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Two chemoattenuated PfSPZ malaria vaccines induce sterile hepatic immunity

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The global decline in malaria has stalled[1], emphasizing the need for vaccines that induce durable sterilizing immunity. Here we optimized regimens for chemoprophylaxis vaccination (CVac), for which aseptic, purified, cryopreserved, infectious *Plasmodium falciparum* sporozoites (PfSPZ) were inoculated under prophylactic cover with pyrimethamine (PYR) (Sanaria PfSPZ-CVac(PYR)) or chloroquine (CQ) (PfSPZ-CVac(CQ))—which kill liver-stage and blood-stage parasites, respectively—and we assessed vaccine efficacy against homologous (that is, the same strain as the vaccine) and heterologous (a different strain) controlled human malaria infection (CHMI) three months after immunization (https://clinicaltrials.gov/, NCT02511054 and NCT03083847). We report that a fourfold increase in the dose of PfSPZ-CVac(PYR) from 5.12×10^4 to 2×10^5 PfSPZs transformed a minimal vaccine efficacy (low dose, two out of nine (22.2%) participants protected against homologous CHMI), to a high-level vaccine efficacy with seven out of eight (87.5%) individuals protected against homologous and seven out of nine (77.8%) protected against heterologous CHMI. Increased protection was associated with Vδ2 γδ T cell and antibody responses. At the higher dose, PfSPZ-CVac(CQ) protected six out of six (100%) participants against heterologous CHMI three months after immunization. All homologous (four out of four) and heterologous (eight out of eight) infectivity control participants showed parasitaemia. PfSPZ-CVac(CQ) and PfSPZ-CVac(PYR) induced a durable, sterile vaccine efficacy against a heterologous South American strain of *P. falciparum*, which has a genome and predicted CD8 T cell immunome that differs more strongly from the African vaccine strain than other analysed African *P. falciparum* strains.

The morbidity and mortality induced by malaria were halved in little more than a decade using drug and anti-vector measures, but progress has stalled[1]. Vaccines that confer sterile protective immunity would prevent deaths and contribute to the elimination of malaria. Pre-erythrocytic-stage vaccines target the clinically silent sporozoite and/or liver stages of the life cycle of the parasite to induce sterile immunity that averts blood-stage infection, disease and transmission.

The most advanced malaria vaccine candidate, RTS,S (also known by the trade name Mosquirix), targets a major surface protein (circumsporozoite protein (CSP)) of *P. falciparum* sporozoites and confers partial protection against clinical malaria[2–4]. Another advanced candidate, Sanaria PfSPZ Vaccine, is composed of radiation-attenuated PfSPZs. PfSPZ Vaccine induces significant protection against homologous (that is, the same strain of *P. falciparum* in the vaccine and the

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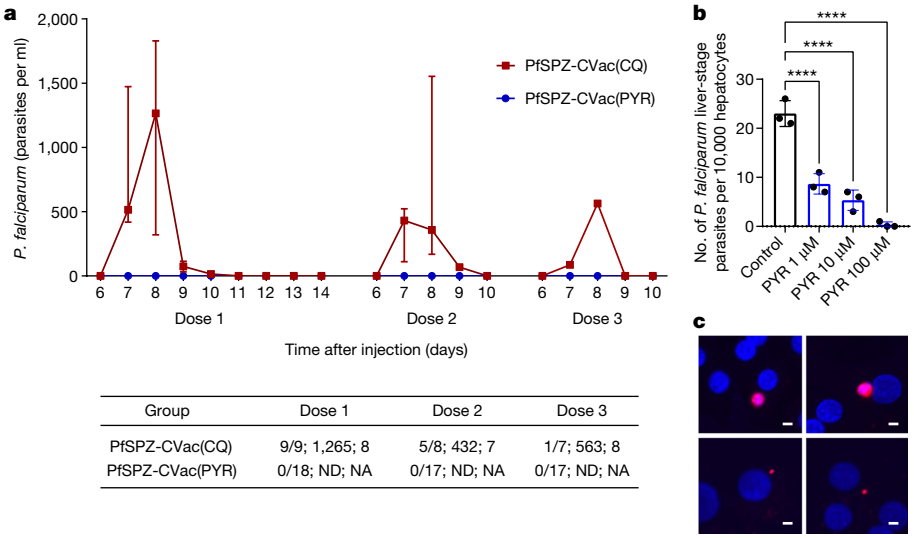

Group	Dose 1	Dose 2	Dose 3
PfSPZ-CVac(CQ)	9/9; 1,265; 8	5/8; 432; 7	1/7; 563; 8
PfSPZ-CVac(PYR)	0/18; ND; NA	0/17; ND; NA	0/17; ND; NA

Fig. 1 | Parasitaemia detected by qPCR after the first, second and third dose of 2×10^5 PfSPZ for the PfSPZ-CVac high-dose study and PYR activity against liver-stage parasites. a, Median parasitaemia values and IQRs are shown for positive PfSPZ-CVac(CQ) participants. Dose 1, PfSPZ Challenge inoculation under CQ or PYR treatment cover with follow-up for 14 days; doses 2 and 3, PfSPZ Challenge inoculation under CQ or PYR treatment cover with follow-up for 10 days. The table shows (from left to right in each cell): the number of participants who were positive by qPCR/the number of injected participants; the median peak parasite density of positive participants (parasites per ml); and the mean day of peak parasite density (positive participants). NA, not applicable; ND, not detected. Individual data are shown in Extended Data Fig. 8b. **b**, **c**, PYR was added to cultures (1 μM, 10 μM or 100 μM) on days 2 and 3 after infection with daily medium changes until day 4, at which point cultures were fixed. **b**, On day 4, the remaining liver-stage parasites were counted. Data are mean ± s.e.m., $n = 3$ independent wells. Statistical significance was determined using a one-way ANOVA with post hoc pairwise comparisons. ****$P < 0.0001$. Parasite diameters are shown in Extended Data Fig. 9c. **c**, Representative images of *P. falciparum* from control (top) and PYR-treated (bottom left, 1 μM; bottom right, 10 μM) groups. Parasites (red) were identified by anti-HSP70. Scale bars, 5 μm.

challenge) and heterologous (a different strain) CHMI[5–8] and against intense natural transmission in Africa[9,10]. Radiation-attenuated PfSPZs are non-replicating parasites and arrest early in the liver stage, as do first-generation genetically attenuated PfSPZs[11,12,13].

For CVac with PfSPZs, non-attenuated PfSPZs (PfSPZ Challenge) are administered under antimalarial drug cover. In seminal studies, blood-stage schizonticides such as CQ[14] or mefloquine[15], given after exposure to mosquitoes carrying *P. falciparum* sporozoites, killed parasites after liver-stage development and brief blood-stage exposure[16], and conferred a high level of homologous vaccine efficacy[14,15] after an immunizing dose of up to 45 infected mosquito bites, much less than the more than 1,000 bites that are required for radiation-attenuated *P. falciparum* sporozoites[17]. This has been ascribed to parasite replication (up to 50,000-fold) in the liver. Similarly, three doses of 5.12×10^4 PfSPZs administered with CQ (Sanaria PfSPZ-CVac(CQ)) protected 100% of the participants (nine out of nine) against homologous CHMI 10 weeks later[18]. This is, to our knowledge, the highest level of durable vaccine efficacy against homologous CHMI that has been achieved. However, a dose of up to 45 infected mosquito bites given under CQ cover induced minimal protection against heterologous CHMI[19,20].

Ideally, PfSPZ-CVac would induce protective immunity without exposure to blood-stage parasites, which cause clinical malaria. Mouse studies of CVac with CQ or PYR, which kills liver-stage parasites, showed that both regimens confer sterilizing homologous immunity[21] with similar efficacy[22]. In humans, the antimalarial drug primaquine, which kills liver-stage parasites, reduced but did not eliminate the breakthrough of blood-stage infections during CVac administration, and CVac (primaquine) with 45 or fewer infected mosquito bites conferred minimal sterilizing immunity against homologous CHMI[23].

Here, we examined PfSPZ-CVac(CQ) and PfSPZ-CVac(PYR) in human trials to assess the requirement for blood-stage parasite exposure and to examine the effects of PfSPZ dose for inducing durable sterilizing immunity.

Safety and efficacy of the low-dose study

In our first trial (https://clinicaltrials.gov/, ID NCT02511054; Extended Data Fig. 1), we compared three monthly doses (days 1, 29 and 57) of 5.12×10^4 PfSPZ-CVac ('low-dose' PfSPZ) and weekly treatment with 500 mg CQ (PfSPZ-CVac(CQ)) against three monthly doses (days 1, 29 and 57) of 5.12×10^4 PfSPZ-CVac ('low-dose' PfSPZ) and treatment with 50 mg PYR on days 2 and 3 (PfSPZ-CVac(PYR)) plus weekly CQ treatment as a safety measure against breakthrough parasitaemia. Vaccinations were safe and well-tolerated (Supplementary Information). PYR killed liver-stage parasites in all recipients (Extended Data Fig. 2). Similar to previous results[18], four out of five PfSPZ-CVac(CQ) recipients (vaccine efficacy = 80%, $P = 0.048$; 95% confidence interval, 1–99%) were protected against homologous CHMI 3 months after the last dose; conversely, only two out of nine (vaccine efficacy = 22.2%, $P = 0.8$) PfSPZ-CVac(PYR) recipients were protected (Extended Data Fig. 3).

Safety and efficacy of the high-dose study

We hypothesized that higher PfSPZ doses would enhance immunity[24] and performed a second study (https://clinicaltrials.gov/, ID NCT03083847). In the dose-escalating pilot phase, doses as high as 2×10^5 PfSPZ Challenge (NF54) ('high dose') were deemed safe for PfSPZ-CVac(CQ) and PfSPZ-CVac(PYR), which was given without weekly CQ (full safety information is provided in the Supplementary Information). Nonsteroidal anti-inflammatory drugs were given on days 7 and 8 after the first PfSPZ inoculation to PfSPZ-CVac(CQ) recipients to reduce symptoms related to brief blood-stage parasitaemia with higher PfSPZ Challenge doses.

In the efficacy phase, 42 participants were allocated to PfSPZ-CVac(PYR) ($n = 20$), PfSPZ-CVac(CQ) ($n = 10$) or no intervention ($n = 12$, infectivity control participants) (Extended Data Fig. 4 and Supplementary Information for further details regarding the study design). Vaccinations were safe and well-tolerated (Supplementary Information). One PfSPZ-CVac(CQ) recipient withdrew because of a serious adverse event that was possibly

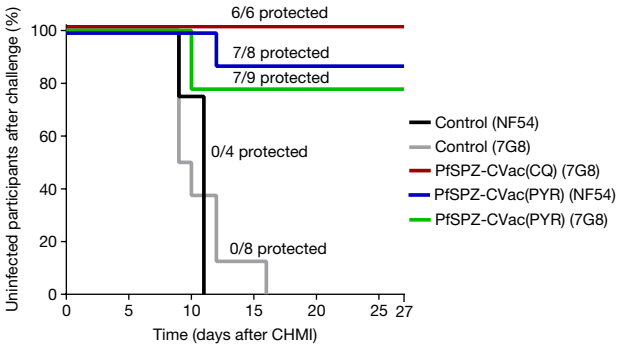

Fig. 2 | Vaccine efficacy of PfSPZ-CVac(PYR) against homologous and heterologous CHMI and PfSPZ-CVac(CQ) against heterologous CHMI, 3 months after the last immunization. Survival curves display the percentage of study participants who remained protected throughout 27 days of follow-up after inoculation with a high dose of PfSPZ (2×10^5). Vaccine efficacy compared the vaccinated to unvaccinated groups for the time to first detectable parasitaemia after inoculation using a log-rank test. In the PfSPZ-CVac(PYR) group, 7/8 participants (vaccine efficacy = 87.5%, $P = 0.003$; 95% confidence interval, 42.5–100%) were protected from homologous CHMI and 7/9 participants (vaccine efficacy = 78%, $P = 0.001$; 95% confidence interval, 39.8–100%) from heterologous CHMI. In the PfSPZ-CVac(CQ) group, 6/6 participants (vaccine efficacy = 100%, $P = 0.001$; 95% confidence interval, 54.1–100%) were protected from heterologous CHMI.

related to CQ (Extended Data Fig. 4). During the vaccination period, no participants had patent parasitaemia detected by microscopy. Subpatent parasitaemia was detected in all PfSPZ-CVac(CQ) recipients using quantitative PCR (qPCR) (which decreased with successive administrations, as described previously[14,18]), but after the third dose of 2×10^5 PfSPZs, only one out of seven vaccinated participants developed any detectable parasitaemia after receiving more than sixty 100% infectious doses, indicating that sterile homologous immunity had developed after only two doses in six out of seven vaccinated participants. As expected, no PfSPZ-CVac(PYR) recipients had detectable subpatent parasitaemia during vaccination (Fig. 1a), due to PYR activity in the liver.

