-cell subsets (Th1/Th2) induced by the heterologous prime-boost strategy. Mice that responded to the CVD 908-*htrA*(pSEC10*tcsp1*) mucosal priming followed by pVR2510 boosting exhibited a predominance of IgG2a, with an IgG2a/IgG1 ratio of 5.2. In contrast, IgG1 prevailed in mice primed and boosted with pVR2510, and the IgG2a/IgG1 ratio was 0.4. All three groups immunized with CVD 908-*htrA* alone or CVD 908-*htrA* carrying pSEC10 or pSEC10*tcsp1* mounted strong serum IgG responses against *Salmonella* serovar Typhi LPS (data not shown).

We assessed the presence of PfCSP-specific T-cell responses by measuring IFN-γ produced by splenocytes upon in vitro stimulation with MHC-matched cells infected with vaccinia virus encoding PfCSP. Mice primed mucosally with CVD 908-*htrA*(pSEC10*tcsp1*) and boosted parenterally with pVR2510 showed the highest frequency of IFN-γ-secreting cells 2 months after the boost (Fig. 2B). In contrast, mice primed with CVD 908-*htrA* alone and boosted with pVR2510 or primed and boosted with pVR2510 exhibited significantly lower IFN-γ responses ($P < 0.001$ and $P = 0.003$, respectively). We also investigated IL-5 production by splenocytes stimulated with recombinant full-length PfCSP. No responses were observed in the vaccinated groups, although IL-5-secreting T cells were found in control wells containing phytohemagglutinin-stimulated cells (data not shown).

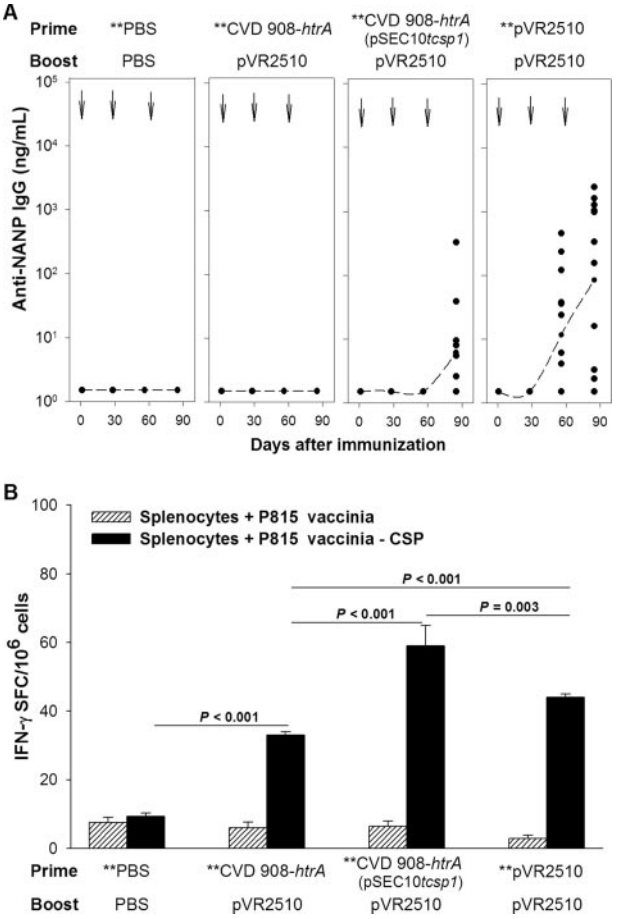

FIG. 2. Immune responses induced by *Salmonella* serovar Typhi expressing ClyA-tCSP1 followed by PfCSP DNA vaccine pVR2510 in a heterologous prime-boost strategy. (A) NANP-specific IgG titers measured by ELISA. Mice were primed i.n. with two doses (two asterisks) of either CVD 908-*htrA* or CVD 908-*htrA*(pSEC10*tcsp1*) on days 0 and 28 and boosted i.m. on day 62 with 100 μg pVR2510, which encodes the full-length PfCSP. Mice primed and boosted with pVR2510 and mice primed and boosted with PBS served as positive and negative controls, respectively. An additional control group received CVD 908-*htrA* carrying empty pSEC10 followed by pVR2510; these mice did not elicit NANP-specific IgG (data not shown). The arrows indicate each immunization. The data are titers from individual animals measured on days 0, 28, 56, and 85 postimmunization; the dashed line was plotted upon the GMT. (B) Frequency of PfCSP-specific IFN-γ-secreting cells measured by the ELISPOT assay. Mice were immunized as described above. Spleens were harvested 2 months after the boost, and splenocytes were restimulated in vitro with irradiated MHC-matched P815 cells infected with PfCSP-encoding vaccinia virus (solid bars) or a vaccinia virus control (cross-hatched bars) as described in Materials and Methods. The bars indicate the mean numbers of IFN-γ SFC per 10^6 cells, and the error bars indicate standard deviations. Statistically significant differences in responses between groups are indicated.

