Attenuated PfSPZ Vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection

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A live-attenuated malaria vaccine, *Plasmodium falciparum* sporozoite vaccine (PfSPZ Vaccine), confers sterile protection against controlled human malaria infection (CHMI) with *Plasmodium falciparum* (Pf) parasites homologous to the vaccine strain up to 14 mo after final vaccination. No injectable malaria vaccine has demonstrated long-term protection against CHMI using Pf parasites heterologous to the vaccine strain. Here, we conducted an open-label trial with PfSPZ Vaccine at a dose of 9.0×10^5 PfSPZ administered i.v. three times at 8-wk intervals to 15 malaria-naive adults. After CHMI with homologous Pf parasites 19 wk after final immunization, nine (64%) of 14 (95% CI, 35–87%) vaccinated volunteers remained without parasitemia compared with none of six nonvaccinated controls ($P = 0.012$). Of the nine nonparasitemic subjects, six underwent repeat CHMI with heterologous Pf7G8 parasites 33 wk after final immunization. Five (83%) of six (95% CI, 36–99%) remained without parasitemia compared with none of six nonvaccinated controls. PfSPZ-specific T-cell and antibody responses were detected in all vaccine recipients. Cytokine production by T cells from vaccinated subjects after in vitro stimulation with homologous (NF54) or heterologous (7G8) PfSPZ were highly correlated. Interestingly, PfSPZ-specific T-cell responses in the blood peaked after the first immunization and were not enhanced by subsequent immunizations. Collectively, these data suggest durable protection against homologous and heterologous Pf parasites can be achieved with PfSPZ Vaccine. Ongoing studies will determine whether protective efficacy can be enhanced by additional alterations in the vaccine dose and number of immunizations.

plasmodium | live-attenuated vaccine | T-cell immunology | malaria | sporozoite

Malaria caused by *Plasmodium falciparum* (Pf) is a major cause of morbidity and mortality, particularly in children in sub-Saharan Africa (1, 2). Travelers, military personnel, and international health care workers are also at risk (3, 4). The ideal vaccine would confer on individuals high-level, sterile protection against infection with Pf and would facilitate elimination efforts by interrupting parasite transmission (5, 6).

The most extensively studied candidate malaria vaccine, RTS,S/AS01, is composed of a truncated form of the main sporozoite (SPZ) surface protein (circumsporozoite protein, PfCSP), and an immune adjuvant (AS01). To our knowledge, this vaccine has never been tested using controlled human malaria infection (CHMI) with a heterologous Pf strain of parasites. However, a phase 3 efficacy trial in Africa using a four-dose regimen on a 0-, 1-, 2-, and 20-mo schedule showed a 26% and 36% reduction in clinical malaria among 6–12-wk-olds and 5–17-mo-olds, respectively, through 3–4 y of follow-up (7). Therefore, alternative vaccine approaches are needed that confer durable and sterile protection against homologous and heterologous strains.

In contrast to recombinant subunit vaccine approaches, attenuated PfSPZ immunization consistently achieves high-level (>80%) sterile protection against CHMI with homologous Pf parasites (8–11). In addition, short-term protection (<8 wk) against a heterologous CHMI with the Pf7G8 clone was demonstrated in five subjects after exposure to >1,000 irradiated PfSPZ-infected mosquitoes (10, 12). Therefore, high-level

Significance

A highly effective malaria vaccine capable of long-term protection against genetically diverse strains is urgently needed. Here, we demonstrate that a three-dose regimen of a live attenuated whole-parasite malaria vaccine conferred durable sterile protection through 33 weeks in ∼50% of subjects against a controlled human malaria infection strain that is heterologous to the vaccine strain. Prior studies by others and us have shown that T cells are critical to mediating sterile protection after live-attenuated malaria vaccination. Here, we provide evidence that this *Plasmodium falciparum* sporozoite vaccine (PfSPZ Vaccine) induces antigen-specific IFN-γ-producing CD8 and CD4 T cells that recognize both the homologous and the heterologous Pf strain.

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Conflict of interest statement: S.C., E.R.J., P.F.B., A.G., A.M., M.L., A.J.R., T.L., A.G.E., R.E.S., N.K., T.M., T.L.R., B.K.L.S., and S.L.H. are salaried employees of Sanaria Inc., the developer and owner of PfSPZ Vaccine and the investigational new drug (IND) application sponsor of the clinical trials. S.L.H. and B.K.L.S. have a financial interest in Sanaria Inc. All other authors declare no conflict of interest.

