

Protection against malaria at 1 year and immune correlates following PfSPZ vaccination

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An attenuated *Plasmodium falciparum* (Pf) sporozoite (SPZ) vaccine, PfSPZ Vaccine, is highly protective against controlled human malaria infection (CHMI) 3 weeks after immunization, but the durability of protection is unknown. We assessed how vaccine dosage, regimen, and route of administration affected durable protection in malaria-naïve adults. After four intravenous immunizations with 2.7×10^5 PfSPZ, 6/11 (55%) vaccinated subjects remained without parasitemia following CHMI 21 weeks after immunization. Five non-parasitemic subjects from this dosage group underwent repeat CHMI at 59 weeks, and none developed parasitemia. Although Pf-specific serum antibody levels correlated with protection up to 21–25 weeks after immunization, antibody levels waned substantially by 59 weeks. Pf-specific T cell responses also declined in blood by 59 weeks. To determine whether T cell responses in blood reflected responses in liver, we vaccinated nonhuman primates with PfSPZ Vaccine. Pf-specific interferon- γ -producing CD8 T cells were present at ~100-fold higher frequencies in liver than in blood. Our findings suggest that PfSPZ Vaccine conferred durable protection to malaria through long-lived tissue-resident T cells and that administration of higher doses may further enhance protection.

In 2015 there were an estimated 214 million clinical cases and 438,000 deaths due to malaria¹, primarily caused by Pf in children in sub-Saharan Africa. A highly effective vaccine is urgently needed to prevent malaria in individuals and to facilitate elimination of malaria from defined geographic areas. To achieve these goals, we established an interim target of >85% sterile protection against Pf infection for >6 months².

There is currently no malaria subunit vaccine that approaches this level of protection. The most extensively studied candidate malaria vaccine, RTS,S (a subunit vaccine based on the Pf circumsporozoite protein (PfCSP)), confers sterilizing protection against controlled

human malaria infection (CHMI) in about 22% of healthy malaria-naïve adults 5 months after vaccination³. In a phase 3 field study, the efficacy of RTS,S against clinical malaria was 26% and 36% in young infants and children between the ages of 5 and 17 months, respectively, through 38–48 months of follow-up following a four-dose regimen on a 0-, 1-, 2-, and 20-month schedule⁴. Therefore, it is necessary to investigate alternative vaccination strategies that confer long-lived sterilizing protection^{5,6}.

Sustained sterilizing immunity against the pre-erythrocytic stages of Pf has been observed in humans immunized by whole-parasite approaches using mosquitoes for vaccination^{7,8}. In a study

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of malaria-naïve adults, 5/6 subjects exposed to >1000 irradiated mosquitoes carrying attenuated PfSPZ were protected when CHMI occurred 23–42 weeks after immunization⁸. To advance from using mosquitoes for inoculation of attenuated PfSPZ toward a clinical product, we previously reported that immunization by intravenous (i.v.) injection of radiation-attenuated, aseptic, purified, cryopreserved PfSPZ, a product called Sanaria PfSPZ Vaccine⁹ (hereafter referred to as PfSPZ Vaccine), was well tolerated and immunogenic (the VRC 312 study)^{10,11}. PfSPZ Vaccine induced a dose-dependent increase in PfSPZ-specific antibodies and frequencies of multifunctional T_H1 cytokine-producing CD4 T cells and $\gamma\delta$ T cells in the blood. For vaccine recipients that underwent CHMI 3 weeks after final immunization, Pf parasitemia was observed in 3/9 and 0/6 subjects who received four or five doses of 1.35×10^5 PfSPZ, respectively, whereas parasitemia was observed in 5/6 unvaccinated controls, demonstrating that PfSPZ Vaccine confers high-level, short-term protection. The next critical milestones for PfSPZ Vaccine were to assess the durability of vaccine efficacy and to investigate the immune correlates and mechanisms of protection.

RESULTS

PfSPZ Vaccine efficacy at 21 weeks

To assess the durability of protection, subjects from the VRC 312 study were re-enrolled for repeat CHMI with the homologous Pf clone 3D7. Because vaccine efficacy (VE) is assessed at multiple time points in the same vaccinated subjects, we defined vaccine efficacy as first VE (VE at first CHMI), subgroup VE (VE among the subgroup of subjects who were not parasitemic after the first CHMI and returned for repeat CHMI), and cumulative VE (first VE $\times$ subgroup VE). VE is calculated as $1 - \text{relative risk}$, where relative risk is the ratio of the infection rate among the vaccinated subjects divided by the infection rate among the controls. Thus, VE is always adjusted to account for those cases in which the infection rate in the controls is not 100%.

Six subjects who had received four or five doses of 1.35×10^5 PfSPZ by i.v. injection and had not developed parasitemia following CHMI at 3 weeks¹⁴ underwent repeat CHMI 21 weeks after the final vaccination. Four of six subjects developed parasitemia, as compared to 6/6 unvaccinated control subjects (**Supplementary Fig. 1a,b**), for a subgroup VE of 33% ($P = 0.23$). For this dosage group, the first VE was 76% (ref. 11), and therefore the cumulative VE was 25%. Thus, 1.35×10^5 PfSPZ administered four or five times did not confer adequate protection at 21 weeks.

Additionally, eight subjects in the VRC 312 study who were parasitemic at a prior CHMI (six immunized subjects and two unvaccinated controls) underwent repeat CHMI, and all of them developed parasitemia (**Supplementary Fig. 1c,d**). Thus, a single previous episode of Pf parasitemia followed by drug treatment did not confer protection to a subsequent CHMI. Therefore, only vaccinated subjects who did not develop parasitemia following their first CHMI underwent repeat CHMI in the following studies.

Study design

We assessed in a new study (VRC 314) whether increasing the dosage of PfSPZ Vaccine from 1.35×10^5 to 2.7×10^5 PfSPZ per dose affected VE. With this higher dose, we tested three-dose (group 1) and four-dose (groups 4 and 5) regimens. A fourth group of subjects received 1.35×10^5 PfSPZ four times, followed by a fifth dose of 4.5×10^5 PfSPZ (group 3), to determine whether a higher final dose improved VE, as compared to that for subjects who received five doses of 1.35×10^5

PfSPZ (**Fig. 1a**). For the subjects in each of these groups, PfSPZ Vaccine was administered by rapid i.v. injection.

The trial also assessed route of administration. Studies in nonhuman primates (NHPs) and humans show that i.v. administration of PfSPZ Vaccine is substantially more immunogenic and protective than subcutaneous (s.c.) or intradermal (i.d.) administration at a dose of 1.35×10^5 PfSPZ^{10,11}. However, studies in rodents show that intramuscular (i.m.) administration of radiation-attenuated SPZs is more protective than s.c. or i.d. administration, although it is considerably less protective than by i.v. administration^{12,13}. Because there may be instances in which administration by i.v. injection is logistically complex, we compared the protective efficacy of administering 2.7×10^5 PfSPZ by i.v. injection versus 2.2×10^6 PfSPZ by the i.m. route (an 8.1-fold higher dose) with the same four-dose regimen (group 2) (**Fig. 1a**).

For the primary efficacy analysis, the results are presented for the first CHMI for each vaccine group as compared to those for the controls. To account for multiple comparisons, $P < 0.01$ was taken as evidence of an effect, and P values between 0.01–0.05 were considered to be suggestive of an effect. This threshold for significance of the primary analysis was specified in the protocol to balance the potential for type 1 error with the conservative Bonferroni approach (Online Methods). Secondary analyses compared the results from the different groups, as well as describe the results of repeated challenges among those subjects who remained parasite free in each group. No formal multiple-comparison adjustment was used for secondary end points.

Daily monitoring by PCR analysis, rather than thick blood smear, was used for diagnosis of parasitemia and treatment. In subjects of the VRC 312 cohort, PCR analysis allowed detection of parasitemia 1–2 d earlier than by blood smear (**Supplementary Table 1**), which enabled clinical monitoring criteria to be changed from overnight stays after day 7 to once-daily outpatient visits.

Study population

Of the 101 subjects who were enrolled, 57 were vaccine recipients, 32 were CHMI controls, and 12 were backup controls (**Supplementary Fig. 2**). Baseline demographic characteristics are shown in **Supplementary Table 2**. 55/57 (96%) subjects completed all scheduled vaccinations and, cumulatively, 224 vaccinations were administered. In total, 52/55 subjects (95%) who completed their vaccinations underwent at least one CHMI. For CHMIs occurring 3 weeks, 21–25 weeks, and 59 weeks after the final vaccination, there were 39, 31, and 5 participating vaccine recipients, respectively (**Supplementary Table 3**).

Adverse events

Vaccinations were well tolerated. Of 57 vaccine recipients, 41 (72%) had no solicited adverse events at the injection site after any vaccination, 15 (26%) had mild symptoms, and one (2%) had moderate symptoms (**Supplementary Table 4**). There were no solicited systemic symptoms for 32 (56%) vaccine recipients, mild ones for 19 (33%) recipients, and moderate ones for six (11%) recipients (**Supplementary Table 5**). There were no serious adverse events attributed to vaccination. Alanine aminotransferase (ALT) levels measured 14 d after each vaccination were not elevated in any dosage group, as compared to those for the same subjects before starting the vaccinations (**Supplementary Fig. 3**).