Optimization of boosting using a mammalian codon-optimized PfCSP-encoding DNA vaccine. To further optimize our prime-boost strategy, we evaluated whether recall responses could be improved using an alternative PfCSP-encoding DNA vaccine, pVR2571, as the boosting agent. This plasmid con-

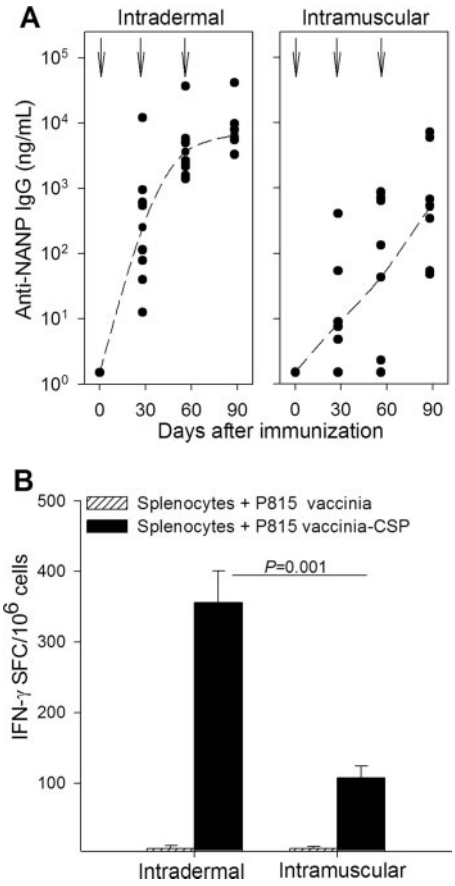

FIG. 3. Immune responses to PfCSP DNA vaccine pVR2571 obtained using different routes and delivery systems. (A) NANP-specific IgG titers. Mice were immunized on days 0, 28, and 56 (indicated by arrows) with 100 μg of pVR2571, which contained the mammalian codon-optimized *csp*, i.d using the Biojector 2000 needle-free jet injector or i.m. using a needle and syringe. The solid circles indicate the titers of individual mice; the dashed lines were plotted upon the GMT. (B) Frequency of PfCSP-specific IFN-γ SFC. Mice were immunized with pVR2571 as described above. Spleens were harvested 2 months after the last immunization, and IFN-γ responses were measured by the ELISPOT assay as described in the legend to Fig. 2. The bars indicate the mean numbers of IFN-γ SFC per 10^6 cells, and the error bars indicate standard deviations. Statistically significant differences are indicated.