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durable sterile protection against heterologous CHMI has not been achieved with any injectable malaria vaccine (10, 13).

Results

Study Design. We recently showed that four i.v. immunizations with PfSPZ Vaccine at a dose of 2.7×10^5 PfSPZ conferred ~55% sterile protection after homologous CHMI up to 14 mo after the final immunization (14). On the basis of the favorable safety profile and dose-dependent durable efficacy against homologous CHMI (11, 14), we continued PfSPZ Vaccine dose escalation to 9.0×10^5 PfSPZ per dose. A three-dose regimen at 8-wk intervals was selected on the basis of data showing that an 8-wk interval between immunizations resulted in enhanced immunogenicity in nonhuman primates (11).

Study Population. Thirty-one volunteers were enrolled: 15 vaccine recipients, 12 CHMI controls, and four back-up controls (*SI Appendix*, Fig. S1). Baseline demographic characteristics are shown in *SI Appendix*, Table S1. Fifteen volunteers (100%) completed all three scheduled vaccinations. Fourteen volunteers underwent CHMI with homologous Pf parasites; one vaccine recipient withdrew before CHMI to attend medical school.

Vaccine Safety. Vaccinations were well tolerated. Solicited local and systemic adverse events (AEs) were generally graded as none or mild (*SI Appendix*, Table S2). After the third immunization, one subject had a temperature of 39.3 °C (graded as severe), which coincided with an acute viral illness. All unsolicited AEs reported during the vaccination period were classified as unrelated to the vaccine. Alanine aminotransferase levels measured 14 d after vaccination were not elevated in any vaccine recipient (*SI Appendix*, Fig. S2). There were no serious AEs attributed to vaccination.

Vaccine Efficacy, Homologous CHMI. Vaccine efficacy was first assessed by CHMI with the Pf clone 3D7 by mosquito bite 19 wk after final immunization. The vaccine is composed of the NF54 strain, which is likely of African origin (15). 3D7 is a clone from the NF54 strain, and thus is homologous to the vaccine (16). 3D7 has been used to evaluate efficacy in the majority of malaria vaccine trials using CHMI (10, 17–21). Nine (64%) of 14 vaccine recipients (95% CI, 35–87%) and none of the six nonvaccinated controls remained without parasitemia after homologous CHMI ($P = 0.012$) (Fig. 1*A* and *SI Appendix*, Table S3). In the five vaccinated volunteers who became parasitemic, the time to first positive malaria PCR ranged from 13 to 15 d compared with 9 to 12 d for the controls ($P < 0.0001$, controls vs. all vaccine recipients by log-rank; *SI Appendix*, Table S4).

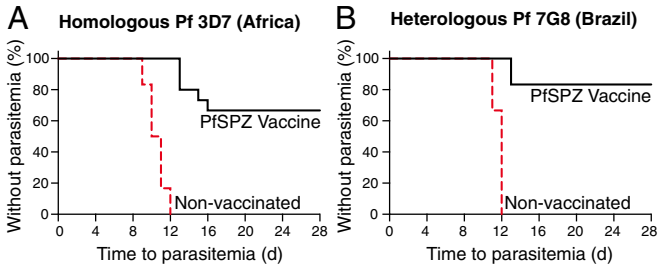

Fig. 1. Efficacy against homologous and heterologous CHMI. Kaplan–Meier curves showing the percentage of volunteers remaining without parasitemia after CHMI with homologous Pf3D7 (*A*) or heterologous Pf7G8 (*B*). Six controls were enrolled for each CHMI. In *A*, nine (64%) of 14 vaccinated volunteers (black, solid line) and none of six controls (red, dashed line) remained without parasitemia. In *B*, five (83%) of six vaccinated volunteers (black, solid line) and none of six controls (red, dashed line) remained without parasitemia. Only vaccinated volunteers who remained without parasitemia after CHMI with the homologous strain, Pf3D7, underwent repeat CHMI with the heterologous strain, Pf7G8.

Vaccine Efficacy, Heterologous CHMI. To assess the durability of vaccine efficacy against heterologous CHMI, six of the nine vaccinated subjects who were not parasitemic after homologous CHMI underwent CHMI by the bite of mosquitoes infected with 7G8, a clone of Brazilian origin (22), ~33 wk after final vaccination. Five (83%) of six vaccine recipients (95% CI, 36–99%) and none of the six nonvaccinated controls remained without parasitemia after heterologous CHMI (Fig. 1*B*). Microsatellite analysis of blood-stage parasites from vaccinated and nonvaccinated individuals confirmed that the parasites detected after heterologous CHMI were genetically distinct from the 3D7 reference clone (*SI Appendix*, Fig. S4).