Vaccine efficacy over 1 year

VE at 3 weeks, by i.v. administration. 4/12 subjects in group 3 (four doses of 1.35×10^5 PfSPZ and a fifth dose of 4.5×10^5 PfSPZ) and 6/9 subjects in group 1 (three doses of 2.7×10^5 PfSPZ) developed

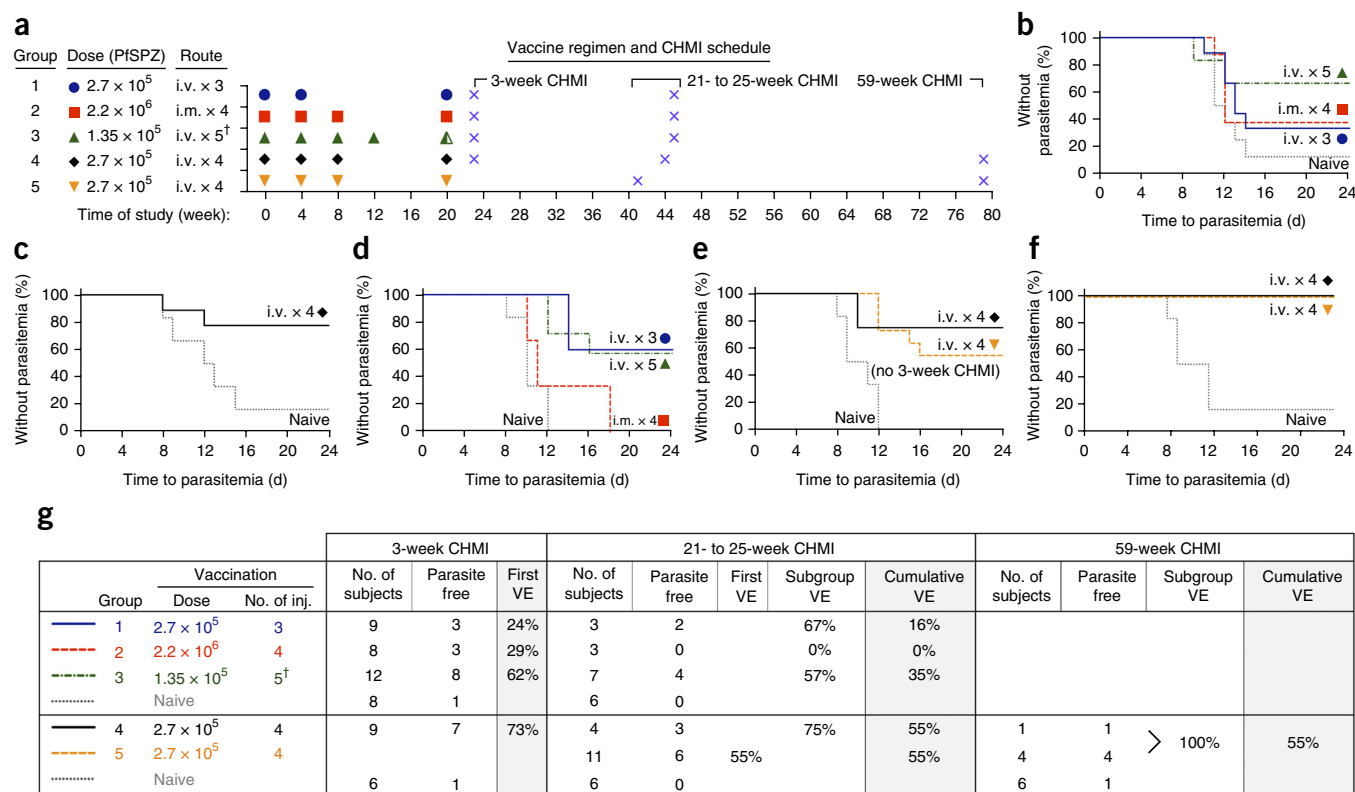


Figure 1 Sterile protection following CHMI at 59 weeks after immunization with PfSPZ Vaccine. **(a)** Timing of immunizations and CHMIs. Vaccinated subjects underwent CHMI 3, 21–25, and/or 59 weeks after final immunization. Half-filled green triangle denotes 4.5×10^5 PfSPZ by i.v. administration. **(b–f)** Kaplan–Meier curves showing the percentage of volunteers who did not develop parasitemia after each of five separate CHMIs at 3 weeks (**b,c**), 21–25 weeks (**d,e**), or 59 weeks (**f**) after the final immunization. New unvaccinated control subjects were enrolled for each CHMI. Day 0 indicates the time of exposure to infected mosquitoes. Monitoring for parasitemia was done by PCR once a day. **(g)** Number of subjects undergoing each CHMI, and vaccine efficacy for each group after each CHMI. In **a,g**, the dagger ($\dagger$) indicates that subjects in group 3 received four doses of 1.35×10^5 PfSPZ each and a fifth dose of 4.5×10^5 PfSPZ.

parasitemia after CHMI, as compared to parasitemia in 7/8 control subjects (**Fig. 1b**). In group 4 (4 doses of 2.7×10^5 PfSPZ), 2/9 vaccinated subjects, as compared to 5/6 controls, developed parasitemia (**Fig. 1c**). First VE was estimated to be 62% for group 3 ($P = 0.025$), 24% for group 1 ($P = 0.34$), and 73% for group 4 ($P = 0.035$).

VE at 21–25 weeks, by i.v. administration. Only vaccine recipients who did not develop parasitemia after their first CHMI underwent repeat CHMI. Seven subjects from group 3 (four doses of 1.35×10^5 PfSPZ and a fifth dose of 4.5×10^5 PfSPZ) underwent repeat CHMI at 25 weeks after final vaccination, and 3/7, as compared to 6/6 controls, developed parasitemia. Subgroup VE was 57%, and cumulative VE was 35% (**Fig. 1d**). These results were similar to the results from the VRC 312 study for CHMI that was performed at 21 weeks (**Supplementary Fig. 1**). Thus, increasing the final dose by 3.3-fold did not improve short- or long-term protection, as compared to that with the five-dose regimen of 1.35×10^5 PfSPZ¹¹. Three subjects in group 1 (three doses of 2.7×10^5 PfSPZ) underwent repeat CHMI at 25 weeks, and 1/3 developed parasitemia (**Fig. 1d**); subgroup VE was estimated at 67%, and cumulative VE was 16%.

Four subjects in group 4 (four doses of 2.7×10^5 PfSPZ) underwent repeat CHMI at 24 weeks. 1/4 vaccinated, and 6/6 control, subjects developed parasitemia (**Fig. 1e**), resulting in a subgroup VE of 75% and cumulative VE of 55%. A second group of subjects who received four doses of 2.7×10^5 PfSPZ (group 5) underwent their first CHMI

at 21 weeks. Five of these 11 vaccinated subjects developed parasitemia, resulting in a first VE of 55% ($P = 0.037$; **Fig. 1e**).

VE at 59 weeks, by i.v. administration. Five subjects who received four doses of 2.7×10^5 PfSPZ, each of whom had undergone a single prior CHMI and had not developed parasitemia, underwent a second CHMI 59 weeks after the final immunization. 0/5 vaccine recipients and 5/6 controls developed parasitemia ($P = 0.013$; **Fig. 1f**). Subgroup VE was 100%, and estimated cumulative VE was 55%, at 59 weeks (**Fig. 1g**).

VE at 3 and 25 weeks, by i.m. administration. 5/8 subjects from group 2 (i.m. vaccination with 2.2×10^6 PfSPZ) developed parasitemia following CHMI 3 weeks after vaccination, resulting in a first VE of 29% (**Fig. 1b**). The three subjects who did not develop parasitemia at 3 weeks underwent repeat CHMI at 25 weeks, and all of them developed parasitemia (**Fig. 1d**). Thus, PfSPZ administered by i.m. injection, even at an 8.1-fold higher dose, was less efficient in inducing protection than by i.v. administration (8/8 parasitemic in group 2 through CHMI at 21–25 weeks versus 5/11 in group 5; $P = 0.018$).

Immunogenicity

Antibody responses. Antibodies induced by vaccination with attenuated SPZs can limit parasites from infecting hepatocytes in animals^{14–16}. Therefore, antibodies to PfCSP and whole PfSPZ were assessed by