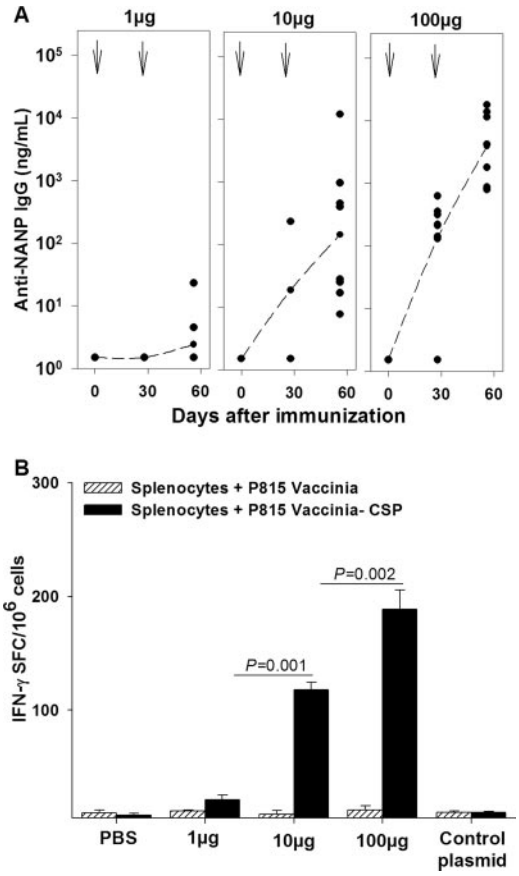

FIG. 4. Dose-response analysis of antibody and IFN-γ responses induced by PfCSP DNA vaccine pVR2571. (A) NANP-specific IgG titers. Mice were immunized with increasing doses of pVR2571 by the i.d. route using the Biojector 2000. Mice immunized with a control plasmid expressing an unrelated antigen (pVR2576) or PBS were included as negative controls; no antigen-specific antibody titers were observed (data not shown). The solid circles indicate the titers of individual mice, and the dashed lines were plotted upon the GMT. Arrows indicate each immunization. (B) Frequency of PfCSP-specific IFN-γ SFC. Mice were immunized with increasing doses of DNA vaccine pVR2571 as indicated above. IFN-γ responses were measured as described in the legend to Fig. 2 at 2 months after the last immunization. The bars indicate the mean numbers of IFN-γ SFC per 10^6 cells, and the error bars indicate standard deviations. Statistically significant differences are indicated.

tains a mammalian codon-optimized allele of *P. falciparum* 3D7 full-length PfCSP rather than the wild-type 3D7 allele present in pVR2510. The rationale for including pVR2571 in these prime-boost studies was to ultimately develop a more effective vaccine approach intended for use in humans. Preliminary experiments comparing the immunogenicities of the two plasmids in mice immunized i.m. revealed that pVR2571 induced higher NANP specific serum IgG titers than pVR2510 (GMT, 513 ng/ml and 84 ng/ml, respectively; $P < 0.05$) and more robust cell-mediated immunity (CMI), measured as PfCSP-specific IFN-γ production (108 and 44 SFC/10^6 cells, respectively; $P < 0.05$ [data not shown]).

We examined whether the responses elicited by pVR2571 could be further enhanced through immunization by the i.d. route, which has been shown to be more effective than the i.m. route for other PfCSP-encoding DNA vaccines (2). Thus, mice were immunized with three doses (100 μg each) of pVR2571 given i.m. via needle or i.d. using the Biojector. Remarkably higher immune responses were generated when the PfCSP-encoding DNA vaccine was delivered i.d. using the Biojector 2000, which elicited NANP-specific serum IgG titers that were 10-fold higher than those achieved by i.m. needle injection (GMT, 6,624 ng/ml and 513 ng/ml, respectively; $P = 0.015$) (Fig. 3A). Similarly, a higher frequency of IFN-γ-secreting cells was observed in mice immunized i.d. with pVR2571 than in mice immunized i.m. (356 versus 108 SFC/10^6 cells; $P = 0.001$) (Fig. 3B).

We also performed a dose-response immunogenicity study in which groups of mice received 1, 10, or 100 μg of pVR2571 i.d. by means of the Biojector on days 0 and 28. The results are shown in Fig. 4A. The mice that received 1 μg of pVR2571