Antibody Responses. Antibody responses against PfCSP after immunization with PfSPZ Vaccine administered at lower doses have been shown to be a sensitive measure of vaccine immunogenicity, have functional activity in vitro and in vivo, and may be useful as an immune correlate of protection (14). After the first vaccination, low-level PfCSP IgG responses were detected in 13/14 subjects [Fig. 2*A*; geometric mean (GM), 213.5]. Such responses were substantially boosted ~40-fold after the second vaccination (14/14 subjects with PfCSP responses; GM, 9,336), with modest boosting after the third vaccination (GM, 13,066). There was a trend toward higher antibody responses among subjects who remained without parasitemia after homologous CHMI [GM, 15,858 (not parasitemic) vs. 8,717 (parasitemic)]. This comparison did not reach the threshold for statistical significance ($P = 0.23$) with this sample size (nine not parasitemic vs. five parasitemic). We also measured antibody responses against whole PfSPZ by the automated immunofluorescence assay and showed that they were highly concordant with PfCSP antibody levels (Spearman's $r = 0.8787$; $P < 0.0001$; Fig. 2*B* and *SI Appendix*, Tables S5 and S6). Finally, we measured the ability of serum to inhibit SPZ invasion of hepatocytes in vitro as a functional assessment of antibodies. Inhibitory activity increased significantly after the three immunizations compared with the preimmunization serum ($P = 0.0012$; *SI Appendix*, Fig. S4). These data showed that at doses of 9.0×10^5 PfSPZ, malaria-specific antibody responses were induced in all subjects, but did not easily discriminate between protected and unprotected subjects (*SI Appendix*, Table S7).

CD8 and CD4 T-Cell Responses. CD8 T cells are the primary cellular effectors that are critical for mediating protection after whole-SPZ vaccination in mice and nonhuman primates (23–25). Here, after PfSPZ vaccination, the frequency of PfSPZ-specific cytokine (IFN-γ, IL-2, or TNF-α) producing memory CD8 T-cell responses peaked after the first immunization [0.38 ± 0.12% (mean ± SEM); $P = 0.002$ compared with prevaccine; Fig. 2*C*], as assessed by intracellular cytokine staining (ICS) after in vitro stimulation with PfSPZ. Of note, IFN-γ^+ CD8 T-cell responses declined markedly after the second immunization and were not different from prevaccine (background) levels 2 wk after the third immunization ($P = 0.12$). The frequency of cytokine-producing PfSPZ-specific memory CD4 T cells also peaked after the first immunization [1.55 ± 0.38% (mean ± SEM); Fig. 2*D*]. Such responses declined to 0.59 ± 0.08% and 0.51 ± 0.07% after the second and third immunizations, respectively. CD8 T-cell responses were detected in 11/14 volunteers, and CD4 T-cell responses were detected in 14/14 volunteers. The magnitude of the total PfSPZ-specific memory T-cell cytokine responses did not significantly differ among vaccinated subjects who did or did not develop parasitemia after homologous CHMI at the assessed points (*SI Appendix*, Table S7).

Analysis of activation markers HLA-DR and CD38 on T cells without ex vivo antigen stimulation has been used as a sensitive