To assess the effects of PYR on liver-stage parasites, we used an in vitro model with micropatterned primary human hepatocytes[25–27]. PYR (1, 10 or 100 μM) was added on days 2 and 3 after infection and significantly reduced the numbers of liver-stage parasites on day 4 in a dose-dependent manner, which suggests that PYR can kill parasites (Fig. 1b); the remaining parasites were significantly smaller, indicating that these parasites showed arrested development (Fig. 1c). Daily PYR 50 mg treatment given orally achieves plasma concentrations of around 300–600 μg ml^{-1} (around 1.2–2.4 μM)[28].

Vaccine efficacy was assessed for homologous (NF54, vaccine strain) and heterologous (7G8, a South American parasite) CHMI with 3.2×10^3 PfSPZ Challenge 3 months after the third vaccination. All 12 unvaccinated infectivity control participants were diagnosed with *P. falciparum* infection by qPCR on days 9–11 (NF54; $n = 4$) or days 9–16 (7G8; $n = 8$). In the PfSPZ-CVac(PYR) groups, seven out of eight participants (87.5%, $P = 0.003$; 95% confidence interval, 42.5–100%) were protected against homologous and seven out of nine participants (77.8%, $P = 0.001$; 95% confidence interval, 39.8–100%) were protected against heterologous CHMI. In the PfSPZ-CVac(CQ) group, six out of six participants (100%, $P = 0.001$; 95% confidence interval, 54.1–100%) were protected against heterologous CHMI (Fig. 2).

Vδ2 γδ T cell expansion in vaccinated participants

PfSPZ Vaccine or PfSPZ-CVac administration expands Vδ2 γδ T cells (hereafter Vδ2 T cells)[29,30]. Vδ2 T cells were measured as a percentage of total T cells using ex vivo flow cytometry throughout the vaccination period (Fig. 3). At baseline, the median percentage of Vδ2 T cells was similar in the PfSPZ-CVac(PYR) and PfSPZ-CVac(CQ) groups (low dose, 2.59 (interquartile range (IQR) = 2.48–2.7) versus 1.42 (IQR: 1.33 – 4.65), $P = 0.3127$, Wilcoxon–Mann–Whitney test; high dose, 2.35 (IQR = 1.96–5.93) versus 2.36 (IQR = 0.88–3.67), $P = 0.6187$, Wilcoxon–Mann–Whitney test) (Extended Data Fig. 5). Subsequent data are expressed as fold change from baseline, either before (unadjusted) or after (adjusted) adding an offset of +1 to the numerator and denominator for calculations of fold change in %Vδ2 T cells (Fig. 3a, b present unadjusted values).

The fold change in the percentage of Vδ2 T cells during PfSPZ-CVac(CQ) was similar ($P = 0.768$, generalized estimating equation model using adjusted fold change values, see Methods) between the low dose (median = 2.01 (IQR = 1.9–2.98)) and the high dose (median = 2.84 (IQR = 2.21–3.04)) from dose 1 to day 84 (28 days after dose 3). All but one of the PfSPZ-CVac(CQ) vaccinated participants were protected during CHMI (in the low-dose study); the one infected vaccinated participant had the lowest fold increase in Vδ2 T cells among PfSPZ-CVac(CQ) recipients (adjusted fold change: 1.78, day 84).

Conversely, the fold change in Vδ2 T cells during the PfSPZ-CVac(PYR) study increased ($P = 0.021$, generalized estimating equation model) from the low dose (median = 1.6 (IQR = 1.18–1.96)) to the high dose (median = 2.02 (IQR = 1.68–3.94)) from dose 1 to day 84 (28 days after dose 3). In the high-dose study, protected PfSPZ-CVac(PYR) recipients (protected against NF54 and 7G8 challenges, $n = 14$) showed a significantly greater expansion of Vδ2 T cells from dose 1 to day 84 (28 days after dose 3) compared with infected vaccinated participants ($P = 0.016$, generalized estimating equation model), but did not differ immediately before CHMI (day 141; protected, median = 2.36 (IQR = 1.94–3.71); infected, median = 2.01 (IQR = 1.01–4.45); $P = 0.45$, Wilcoxon–Mann–Whitney test).

Antibody responses induced by PfSPZ-CVac

Two weeks after the last vaccination (day 70), all PfSPZ-CVac(PYR) or PfSPZ-CVac(CQ) recipients met the criteria for seroconversion according to IgG PfCSP levels analysed by enzyme-linked immunosorbent assay (ELISA). IgG levels 2 weeks after the last administration of 5.12×10^4 PfSPZ-CVac(CQ) (low-dose study) were similar in this trial (median net optical density (OD) 1.0 = 2,480) (Fig. 4a) and a previous trial (median net OD 1.0 = 3,844)[18]. IgG levels 2 weeks after vaccination were significantly higher in recipients of high-dose versus low-dose PfSPZ-CVac(PYR) (3,768 versus 255, respectively ($P < 0.001$)), but not in recipients of high-dose versus low-dose PfSPZ-CVac(CQ) (8,060 versus 2,480 ($P = 0.178$)) (Fig. 4a); IgG levels were higher in PfSPZ-CVac(CQ) than PfSPZ-CVac(PYR) participants for the low dose (2,480 versus 255 ($P = 0.012$)) but not the high dose (8,060 versus 3,768 ($P = 0.280$)). Similar relationships were seen before CHMI, 3 months after vaccination (Fig. 4b).

In general, median IgG levels were higher in protected versus infected vaccinated participants within study arms; small sample sizes precluded or limited subgroup statistical analyses. Within low-dose PfSPZ-CVac(PYR) participants, the two protected participants had higher median levels compared with the seven infected participants (median 1,457 versus 147 ($P = 0.057$)); within high-dose PfSPZ-CVac(PYR) participants, 14 protected participants had higher levels versus 3 infected participants (4,215 versus 829 ($P = 0.065$)) (Fig. 4). Among PfSPZ-CVac(PYR) participants, IgG levels 3 months after vaccination (before CHMI) were not related to protection in the low-dose (protected, 608; infected, 47; $P = 0.306$) or high-dose (protected, 3,788; infected, 2,170; $P = 0.439$) groups.

IgM PfCSP levels analysed by ELISAs 2 weeks after the third dose and before CHMI did not differ between high-dose PYR and CQ groups, with too few infected participants to assess the association with vaccine

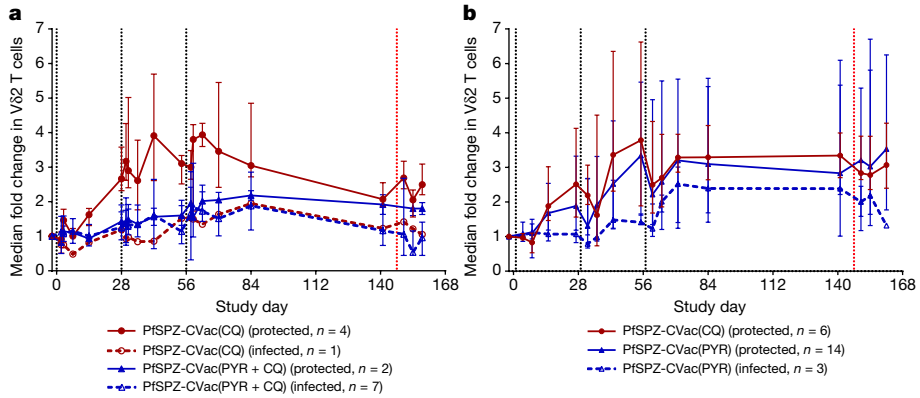

Fig. 3 | Vδ2 γδ T cells related to protective immunity. Vδ2 γδ T cells in vaccinated individuals stratified by protection status. Median fold changes and IQRs using unadjusted %Vδ2 values are shown. **a**, For the low-dose study, $n = 4$ PfSPZ-CVac(CQ) protected vaccinated participants; $n = 1$ PfSPZ-CVac(CQ) infected vaccinated participant; $n = 2$ PfSPZ-CVac(PYR + CQ) protected vaccinated participants; $n = 7$ PfSPZ-CVac(PYR + CQ) infected vaccinated participants. **b**, For the high-dose study, $n = 6$ PfSPZ-CVac(CQ) protected vaccinated participants; $n = 14$ PfSPZ-CVac(PYR) protected vaccinated participants; $n = 3$ PfSPZ-CVac(PYR) infected vaccinated participants. Black dashed lines indicate vaccination days; red dashed line indicates day of PfSPZ Challenge inoculation.

efficacy (Extended Data Fig. 6). In the automated immunofluorescence assay and automated inhibition of sporozoite invasion assay, the high-dose groups generally had higher levels of activity than low-dose groups (Extended Data Fig. 7).

Discussion

Progress in malaria control has plateaued, and new interventions are needed[1]. No human parasite vaccines have marketing authorization (licensure) in the USA or Europe. Furthermore, the complexity of the PfSPZ vaccines (like any eukaryotic cell vaccine) for which there are no suitable models in animals to test efficacy have required multiple human trials to assess the dose, schedule, route of administration and method of attenuation. Here, we report a culmination of that empirical process and show sterilizing immunity against heterologous *P. falciparum* CHMI for at least 3 months after vaccination. Although PfSPZ-CVac(CQ) was previously shown to induce long-lived protection, this was thought to be limited to homologous parasites[19,20] and to require exposure to blood-stage parasites[31,32]. Our findings overturn both of these notions and strongly suggest that PfSPZ-CVac will confer broadly protective sterilizing immunity against *P. falciparum* in the field.

The *P. falciparum* 7G8 strain used for heterologous CHMI originates from Brazil. *P. falciparum* 7G8 is more divergent at the levels of the genome, proteome and CD8 T cell immunome than any of more than 400 *P. falciparum* isolates studied from east, west and central Africa, including the West African NF54 strain used for PfSPZ-CVac[33]. We previously reported that the field efficacy of a PfSPZ Vaccine regimen in adults in Mali against 6 months of intense *P. falciparum* transmission[9] equalled or exceeded its vaccine efficacy at 6 months against CHMI with *P. falciparum* 7G8 in adults in the USA[5]. In a previous trial, participants who received chemoprophylaxis and sporozoites vaccination that induce high levels of homologous protection showed minimal heterologous protection against NF135.C10 (Cambodia) and NF166.C8 (Guinea) parasites[20]; future studies of different parasite challenge strains are warranted to predict field vaccine efficacy in Africa or other regions. The 100% vaccine efficacy against heterologous CHMI in PfSPZ-CVac(CQ) recipients here may have resulted from the fourfold increase in dosage above that required to achieve similar homologous protection, whereas CVac studies of individuals immunized by mosquito bite that conferred minimal heterologous protection had not increased the dosage above that required for homologous CHMI protection[20]. Whether doses above the 2×10^5 PfSPZ Challenge might further enhance sterilizing immunity requires further study, using more rigorous tests such as CHMI at later time points or field efficacy trials. The effect of the fourfold increase in dose was even more notable for PfSPZ-CVac(PYR): at 5.12×10^4 PfSPZs, vaccine efficacy was only 22% against homologous CHMI; at 2×10^5 PfSPZs, vaccine efficacy was 88% for homologous CHMI ($P = 0.009$, two-tailed Barnard's test) and 78% for heterologous CHMI ($P = 0.03$, two-tailed Barnard's test).