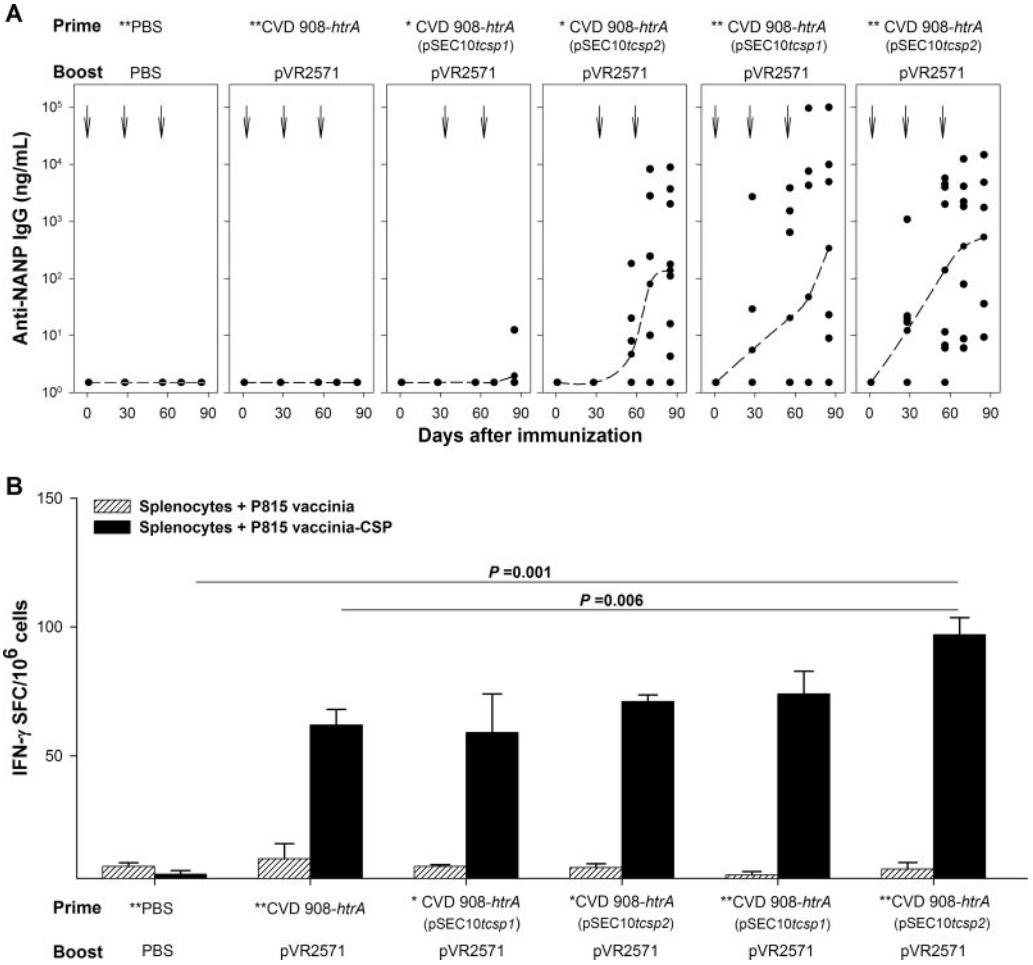

FIG. 5. Immune responses in mice primed with *Salmonella* serovar Typhi expressing ClyA-tCSP2 and boosted with PfCSP DNA vaccine pVR2571. (A) NANP-specific IgG titers. Mice were primed with one dose (one asterisk) (day 28) or two doses (two asterisks) (days 0 and 28) of CVD 908-*htrA*, CVD908-*htrA*(pSEC10*tcsp1*), or CVD908-*htrA*(pSEC10*tcsp2*) and boosted i.d. with 20 μg of pVR2571. Mice that received three doses of PBS served as negative controls. The solid circles indicate the antibody titers in individual mice, and antibody production curves are plotted upon the GMT. Arrows indicate each immunization. (B) Frequency of PfCSP-specific IFN-γ SFC. Mice were immunized as described above. Spleens were harvested 2 months after the boost, and IFN-γ responses were measured by the ELISPOT assay as described in the legend to Fig. 2. The bars indicate the mean numbers of SFC per 10^6 cells, and the error bars indicate standard deviations. Statistically significant differences between IFN-γ responses in mice primed twice with CVD 908-*htrA*(pSEC10*tcsp2*) and IFN-γ responses in mice primed with CVD 908-*htrA* or PBS are indicated.

exhibited negligible antibody responses (only two of eight mice seroconverted after three doses; GMT, 2.4 ng/ml; $P = 0.442$ for a comparison with the PBS control), whereas those that received 10 μg achieved high IgG anti-NANP antibody titers (all seroconverted; GMT, 145 ng/ml; $P = 0.001$ for a comparison with the PBS control). Mice immunized with 100 μg of pVR2571 exhibited the highest antibody responses (GMT, 3,937 ng/ml; $P < 0.001$ for a comparison with the PBS control).