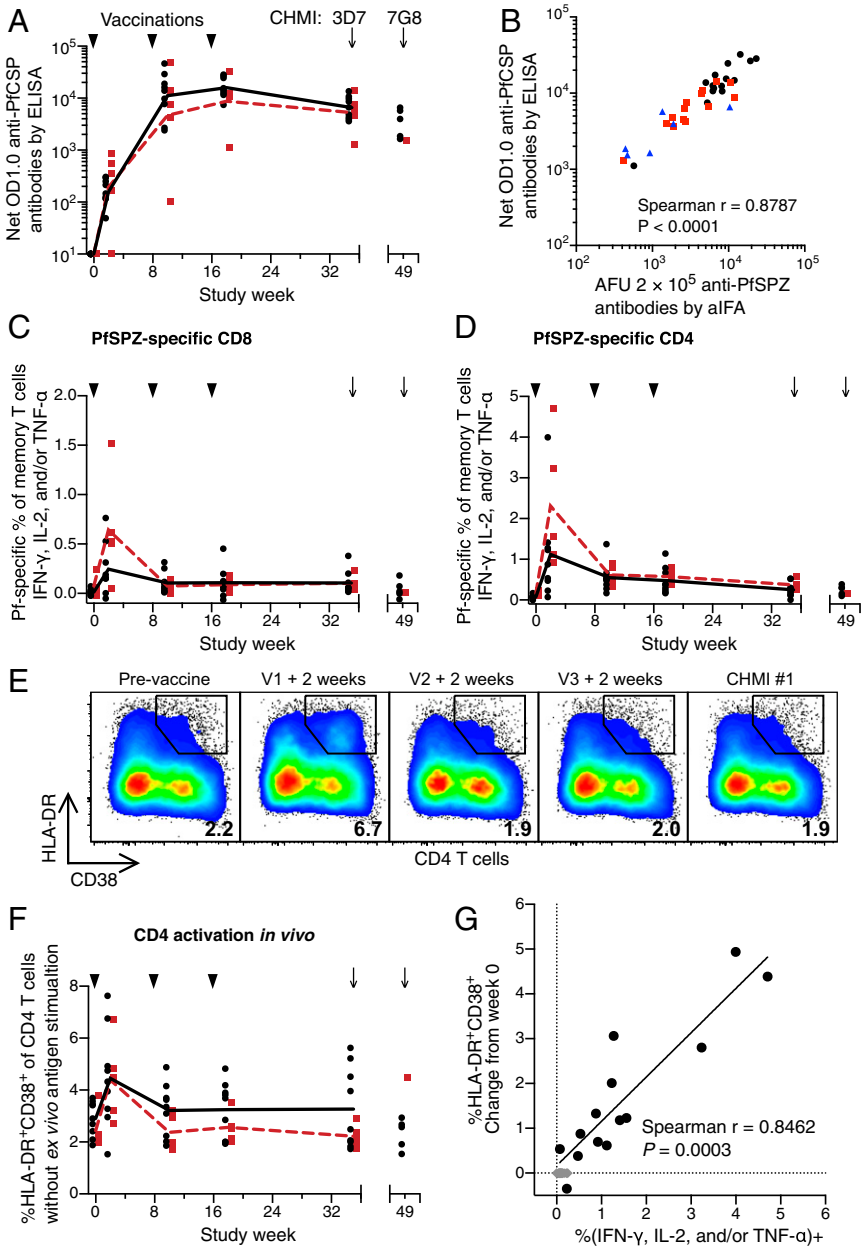

Fig. 2. PfSPZ-specific antibody, CD8, and CD4 responses. (*A*) Antibody responses to PfCSP 2 wk after each vaccination and at the time of homologous (3D7) and heterologous (7G8) CHMI. Subjects who were parasitemic (red, dashed line and red squares) or not parasitemic (black, solid line and black circles) are shown. (*B*) Antibody responses to whole PfSPZ (NF54) compared with PfCSP responses. Spearman's *r* was calculated using data from the three points combined: $n = 15$ for study week 18 (black circles), $n = 14$ for study week 35 (red squares), $n = 6$ for study week 49 (blue triangles). (*C*) Frequency of PfSPZ-specific cytokine-producing memory CD8 T cells. Results are the percentage of memory CD8 T cells producing IFN-γ, IL-2, and/or TNF-α after PfSPZ stimulation minus the percentage of cells after stimulation with vaccine diluent (1% human serum albumin in media). (*D*) Frequency of PfSPZ-specific cytokine-producing memory CD4 T cells. Results are calculated as in *C*. (*E*) Representative flow cytometry gating of the T-cell activation markers HLA-DR and CD38 over time showing memory CD4 T cells from a vaccinated subject without additional in vitro antigen stimulation. (*F*) CD4 T-cell activation in vivo. Results are the percentage of memory CD4 T cells expressing HLA-DR and CD38, as measured on PBMCs without antigen stimulation. (*G*) Correlation of CD4 T-cell activation in vivo and frequency of PfSPZ-specific memory CD4 T cells. The *y* axis shows the change in percentage of memory CD4 T cells expressing HLA-DR and CD38 from prevaccine to 2 wk after vaccination #1. The *x* axis shows the percentage of memory CD4 T cells expressing IFN-γ, IL-2, and/or TNF-α determined by intracellular cytokine staining after ex vivo stimulation with PfSPZ. Black circles denote the frequency 2 wk after vaccination #1; gray diamonds denote the frequency prevaccination. Spearman's *r* was calculated using postvaccination data. For *A*, *C*, *D*, and *F*, $n = 14$ for study week 0 through 35 and $n = 6$ at week 49. Arrowhead designates PfSPZ Vaccine administration, arrows designate time of CHMI with 3D7 or 7G8. For weeks 0–35, data are stratified by outcome after homologous CHMI at study week 35. Data at week 49 are stratified by outcome after heterologous CHMI at study week 49. Individual points represent one subject. Line is geometric mean (*A*) or mean (*C*, *D*, *F*).