We show that durable, sterile vaccine efficacy against heterologous CHMI can be achieved without blood-stage parasite exposure during PfSPZ-CVac(PYR). The vaccine efficacy of PfSPZ-CVac(CQ) has been attributed in part to exposure to blood-stage parasites[32], even though vaccinated participants were not protected against CHMI with blood-stage parasites[34]. Instead, blood-stage parasite exposure has been hypothesized to enhance pre-erythrocytic immunity by inducing protective cross-stage responses[31]. In a mouse model of *Plasmodium yoelii* infection, sterilizing immunity induced by CVac(CQ) included activity against liver- or blood-stage infection, and at higher doses was mediated primarily by activity against liver-stage parasites[35]. Our data do not exclude the possibility that blood-stage parasite exposure could enhance pre-erythrocytic immunity (PfSPZ-CVac(CQ) results), but do demonstrate that durable heterologous immunity is achieved without it (PfSPZ-CVac(PYR) results).

We were surprised that heterologous vaccine efficacy at 3 months of PfSPZ-CVac(PYR) with the 2×10^5 PfSPZ dose (78%) exceeded that of radiation-attenuated PfSPZs (PfSPZ Vaccine) with the 9×10^5 PfSPZ dose (20%)[36]. Radiation-attenuated PfSPZs are thought to develop normally for around 2 days before halting development, and never replicate. Using micropatterned primary human hepatocyte cultures, we found that PYR at clinically relevant concentrations[28] on days 2–4 significantly reduced parasite numbers and size on day 4 (Fig. 1b, c), which suggests that the drug induces killing and developmental arrest of the parasite. The results are in agreement with our clinical finding that no parasites emerged in blood (Fig. 1a), and suggest that fine differences in attenuated PfSPZs, such as the timing of arrest and/or antigen repertoire, can substantially affect the efficacy of the vaccine.

We[9] and others[29] previously associated Vδ2 T cells with PfSPZ Vaccine protection. In mice that received SPZ vaccines, a subset of γδ T cells was required to induce protective $CD8^+$ T cells that kill intrahepatocytic parasites, but γδ T cells did not directly mediate protection[30]. $CD8^+$ T cells—presumably resident memory T cells—may eliminate parasite-infected hepatocytes directly or through IFNγ to kill the parasite[37]. These effector processes take place in the liver and have been difficult to study in humans[38]. In this study, Vδ2 T cell responses and vaccine

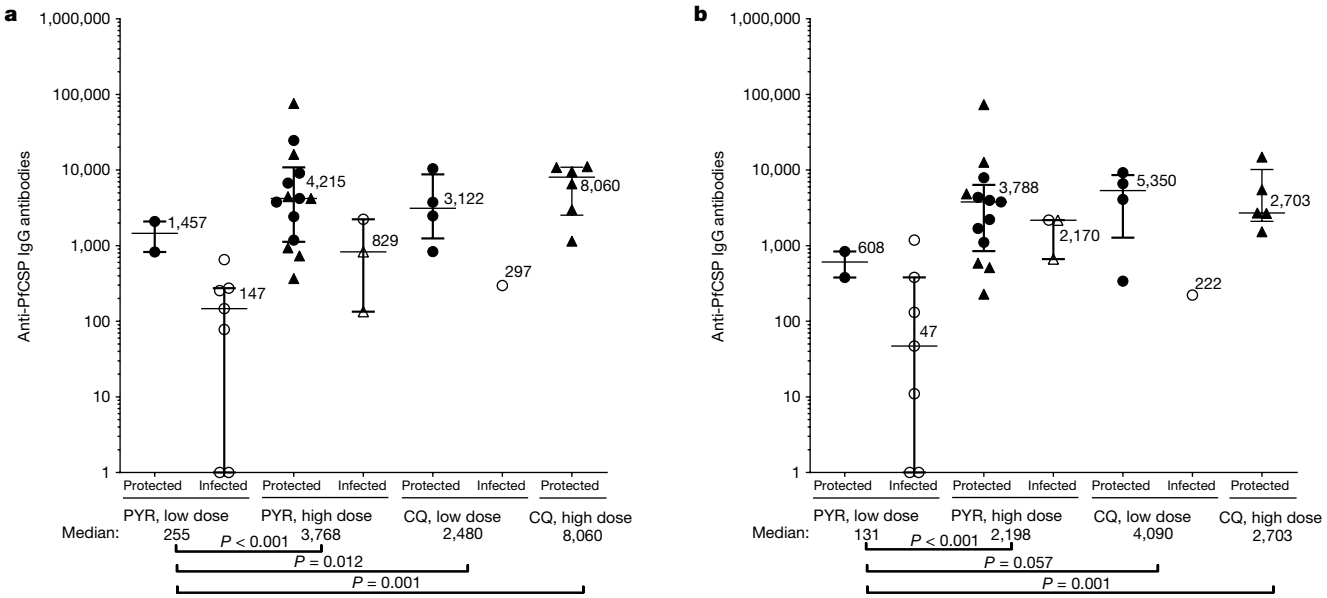

Fig. 4 | Anti-PfCSP IgG levels by ELISA in vaccinated participants of the low-dose and high-dose studies. Filled circles are protected participants and open circles are infected participants in homologous CHMI; filled triangles are protected participants and open triangles are infected participants in heterologous CHMI. Median values and IQRs are displayed. *P* values were calculated using two-sided Wilcoxon–Mann–Whitney tests and group differences with $P > 0.07$ are not indicated. **a**, **b**, IgG antibodies against PfCSP (net OD 1.0) 2 weeks (day 70) (**a**) or approximately 3 months (before CHMI) after the third PfSPZ-CVac dose (**b**). PYR low-dose study, $n = 2$ protected and $n = 7$ infected vaccinated participants; PYR high-dose study, $n = 14$ protected and $n = 3$ infected vaccinated participants; CQ low-dose study, $n = 4$ protected and $n = 1$ infected vaccinated participant; CQ high-dose study, $n = 6$ protected vaccinated participants in **a** (day 70) and $n = 5$ protected vaccinated participants in **b** (before CHMI). No vaccinated participants were infected in the CQ high-dose study.

efficacy increased with PfSPZ dose among PfSPZ-CVac(PYR) recipients. At the lower dosage (5.12×10^4), Vδ2 T cell increases (and vaccine efficacy) were significantly greater in PfSPZ-CVac(CQ) recipients than PfSPZ-CVac(PYR) recipients; at modestly higher dosage (2×10^5), Vδ2 T cell increases (and vaccine efficacy) were similar between PfSPZ-CVac groups. Among all vaccinated participants, those protected had significantly greater Vδ2 T cell expansion during immunizations. We surmise that vaccine-induced Vδ2 T cell responses may correspond to enhanced CD8 T cell responses in the liver that mediate protection against PfSPZ Challenge. More studies are needed to evaluate the immune responses including Vδ2 T cells that contribute to or correlate with protection.

Despite non-human data indicating that the protective immunity is mediated by T cells, several PfSPZ Vaccine trials associated PfCSP antibody levels and protection in malaria-naive[8] and malaria-experienced[9] individuals. Similarly, we find here a significant relationship between PfCSP levels and regimens that protect from CHMI. Notably, PfCSP antibody levels induced by the high-efficacy CVac regimens tested here (median net OD 1.0 levels of 8,060 and 4,215) were substantially lower than PfSPZ Vaccine regimens that conferred minimal heterologous protection[5,36]. We continue to think that the antibody responses are a correlate, but not a primary mediator of vaccine efficacy, although the antibodies probably have a role in the protection in some individuals.

PfSPZ-CVac(PYR) is attractive for future use in Africa for several reasons. Killing of the parasites during the clinically silent liver stages by PYR rather than during blood-stage development as occurs with CQ treatment offers a wider safety margin. In Africa and other areas of high malaria transmission, pregnant women already take PYR monthly (in combination with sulfadoxine) after the first trimester for malaria control. Millions of children in the Sahel receive PYR in a monthly drug combination (sulfadoxine–PYR + amodiaquine) during the malaria season[39]. The widespread use and excellent safety profile of PYR will facilitate the deployment of PfSPZ-CVac(PYR), in particular among pregnant women and children who bear the greatest burden of disease. Notably, antibody and T cell responses to PfSPZ Vaccine in children in Africa are comparable to those of non-immune individuals in the USA and Europe, and superior to those of adults in Africa[40]. A programme to assess PfSPZ-CVac(PYR) field efficacy has already begun by first assessing the vaccine efficacy against *P. falciparum* in adults in Mali (https://clinicaltrials.gov/, ID NCT03952650) before moving to children, and is exploring same-day administration of PYR and PfSPZ to improve feasibility and safety. PfSPZ-CVac could also be an excellent preventive vaccine for deployed military personnel and non-immune travellers: more than 90% of travellers to Africa stay fewer 10 weeks, and we have demonstrated up to 100% vaccine efficacy for 3 months against a 100% infectious dose in heterologous CHMI. Furthermore, it has been reported that all three doses can be administered within four weeks[41].

Alternative whole-PfSPZ vaccine approaches are currently being developed, including genetically attenuated parasites that arrest at a specific lifecycle time point. This is hypothetically attractive as it obviates the need for antimalarial drugs, and late liver-stage-arresting parasites enhance sterilizing immunity compared with early-arresting parasites in mice[11]. However, breakthrough infections occurred with some genetically attenuated parasites in the clinic[42]. A recent genetically attenuated *P. falciparum* strain has been developed to arrest late in liver-stage development[43] and therefore display a wider repertoire of antigens although many of these antigens are shared with blood-stage parasites and are highly variant. Future efficacy trials in malaria-experienced populations should examine the relative benefits of parasites that arrest earlier versus later in development, and PfSPZ-CVac(CQ) versus PfSPZ-CVac(PYR) models offer one such opportunity.

We have established that we can achieve a high level of protection for at least 3 months against heterologous *P. falciparum* parasites by immunizing with chemo-attenuated PfSPZ. Vaccines are the most efficient way to control any infectious disease. These data indicate that immunization can provide the level of protection against malaria needed for the control and elimination of malaria caused by *P. falciparum*.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-021-03684-z.

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Methods

The sample size was predetermined to identify safety concerns associated with investigational product administration, specifically severe adverse events. The main phase groups in both clinical studies were randomized: the CVac arms were randomized to either PfSPZ-CVac(PYR) or PfSPZ-CVac(CQ); the infectivity control participants were randomized to CHMI with either NF54 or 7G8 in the high-dose study (in the low-dose study, only CHMI NF54 was used). The pilot groups in both studies were not randomized. Laboratory investigators but not clinical investigators or participants were blinded to allocation during experiments and outcome assessment.

Clinical trial design

The clinical trial design for the initial low-dose study (https://clinicaltrials.gov/, ID NCT02511054) can be found in the Supplementary Information. The clinical trial design for the second, high-dose study is described below.

This was an open-label, randomized clinical trial conducted at the National Institutes of Health Clinical Center (NIH CC) in Bethesda, Maryland, USA (https://clinicaltrials.gov/, ID NCT03083847) from June 2017 to March 2019. The study adhered to Good Clinical Practice guidelines and US NIH guidelines and procedures. All participants provided written informed consent. The study was reviewed and approved by the US National Institute of Allergy and Infectious Diseases, NIH institutional review board and conducted under an FDA IND application.

The study objectives were to investigate the safety, tolerability, immunogenicity and vaccine efficacy of direct venous inoculation (DVI) with three doses at four-week intervals of aseptic, purified, cryopreserved PfSPZ (Sanaria PfSPZ Challenge (NF54))[44,45], combined with either PYR or CQ prophylaxis, known as PfSPZ chemoprophylaxis vaccination (PfSPZ-CVac). In addition, we sought to determine whether pre-erythrocytic stage immunity (induced by the PYR arm) was sufficient to protect against homologous (NF54) and heterologous (7G8) CHMI, or whether erythrocytic stage immunity (induced by the CQ arm) might be required for protection.