In addition, the T-cell responses in relation to the escalating doses of PfCSP DNA vaccine were measured by determining the frequency of IFN-γ-secreting cells (Fig. 4B). Mice that received 1 μg of pVR2571 had insignificant IFN-γ production, whereas mice that received 10 μg or 100 μg showed higher frequencies of IFN-γ-secreting cells (118 and 189 SFC/10^6 cells, respectively, 2 months after the second dose; $P = 0.001$ and $P = 0.002$ for 10 μg and 100 μg compared with the 1-μg dose, respectively). Based on these results, we selected 20 μg (equivalent to two 10-μg doses) and the i.d. route of immunization for subsequent prime-boost experiments. We reasoned that the use of a suboptimal dose of DNA to boost would enable us to more clearly assess the priming capacity of the live vectors, as opposed to a higher dose (such as 100 μg) that was already highly immunogenic and would likely override the priming effect.

Immunogenicity of *Salmonella* serovar Typhi-exported ClyA-tCSP2 in an optimized heterologous prime-boost regimen. Having optimized the conditions for boosting with PfCSP DNA vaccine, we compared the priming capacities of CVD 908-*htrA*(pSEC10*tcsp1*) and CVD 908-*htrA*(pSEC10*tcsp2*) followed by a 20-μg dose of pVR2571 delivered i.d. via the Biojector 2000. In addition, we examined whether there was a difference in the immune responses when we primed with one or two doses of live vector. The results are shown in Fig. 5. A single priming dose of CVD 908-*htrA*(pSEC10*tcsp1*) failed to

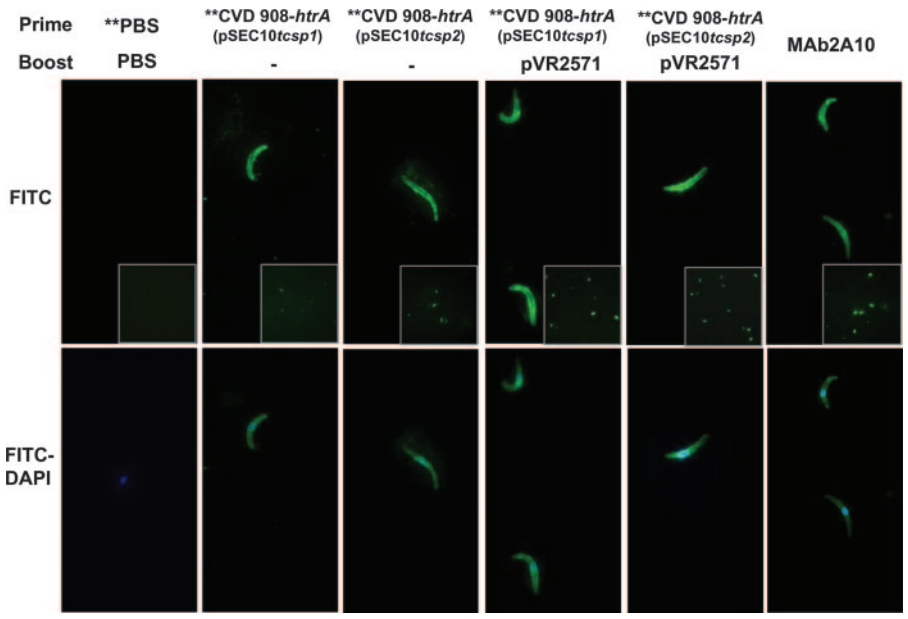

FIG. 6. Binding of PfCSP antibodies elicited by *Salmonella* serovar Typhi ClyA-tCSP priming and PfCSP DNA boosting to *P. falciparum* sporozoites. Antibodies recognizing PfCSP in the *P. falciparum* sporozoite were detected by an immunofluorescence assay using FITC-labeled anti-mouse IgG (upper panels); parasite nuclei were stained with DAPI (superimposed images in lower panels). MAb 2A10 was used as a positive control. The images were obtained with a ×100 objective and a ×40 objective (insets).