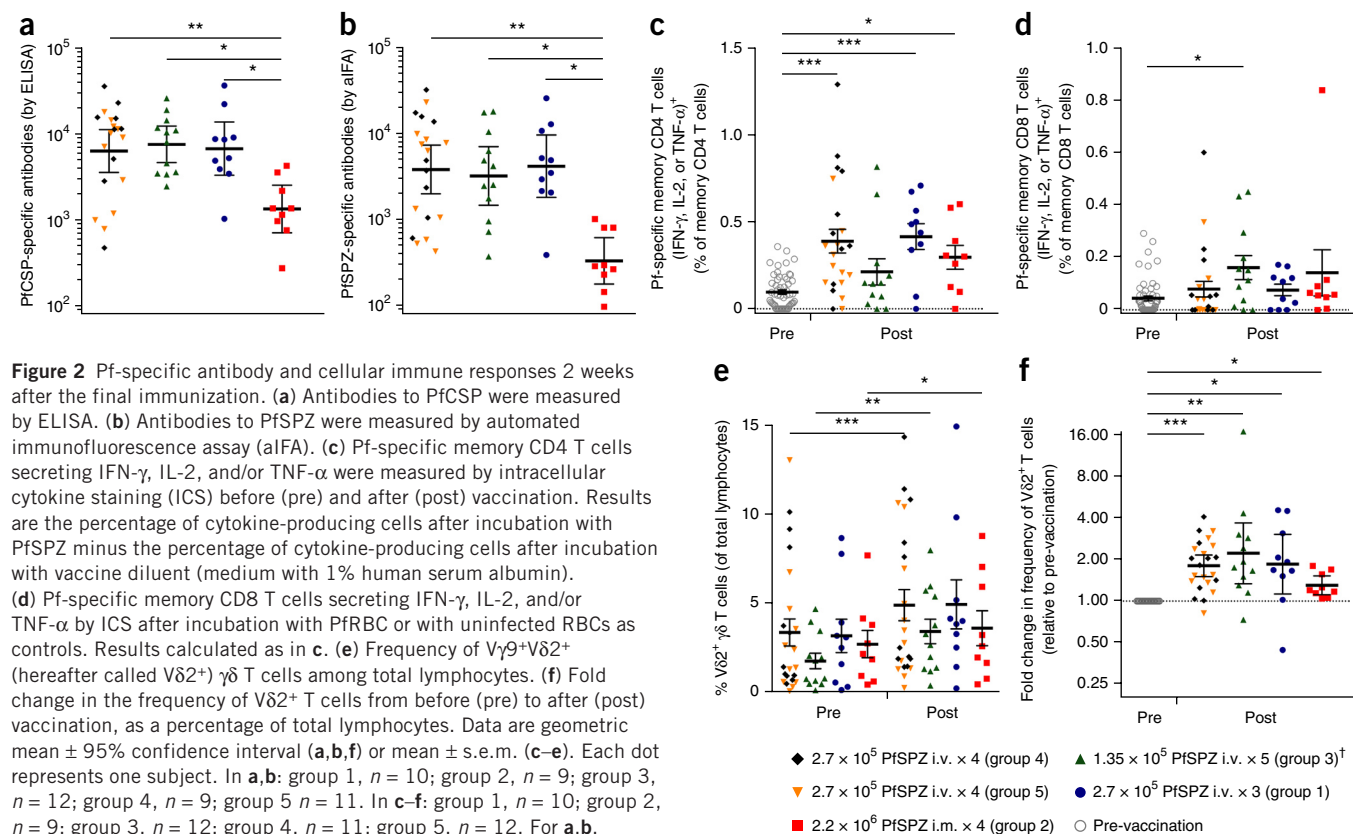


Figure 2 Pf-specific antibody and cellular immune responses 2 weeks after the final immunization. **(a)** Antibodies to PfCSP were measured by ELISA. **(b)** Antibodies to PfSPZ were measured by automated immunofluorescence assay (aIFA). **(c)** Pf-specific memory CD4 T cells secreting IFN- γ , IL-2, and/or TNF- α were measured by intracellular cytokine staining (ICS) before (pre) and after (post) vaccination. Results are the percentage of cytokine-producing cells after incubation with PfSPZ minus the percentage of cytokine-producing cells after incubation with vaccine diluent (medium with 1% human serum albumin). **(d)** Pf-specific memory CD8 T cells secreting IFN- γ , IL-2, and/or TNF- α by ICS after incubation with PfRBC or with uninfected RBCs as controls. Results calculated as in **c**. **(e)** Frequency of V δ 2⁺ $\gamma\delta$ T cells among total lymphocytes. **(f)** Fold change in the frequency of V δ 2⁺ T cells from before (pre) to after (post) vaccination, as a percentage of total lymphocytes. Data are geometric mean $\pm$ 95% confidence interval (**a,b,f**) or mean $\pm$ s.e.m. (**c–e**). Each dot represents one subject. In **a,b**: group 1, $n = 10$; group 2, $n = 9$; group 3, $n = 12$; group 4, $n = 9$; group 5, $n = 11$. In **c–f**: group 1, $n = 10$; group 2, $n = 9$; group 3, $n = 12$; group 4, $n = 11$; group 5, $n = 12$. For **a,b**, between-group differences were assessed by the Kruskal–Wallis test with Dunn's correction for multiple comparisons. For **c–e**, there were no differences between vaccine groups in the post-vaccination responses by the Kruskal–Wallis test. Differences from the pre-vaccination levels were assessed by the Wilcoxon signed-rank test (**c–e**) or one-sample Student's *t*-test (**f**). For **c–f**, *P* values were corrected by the Bonferroni method. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Dagger (†) indicates to see **Figure 1** for doses.

enzyme-linked immunosorbent assays (ELISAs) and automated immunofluorescence assays (aIFAs), respectively, 2 weeks after the final vaccination. There were no differences in antibody levels among the groups that received the different i.v. regimens. However, subjects immunized by the i.m. route had lower antibody responses than did subjects immunized by i.v. injection (**Fig. 2a,b**).

Cellular responses. Cellular immunity is critical for protective efficacy by vaccination with live-attenuated SPZs in rodent and NHP models^{10,17–19}. Although interferon (IFN)- γ -producing CD8 T cells are necessary and sufficient for protection in the majority of mouse and NHP studies²⁰, NK, $\gamma\delta$, and CD4 T cells also influence protection^{19,21}. Pf-specificity was determined by stimulating peripheral blood mononuclear cells (PBMCs) with PfSPZ Vaccine (for CD4 T cell responses) or with Pf-infected erythrocytes (PfRBC) (for CD8 T cell responses) (**Supplementary Figs. 4 and 5** and **Supplementary Table 6**). There is considerable overlap in the proteomes of PfSPZ and PfRBC. Although there was an increase in PfSPZ-specific CD4 T cell cytokine responses after the final immunization in subjects from groups 1, 2, 4, and 5, there were no differences between the vaccine groups (including the i.m. group) in the magnitude (**Fig. 2c**) or quality of PfSPZ-specific CD4 cytokine responses (**Supplementary Fig. 6**). The PfRBC-specific CD8 T cells in blood 2 weeks after the final immunization were no different from those observed before administration of the vaccine (background) in most vaccine groups (**Fig. 2d**). Finally, there was a vaccine-induced increase in the frequency of total $\gamma\delta$ T cells, but no differences between the groups

(**Fig. 2e,f**). The expansion was restricted to the V δ 2⁺ family (hereafter referred to as V δ 2⁺), which comprises ~75% of $\gamma\delta$ T cells in blood (**Supplementary Fig. 7a–f**). A higher percentage of $\gamma\delta$ T cells expressed the activation markers CD38, the cytotoxic molecule perforin, and the liver-homing chemokine receptor CXCR6 (ref. 22) after vaccination (**Supplementary Fig. 7g–i**). Of note, $\gamma\delta$ T cells from PBMCs analyzed before vaccination expressed IFN- γ when stimulated *ex vivo* with live-attenuated PfSPZ (**Supplementary Fig. 7m**).

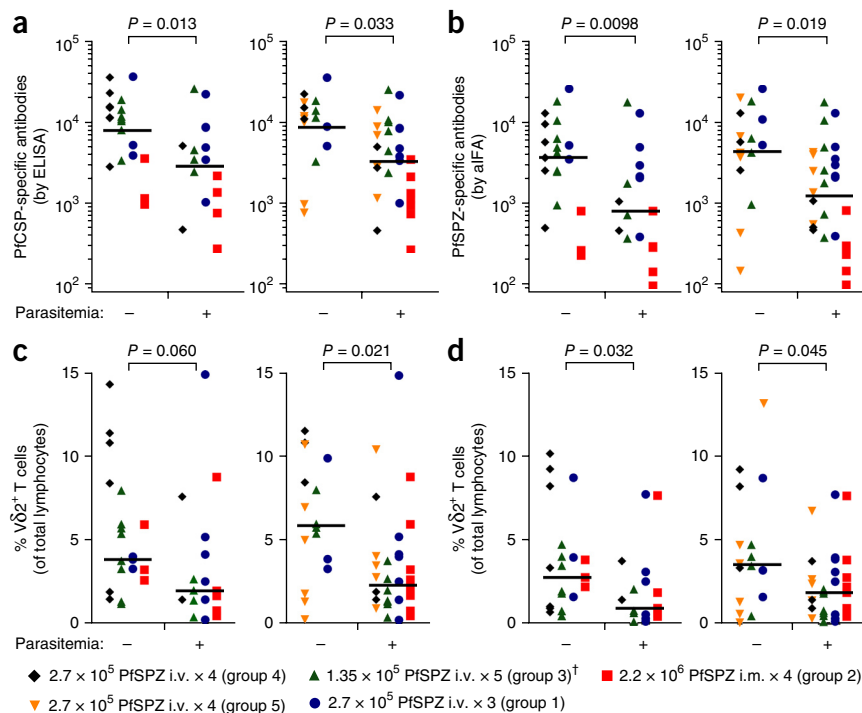
Immune correlates

To identify potential immune correlates of protection²³, we assessed whether there were associations between the immune responses measured 2 weeks after the last immunization and the outcome after each CHMI (parasitemia versus no parasitemia).

Antibody correlates. PfCSP- and PfSPZ-specific antibody levels correlated with outcome for CHMIs that were done 3 weeks and 21–25 weeks after the final immunization (**Fig. 3a,b**). For this analysis, we assumed that subjects who developed parasitemia after the 3-week CHMI would also develop parasitemia after the 21- to 25-week CHMI, on the basis of our earlier findings (**Supplementary Fig. 1**).

Cellular correlates. The percentage of Pf-specific CD4 and CD8 T cells in the blood that produced IFN- γ , IL-2 and/or TNF- α did not correlate with outcome at the 3-week or the 21- to 25-week CHMI (**Supplementary Fig. 8**). However, the absolute frequency of unstimulated V δ 2⁺ T cells as a percentage of total lymphocytes correlated with outcome at the 21- to 25-week CHMI (**Fig. 3c**). Notably, the frequency

Figure 3 Correlation between immune responses and CHMI outcome. (a) Antibodies to PfCSP, as measured by ELISA, 2 weeks after final immunization in subjects who did (+) or did not (–) develop parasitemia at the 3-week (left) and 21- to 25-week (right) CHMIs. (b) Antibodies to PfSPZ, as measured by aIFA (arbitrary fluorescence units (AFU) = 2×10^5), 2 weeks after final immunization in subjects who did (+) or did not (–) develop parasitemia at the 3-week (left) and 21- to 25-week (right) CHMIs. (c) Frequency of Vδ2⁺ T cell subset among total lymphocytes 2 weeks after final immunization in subjects who did (+) or did not (–) develop parasitemia at the 3-week (left) and 21- to 25-week (right) CHMIs. (d) Frequency of Vδ2⁺ T cells among total lymphocytes before immunization in subjects who did (+) or did not (–) develop parasitemia at the 3-week (left) and 21- to 25-week (right) CHMIs. In a–d, $n = 21$ (–) and $n = 17$ (+) for left-hand graphs; in a–c, $n = 16$ (–) and $n = 30$ (+) for right-hand graphs; in d, $n = 16$ (–) and $n = 31$ (+) for right-hand graph. For a–d, because we expected subjects who were parasitemic at 3 weeks to again be parasitemic at 21–25 weeks, the immune data from individuals who were parasitemic at 3 weeks were included in the analysis for 21- to 25-week CHMI. Statistical significance for all correlations was determined by stratified Wilcoxon test with vaccine regimen as covariate. Bar denotes geometric mean (a,b) or median (c,d). Each dot represents one subject. Dagger (†) indicates to see Figure 1 for doses.



of the Vδ2⁺ T cell subset measured before the first vaccination also correlated with outcome at the 3-week CHMI and the 21- to 25-week CHMI (Fig. 3d).