A total of 56 healthy malaria-naive adults were enrolled in this study (Extended Data Fig. 1). The study did not enrol women who were pregnant or nursing. Medical history, physical exams and safety laboratory (haematological, creatinine, alanine transferase and urinalysis) evaluations were performed at a screening visit. All volunteers had negative screening tests for human immunodeficiency virus (HIV), and hepatitis B and C. The study began with the dose-escalating pilot phase of the study to assess the safety and determine the optimal dosing of PfSPZ-CVac for the proposed regimens. In the PYR pilot study ($n = 8$), participants received one exposure of PfSPZ Challenge (NF54), in a dose-escalating manner of 5.12×10^4 ($n = 2$), 1×10^5 ($n = 2$) or 2×10^5 ($n = 4$) PfSPZ, along with PYR treatment 2 and 3 days after injection of the PfSPZ Challenge. All PYR pilot arms were safe and prevented detectable subpatent parasitaemia by sensitive qPCR; therefore, the highest dose (2×10^5 PfSPZ) was used during the main study. In the CQ pilot study ($n = 6$), participants received one exposure of the PfSPZ Challenge (NF54) either at 1×10^5 ($n = 2$) or 2×10^5 PfSPZ ($n = 4$) and CQ with a loading dose (1,000 mg) 2 days before the first DVI followed by a single maintenance dose (500 mg) administered 5 days after DVI. The CQ pilot group demonstrated that this regimen was safe, but tolerability in the higher dose pilot was less than expected, with more malaria-related symptoms experienced during days 7 and 8 after DVI. However, symptoms improved quickly with the co-administration of nonsteroidal anti-inflammatory drugs on the days of peak parasitaemia. Therefore, in consultation with an independent safety-monitoring committee, it was determined that the goal dose (2×10^5 SPZ) could be used during the main study with the addition of nonsteroidal anti-inflammatory drugs preemptively during days 7 and 8 after DVI for PfSPZ-CVac(CQ). Participants in the pilot group did not join the main study nor undergo CHMI.

In the main phase, the protocol was initially designed to enrol participants in either PfSPZ-CVac(PYR) with homologous CHMI with NF54 ($n = 17$) or PfSPZ-CVac(PYR) with heterologous CHMI with 7G8 ($n = 10$) or PfSPZ-CVac(CQ) with heterologous CHMI with 7G8 ($n = 10$). Owing to logistical reasons, the main study was conducted in two consecutive cohorts between January 2018 and March 2019, with a portion of each of the three PfSPZ-CVac groups enrolled in the first cohort while only the heterologous CHMI groups were enrolled in the second cohort. With the considerable evidence of the development of protective efficacy against both homologous and heterologous PfSPZ Challenge doses, the protocol was amended such that enrolment into a third cohort did not proceed, and the PYR arm undergoing homologous CHMI finished with 10 enrolled participants instead of the expected 17.

By the end of study, 17 of the 20 enrolled individuals who received PfSPZ-CVac(PYR) completed all three vaccinations (2×10^5 PfSPZ + 50 mg PYR on days 2 and 3 after DVI) (Fig. 1). Two participants withdrew before any receipt of the PfSPZ Challenge or PYR but had undergone enrolment (1 non-compliance, 1 secondary to acute illness) and 1 participant had to withdraw secondary to a scheduling conflict.

Ten participants were initially enrolled into the PfSPZ-CVac(CQ) arm, of which 7 completed all three vaccinations (2×10^5 PfSPZ + CQ loading dose of 1,000 mg 2 days beforehand + a weekly CQ maintenance dose of 500 mg for a total of 9 CQ doses). One individual who received PfSPZ-CVac(CQ) withdrew because of a serious adverse events (possibly related to CQ, mental status changes), one due to pregnancy, and one secondary to a scheduling conflict. One additional individual withdrew after completion of CVac, but before the CHMI phase, due to a serious adverse event unrelated to study participation (pneumothorax).

The CHMI phase was conducted with 3.2×10^3 PfSPZ of either PfSPZ Challenge NF54 or 7G8[46] administered by DVI approximately 13 weeks after the receipt of the third PfSPZ-CVac dose. Of those who received PYR, $n = 8$ underwent homologous NF54 CHMI, and $n = 9$ underwent heterologous 7G8 CHMI; from the CQ arm, $n = 6$ underwent heterologous 7G8 CHMI (one participant had withdrawn secondary to pregnancy before PfSPZ CHMI).

Infectivity controls were enrolled to undergo PfSPZ Challenge with 3.2×10^3 SPZ of either NF54 ($n = 4$) or 7G8 ($n = 8$). Participants were followed daily starting 6 days after CHMI for evaluation of the development of malaria parasitaemia defined as one positive NIH CC clinical diagnostic malaria qPCR (NIH CC qPCR) assay or one positive thick blood smear (TBS). Follow-up was completed either at the time of malaria diagnosis or at the protocol-defined end of study (27 days after CHMI).

All participants were treated with a 3-day standard treatment course of Malarone (also known as atovaquone/proguanil) either at the time of malaria diagnosis or at the end of study if they were not diagnosed with malaria during the follow-up period.

Research parasitaemia determination by qPCR

For the low-dose study, a modified DNA-based qPCR was used to detect subpatent parasitaemia starting days 6 to 10 (or day 14 for dose 1) after each DVI with PfSPZ Challenge (NF54) as previously described[47]. This method has been validated in our laboratory with the lowest limit of detection of approximately 200 parasites per ml.

For the high-dose study, starting days 6 to 10 (or day 14 for dose 1) after each DVI with PfSPZ Challenge (NF54), a modified RNA-based qPCR was used to detect subpatent parasitaemia as previously described[48]. This assay has been validated in our laboratory with the lowest limit of detection of approximately 15 parasites per ml based on a plasmid standard. Standard quantities were defined using reference samples with known microscopic values. Samples with resulting values of less than 15 parasites per ml are reported as negative (below the limit of detection/quantification) using this assay. More details of the primers and cycling conditions for both assays are reported in the Supplementary Information.

Malaria diagnostic qPCR (NIH CC qPCR)

The established NIH CC malaria diagnostic test in the CLIA-certified clinical laboratory, Malaria Genus Species (4-plex) PCR, was used for real-time safety monitoring during the vaccination period as well as for malaria diagnosis during CHMI[8,49,50]. NIH Malaria Genus Species (4-plex) PCR is a DNA-based test with a sensitivity of 500 parasites per ml of whole blood. NIH Malaria Genus Species 4-plex qRT–PCR was used for malaria diagnosis after administration of the Sanaria PfSPZ Challenge during vaccination (from 6 to 10 days (or 14 days after dose 1) after DVI) and CHMI phases (from 6 to 27 days after DVI or until malaria diagnosis) and for any visits during which a partipcipant presented as symptomatic. The turnaround was approximately 6 h. During the low-dose study, two consecutive positive NIH Malaria Genus Species (4-plex) PCR results or a single patent parasitaemia by TBS after PfSPZ Challenge were used for diagnosis and initiation of treatment with Malarone. For enhanced safety in the high-dose study, only one positive NIH Malaria Genus Species (4-plex) PCR result or a single patent parasitaemia by TBS after PfSPZ Challenge was used for diagnosis and initiation of treatment with Malarone.

TBS analyses

TBSs were prepared in duplicate and stained with Giemsa according to standard malaria CHMI procedures and evaluated by trained study microscopists from 6 to 10 days (or 14 days after dose 1) after each DVI during the vaccination phase. During the CHMI phase, TBSs were only performed when malaria diagnostic qPCR could not be reported on the same day or if a participant was symptomatic and met evaluation criteria per the study standard operating procedures. The results were reported to the study principal investigator within 24 h and were prioritized to be read immediately if a participant was symptomatic. At least 0.5 µl was scanned for the presence of malaria parasites, giving a theoretical limit of detection of 2 parasites per µl of blood. For symptomatic participants, at least 1.5 µl was evaluated before calling the TBS negative. Slides were considered positive if at least two unambiguous parasites per slide were identified and confirmed by a second microscopist.

Assessing in vitro PYR activity against liver-stage parasites

To assess in vitro PYR activity against liver-stage parasites, micropatterned co-cultures were generated as described previously[26]. In brief, glass-bottom 96-well plates were coated with rat-tail type-I collagen (50 mg ml^{-1}) and subjected to soft lithographic techniques to pattern the collagen into microdomains (islands of 500 mm) that mediate selective hepatocyte adhesion. To create micropatterned co-cultures, cryopreserved primary human hepatocytes (Bioreclamation IVT, BGW lot, male, age 50) were pelleted by centrifugation and then seeded on collagen-micropatterned plates. 3T3-J2 mouse embryonic fibroblasts were seeded 1 day later.

The next day, 2×10^5 aseptic, purified cryopreserved PfSPZs were overlaid onto micropatterned co-cultures (that had been seeded the day before) in hepatocyte medium and kept at 37 °C and 5% CO_2 for 3 h for infection to occur. After infection, wells were washed twice and fresh medium containing fibroblasts was added. Cultures were fixed on day 4 with ice-cold methanol for analysis by immunofluorescence.

On days 2 and 3, fresh medium containing 0 µM, 1 µM, 10 µM or 100 µM PYR was added to the cultures. Cultures were fixed on day 4 as described above.

The numbers of parasites per well were assessed by staining with a monoclonal antibody against the 70 kDa heat shock protein (HSP70) (clone 4C9, 5 µg ml^{-1}, gift from F. Zavala, Johns Hopkins Universtiy) using a Nikon Eclipse Ti fluorescence microscope. Results are reported as the number of parasites per 10^4 hepatocytes.

Ex vivo flow cytometry

Freshly isolated whole blood was collected from each study participant and was used to enumerate the percentage and subsets of γδ T cells, Vδ2 T cells, CD4 T cells, CD8 T cells and NK cells (the gating strategy is shown in Extended Data Fig. 10). Activated CD4 and CD8 T cells were defined by co-expression of HLA-DR. Samples were collected at enrolment and at days 2, 3, 7, 14 and 28 after each vaccination. In addition, whole blood was also collected immediately before CHMI and days 3, 7 and 14 after CHMI. In brief, 200 µl of whole blood from each participant was used to stain with a cocktail of conjugated monoclonal antibodies. The samples were washed, red blood cells lysed and washed again before acquisition on a BD LSRII flow cytometer equipped with a blue, red and violet laser, using BD FACSDiva version 8 software. All events in the tubes were acquired and analysed using FlowJo v.10.4.1 software.

Antibody assays

IgG antibodies against PfCSP were assessed by ELISA, IgG antibodies against PfSPZs were assessed using an automated immunofluorescence assay and the capacity of sera to inhibit the invasion of hepatocytes (HC-04 cell line) by PfSPZs was assessed using an automated inhibition of sporozoite invasion assay, all of which have been described previously[18]. In addition, IgM antibodies against PfCSP were assessed by ELISA. In brief, 96-well plates (Nunc Maxisorp Immuno Plate) were coated overnight at 4 °C with 2.0 µg recombinant PfCSP in 50 µl coating buffer (KPL) per well. Plates were washed three times with 2 mM imidazole, 160 mM NaCl, 0.02% Tween-20, 0.5 mM EDTA and blocked with 1% bovine serum albumin blocking buffer (KPL) containing 1% non-fat dry milk for 1 h at 37 °C. Plates were washed three times and serially diluted serum samples (in triplicate) were added and incubated at 37 °C for 1 h. After three washes, a peroxidase-labelled goat anti-human IgM antibody (KPL) was added at a dilution of 2.0 µg ml^{-1} and incubated at 37 °C for 1 h. Plates were washed three times, ABTS 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 v.5 and fit to a four-parameter logistic curve to calculate the serum dilution at OD 1.0. A negative control (pooled serum from non-immune individuals from malaria-free area) was included in all assays. Serum from an individual with anti-PfCSP antibodies was used as a positive control. The antibody assays were conducted on sera obtained before immunization, 2 weeks after the third immunization and before CHMI. Definitions for a positive response were taken relative to the pre-dose 1 measurement. For ELISA, samples were considered positive if the difference between the post-immunization OD 1.0 and the pre-immunization OD 1.0 (net OD 1.0) was ≥50 and the ratio of the post-immunization OD 1.0 to pre-immunization OD 1.0 (ratio) was ≥3.0. For automated immunofluorescence assays, participants with a net reciprocal serum dilution for 2×10^5 arbitrary fluorescence units (AFU) of ≥150 and a ratio reciprocal serum dilution of ≥3.0 were considered positive. For the automated inhibition of sporozoite invasion assay, participants with a net reciprocal serum dilution for 80% inhibition of ≥10 in the inhibition of sporozoite invasion assay and a ratio reciprocal serum dilution for 80% inhibition of ≥3.0 in the inhibition of sporozoite invasion assay were considered positive. A non-parametric Wilcoxon–Mann–Whitney test was used to determine statistical significance for fold change values of antibody levels.