induce NANP-specific antibodies, whereas humoral responses became visible after two priming doses of CVD 908-*htrA*(pSEC10*tcsp1*) followed by a pVR2571 boost; five of eight mice seroconverted with a peak GMT of 338 ng/ml ($P = 0.008$ for a comparison with priming with CVD 908-*htrA* alone) (Fig. 5A). One priming dose of CVD 908-*htrA*(pSEC10*tcsp2*) followed by pVR2571 elicited high anti-NANP titers; seven of eight mice seroconverted with a peak GMT of 138 ng/ml ($P = 0.002$ for a comparison with priming with CVD 908-*htrA* alone) (Fig. 5A). The highest antibody responses were observed when mice were primed twice with CVD 908-*htrA*(pSEC10*tcsp2*); seven of eight mice seroconverted after priming, and all mice seroconverted after the boost, showing a GMT of 529 ng/ml ($P = 0.002$ for a comparison with priming with CVD 908-*htrA* alone). The IgG2a/IgG1 ratios in mice primed twice with CVD 908-*htrA*(pSEC10*tcsp1*) or CVD 908-*htrA*(pSEC10*tcsp2*) and boosted with pVR2571 were 5.2 and 1.8, respectively. No responses were observed in mice primed with CVD 908-*htrA* alone and boosted with pVR2571 or primed and boosted with PBS. All mice primed with either one or two doses of live vectors mounted antibody responses to *Salmonella* serovar Typhi LPS (data not shown).

The frequency of IFN-γ-secreting cells was measured by an ELISPOT assay in spleens from vaccinated and control mice upon in vitro stimulation with MHC-matched cells expressing PfCSP (Fig. 5B). Mice primed with two doses of CVD 908-*htrA*(pSEC10*tcsp2*) and boosted with pVR2571 mounted a PfCSP-specific IFN-γ response that was significantly higher than the response of control mice primed with CVD 908-*htrA* alone and boosted with pVR2571 ($P = 0.006$).

The capacity of anti-PfCSP antibodies elicited by a *Salmonella* serovar Typhi prime-pVR2571 boost regimen to recognize the native CSP protein in the membrane of the *P. falciparum* sporozoite was examined by immunofluorescence (Fig. 6). In agreement with the ELISA results, positive responses to PfCSP measured by immunofluorescence were demonstrated after mucosal priming with two doses of CVD 908-*htrA*(pSEC10*tcsp1*) or CVD 908-*htrA*(pSEC10*tcsp2*), which increased further after the pVR2571 boost (Fig. 6). Mucosal priming with CVD 908-*htrA*(pSEC10*tcsp2*) followed by pVR2571 boosting elicited the highest titers (1:2,560), as opposed to priming with CVD 908-*htrA*(pSEC10*tcsp1*) (1:640) or with CVD 908-*htrA* alone (1:40).

DISCUSSION

There have been few reports describing attenuated *S. enterica* live vectors as delivery vehicles for protective malarial antigens. The bane of this approach is that eukaryotic antigens are notoriously difficult to express in prokaryotes. Early attempts using plasmid-based expression of full-length PfCSP in attenuated *Salmonella* serovar Typhimurium resulted in partially protective CMI to sporozoites in a murine malaria model but did not elicit significant antibody responses (43). Further attempts involving integration of the full-length CSP gene into the *Salmonella* serovar Typhimurium chromosome likewise produced CMI in the absence of antibodies (1). However, integration of full-length PfCSP into the chromosome of attenuated *Salmonella* serovar Typhi resulted in modest levels of antisporozoite antibodies in mice, as well as modest serologic and CMI responses in humans (22). A recent attempt to improve the immunogenicity of PfCSP delivered by *Salmonella* live-vector vaccines included display of the immunodominant NANP B-cell epitope as tandem repeats on the bacterial surface and their release by fusion to the autotransporter MisL. The immunogenicity of such constructs, however, was negligible (42). Interestingly, the expression in *Salmonella* serovar

Typhimurium of another sporozoite surface protein, SSP-2, was dramatically improved by secretion out of the live vector as a fusion with HlyA through the type I secretion system of uropathogenic *E. coli*, inducing PfSSP-2-specific IFN-γ-secreting T cells (21). Here, we describe for the first time the development of *Salmonella* serovar Typhi live-vector strains capable of expressing and exporting PfCSP using the *Salmonella* serovar Typhi ClyA secretion system. We also demonstrate that these live-vector strains induce PfCSP-specific antibodies and CMI when they are delivered mucosally, followed by parenteral boosting with PfCSP-encoding DNA vaccines in a "heterologous prime-boost" regimen.