Role of PfSPZ antibodies in protection

The finding that PfCSP- and PfSPZ-specific antibody levels 2 weeks after the final immunization correlated with outcome of the 3-week and 21- to 25-week CHMIs suggested that antibodies were a biomarker of a successful vaccine response; however, antibodies could also have a functional role in mediating protection^{14–16}. To investigate this we assessed the levels of antibodies at the times of the 21- to 25-week CHMIs and the 59-week CHMIs. At 59 weeks, PfCSP-specific antibody levels in the five subjects who did not develop parasitemia were 8-fold lower (PfCSP geometric mean (GM) = 1,640) than the responses among subjects who did not develop parasitemia at 3 weeks (PfCSP GM = 13,200; $P = 0.0051$; Fig. 4a). Of note, the levels of antibodies to PfCSP in the five subjects at 59 weeks (PfCSP GM = 1,640) were no different than those in subjects immunized with the same regimen and who developed parasitemia following CHMI at 21–24 weeks (PfCSP GM = 1,860; $P = 0.87$; Fig. 4a). Assessment of antibody responses to whole PfSPZ (Fig. 4b) also revealed a decline in Pf-specific antibody levels over time.

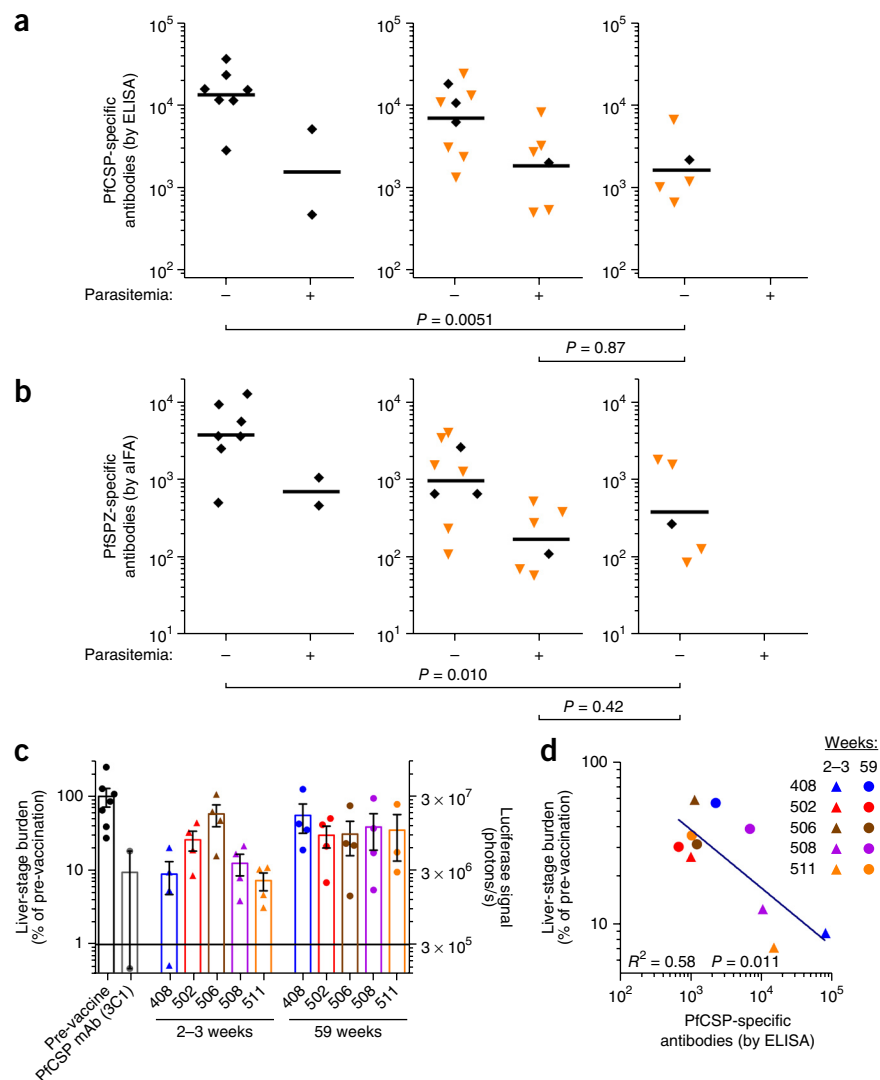
To assess the *in vivo* functional activity of Pf-specific antibodies, we used *Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}* (FRG) mice (which are deficient in T cells, B cell, and NK cells) reconstituted with human hepatocytes (which we refer to as FRG-huHep mice), which are capable of supporting liver-stage development of Pf (ref. 24). Total IgG from the five vaccine recipients that did not develop parasitemia following CHMI at 59 weeks was purified from plasma and serum. The IgG was passively transferred to FRG-huHep mice, and each mouse was challenged by exposure to 50 mosquitoes infected with luciferase-expressing PfSPZ. Liver-stage burden was quantified by whole-animal imaging.

The negative control consisted of pooled IgG obtained from the blood of the five subjects before vaccination. Passive transfer of 150 μg of the PfCSP-specific monoclonal antibody 3C1, which was used as the positive control, reduced liver-stage burden by ~90% as compared to that by transfer of the negative control. Purified IgG from the five vaccinated subjects taken 2–3 weeks after vaccination reduced liver-stage burden by 88% (median), and IgG taken at 59 weeks after the final immunization reduced liver-stage burden by 65% (median) (Fig. 4c). There was higher inhibitory activity in IgG taken at 2–3 weeks from 4/5 subjects as compared to that taken at 59 weeks, but the difference did not reach the level of statistical significance ($P > 0.05$ by Wilcoxon signed-rank test). However, liver-stage burden did correlate inversely with anti-PfCSP levels (Fig. 4d).

PfSPZ-specific T cell activation is dependent on the timing of CHMI

To provide additional insight into the potential mechanisms of protection, we assessed how CHMI affected PfSPZ-specific T cell responses *in vivo* after each CHMI. For this analysis, we identified PfSPZ-specific T cells by the expression of IFN-γ, interleukin (IL)-2, and tumor necrosis factor (TNF)-α that was induced by *ex vivo* stimulation with PfSPZ antigens, and we simultaneously measured the expression of Ki-67 (a marker used to detect recent lymphocyte cell division²⁵; Fig. 5a). One week after the subjects in the VRC 312 (Fig. 5b) and VRC 314 (Fig. 5e) clinical trials received their final immunizations, ~20–30% of PfSPZ-specific CD4 T cells were Ki-67⁺, demonstrating that vaccination induced T cell division. Ki-67 was only detected in PfSPZ-specific CD4 T cells and not in total memory CD4 T cells (Fig. 5b–h), providing evidence for PfSPZ specificity. One to two weeks following the 3-week CHMI, Ki-67 was detected in ~40–60% of PfSPZ-specific CD4 T cells from subjects with parasitemia (both vaccinated and unvaccinated subjects), showing that malaria infection was associated with activation of a high proportion of PfSPZ-specific

Figure 4 Functional role of PfSPZ-specific antibodies. **(a)** Antibodies to PfCSP, as measured by ELISA, at the time of CHMI in subjects who received four doses of 2.7×10^5 PfSPZ/dose by i.v. injection and who subsequently did (+) or did not (–) develop parasitemia at the 3-week (left), the 21- to 24-week (middle), or the 59-week (right) CHMI. **(b)** Antibodies to PfSPZ (AFU = 2×10^5), as measured by aIFA, at the time of each CHMI in subjects who received four doses of 2.7×10^5 PfSPZ/dose by i.v. injection and who subsequently did (+) or did not (–) develop parasitemia at the 3-week (left), 21- to 24-week (middle), or 59-week (right) CHMI. **(c)** Effect of passive transfer of purified IgG on liver-stage burden, as assessed by whole animal imaging. The experiment was performed once. Data are mean $\pm$ s.e.m. y axis on the right denotes the raw luciferase signal. y axis on the left denotes the percentage of signal in mice that received the IgG from each of the five subjects (identified by the identification numbers on the x axis) at each of the two time points relative to the signal in the mice that received pre-vaccination IgG, which was set to 100%. **(d)** Correlation between PfCSP antibody abundance (as determined by ELISA) and liver-stage burden (as measured by luciferase expression) in the FRG-huHep mice. In **a,b**, $n = 7$ (–) and $n = 2$ (+) for left-hand graphs; $n = 9$ (–) and $n = 6$ (+) for middle graphs; $n = 5$ (–) for right-hand graphs. In **c**, $n = 7$ for pre-vaccine, $n = 2$ for PfCSP mAb, and $n = 4$ for each IgG sample, except 511 ($n = 3$). In **d**, $n = 10$ for IgG samples. In **a,b**, bar denotes geometric mean, and comparison (P values) between groups was assessed by the Mann-Whitney U -test. In **d**, Pearson correlation coefficient was used to determine the relationship between Pf liver-stage burden and PfCSP level.



CD4 T cells (**Fig. 5c,d**). In contrast, there were low to undetectable numbers of Ki-67⁺ PfSPZ-specific CD4 T cells in PBMCs from the 25 subjects in the VRC 312 ($n = 12$; **Fig. 5c**) and VRC 314 ($n = 13$; **Fig. 5f**) studies who did not develop parasitemia after the 3-week CHMI. One to two weeks after the 21- to 25-week and 59-week CHMIs, ~10–20% of PfSPZ-specific CD4 T cells from the subjects who did not develop parasitemia were Ki-67⁺ (**Fig. 5d,g,h**), as compared to the 0–1% of PfSPZ-specific CD4 T cells that were present on the day of the CHMI. These data suggest that there was differential antigen exposure *in vivo* when CHMI was done at 3 weeks versus 21–25 or 59 weeks among subjects who remained without parasitemia. The percentage of Pf-specific CD4, CD8, or $\gamma\delta$ T cells in blood did not change after CHMI at any time in subjects who did not have parasitemia (**Supplementary Fig. 9a–f**), nor did PfCSP-specific antibody levels (**Supplementary Fig. 9g**).