Statistical analysis

For the primary objective of assessing safety, the proportion of participants with at least one adverse event was compared by immunization schedule and dose group. Fisher's exact tests were performed to assess whether groups differed with respect to these proportions. A Wilcoxon signed-rank test was performed to analyse differences in adverse events between initial immunizations and subsequent immunizations. To assess vaccine efficacy, the unvaccinated group was compared with the vaccine group for time to first detectable parasitaemia after the final challenge using a log-rank test, and the presence or absence of parasitaemia by a two-tailed Barnard's test. To assess subpatent parasitaemia

during the vaccination period, the two vaccine groups were compared using an unconditional exact test by the ORRT procedure[51]. Vaccine efficacy analyses were performed in R. The non-parametric Wilcoxon–Mann–Whitney test was used to determine statistical significance for group differences in levels or fold changes in Vδ2 T cells and in antibody levels. Subsequent Vδ2 data were expressed as fold change from baseline, with (adjusted) or without (unadjusted) addition of an offset of +1 to numerator and denominator values of the percentage of Vδ2 T cells. Generalized estimating equation models—using the log-transformed fold change in the adjusted percentage of Vδ2 T cells as the outcome variable—were employed to analyse the dynamics of Vδ2 T cells in the low-dose and high-dose trials in the PfSPZ-CVac(PYR) and PfSPZ-CVac(CQ) groups during the vaccination phase (study days −7 to 84) using Stata software v.16.1 (US).

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

The data supporting the findings of this study are available within the Article and its Supplementary Information and from the corresponding author upon reasonable request.

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Author contributions S.A.H., S.L.H. and P.E.D. conceived and designed the study. A.M.-O., S.A.H., J.L., D.M.C., S.K., C.W., A.K., O.M.-S., A.I., L.K.D., H.D. and J.E.J. executed the clinical trials, with safety oversight by L.W.C.P. and T.L.R. T.M., A.M., A.G., B.K.L.S., P.F.B. and E.R.J. prepared the investigational product and coordinated regulatory affairs. M.N. analysed the clinical trial results. J.D., J.N., J.R., G.L., J.C.C.H., N.K., S.C., I.I., C.A., S.C.M., S.M., S.N.B. and I.Z. designed and performed laboratory studies. A.M.-O., S.L.H. and P.E.D. prepared the initial manuscript draft; all authors reviewed and edited the manuscript. A.M.-O. and S.A.H. supervised the trials and S.L.H. and P.E.D. supervised the study teams.

Competing interests T.M., L.W.P.C., A.M., A.G., B.K.L.S., N.K.C., S.C., P.F.B., E.R.J., T.L.R. and S.L.H. are salaried, full-time employees of Sanaria, the developer and sponsor of Sanaria PfSPZ Vaccine. S.L.H. and B.K.L.S. also have financial interests in Sanaria. B.K.L.S. and S.L.H. are inventors on patents and patent applications that have been assigned to Sanaria. All other authors declare no competing interests.

Additional information

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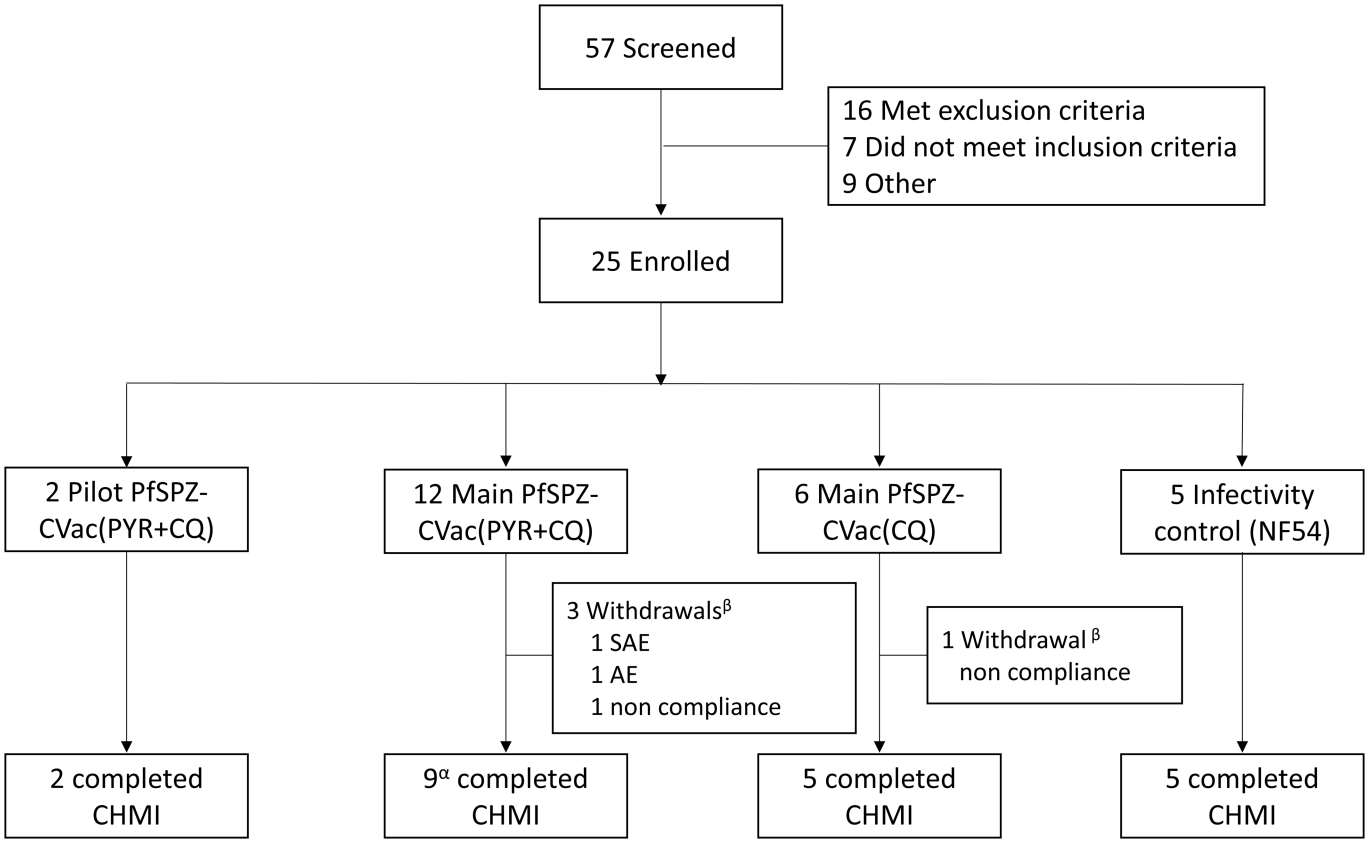
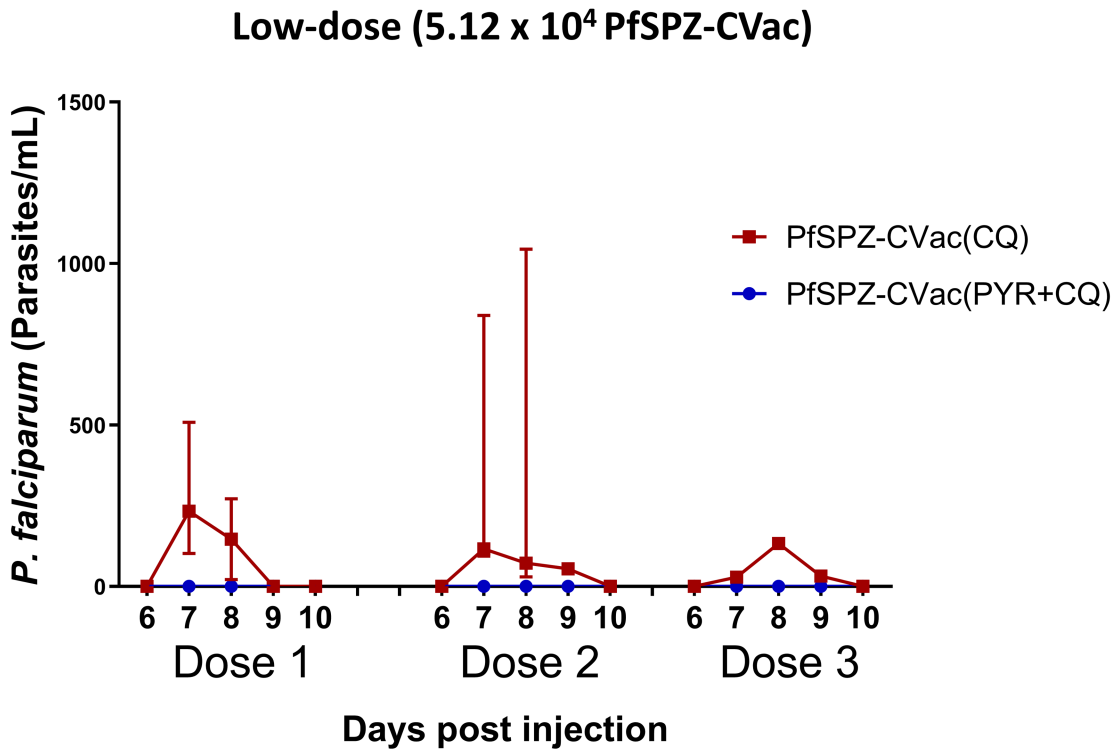
Correspondence and requests for materials should be addressed to P.E.D.

Peer review information *Nature* thanks Stefan Kappe and Laurent Renia for their contribution to the peer review of this work. Peer reviewer reports are available.

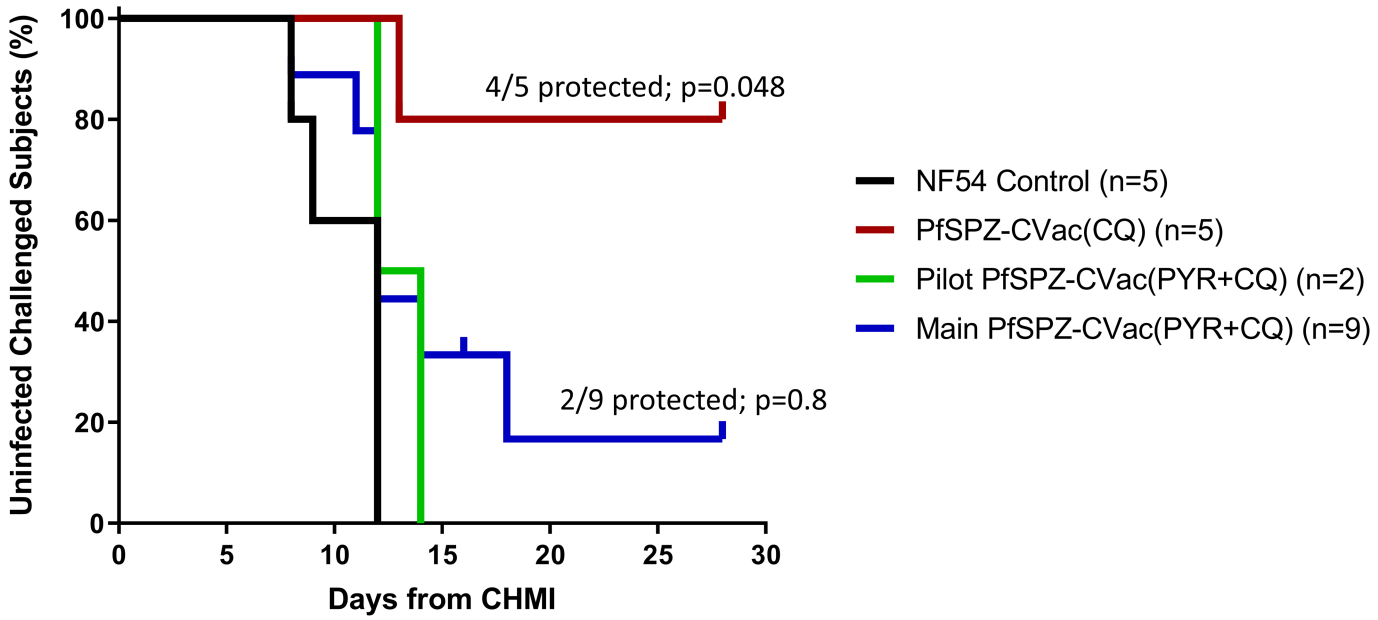


Extended Data Fig. 1 | Trial profile for PfSPZ-CVac low-dose study. [α]One participant was treated at day 16 after CHMI although that participant did complete the end-of-study visit. [β]The serious adverse event (SAE) and adverse event (AE) in the PYR/CQ group were encephalopathy and eye irritation, respectively. The non-compliance incidents include a participant's unwillingness to remain on pregnancy prevention (PYR/CQ group) and a work conflict preventing the participant from attending scheduled visits (CQ group).