measure of T-cell activation in vivo after immunization (Fig. 2*E*) (26, 27). The frequency of HLA-DR$^+$CD38$^+$ memory CD4 T cells increased by 1.69 ± 0.42 percentage points (mean ± SEM; $P < 0.001$; Fig. 2*F*) 2 wk after first immunization compared with baseline (prevaccine). The mean increase was similar to the level of memory CD4 T cells that were determined to be PfSPZ-specific by the ICS assay (Fig. 2*D*; i.e., 1.55%), and individual responses were highly correlated (Spearman's $r = 0.8462$; $P = 0.0003$;

Fig. 2*G*), suggesting the ICS assay used to assess the frequency of PfSPZ-specific T-cell responses after in vitro stimulation with PfSPZ accurately reflects the absolute magnitude of in vivo CD4 T-cell activation by the first PfSPZ vaccination. Interestingly, although circulating PfSPZ-specific CD4 T cells were detected at >0.5% by in vitro PfSPZ stimulation after the second and third vaccination, the frequency of HLA-DR$^+$CD38$^+$ memory CD4 T cells 2 wk after second and third immunizations were only 0.17 ± 0.15 percentage points (mean ± SEM) and 0.25 ± 0.18 percentage points, respectively, above the prevaccination frequencies. These findings indicate that the second and third vaccinations resulted in a dramatic reduction in in vivo activation of circulating PfSPZ-specific CD4 T cells compared with the first vaccination.

γδ T-Cell Responses. Prior studies have shown that γδ T cells expand after immunization with PfSPZ Vaccine, and the frequency of the Vδ2$^+$ subfamily of γδ T cells is a potential immune correlate of protection (11, 14). Here, we extend these findings and show that γδ T cells have a high level of in vivo activation after vaccination, as measured by HLA-DR$^+$CD38$^+$ coexpression (Fig. 3*A*). Activation peaked at a mean of 34% (compared with 6.4% at prevaccination) 2 wk after second immunization (Fig. 3*B*). The frequency of circulating Vδ2$^+$ T cells increased after each vaccination (Fig. 3*C*). The frequency was 2.8-fold higher than prevaccination, and this increase persisted through follow-up (2.5-fold increase at the time of CHMI with 3D7).

T-Cell Responses Against 7G8. Because we observed protection against homologous and heterologous CHMI in subjects that received only the homologous NF54 vaccine strain, we assessed the PfSPZ-specific T-cell cytokine producing responses against the heterologous 7G8 strain and compared them with responses against the homologous NF54 vaccine strain. There was a significant correlation in the magnitude of CD8 T-cell cytokine responses to NF54 and 7G8 PfSPZ (Spearman $r = 0.8052$; $P = 0.0005$; Fig. 4*A*) 2 wk after the first immunization, when such responses were measurable at detectable frequencies. CD8 T-cell responses were also assessed by expression of the TNF-receptor superfamily member 4-1BB (a marker of T-cell activation also known as CD137). This marker has been used to identify antigen-specific CD8 T cells after ex vivo antigen stimulation (28). Using this alternative assessment, CD8 T-cell responses were also highly correlated (Spearman's $r = 0.9033$; $P < 0.0001$; Fig. 4*B*). CD4 T-cell cytokine responses were significantly correlated between the two strains (Fig. 4*C*). Finally, γδ T-cell activation by the two strains (as measured by 4-1BB) was also highly correlated (Fig. 4*D*). Expression of 4-1BB in the γδ T-cell compartment in response to PfSPZ was evident in the prevaccine sample, as expected, as this lineage of cells is prevalent before vaccination, albeit at lower absolute frequencies than found after vaccination.

Discussion

The data presented here show that PfSPZ Vaccine administered three times at a dose of 9.0×10^5 PfSPZ was safe and immunogenic. The finding that nine (64%) of 14 volunteers remained without parasitemia on homologous CHMI at 19 wk after final vaccination with a three-dose regimen is higher than that previously achieved by four or five doses of 1.35×10^5 or 2.7×10^5 PfSPZ and further demonstrates the dose-dependent efficacy of PfSPZ Vaccine (11, 14, 29). The results after heterologous CHMI 33 wk after final immunization provide the first evidence that durable protection can be induced against heterologous CHMI with an injectable malaria vaccine. These data support our hypothesis that higher PfSPZ Vaccine doses are required for durable heterologous protection than are required for durable homologous protection (14, 29).