Tissue distribution of Pf-specific T cells

The low amount of Pf-specific antibody at 59 weeks for all five vaccinated subjects who were not parasitemic after CHMI (**Fig. 4a,b**) suggested that antibodies did not probably have a major role in mediating protection at this time point. Therefore, PfSPZ-specific T cell responses were assessed in blood throughout the course of vaccination and following each CHMI.

Longitudinal cellular responses in humans. In PBMCs, cytokine-producing PfSPZ-specific CD4 T cell responses declined significantly over the course of 59 weeks in vaccinated subjects who remained without parasitemia ($P = 0.047$; **Fig. 6a**), and cytokine-producing PfrBC-specific CD8 T cell responses were induced by vaccination; however, they returned to pre-vaccine levels shortly after the final vaccination (**Fig. 6b**). Thus, neither antibodies nor Pf-specific T cells were readily detected in the blood of the five vaccinated subjects who were not parasitemic after CHMI at 59 weeks. We hypothesized that the T cells resident in the liver might have a critical role in protection^{10,26}.

Tissue-resident T cell responses in NHPs. Because liver-resident cellular immunity could not be directly assessed in the human subjects, such responses were assessed in NHPs following immunization with PfSPZ (**Supplementary Fig. 10** and **Supplementary Table 6**). Immunogenicity studies with PfSPZ Vaccine in NHPs previously provided immune data that guided successful translation to human studies^{10,11}. We had previously shown that 1.35×10^5 PfSPZ administered by the s.c. route did not induce detectable T cell responses in the blood or liver of NHPs¹⁰, so we administered tenfold more PfSPZ in each of five doses that were administered by the i.m. or i.d. route and compared the T cell responses to those resulting from five i.v. doses

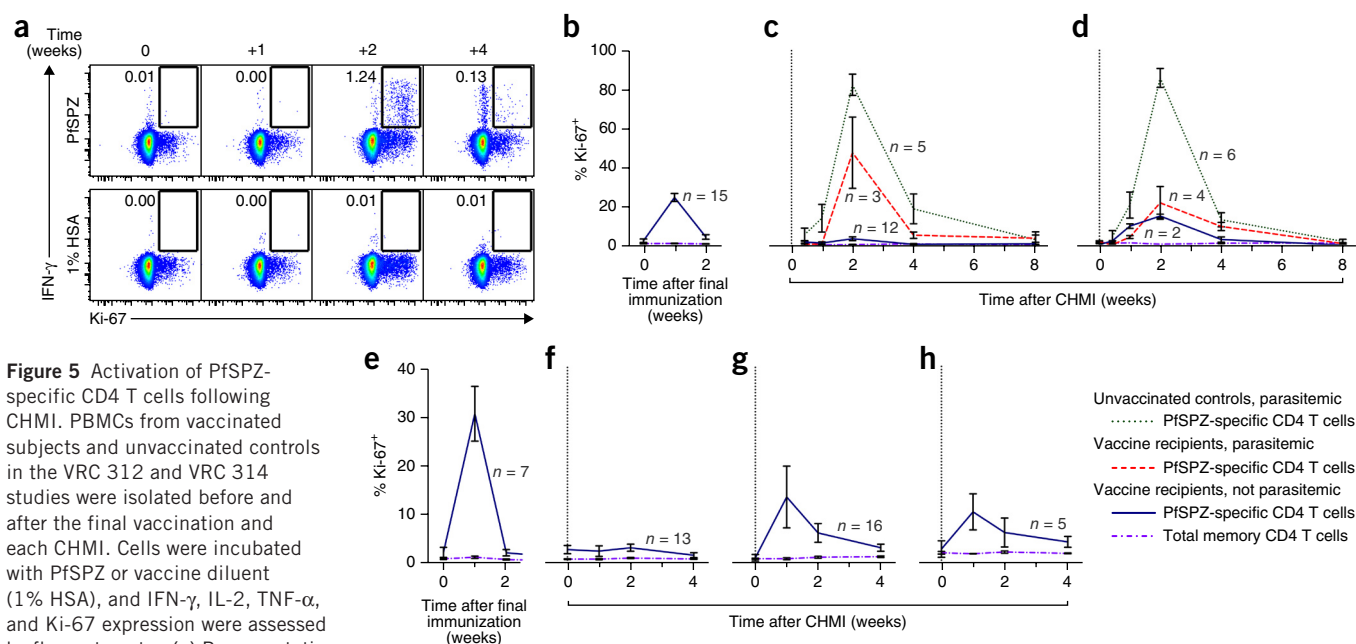


Figure 5 Activation of PfSPZ-specific CD4 T cells following CHMI. PBMCs from vaccinated subjects and unvaccinated controls in the VRC 312 and VRC 314 studies were isolated before and after the final vaccination and each CHMI. Cells were incubated with PfSPZ or vaccine diluent (1% HSA), and IFN- γ , IL-2, TNF- α , and Ki-67 expression were assessed by flow cytometry. **(a)** Representative flow cytometry plots showing memory showing gating for CD4 T cells at 0, 1, 2, and 4 weeks following CHMI in an unvaccinated control subject who developed parasitemia. PfSPZ-specific cells were identified by expression of IFN- γ following PfSPZ stimulation, and cells undergoing replication were identified by Ki-67 expression. **(b)** Percentage of either total memory CD4 T cells (purple dashed line) expressing Ki-67 or PfSPZ-specific CD4 T cells (blue solid line) expressing Ki-67 after final immunization in vaccine recipients who received 1.35×10^5 PfSPZ by i.v. injection in the VRC 312 study. **(c,d)** Frequency of cells (see key) expressing Ki-67 in vaccine recipients who did or did not develop parasitemia following CHMI at 3 **(c)** or 21 **(d)** weeks after the final vaccination. Unvaccinated controls are also shown. Subjects were from the VRC 312 study. **(e)** Analysis as in **b** for subjects from the VRC 314 study who were vaccinated by the i.v. route. **(f–h)** Analyses as in **c,d** for subjects from the VRC 314 study who were vaccinated by the i.v. route and for who CHMIs were done 3 weeks **(f)**, 21- to 25-weeks **(g)**, and 59 weeks **(h)** after the final vaccination. For **b–h**, PfSPZ-specific cells were identified by any combination of IFN- γ , IL-2, or TNF- α expression. Throughout, data are mean $\pm$ s.e.m. The number of subjects in each group is indicated on the respective graph.

of 1.35×10^5 PfSPZ and four i.v. doses of 2.7×10^5 PfSPZ (**Fig. 6c**). PfSPZ-specific CD4 T cell responses in PBMCs were modestly higher in NHPs vaccinated by the peripheral (i.m. or i.d.) routes than by the systemic (i.v.) route ($P = 0.046$), and there were no differences in PfrBC-specific CD8 T cell responses in PBMCs (**Fig. 6d**). In contrast, i.v. administration induced twofold higher frequencies of PfrBC-specific CD8 T cells in the liver, as compared to those induced by the 6- to 10-fold higher total doses of PfSPZ that were administered by the i.m. or i.d. route (**Fig. 6e**). Notably, the frequency of PfrBC-specific CD8 T cell responses in the liver were ~ 100 -fold higher than in PBMCs. Moreover, whereas multiple populations of IFN- γ -producing lymphocytes—including NK cells, $\gamma\delta$ T cells, and CD4 T cells—were identified in the livers of NHPs, the majority ($\sim 60\%$) were CD8 T cells (**Fig. 6f** and **Supplementary Fig. 11**). The composition and differentiation state (i.e., memory phenotype) of lymphocytes in NHP livers largely reflect that of unvaccinated human liver samples, underscoring the biological relevance of the NHP model (**Supplementary Fig. 11**).

DISCUSSION

This was the first clinical trial of a malaria vaccine in which the first CHMI in one of the vaccine groups was done more than 4 weeks after the final immunization, thus representing a shift beyond a search for short-term protection toward trials that focus on dose and regimen optimization for durable sterilizing protection. At 59 weeks after four doses of 2.7×10^5 PfSPZ each, the cumulative VE against CHMI was estimated to be 55%—the most durable efficacy against CHMI reported to date with an injectable malaria vaccine. In the vaccination

regimens tested here, the efficacy of PfSPZ Vaccine depended on both the dose per vaccination and the number of vaccinations. The estimated VE against CHMI done 3 weeks after immunization with three doses of 2.7×10^5 PfSPZ was 24%, as compared to 73% with four doses of 2.7×10^5 PfSPZ. Similarly, four or five doses of 1.35×10^5 PfSPZ conferred an estimated 25% efficacy against CHMI at 21 weeks, as compared to 55% with four doses of 2.7×10^5 PfSPZ. On the basis of these data, we hypothesize that additional increases in the dosage of PfSPZ Vaccine will further increase the magnitude and durability of protective efficacy. Ongoing studies using 4.5×10^5 to 2.7×10^6 PfSPZ per dose are assessing this for homologous CHMI, heterologous CHMI, and natural exposure in all age groups.

The route of administration influenced VE, further underscoring the importance of PfSPZ administration by the i.v. route in achieving protection with this vaccine. Completed studies in Mali, Tanzania, and Equatorial Guinea have provided the first data for the feasibility of direct venous inoculation (DVI) of PfSPZ Vaccine to adults in Africa (M. Sissoko (International Center for Excellence in Research, Mali), S. Healy (NIH), S. Shekalaghe & A. Olotu (both from Ifakara Health Institute, Tanzania), personal communication), and field studies in Africa have begun to determine the safety, efficacy, and feasibility of DVI administration in young children and infants.