We have hypothesized that an optimal immune response against both the live vector and the foreign antigen requires a proper balance between adequate levels of antigen expression to prime the immune system and minimization of the bacterial metabolic burden (18, 19). We reasoned that this could be accomplished through export of foreign antigens out of the live vector. Thus, we engineered the cryptic hemolysin ClyA from *Salmonella* serovar Typhi Ty2 to export PfCSP out of *Salmonella* serovar Typhi vaccine strain CVD 908-*htrA* into the surrounding medium. Two versions of truncated codon-optimized *csp* (t-*csp1* and t-*csp2*) were cloned into a low-copy-number genetically stabilized expression plasmid, pSEC10, and introduced into attenuated *Salmonella* serovar Typhi CVD 908-*htrA*, generating vaccine strains CVD 908-*htrA*(pSEC10*tcsp1*) and CVD 908-*htrA*(pSEC10*tcsp2*). Both constructs were tested in mice in a series of heterologous prime-boost experiments in which mucosal priming with PfCSP-expressing *Salmonella* serovar Typhi live vectors was followed by parenteral boosting with PfCSP-encoding DNA vaccine.

Heterologous prime-boost regimens have been efficient at raising immune responses against poorly immunogenic plasmodial antigens (14, 33). Upon antigenic reexposure, primed memory T and B cells are believed to rapidly expand, mounting enhanced and broadened effector responses. Studies in nonhuman primates (26, 41) and more recently in adult volunteers (15, 58) suggest that prime-boost strategies could be useful for inducing more robust immunity against malaria.

Preliminary "proof-of-principle" experiments combining mucosal delivery of CVD 908-*htrA*(pSEC10*tcsp1*) and then parenteral boosting with the PfCSP DNA vaccine pVR2510 enabled us to confirm the usefulness of the live-vector priming-DNA vaccine boosting regimen to induce CSP-specific humoral immunity and CMI. Previous studies in humans showed that pVR2510 was able to induce CSP-specific $CD8^+$ CTL and IFN-γ responses (56, 57), although it failed to elicit antibody responses (15). One possible explanation given was the low level of PfCSP expressed by pVR2510-transfected cells in vitro (15).

To improve our boosting step, we performed a series of experiments comparing the immune response achieved with pVR2510 with that induced by another PfCSP DNA vaccine candidate, pVR2571, using different methods and routes of plasmid delivery. An advantage of pVR2571 over pVR2510 is that it contains a mammalian codon-optimized *csp* allele; the use of codon-optimized genes for malaria antigens has been shown to enhance the levels of protein expression in vitro and immune responses in vivo (36, 45). We observed that (i) superior PfCSP-specific immune responses, including both NANP antibodies and IFN-γ-secreting cells, were elicited by pVR2571 in comparison with pVR2510 and (ii) pVR2571 was more immunogenic when it was delivered i.d. using jet injection than when it was delivered i.m. using a needle and syringe. The use of the Biojector 2000 for i.m. or combined i.m. and i.d. DNA delivery has previously been shown to dramatically improve antibody responses to PfCSP DNA vaccines in rabbits (2). Our results confirm the superiority of this DNA delivery system using the i.d. route in mice. The enhanced immunity observed after i.d. jet injection could be attributed to a wider distribution of the DNA within the dermis, made possible by the jet injector, in contrast to the localized deposit after needle injection (40). This probably leads to more efficient uptake of DNA by the host's cells, enhanced antigen expression, and more efficient antigen presentation and cross presentation by dendritic cells (9, 40). It is also possible that administration of the DNA vaccine i.d. under high pressure (as opposed to low-pressure administration with a needle and syringe) results in a physical "danger signal" that activates the innate immune system, thereby enhancing the adaptive immune responses. We were able to select a booster dose of DNA vaccine that was suboptimal to induce an immune response by itself yet was sufficient to boost already primed immune responses. An appealing live-vector vaccine for a prime-boost regimen in humans would be a vaccine that can engender high recall responses upon reexposure to small amounts of boosting antigen (59).