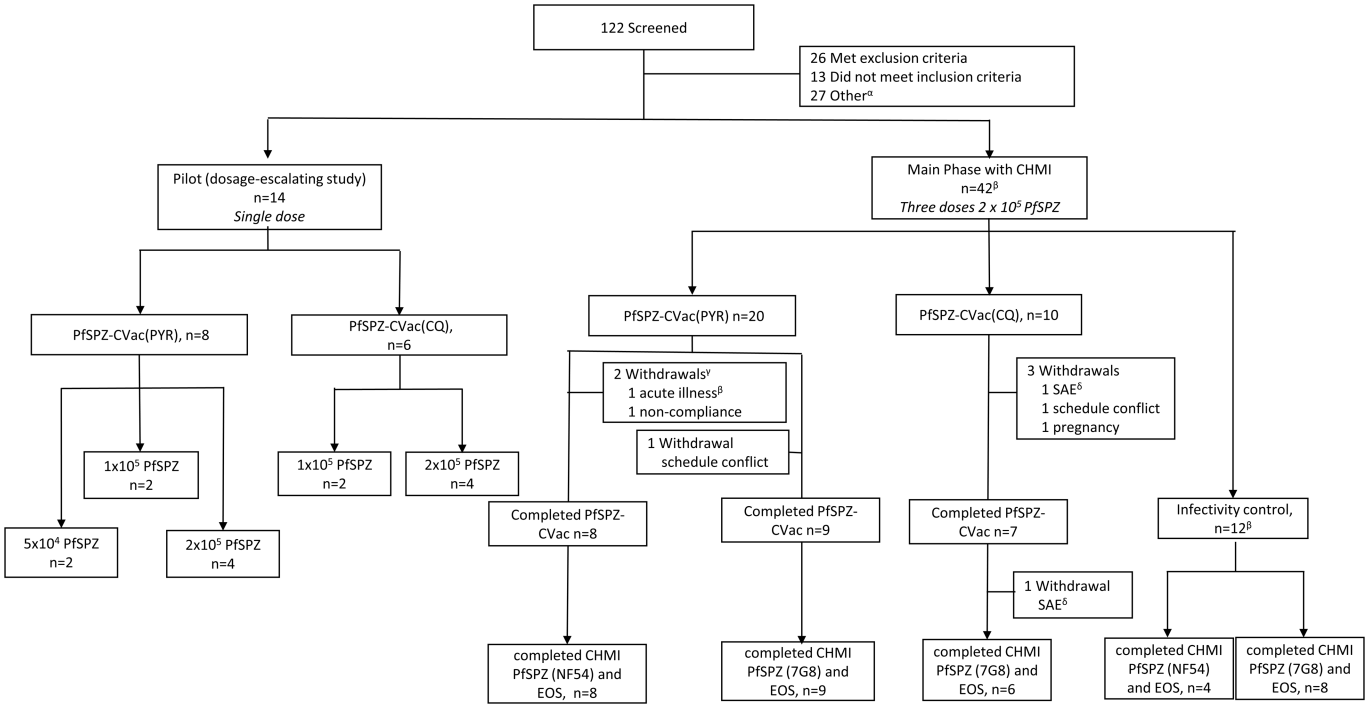


Group	Dose 1	Dose 2	Dose 3
PfSPZ-CVac(CQ)	5/5; 233; 7	3/5; 116; 7	1/5; 133; 8
PfSPZ-CVac(PYR+CQ)	0/12; ND; n/a	0/12; ND; n/a	0/11; ND; n/a

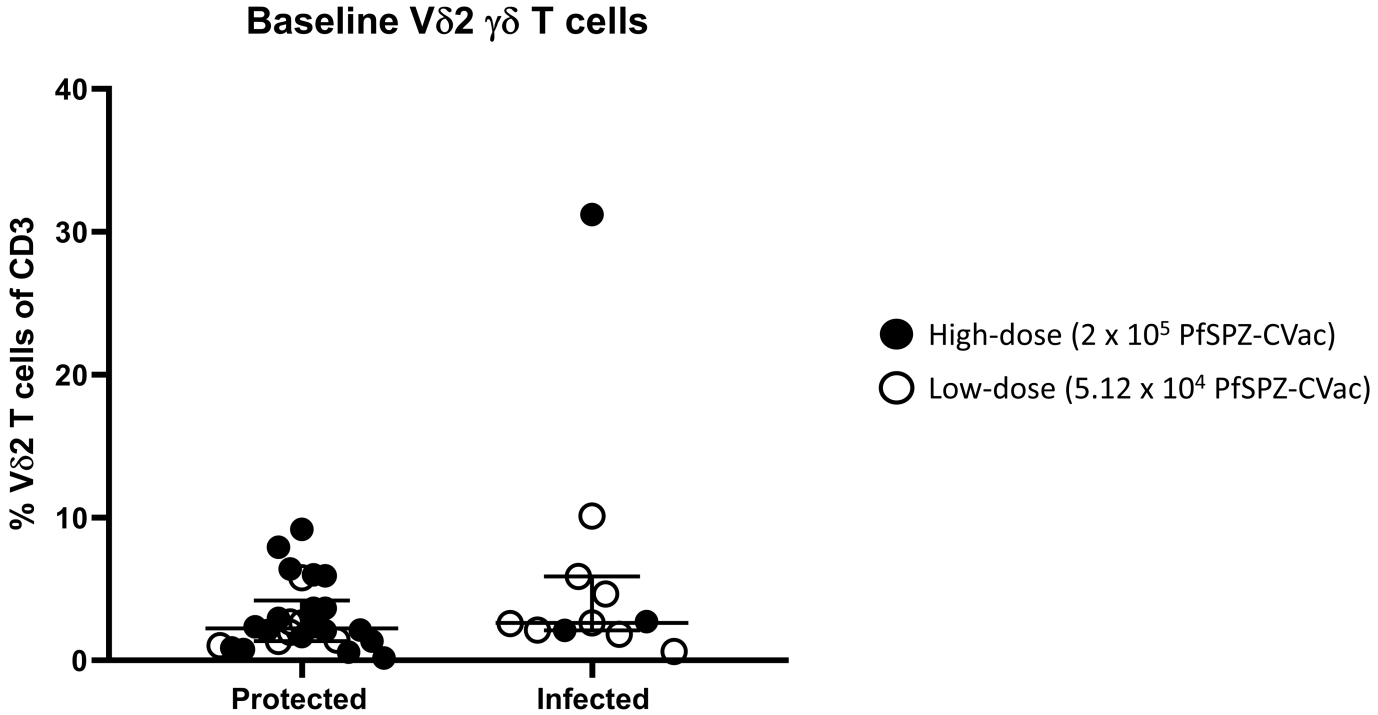
Extended Data Fig. 2 | Detection of parasitaemia in volunteers by qPCR after low-dose PfSPZ-CVac. Parasitaemia was measured in participants on days 6–10 after each PfSPZ-CVac dose. The median parasitaemia is displayed for positive participants and error bars indicate the IQR. Vax, PfSPZ Challenge + CQ or PYR/CQ administration and follow-up until day 14 after PfSPZ Challenge 1 and day 10 after PfSPZ Challenge doses 2 and 3. The table shows (from left to right in each cell): the number of participants who were positive by qPCR/the injected number of participants; the median peak parasite density of positive participants (parasites per ml); and the average day of peak parasite density after each dose of PfSPZ-CVac. ND, not detected; n/a, not applicable.



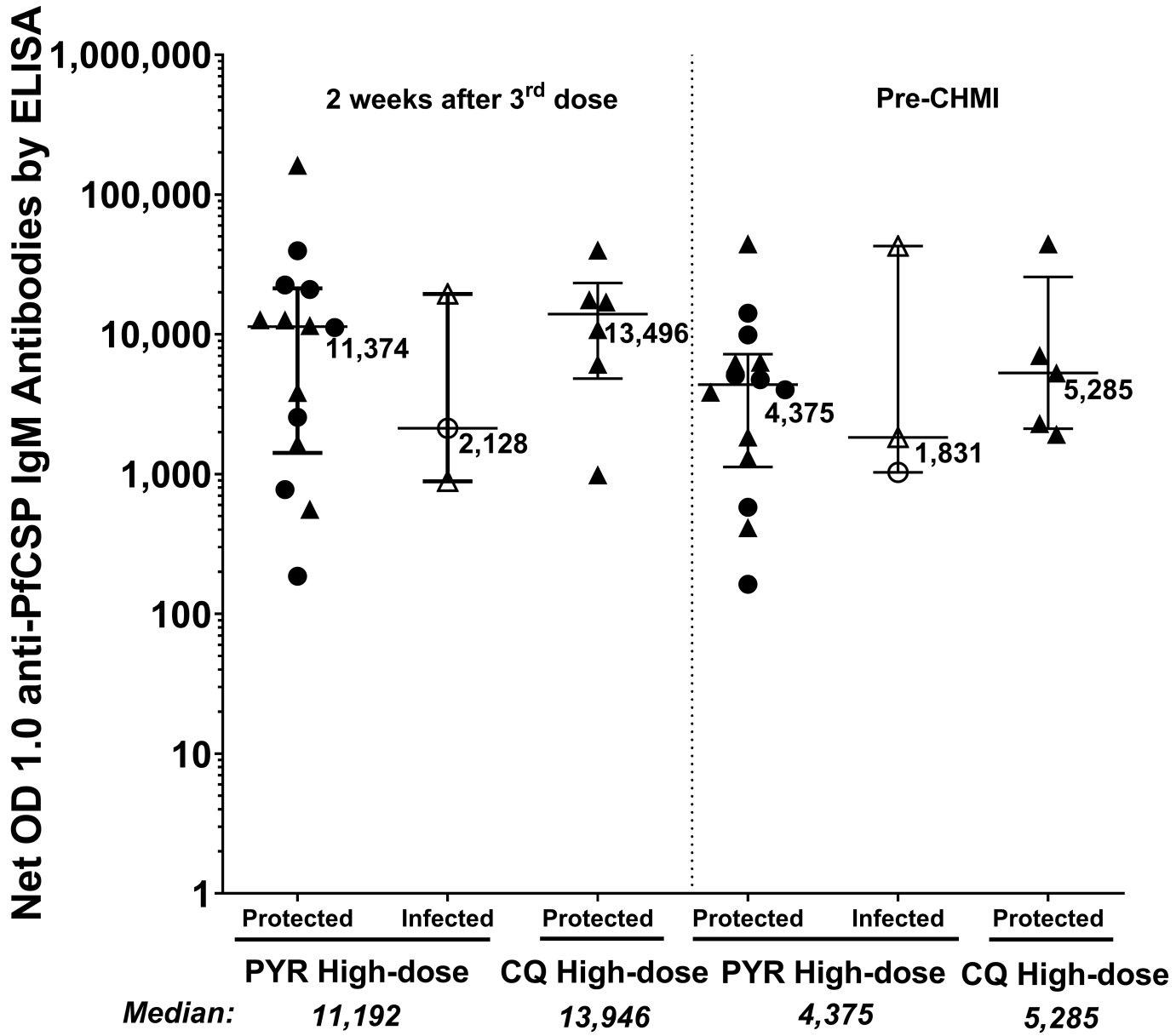
Extended Data Fig. 3 | Protective efficacy of PfSPZ-CVac(CQ) and PfSPZ-CVac(PYR + CQ) against homologous PfSPZ CHMI in the PfSPZ-CVac low-dose study. Participants were followed for 27 days after CHMI, and a survival curve is presented displaying the number of participants that remained protected throughout follow-up after homologous CHMI. To assess vaccine efficacy, the unvaccinated group was compared with the vaccine group for time to first detectable parasitaemia after the final challenge using a log-rank test, and the presence or absence of parasitaemia using a two-tailed Barnard's test. In the pilot PfSPZ-CVac(PYR + CQ) group (to test safety), 0/2 participants were protected; in the main PfSPZ-CVac(PYR+CQ) group, 2/9 (22.2%, $P = 0.8$) participants were protected; in the PfSPZ-CVac(CQ) group, 4/5 (80%, $P = 0.048$; 95% confidence interval, 1–99%) participants were protected.