We assessed heterologous efficacy after homologous CHMI in subjects that were previously protected. Such sequential CHMIs have been used in the majority of studies to assess durable vaccine efficacy (9, 13, 14, 19, 20, 29). In terms of whether a CHMI may influence immunity, we have previously demonstrated that there is no measurable boosting of PfSPZ-specific T-cell or antibody responses after homologous CHMI in protected subjects (14). In addition, in our recent report, the estimated vaccine efficacy against homologous CHMI at 5 mo was 55% in subjects who underwent sequential CHMI at 3 wk and 5 mo, which was the same as those who had their first CHMI at 5 mo (14). Collectively, these data suggest that the single homologous CHMI did not likely influence immunity or protection against heterologous CHMI.

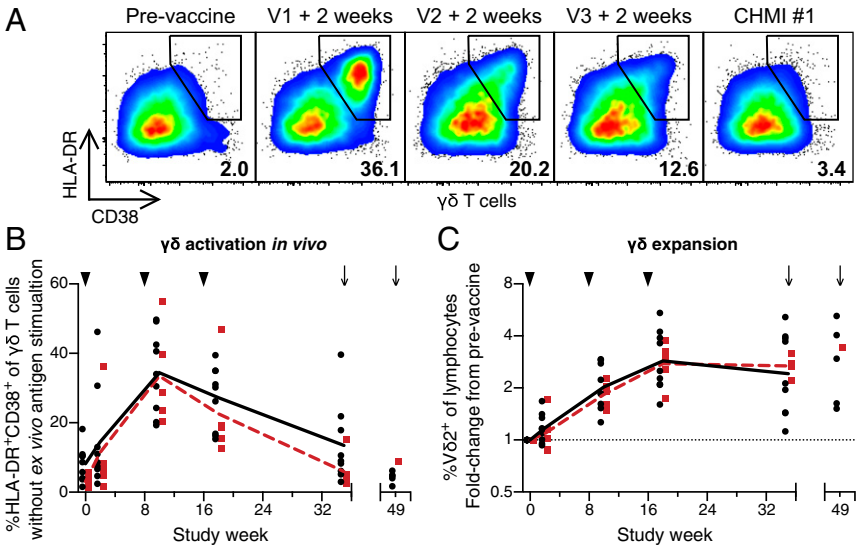

Fig. 3. PfSPZ-specific γδ T-cell responses. (*A*) Flow cytometry gating showing γδ T-cell activation in vivo. PBMCs were stained as in Fig. 2*E*. (*B*) γδ T-cell activation in vivo. Results are the percentage of memory γδ T cells expressing HLA-DR and CD38 as measured on PBMCs without antigen stimulation. (*C*) Frequency of Vδ2$^+$ T cells after immunization. Results are expressed as fold-change from the prevaccine frequency. For each graph, $n = 14$ for study week 0 through 35 (time of homologous CHMI) and $n = 6$ at week 49 (time of heterologous CHMI). Arrowhead designates PfSPZ Vaccine administration, arrows designate time of CHMI with 3D7 or 7G8. For *B* and *C*, data points are stratified as Fig. 2*A*, and lines are mean (*B*) and geometric mean (*C*).