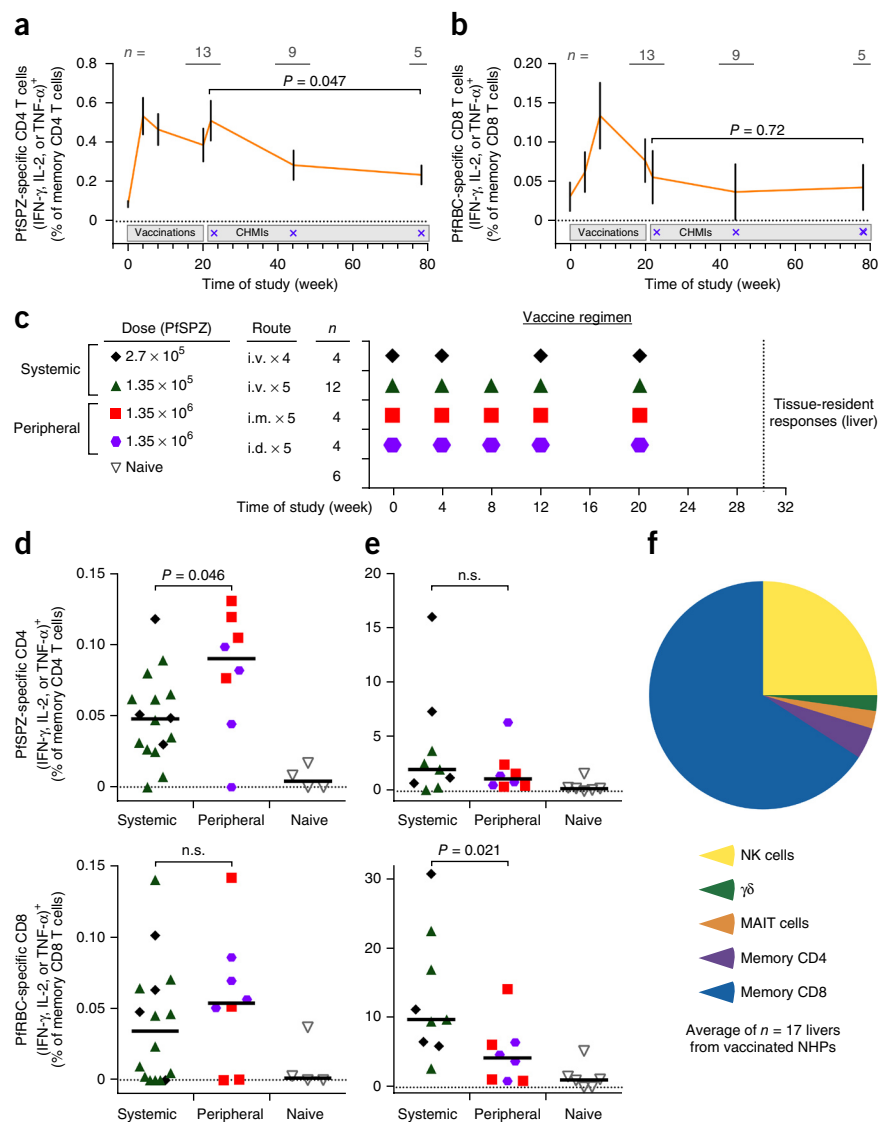
An unexpected immunological finding was that the pre-vaccination frequency of circulating $V\delta 2^+$ T cells correlated with outcome of CHMI. $V\delta 2^+$ T cells recognize intracellular lipid metabolites (such as hydroxyl-methyl-butenyl-pyrophosphate (HMBPP)) from *Plasmodium*^{27,28}. It is possible that these cells have a role in the initial priming of adaptive immune responses through rapid Pf recognition,

Figure 6 Circulating and liver-resident Pf-specific T cells in humans and NHPs.

(a,b) Longitudinal assessment of PfSPZ-specific CD4 T cell responses (a) and PfrBC-specific CD8 T cell responses (b) in subjects who received four doses of 2.7×10^5 PfSPZ/dose by i.v. injection and who did not develop parasitemia at the 3-week, 21-to 24-week, and 59-week CHMIs. The numbers of subjects included at each time point (marked by the blue 'x') are indicated on the graphs. Data are mean $\pm$ s.e.m. *P* values are for comparisons between immune responses at 2 and 59 weeks after the final immunization and were calculated by the Mann–Whitney *U*-test. (c) Schematic of NHP study design. (d) PfSPZ-specific CD4 (top) and PfrBC-specific CD8 (bottom) T cell responses in PBMCs of NHPs 2 weeks after the final vaccination for the subject groups outlined in c. (e) PfSPZ-specific CD4 (top) and PfrBC-specific CD8 (bottom) T cell responses in livers of NHPs 10 weeks after the final vaccination. In d, *n* = 16 for systemic, *n* = 8 for peripheral, *n* = 4 for naive; in e, *n* = 9 for systemic, *n* = 8 for peripheral, *n* = 6 for naive. In d,e, bar denotes median, and between-group comparisons were determined by the Mann–Whitney *U*-test. n.s., not significant (*P* > 0.05). (f) Lineage of liver-resident lymphocytes in vaccinated NHPs expressing IFN- γ following stimulation with PfrBC. MAIT, mucosally associated invariant T cell.

IFN- γ production (Supplementary Fig. 7m), and/or by serving as antigen-presenting cells²⁹ at the time of immunization, leading to enhanced CD8 T cell priming³⁰. $\gamma\delta$ T cells have been shown to provide help to dendritic cells to facilitate protective responses to blood-stage malaria³¹, and IFN- γ -secreting $\gamma\delta$ T cells are a correlate of protection against blood-stage malaria³². It is possible V δ 2⁺ T cells also mediate direct effector functions in the liver. A high frequency of circulating $\gamma\delta$ T cells express CXCR6, a chemokine receptor that facilitates surveillance of the liver sinusoids²², and produce IFN- γ and perforin (Supplementary Fig. 7i–m), two potent effector molecules shown to mediate killing of intracellular Pf. $\gamma\delta$ T cells have been shown to contribute to protection against pre-erythrocytic malaria in mouse models²¹, and these T cells expand in humans following whole-SPZ immunization³³. The correlation between $\gamma\delta$ T cell frequency and CHMI outcome described herein suggests that PfSPZ Vaccine induces protective immunity not only through induction of conventional CD8 and CD4 T cell responses but also through induction of $\gamma\delta$ T cells. Such broad-based T cell responses that are induced by live-attenuated vaccines may not occur after immunization with the most commonly used protein- or virus-based subunit vaccines, highlighting one of many important differences between these approaches.

We previously showed a dose-dependent increase in Pf-specific antibodies and multifunctional cytokine-producing CD4 T cells with doses between 7.5×10^3 and 1.35×10^5 PfSPZ that were administered by i.v. injection, but we could not assess correlates of protection because of the high-level of protection at the 3-week CHMI¹¹. The clear dose response in this prior study¹¹ probably reflects the wider range (18-fold) of doses given. In the present study, there was a



smaller range of doses that were given by the i.v. route, which probably accounts for the narrower range of antibody and T cell responses. The correlates of protection described here are hypothesis-generating and require validation in large prospective studies. Moreover, correlates of protection may differ depending on the dose per vaccination, the number of vaccinations, the time of sampling, and history of malaria exposure.

The data presented here are consistent with the following role for antibodies: at the time of early CHMIs (3 weeks), antibody responses were highest, and subjects may have reduced the numbers of PfSPZ that successfully invaded hepatocytes (Fig. 4c,d)^{14–16}, allowing for rapid elimination of the remaining parasites in the liver by cellular responses^{34,35}, thereby resulting in low Ki-67 expression by PfSPZ-specific CD4 T cells in the protected subjects. At the time of the later CHMIs (21–25 weeks and 59 weeks), antibody levels were considerably lower, resulting in more parasites reaching the liver after CHMI, thus leading to greater activation (based on Ki-67 expression) of PfSPZ-specific CD4 T cells during the clearance of liver-stage parasites (Fig. 5d,g,h).

Circulating Pf-specific T cell responses were low in blood at 59 weeks in all of the subjects who were not parasitemic after CHMI

(Fig. 6a,b). Thus, if T cells are contributing to protection, then it may be due to an unmeasured aspect of the response in blood or to differential responses in the liver. Indeed, tissue-resident Pf-specific CD8 T cell responses in the liver of PfSPZ-vaccinated NHPs were highest after i.v. administration despite using 6- to 10-fold higher doses with the i.m. or i.d. route. Infectivity studies in mice show that >90% of SPZs that are administered by the i.m. or i.d. route fail to reach the liver¹², and the parasite antigens are limited to muscle- or skin-draining lymph nodes³⁶. Intravenous administration, in contrast, results in the efficient delivery of attenuated PfSPZ to the liver, where they presumably mediate priming of T cells that are programmed to remain as non-recirculating, liver-resident cells²⁶. For PfSPZ Vaccine, efficacy is critically dependent on the route of vaccination. The data from the NHP studies indicate this is likely because i.v. administration induces robust tissue-resident responses in the liver, whereas peripheral (i.m. or i.d.) administration results in T cell responses in blood but more limited responses in the liver. Thus, for vaccines seeking to induce protective T cells, vaccine development efforts may be misled by focusing solely on maximizing T cell responses in the blood without considering how the vaccination strategy affects T cell responses at the infection site. In summary, the immune data presented here support the conclusion that whereas antibodies may have some contribution to protection early after final immunization, tissue-resident CD8 T cells are probably necessary for durable sterile protection^{10,17–20,26,37,38}.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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was prepared as part of their official duties. Title 17 U.S.C. §101 defines a US Government work as a work prepared by a military service member or employee of the US Government as part of that person's official duties.