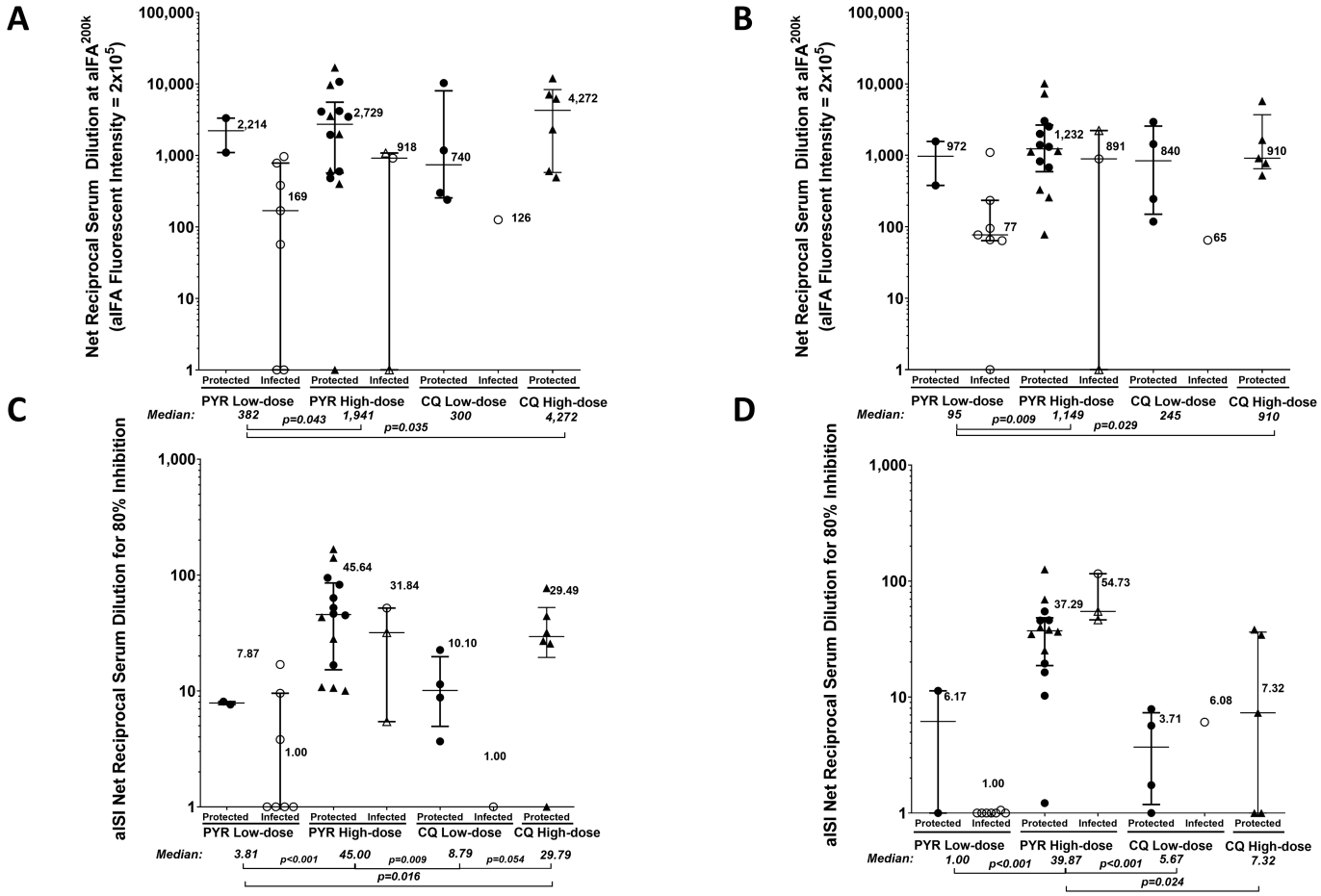
Extended Data Fig. 4 | Trial profile for the PfSPZ-CVac high-dose study. [α]Other, 15 withdrew consent, 6 deemed eligible after study was fully enrolled, 3 lost to follow-up, 2 schedule conflict, 1 withdrawn before randomization. [β]One individual enrolled in the PYR group was withdrawn before receiving the first vaccination owing to acute illness, and enrolled in the control group. This person is counted twice. [γ]Both participants were withdrawn before receiving vaccination 1. [δ]The two serious adverse events in the CQ group were mental status change and pneumothorax.



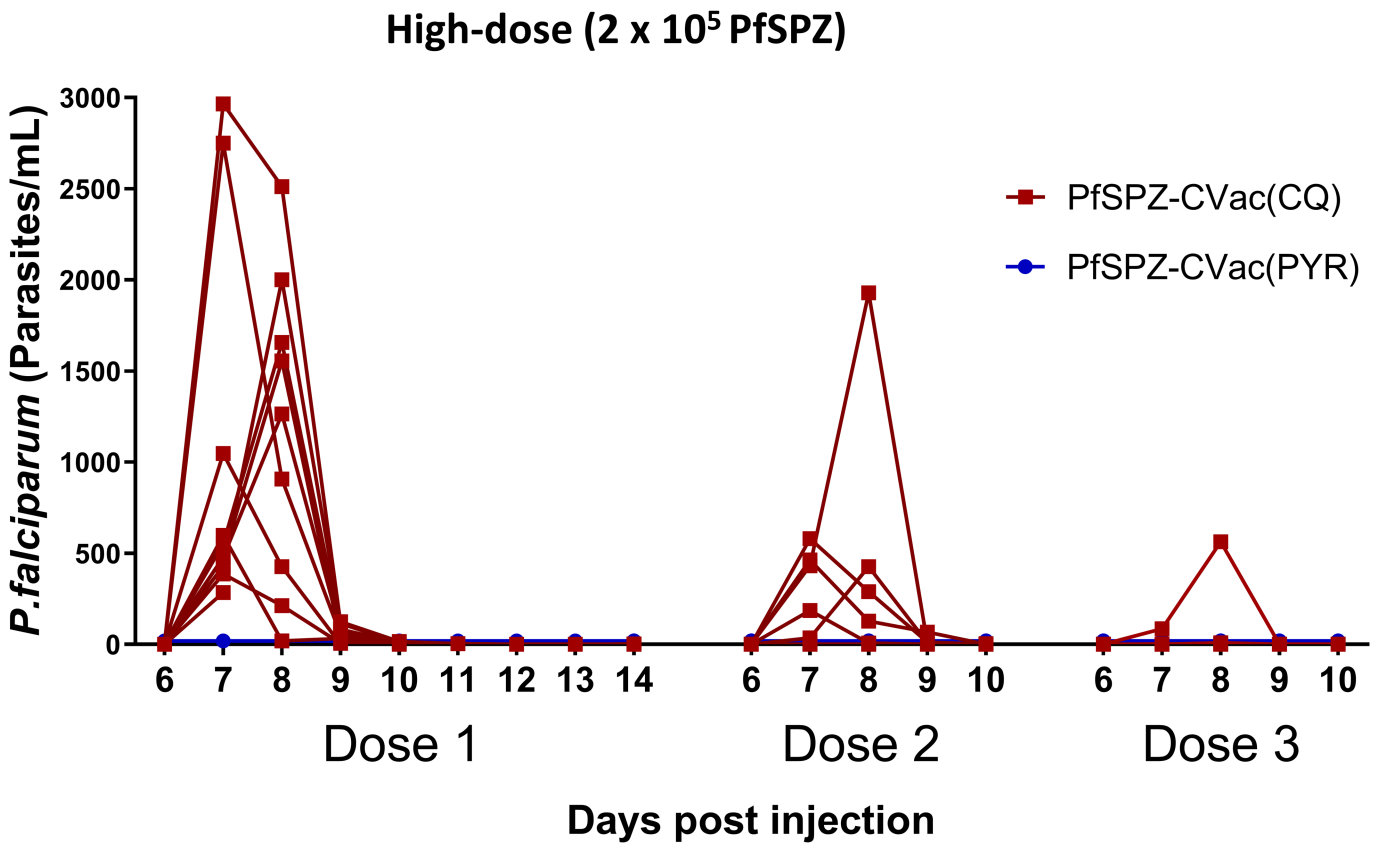
Extended Data Fig. 5 | Vδ2 γδ T cells at baseline in protected and infected individuals who received the vaccine. Filled circles indicate vaccinated participants in the high-dose study, and open circles indicate vaccinated participants in the low-dose study. Median values are displayed and error bars indicate the IQR. For protected individuals in the high-dose study, $n = 20$; for protected individuals in the low-dose study, $n = 6$. For infected individuals in the high-dose study, $n = 3$; for infected individuals in the low-dose study, $n = 8$.



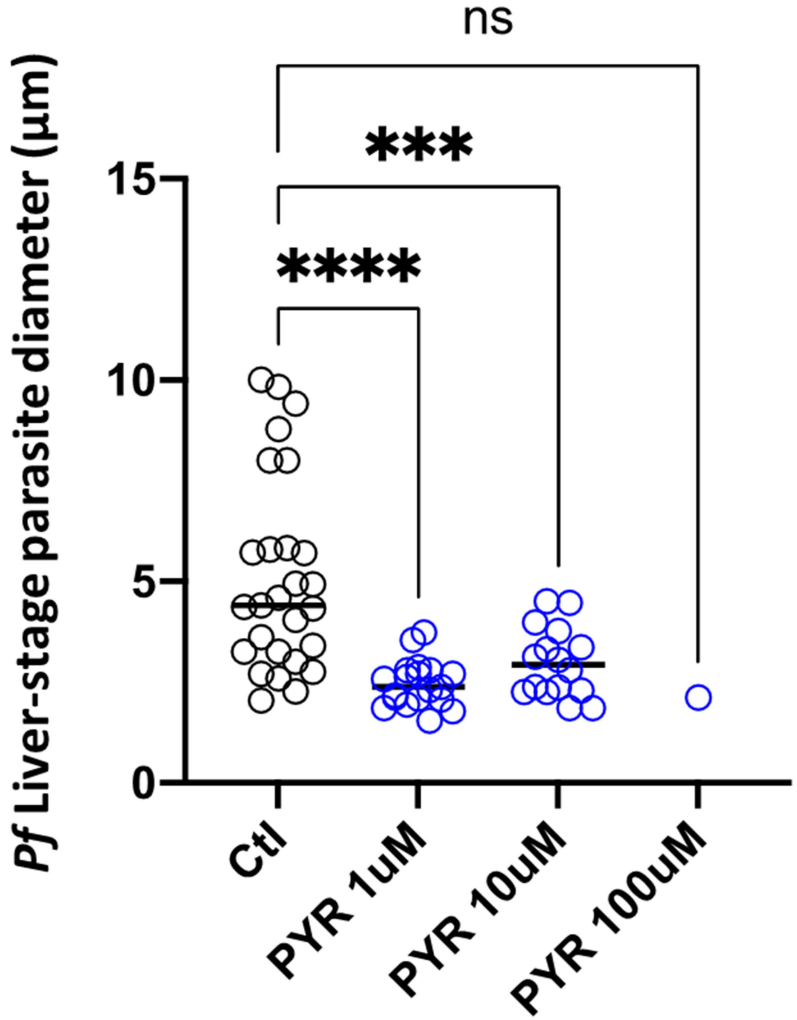
Extended Data Fig. 6 | Anti-PfCSP IgM antibodies in vaccinated participants in the PfSPZ-CVac high-dose study analysed using ELISA. IgM antibodies against PfCSP (net OD 1.0) 2 weeks after the third dose of PfSPZ-CVac(PYR) and PfSPZ-CVac(CQ); and immediately before CHMI. Median values are displayed and error bars indicate the IQR. At both time points, sample sizes for the PYR high-dose study, $n = 14$ for protected and $n = 3$ for infected vaccinated participants; for the CQ high-dose study, $n = 6$ for protected vaccinated individuals at 2 weeks after the third CVac and $n = 5$ for protected vaccinated individuals at before CHMI. No vaccinated participants were infected in the CQ high-dose study.