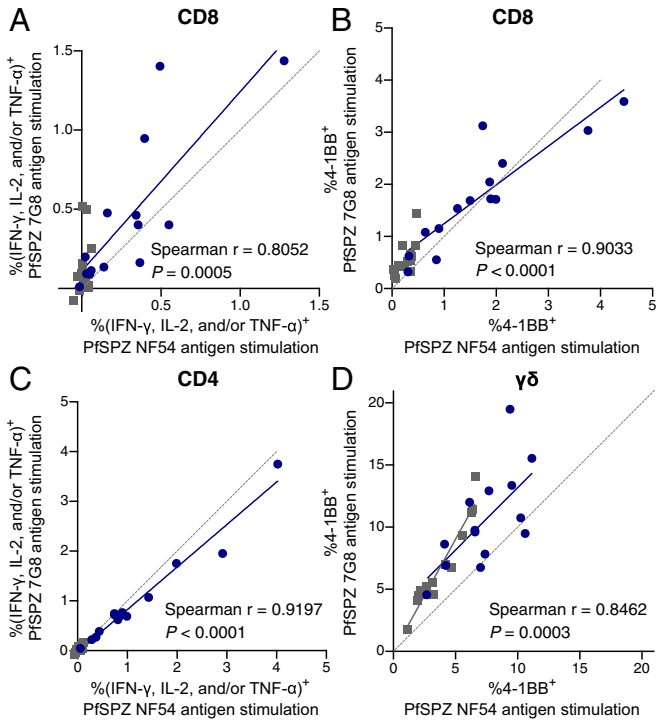

Fig. 4. T-cell responses to PfSPZ NF54 and 7G8. (*A*) Memory CD8 T-cell cytokine production after in vitro PfSPZ 7G8 antigen stimulation compared with PfSPZ NF54. Results are the percentage of memory CD8 T cells producing IFN-γ, IL-2, and/or TNF-α after stimulation with PfSPZ 7G8 (*y* axis) or PfSPZ NF54 (*x* axis) after subtracting the percentage of cytokine-producing cells in PBMCs incubated with the vaccine diluent (1% human serum albumin in media). (*B*) Percentage of memory CD8 T cells expressing the activation marker 4-1BB after PfSPZ antigen stimulation as in *A*. (*C*) Memory CD4 T-cell cytokine responses as in *A*. (*D*) Percentage of memory γδ T cells expressing 4-1BB after PfSPZ antigen stimulation as in *B*. $n = 14$ for each point. Each symbol represents an individual volunteer. Blue circles are samples 2 wk after vaccination #1, and gray squares are prevaccine samples. Blue line is linear regression on postvaccination sample percentages. *P* value is by Spearman's correlation on postvaccination sample percentages.

The consistent high-level protection observed with whole-PfSPZ Vaccine approaches in CHMI studies suggest several critical differences in the immune responses induced by attenuated PfSPZ compared with subunit protein or viral vector vaccines. PfSPZ Vaccine primes antigenically diverse T-cell responses (11) and strain-transcending CD8 and CD4 T cells (Fig. 4). In addition, PfSPZ Vaccine expands γδ T cells, which are not induced by current subunit malaria vaccines. γδ T cells recognize an invariant antigen from intracellular PfSPZ (phosphoantigens) (11, 14, 30). Together, these characteristics of both antigenic and effector cell breadth may help limit vaccine escape mutants that may reduce protective efficacy of vaccines where immune responses are directed against a single protein (31).

Pf-specific T-cell responses were induced in all vaccinated volunteers and were significantly higher than we previously reported, with lower doses of PfSPZ Vaccine (11, 14). CD8 and CD4 T-cell responses in the blood were highest after the first immunization, with limited boosting after the second and third immunizations. The goal of PfSPZ Vaccine administration is to induce immunity that interferes with the development of naturally transmitted Pf sporozoites. Therefore, rapid induction of anti-PfSPZ immunity may limit boosting by subsequent administrations of PfSPZ Vaccine, especially at the higher vaccine doses used here compared with prior studies. Indeed, limited boosting after irradiated SPZ vaccination has been demonstrated in mouse models (32, 33). These data raise the question of whether equivalent protective efficacy may be possible with fewer than three immunizations, especially with higher doses of the vaccine. Another possibility is that the second and third vaccinations were expanding nonrecirculating liver-resident CD8 T cells (14, 34).

The frequency of PfSPZ-specific cytokine-producing CD8 and CD4 T-cell responses were not different between subjects who were not parasitemic and parasitemic at the peak after vaccination or before the homologous CHMI. The lack of a significant correlation may be a result of the small sample size studied here. Furthermore, protection may be mediated primarily by nonrecirculating liver-resident T cells that cannot be directly measured in the blood (14, 35).

This study was performed in malaria-naive US adults representative of travelers or military personnel who would benefit from a highly protective vaccine. Important considerations for successfully using this vaccine in Africa and other endemic areas include how preexisting malaria exposure, age, and baseline health status influence immunogenicity and protection. Future studies using additional heterologous CHMI strains in conjunction with field studies against natural exposure will further establish the breadth of protective efficacy conferred by PfSPZ Vaccine.

The findings reported here suggest that sterile protection against homologous and heterologous CHMI can be achieved in malaria-naive adults by a three-dose regimen of PfSPZ Vaccine. On the basis of the favorable safety profile of the doses tested to date and the immune data reported here, further dose increases as well as fewer immunizations will be tested to determine whether higher protective efficacy can be achieved, which will be necessary for travelers and for mass vaccination strategies aimed at interrupting transmission in endemic regions.