AUTHOR CONTRIBUTIONS

A.S.I., K.E.L., T.L.R., B.K.S., M.C.N., B.S.G., M.R., B.K.L.S., S.L.H., J.E.L., and R.A.S. designed the research studies; A.S.I., K.E.L., A.D., A.A.B., T.L.R., F.H.M., M.E.E., I.J.G., L.-J.C., U.N.S., K.L.Z., L.A.H., E.R.J., P.F.B., A.G., S.C., A.M., M.L., A.J.R., T.L., A.G.E., R.E.S., N.K., T.M., H.D., S.H.P., C.S.H., L.N., P.J.M.C., J.G.S., M.B.L., C.V.P., B.F., W.R.W., J.P.T., J.N., S.R., K.S.-D., G.M.L., J.E.E., M.A.K., G.A.F., S.A.M., M.F., B.K.S., S.H.I.K., S.A.D., L.S.G., N.K.B., M.C.N., B.S.G., M.R., B.K.L.S., S.L.H., J.E.L., and R.A.S. performed the research, analyzed data, and discussed the results and implications; and A.S.I., K.E.L., S.L.H., and R.A.S. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the [online version of the paper](#).

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ONLINE METHODS

VRC 312 study. VRC 312 (<https://clinicaltrials.gov>; NCT01441167) was a phase 1, open-label, dose-escalation trial with CHMI to assess the safety, immunogenicity, and protective efficacy of PfSPZ Vaccine^{9,10} that was administered by intravenous (i.v.) injection. The VRC 312 clinical trial protocol, funding, study oversight, and data reporting have been previously described¹¹. Subjects from the VRC 312 study were re-enrolled for repeat CHMI. PCR and thick blood smear (TBS) for detection of parasitemia were assessed in parallel. The criteria for initiation of treatment for malaria were: (i) a positive TBS, (ii) two consecutive positive PCRs, or (iii) one positive PCR with symptoms or signs consistent with malaria. Upon diagnosis of parasitemia, a 3-d course of atovaquone and proguanil (Malarone) was used for treatment.

VRC 314 study. VRC 314 (<https://clinicaltrials.gov>; NCT02015091) was a multi-institution, phase 1, open-label, dose-escalation trial with CHMI, designed to assess the safety, immunogenicity, and protective efficacy of PfSPZ Vaccine that was administered by i.v. or intramuscular (i.m.) injection. Complete inclusion and exclusion criteria are available at <https://clinicaltrials.gov>. Briefly, malaria-naïve, healthy US adults, 18–45 years of age, were screened for good health, including laboratory testing for hepatitis B and C, HIV, and for pregnancy testing for women of child-bearing potential. Baseline complete blood counts, creatinine, and alanine aminotransferase (ALT) were screened, and only volunteers with normal values were enrolled. Volunteers with significant cardiovascular risk were excluded (i.e., 5-year risk of >10%)³⁹. Exclusion criteria included known history of malaria infection or vaccination, the presence of hemoglobin S by electrophoresis, and splenectomy. The Vaccine Research Center (VRC) Clinical Trials Core at the National Institutes of Health (NIH) Clinical Center (CC) developed the protocol. The University of Maryland, Baltimore (UMB), Center for Vaccine Development (CVD) assisted with protocol development and study design. VRC and UMB CVD performed clinical duties. Sanaria produced, characterized, and prepared the syringes of PfSPZ Vaccine. The Walter Reed Army Institute of Research raised and infected mosquitoes, and assisted with the conduct of the CHMIs.

Study oversight and safety surveillance. Study Oversight and Safety Surveillance was approved by the Intramural Institutional Review Board (IRB) of the National Institute of Allergy and Infectious Diseases (NIAID). US Department of Health and Human Services guidelines for conducting clinical research were followed. Each step was reviewed and approved by a Safety Monitoring Committee and submitted to the Food and Drug Administration (FDA). The protocol was reviewed by the FDA and conducted under IND 14826. Oral and written informed consent was obtained from all subjects.

Local injection-site and systemic reactogenicity parameters were recorded through 7 d after vaccination as solicited adverse events (AEs). Unsolicited AEs were recorded until day 28 post-CHMI. Serious AEs and new chronic medical conditions were recorded through 24 weeks following the last vaccination. AEs were graded using a Toxicity Grading Scale for Adults, adapted from FDA Guidance for Industry 2007 (ref. 40). Malaria infection was recorded as a separate study end point. Therefore, associated signs and symptoms were not included in unsolicited AE listings.

Vaccine and vaccination. PfSPZ Vaccine, which is composed of aseptic, purified, cryopreserved, metabolically active, radiation-attenuated *P. falciparum* sporozoites, was manufactured as described⁹ using Pf NF54 parasites and met all quality control release- and stability-assay specifications. Cryovials containing PfSPZ Vaccine were thawed and formulated using phosphate-buffered saline (PBS) and human serum albumin (HSA) as diluent. Vaccines were administered by rapid intravenous injection of 1-ml volume into an antecubital vein or by intramuscular injection in two divided doses of 0.25 ml in the deltoid muscle.

CHMI. CHMI was achieved by the bites of five PfSPZ-infected *Anopheles stephensi* mosquitoes, which met standard infectivity criteria^{41–43}. All challenges were performed with the 3D7 clone. Subjects were monitored from days 7–28 after CHMI until diagnosis and cure of parasitemia was documented. Blood draws for PCR were performed and analyzed daily as previously described¹¹. Treatment with atovaquone and proguanil (Malarone) was initiated when two PCR results were positive for Pf parasitemia. For the week 3 CHMI for groups 1–3,

based on the large number of vaccinated subjects and controls, half of the subjects in each vaccine group were equally randomized and four unvaccinated controls underwent CHMI on two consecutive days (total of eight controls).

Efficacy analysis. For the primary efficacy analysis, the results of the first CHMI for each vaccine group were compared to controls who underwent CHMI concurrently. To account for multiple comparisons, $P < 0.01$ was taken as evidence of an effect and P values between 0.01–0.05 were taken as suggestive of an effect. This threshold for significance of the primary analysis was specified in the protocol to partially address the potential for type-1-error-rate inflation. A strict Bonferroni adjustment would have been overly conservative, as the study design has several features, including the sequential and dependent nature of the groups and the use of new controls for each CHMI that resembles a sequence of small trials rather than a traditional parallel multi-arm trial. A fixed threshold that did not depend on the final number of groups was preferable in this case where results of some CHMIs were available before the next arm had been finalized. Secondary efficacy analyses compared the results from the different groups, as well as described the results of repeated challenges among those subjects who remained parasite free in each group. No formal multiple-comparison adjustment was used for secondary end points.

For determining vaccine efficacy (VE), first VE was the VE ($VE = 1 - \text{relative risk}$) for the first CHMI. In instances in which a group underwent two CHMIs, vaccine recipients who developed parasitemia during the first CHMI did not undergo repeat CHMI. This was based on data in **Supplementary Figure 1** showing that vaccinated subjects who were parasitemic following CHMI and who were treated remained parasitemic following a second CHMI. Subgroup VE was VE among the subgroup of subjects who were not parasitemic at the prior CHMI. Cumulative VE is first VE multiplied by subgroup VE.

PfCSP ELISA. For ELISA measurement of IgG against PfCSP, a recombinant Pf circumsporozoite protein (rPfCSPv2) was used. The rPfCSP (3D7 clone) is a 48-kDa protein, which begins at amino acid residue 50 of the native protein sequence. The first six amino acids of rPfCSP are S-L-G-E-N-D. The central repeat region of this rPfCSP is composed of 22 N-A-N-P and 4 N-V-D-P tandem repeat sequences. 96-well plates (Nunc MaxiSorp Immuno Plate) were coated overnight at 4 °C with 2.0 µg rPfCSP/ml in 50 µl per well in coating buffer (KPL). Plates were then washed three times with 1× imidazole-based wash solution containing 2 mM imidazole, 160 mM NaCl, 0.02% Tween-20, 0.5 mM EDTA and were blocked with 1% bovine serum albumin (BSA) blocking buffer (KPL) containing 1% non-fat dry milk for 1 h at 37 °C. Plates were washed three times, and serially diluted samples (in triplicates) were added and incubated at 37 °C for 1 h. After washing three times, peroxidase-labeled goat anti-human IgG (KPL) was added at a dilution of 0.1 µg/ml and incubated at 37 °C for 1 h. After washing three times, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt) peroxidase substrate was added for plate development, and the plates were incubated for 75 min at 22 °C. The plates were read with a Spectramax Plus384 microplate reader (Molecular Devices) at 405 nm. The data were collected using Softmax Pro GXP v5. Data were fit to a 4-parameter sigmoidal curve, and the reciprocal serum dilution at which the optical density was 1.0 (OD1.0) was calculated. To serve as assay controls, a negative control (pooled serum from non-immune individuals from a malaria-free area) and a positive control (serum from an individual with anti-PfCSP antibodies) were always included. The results are reported as net OD1.0, which was the OD1.0 of the post-immunization serum minus the OD1.0 of the pre-immunization serum. Samples were considered positive if the difference between the post-immunization OD1.0 and the pre-immunization OD1.0 (net OD1.0) was >50 and the ratio of post-immunization OD1.0 to pre-immunization OD1.0 (ratio) was >2.5.

Supplementary Table 7 lists all PfCSP antibody levels 2 weeks after final vaccination.

PfSPZ aIFA. Purified PfSPZ (NF54 strain) from aseptic *A. stephensi* mosquitoes, which was produced by Sanaria, were resuspended in PBS (pH 7.4). 0.5×10^4 PfSPZ in 40 µl were added to each well of Greiner CELLSTAR clear-bottom black 96-well plates (Sigma-Aldrich). After addition of the suspension, plates were left at room temperature for 12–18 h for air-drying. 50 µl of sera diluted in PBS with 2% BSA were added to each well containing air-dried PfSPZ. Sera samples

were added at twofold dilutions. Plates were incubated at 37 °C for 1 h. Plates were washed in PBS three times. Alexa-Fluor-488-conjugated goat anti-human IgG (Molecular Probes) was diluted to 1:250 in PBS with 2% BSA, and 40 µl was added to each well. The plates were then incubated for 1 h at 37 °C. Plates were washed three times with PBS. 100 µl PBS was added to each well. Samples were assessed by scanning the entire surface of each well using an Acumen eX3 laser-scanning imaging cytometer. The positive control was pooled human serum taken 2 weeks after the last immunization from 12 protected volunteers immunized four or five times with PfSPZ Vaccine in the VRC 312 clinical trial¹¹. The data was plotted to fit a four-parameter sigmoidal curve. Sera from malaria-naïve volunteers in the USA and Europe, including pre-immune sera, always register an AFU (arbitrary fluorescence units) value less than 2.0×10^5 , even at the highest concentration used in this assay (1:50 dilution). For sera that do bind to PfSPZ, 2.0×10^5 AFU falls in the exponential portion of the sigmoidal curves. Therefore 2.0×10^5 was chosen as the threshold in the aIFA assay, and the level of anti-PfSPZ antibodies for each volunteer is reported as the reciprocal serum dilution at which fluorescence intensity was equal to 2.0×10^5 AFU.