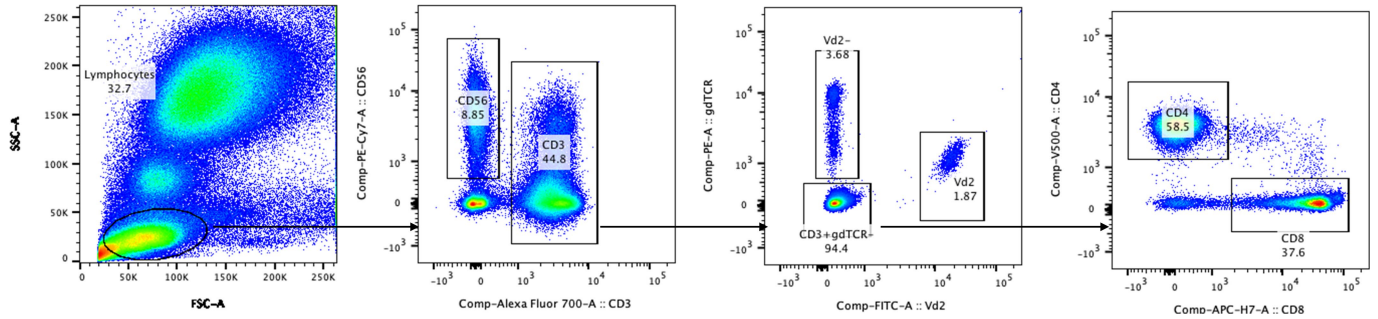
Extended Data Fig. 7 | Antibody responses in vaccinated participants of the PfSPZ-CVac low-dose and PfSPZ-CVac high-dose studies analysed using automated immunofluorescence and automated inhibition of sporozoite invasion assays. For each panel, filled circles are protected participants, open circles are infected participants who received homologous CHMI, filled triangles are uninfected (protected) participants and open triangles are infected participants who received heterologous CHMI. Median values are displayed and error bars indicate the IQR. *P* values were calculated using two-sided Wilcoxon–Mann–Whitney tests and group differences with $P > 0.07$ are not indicated. **a**, **b**, Antibodies against PfCSP by automated immunofluorescence assay were analysed 2 weeks after the third dose of PfSPZ-CVac(PYR) and PfSPZ-CVac(CQ) (**a**) and immediately before CHMI (**b**). **c**, **d**, Antibodies against PfCSP by automated inhibition of sporozoite invasion were analysed 2 weeks after the third dose of PfSPZ-CVac(PYR) and PfSPZ-CVac(CQ) (**c**) and immediately before CHMI (**d**). At both time points, sample sizes for the PYR low-dose study, $n = 2$ for protected and $n = 7$ for infected vaccinated participants; for the PYR high-dose study, $n = 14$ for protected and $n = 3$ for infected vaccinated participants; for the CQ low-dose study, $n = 4$ for protected and $n = 1$ for infected vaccinated participants; for the CQ high-dose study, $n = 6$ for protected participants in **a** and **c** (2 weeks after the third CVac), and $n = 5$ for protected participants in **b** and **d** (before CHMI). No vaccinated participants were infected in the CQ high-dose study. Anti-PfCSP antibodies at both 2 weeks after the last dose (high-dose 1,941 versus low-dose 382, $P = 0.043$) and immediately before CHMI (high-dose 1,149 versus low-dose 95, $P = 0.009$) were significantly higher in the high-dose PfSPZ-CVac(PYR) participants compared with the low-dose PfSPZ-CVac(PYR) participants. In PfPSZ-CVac(CQ) participants, the anti-PfSPZ antibodies were higher but not significant (2 weeks after the third dose, 300 versus 4,272, $P = 0.176$; before CHMI, 245 versus 910, $P = 0.310$). The inhibition of sporozoite invasion assay showed a statistically significant increase after vaccination for the high-dose PfSPZ-CVac(PYR) versus low-dose PfSPZ-CVac(PYR) when measured 2 weeks after the third dose (45.00 versus 3.81, $P < 0.001$) and immediately before CHMI (39.87 versus 1.00, $P < 0.001$). In PfPSZ-CVac(CQ) participants, the anti-PfCSP antibodies were significantly higher in the high-dose group when measured 2 weeks after the third dose (29.79 versus 8.79, $P = 0.054$) but not significantly different before CHMI (5.67 versus 7.32, $P = 0.671$).



Extended Data Fig. 8 | Detection of parasitaemia by qPCR in each individual after the first, second and third dose of PfSPZ-CVac in the high-dose study. Parasitaemia was measured in participants on days 6–10 after each PfSPZ-CVac dose. Vax, PfSPZ Challenge + CQ or PYR administration and follow-up until day 14 after PfSPZ Challenge 1 and day 10 after PfSPZ Challenge doses 2 and 3.



Extended Data Fig. 9 | In vitro PYR activity against liver-stage parasites. PYR was added to the cultures (blue) at three concentrations (1 µM, 10 µM and 100 µM), starting 2 days after infection and replaced with daily medium changes until day 4, at which point the cultures were fixed. The diameter of representative parasites of each group on day 4 are shown. Ctl, control. Statistical significance was determined by one-sided ANOVA. ***$P < 0.001$ (exact $P = 0.0006$); ****$P < 0.0001$; ns, not significant (exact $P = 0.2335$).



Extended Data Fig. 10 | Flow cytometry gating strategy used to enumerate T cell subsets using flow cytometry ex vivo staining of whole blood. The FSC/SSC parameters were used to define the lymphocyte gate as the first gate in the whole blood ex vivo assay. Within the lymphocyte gate, CD3-Alexa-700-positive events (T cell gate) were gated against CD56-PE-Cy7-positive events (NK cells). In the T cell gate, plotting Vδ2-FITC versus gdTCR-PE identified discrete $V\delta2^+$, $V\delta2^-$ and $CD3^+\gamma\delta TCR^-$ populations. The T cell gate was used to further delineate CD4- and CD8-positive events.

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒	A description of all covariates tested
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☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	n/a
Data analysis	n/a

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Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
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The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials, and are available from the corresponding author, PED, upon reasonable request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	A total of 81 healthy malaria-naive adults were enrolled in these studies.
Data exclusions	No data were excluded.
Replication	Not applicable. This was a clinical trial.
Randomization	The main phase groups in both studies were randomized: the CVac arms were randomized to either CVac-(PYR) or CVac-(CQ); the infectivity controls were randomized to CHMI with either NF54 or 7G8 in the high-dose study (in the low-dose study, only CHMI NF54 was used). The pilot groups in both studies were not randomized.
Blinding	Subjects and investigators were aware of Arm assignments in this Phase 1 safety and efficacy trial. Laboratory staff performing efficacy endpoint assays (detection of P. falciparum parasites in blood samples) or immunology endpoint assays were blinded to CVac group assignments; infectivity controls were enrolled separately, and in the high-dose study, laboratory staff were blinded to CHMI assignment (NF54 vs 7G8).

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☐	☒ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	CD3 (Clone UCHT1, BD Pharmingen); CD4 (Clone SK3, BD Pharmingen); CD8 (Clone SK1, BD Pharmingen); gdTCR (Clone B1.1, Thermofisher); Vd2 (Clone B6, BD Pharmingen); CD45RO (Clone UCHL1, BD Pharmingen); CD28 (Clone CD28.2, BD Pharmigen); CCR7 (G043H7, Biolegend); CD56 (Clone B159, Thermofisher); HLADR (Clone G46-6, BD Pharmingen).
Validation	Per the BD Pharmingen website and product notice: All antibodies were developed for use in flow cytometry. The production process underwent stringent testing and validation to assure that it generates a high-quality conjugate with consistent performance and specific binding activity. From the Biolegend website: BioLegend's antibody validation process involves testing and qualification at all stages of development, including hybridoma clone verification, applications testing, and quality control. For monoclonal antibodies (not IgM), antibody purity is always >95% and guaranteed to be low in aggregates. Additional testing includes verification of the isotype by ELISA. Once an antibody reaches the application testing stage, clone specifications need to be met to advance the product. The specifications for flow cytometry testing involves titration of the antibody and staining on positive and negative cell populations to confirm specificity. From Thermofisher website: To help ensure superior antibody results, we've expanded our specificity testing methodology using a 2-part approach for advanced verification. 1) Target specificity verification helps ensure the antibody will bind to the correct target. Our antibodies are being tested using at least 1 of 9 different methods to ensure proper functionality in researcher's experiments; 2) Functional application validation helps ensure the antibody works in a particular application(s) of interest, which may include (but not limited to): Western blotting, Flow cytometry, ChIP, Immunofluorscence imaging, Immunohistochemistry.

Human research participants

Policy information about studies involving human research participants

Population characteristics	The study was conducted at the NIH Clinical Center (outpatient facilities) in Bethesda, Maryland. NIH Clinical Center has successfully supported healthy volunteer subject clinical trials, including malaria vaccine and controlled human malaria infection trials. Healthy malaria-naïve subjects aged 18 to 50 years old were recruited from the surrounding community. Subjects were in good general health and without clinically significant medical history. All subjects passed a malaria comprehension exam (with a score of ≥80% or per investigator's discretion) reviewed prior to enrollment. Volunteers had reliable access to the clinical trial center and availability to participate for duration of study, and females of childbearing potential agreed to use reliable contraception (as defined in the approved protocol) from 21 days prior to study day -2 to 28 days following last Sanaria® PfSPZ Challenge exposure.
Recruitment	Healthy adult male and non-pregnant female subjects were recruited from a variety of sources including those previously screened or enrolled in other vaccine trials at the NIH clinical center or by the use of an Institutional Review Board (IRB)-approved screening protocol and study- specific print or media advertising. After an initial phone screen (using an IRB approved Phone Screen), a screening visit was scheduled. During the screening process, the subject read the consent form, was encouraged to ask questions, and then completed a written comprehension evaluation questionnaire (Malaria Comprehension Exam). The questionnaire was used to identify the areas of the study and consent that the subject may not fully understand. The person administering consent reviewed the answers with the subject. If the subject answered a question wrong, the person administering consent reviewed the portion of the consent form that related to that particular question with the subject. The subject could either sign the consent form during the screening visit, or return after further consideration.
Ethics oversight	The study was reviewed and approved by the US National Institute of Allergy and Infectious Diseases, NIH institutional review board and conducted under FDA IND 16650.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration	NCT02511054; NCT03083847
Study protocol	The full study protocol is available upon request.
Data collection	Access to stored research samples was limited using either a locked room or a locked freezer. Samples were stored at the LMIV in Bethesda, MD or at LMIV's designated repository, Thermo Scientific, Rockville, MD. Samples and data were stored using codes assigned by the investigators or their designees. Data were kept in password-protected computers. Only investigators or their designees had access to the samples and data.
Outcomes	Primary Outcomes: Safety • Incidence and severity of local and systemic adverse events (AEs) and serious adverse events (SAEs) occurring after PfSPZ-CVac. • P. falciparum blood stage infection defined as detection of P. falciparum parasites by qPCR (real time NIH qPCR and sensitive retrospective LMIV qPCR) following Sanaria® PfSPZ Challenge. • Incidence of clinical malaria diagnosis occurring after PfSPZ-CVac – chloroquine requiring treatment with additional antimalaria Secondary Outcomes: Protective Efficacy • P. falciparum blood stage infection defined as detection of at least 2 P. falciparum parasites by microscopic examination of 0.5 µL of blood or one positive real time NIH qPCR after homologous PfSPZ CHMI (NF54) via direct venous inoculation (DVI). • P. falciparum blood stage infection defined as detection of at least 2 P. falciparum parasites by microscopic examination of 0.5 µL of blood or one positive real time NIH qPCR after heterologous PfSPZ CHMI (7G8) via DVI.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Fresh whole blood collected in heparin tubes; used within 2 hours of collection
Instrument	BD LSR2 equipped with blue/red/violet laser
Software	FlowJo for analysis
Cell population abundance	n/a, sorting not performed
Gating strategy	The FSC/SSC parameters were used to define the lymphocyte gate as the first gate in the whole blood ex vivo assay. Within the lymphocyte gate, CD3-Alexa 700 positive events (T cell gate) were gated against CD56-PE-Cy7 positive events (NK cells). In the T cell gate, plotting Vd2-FITC versus gdTCR-PE identified discrete Vd2+ , Vd2- and CD3+gdTCR- populations. The latter gate was used to further delineate CD4 and CD8 positive events.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.