Consistently, in all prime-boost experiments, mice that had been primed with PfCSP exported from *Salmonella* serovar Typhi responded with NANP antibodies after the boost with PfCSP-encoding DNA vaccines. In the absence of PfCSP live-vector priming, single boosting doses of pVR2510 or pVR2571 were unable to induce serological responses (Fig. 2 and 5). The magnitude of the immune responses after the boost also correlated with the efficiency of priming. For example, higher postboost responses were observed in mice primed with two doses of CVD 908-*htrA*(pSEC10*tcsp1*) or CVD 908-*htrA*(pSEC10*tcsp2*) than in mice that received a single dose of live vector. Moreover, *Salmonella* serovar Typhi expressing ClyA-tCSP2 had a better priming capacity than ClyA-tCSP1, in agreement with the superior ability of this vaccine strain to express and export PfCSP observed in vitro.

The use of *Salmonella* serovar Typhi expressing PfCSP for priming followed by a PfCSP DNA boost induced both serum antibodies and CMI, which are considered immunologic prerequisites for an effective preerythrocytic malaria vaccine candidate. Antisporozoite antibodies directed towards the immunodominant epitope of CSP, $(NANP)_n$, can protect by blocking sporozoites from entering hepatocytes or from developing further in hepatocytes (46, 47). *Salmonella* serovar Typhi expressing ClyA-tCSP2, in particular, stimulated high levels of NANP-specific serum IgG antibodies after sequential immunization with two doses of mucosally delivered live vector followed by PfCSP DNA vaccine pVR2571 delivered i.d. These antibodies were capable of recognizing PfCSP in its native form, as expressed on the surface of the plasmodium sporozoite (Fig. 6). Analysis of the IgG subclasses of the NANP-specific response showed noticeably higher levels of IgG2a in mice primed with ClyA-tCSP1 or ClyA-tCSP2, whereas similar levels of IgG2a and IgG1 were seen in mice that received only PfCSP DNA vaccines. This difference is likely due to the vigorous Th1-driving force of *Salmonella* serovar Typhi (38). The presence of

IgG2a has been linked with more efficient clearing of intracellular parasite infection (29, 48). The induction of a predominant Th1-type response against CSP in our prime-boost system was confirmed by the observation of splenocyte-derived CSP-specific IFN-γ responses in vaccinated mice in the absence of IL-5, most likely by $CD8^+$ T cells, as discussed below.

It is well accepted that even though antibodies interfere with sporozoite access to the liver, protection at the preerythrocytic phase of malaria infection is mediated largely by cellular immunity. In our prime-boost strategy, mice mucosally primed with *Salmonella* serovar Typhi expressing ClyA-tCSP and boosted with PfCSP vaccine exhibited a high frequency of PfCSP-specific T cells that produced IFN-γ upon antigen stimulation. These responses were detected after in vitro restimulation of splenocytes with PfCSP-expressing P815 cells, which display only MHC class I molecules on the cell surface. Thus, the secretion of IFN-γ is likely derived from $CD8^+$ T cells that uniquely recognize PfCSP expressed in the context of MHC class I molecules. Differential activation of Th1 and Th2 T-cell subsets has been implicated in modulating the course of infection during different stages of the parasite life cycle (53). Although IFN-γ responses to CSP peptides in humans have been associated with $CD4^+$ and $CD8^+$ T cells, depletion of $CD8^+$ cells abrogated or significantly reduced IFN-γ production (57).

Contrary to the requirement of priming for the production of antibodies, IFN-γ-secreting cells were observed after the boost even in the absence of priming, indicating the high capacity of DNA vaccines to elicit de novo T-cell responses. Nevertheless, higher IFN-γ responses were achieved in groups primed with the PfCSP-recombinant live vectors. IFN-γ responses were still strong 2 months after prime-boost immunization, suggesting that this is an active and persistent effector T-cell mechanism.

Taken together, the data presented here suggest that a heterologous prime-boost regimen involving the sequential mucosal delivery of recombinant *Salmonella* serovar Typhi exporting a malaria antigen followed by a parenteral DNA vaccine could be a promising strategy in the development of a malaria vaccine for humans. This strategy generates strong and balanced immune responses and may represent a more practical, more versatile, and less expensive alternative than other vaccine strategies. The approach could also be effective for protection against other viral, bacterial, or parasitic pathogens.

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