Supplementary Table 7 lists all PfSPZ antibody levels 2 weeks after final vaccination.

T cell assays. Assessment of cellular immune responses using multi-parameter flow cytometry was done from PBMCs on cryopreserved samples at the completion of the study. After thawing, PBMCs were rested for 8 h in complete RPMI (RPMI-1640 medium containing 2 mM L-glutamine (GE Life Sciences), 10% vol./vol. heat-inactivated FCS (Atlanta Biologicals), 100 U/ml penicillin (Life Technologies), 100 µg streptomycin (Life Technologies), 25 mM HEPES buffer (Life Technologies), 0.1% vol./vol. 2-mercaptoethanol (Sigma)) containing 25 U/ml Benzoxase (EMD Biosciences), and plated in 200 µl of medium at 1.5×10^6 cells per well in a 96-well V-bottom plate and stimulated for 17 h with: (i) PfSPZ Vaccine diluent (1% human serum albumin; CSL Behring); (ii) 1.5×10^5 viable, irradiated, aseptically purified, cryopreserved PfSPZ from a single production lot; (iii) 2×10^5 lysed, infected RBCs consisting of >90% parasitemic late-stage schizonts (PfRBC) from a single production lot; or (iv) a single lot of donor-matched uninfected erythrocytes (uRBCs). PfSPZ and PfRBC were titrated for optimal sensitivity and specificity of detection of Pf-specific responses. For the last 5 h of the stimulation, 10 µg/ml brefeldin A (BD Biosciences) was added to the culture. A positive control sample from a subject vaccinated with five doses of 1.35×10^5 PfSPZ by i.v. injection and a negative malaria-naïve control were included for each day the subjects were analyzed to determine the reproducibility of antigen stimulation.

After stimulation, cells were stained as previously described⁴⁴. The staining panels are shown in **Supplementary Table 6**; the gating tree is shown in **Supplementary Figures 4 and 5**, and the antibody clones and manufacturers are shown in **Supplementary Table 6**. Briefly, cells were surface-stained for the chemokine receptor CCR7 at 37 °C for 20 min. Dead cells were identified by Aqua Live/Dead dye (Invitrogen) as per the manufacturer's instructions. This was followed by a 15-min surface-staining at room temperature for the markers CD4, CD8, CD14, CD20, CD38, CD45RA, CD56, TCR Vα-7.2, CD161, TCR-γδ, TCR-Vδ1, TCR-Vδ2, TCR-Vγ9, or CXCR6. Cells were washed, fixed, and permeabilized using Cytofix/Cytoperm kit (BD Biosciences) and were stained intracellularly for CD3, IFN-γ, IL-2, TNF-α, perforin, or Ki-67. Cells were washed, fixed in 0.5% paraformaldehyde, and signals were acquired on a modified LSR II (BD Biosciences). All antigen-specific cytokine frequencies are reported after background subtraction of identical gates from the same sample incubated with the control antigen stimulation (HSA or uRBC).

Human liver samples. Human liver samples were obtained under a clinical protocol unrelated to the PfSPZ vaccination protocol. The Regional Ethical Review Board in Stockholm, Karolinska Institutet, Stockholm, Sweden, approved the study. Oral and written informed consent was obtained from all subjects. Livers were obtained during partial hepatectomy from living donors undergoing therapeutic tumor excision where only tumor-free unaffected tissue was used for isolation of immune cells. Immune cells were isolated using a previously described protocol⁴⁵.

Functional assessment of vaccine-induced IgG. Total IgG serum antibodies were purified from serum or plasma by affinity chromatography using Pierce

Protein G Plus Agarose (Thermo Scientific) according to manufacturer's instructions. Column-eluted IgG was concentrated and buffer-exchanged into phosphate-buffered saline (pH 7.4) using Amicon Ultra 30,000 MWCO centrifugal filters (EMD Millipore). Protein concentration of the purified IgG was calculated by the BCA assay (Thermo Scientific).

Human hepatocyte donor-matched FRG-huHep mice (male and female, aged 5–9 months) were purchased from Yecuris, Inc. All animals were cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals⁴⁶. The Institutional Animal Care and Use Committee of the Center for Infectious Disease Research, Seattle, WA approved all animal procedures. Animals were randomly allocated to experimental and control groups.

Mice were intravenously injected with 8 mg/mouse of total IgG purified from vaccinated subjects or 150 µg of anti-PfCSP mouse monoclonal antibody (IgG3; clone 3C1; Rockefeller University) 16 h before challenge. For the mosquito bite challenge, *Anopheles* mosquitoes infected with *P. falciparum* expressing GFP-luciferase were generated as previously described⁴⁷. Mosquito infection was quantified at day 10 after blood meal and were used only if infection was >50% prevalence with an average of >10 oocysts/midgut. All qualifying mosquitoes were then pooled and distributed into cages with ~50 mosquitoes/mouse (up to 250 mosquitoes). Mice, in groups of up to five, were then anesthetized with isoflurane and placed on a mesh screen covering the container of mosquitoes while under isoflurane anesthesia. Mosquitoes were then allowed to feed for 10 min, with lifting of mice once every minute to encourage probing and injection of sporozoites rather than blood feeding. After 10 min, the mice were returned to normal activity.

At day 6 post-infection (peak of liver burden), mice were imaged for liver-stage burden using bioluminescence and IVIS imaging as previously described^{24,48}. Briefly, mice were intraperitoneally injected with 100 µl of Rediject D-Luciferin (PerkinElmer) and, after 5 min, were imaged with a 5-min exposure. Liver-stage burden was assessed by placing a region of interest (ROI) around the liver of each mouse and measuring total flux in photons/s. An identical ROI was placed over the thorax, which was used to subtract background signal. Liver-stage burden of all mice was normalized to the mean of the negative control group, which received pre-immune IgG. Sample size was based on prior passive transfer experiments and calculated using Prism (GraphPad) and JMP Design of Experiment functionality (SAS). Pearson correlation coefficient was used to determine the relationship between Pf liver-stage burden and PfCSP level. Investigators were blinded to treatment group during passive transfer of IgG, Pf infection, and measurement of luminescence.

NHP vaccinations. Thirty healthy male and female rhesus macaques of Indian origin (*Macaca mulatta*) with a mean (s.d.) age and weight of 6.1 (1.5) years and 8.7 (3.1) kg, respectively, were singly housed in animal biosafety level 2 facilities and were monitored throughout the study for physical health, food consumption, body weight, and temperature. All animals were cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals⁴⁶. The Institutional Animal Care and Use Committee of the Vaccine Research Center, NIH approved all animal procedures.

Study groups were balanced with respect to age, weight, and gender (equal numbers of males and females in each group). Sample size was based on prior NHP immunogenicity studies and calculated using Prism (GraphPad) and JMP Design of Experiment functionality (SAS). PfSPZ Vaccine was formulated and administered as for human injection with the following exceptions. For i.v. groups, PfSPZ in 500 µl was administered by direct venous inoculation into the saphenous vein. For the i.m. group, PfSPZ was administered to the left and right deltoid, and each injection was 500 µl in volume. For the i.d. group, PfSPZ was administered in the skin over the left and right deltoid, and each injection was 100 µl in volume. Immunizations and blood sampling occurred with the animal under anesthesia (10 mg per kg body weight ketamine HCl).

NHP T cell assays. Immune assays on PBMCs were batch-analyzed on cryopreserved samples at the completion of the study. PBMCs were isolated by density-gradient centrifugation from Acid-citrate-dextrose-anti-coagulated whole blood. Ten weeks after final immunization, animals were euthanized, and liver tissue was processed as previously described¹⁰. For immune analysis, samples were thawed, rested, stimulated, stained, and analyzed using identical reagents,



stimulation antigens, and protocol as the intracellular cytokine staining assay used for human PBMC samples (described above). The staining panel is shown in **Supplementary Table 6**; the gating tree is shown in **Supplementary Figure 10**; and the antibody clones and manufacturers are shown in **Supplementary Table 6**. Investigators were blinded to the treatment group during lymphocyte stimulations and flow cytometry gating. All antigen-specific cytokine frequencies are reported after background subtraction of identical gates from the same sample incubated with the control antigen stimulation (HSA or uRBC).

Statistical analyses. Flow cytometry data was analyzed using FlowJo v9.8.5 (Tree Star). Statistical analyses were performed with Pestle v1.7 and SPICE v5.3 (M. Roederer)⁴⁹, JMP 11 (SAS), Prism 6 (GraphPad), and R v3.2.2 with RStudio v0.99.483.

For vaccine immunogenicity, comparisons between groups were performed using Kruskal–Wallis with Dunn’s post-test correction for multiple comparisons. If no differences between vaccines groups were identified by Kruskal–Wallis, then differences from pre-vaccination were assessed by Wilcoxon matched-pairs signed rank test with Bonferroni correction for multiple comparisons, as specified in the figure legends.

Immune responses were assessed 2 weeks after the final immunization or pre-vaccination (as specified in the figure legends) and were compared to outcome at either 3-week CHMI or 21- to 25-week CHMI. Assessment of immune responses that correlate with outcome at CHMI (parasitemia or no parasitemia) was made using a stratified Wilcoxon test controlling for vaccine regimen as a covariate.

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