Materials and Methods

Study Design. VRC 314 was an open-label, dose-escalation trial with CHMI to assess safety, immunogenicity, and protective efficacy of PfSPZ Vaccine (34, 36). The Vaccine Research Center Clinical Trials Core at the NIH Clinical Center and the University of Maryland, Baltimore, developed the protocol and conducted the clinical trial. The trial is registered at ClinicalTrials.gov (NCT02015091). Briefly, malaria-naive, healthy adults 18–45 y of age were enrolled after obtaining written informed consent. Results of the initial dose-escalation phase were reported previously (14). On the basis of the favorable safety profile and data consistent with dose-dependent efficacy, an amendment to the protocol was made, and an additional group of volunteers was enrolled to receive 9.0×10^5 PfSPZ three times at 8-wk intervals. The vaccine developer and investigational new drug (IND) application sponsor, Sanaria Inc., participated in protocol design.

Study Oversight and Safety Surveillance. VRC 314 was approved by the Intramural Institutional Review Board of the National Institute of Allergy and Infectious Diseases and the University of Maryland, Baltimore, operated under a Reliance Agreement with National Institute of Allergy and Infectious Diseases. Each step was reviewed and approved by a Safety Monitoring Committee. Intravenous site and systemic reactogenicity parameters were recorded through 3 d after vaccination as solicited AEs. Unsolicited AEs were recorded until day 28 post-CHMI. Serious AEs and new chronic medical conditions were recorded through 24 wk after the last vaccination. AEs were graded using a table adapted from US Food and Drug Administration Guidance for Industry (37). Malaria-associated symptoms and signs after CHMI were not included in unsolicited AE listings because Pf infection was recorded as a separate study endpoint.

Vaccine and Vaccination. Sanaria PfSPZ Vaccine, composed of radiation-attenuated PfSPZ of the NF54 strain, was manufactured as described (34, 36). PfSPZ Vaccine was thawed and diluted in 1 mL PBS containing 1% human serum albumin. The vaccine (1 mL) was administered by rapid injection through an intravenous catheter.

CHMI. CHMI was by the bites of five PfSPZ-infected *Anopheles stephensi* mosquitoes that met standard infectivity criteria (19, 34, 38–40). Volunteers underwent CHMI with 3D7 an average of 19 wk (range, 17–20 wk; *SI Appendix*, Table S3) after final immunization. CHMI with 7G8 was performed an average of 33 wk (range, 32–35 wk; *SI Appendix*, Table S3) after final immunization. Volunteers were monitored as outpatients on days 7–18, 20,

22, 24, and 28 after CHMI. PCR was performed at each visit to detect parasite DNA in blood and was used for primary clinical diagnosis and treatment using established methods (14). Treatment with combination atovaquone (1,000 mg) and proguanil (400 mg) tablets by mouth daily for 3 d was initiated upon two positive PCR results. Volunteers were considered protected if the PCRs were negative through day 28 post-CHMI.

PfSPZ-Specific Antibody Responses. Sera were assessed for antibodies against recombinant PfCSP by enzyme-linked immunosorbent assay and against whole PfSPZ by automated immunofluorescence assay, as described (14). Sera were evaluated for functional activity by the inhibition of sporozoite invasion (ISI) assay (*SI Appendix*).

PfSPZ-Specific T-Cell Responses. PfSPZ-specific T-cell responses were analyzed by flow cytometry on cryopreserved peripheral blood mononuclear cells (PBMCs). PfSPZ-specific T cells were identified by stimulating PBMCs with attenuated PfSPZ or PfSPZ Vaccine diluent and assessing cytokine production by ICS, as described (14) (*SI Appendix*, Tables S8 and S9).

Statistical Analysis. The primary analysis compared the results of the vaccine group's first CHMI with those of nonvaccinated controls who underwent CHMI concurrently, and was based on a Fisher's exact test, one-tailed. The threshold for significance for this primary analysis was set to 0.01 in the protocol to account for analyzing results from multiple vaccine groups. These earlier groups, which assessed lower vaccine doses and the intramuscular route of immunization, had completed primary follow-up before the design, screening, enrollment, or dosing of the present group. Results from the prior groups are reported elsewhere (14). All other analyses were based on nonparametric tests: log-rank test for delay in parasitemia, and Mann–Whitney *U* test, Wilcoxon's signed rank test, and Spearman's rank correlation for immune responses, as specified in the text.

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