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

Andrew S Ishizuka1,13, Kirsten E Lyke2,13, Adam DeZure1, Andrea A Berry2, Thomas I Richie3, Floreliz H Mendoza1, Mary E Enama1, Ingelise J Gordon1, Lee-Jah Chang1,12, Uzma N Sarwar1,12, Kathryn L Zephir1, LaSonji A Holman1, Eric R James3, Peter F Billingsley3, Anusha Gunasekera3, Sumana Chakravarty3, Anita Manoj3, MingLin Li3,4, Adam J Ruben3, Tao Li3, Abraham G Eappen3, Richard E Stafford3,4, Natasha K C3,4, Tooba Murshedkar3, Hope DeCederfelt5, Sarah H Plummer1, Cynthia S Hendel1, Laura Novik1, Pamela J M Costner1, Jamie G Saunders1, Matthew B Laurens1, Christopher V Plowe2, Barbara Flynn1, William R Whalen1, J P Todd1, Jay Noor1, Srinivas Rao1,12, Kailan Sierra-Davidson1, Geoffrey M Lynn1, Judith E Epstein6, Margaret A Kemp7, Gary A Fahle7, Sebastian A Mikolajczak8, Matthew Fishbaugh8, Brandon K Sack8, Stefan H I Kappe8, Silas A Davidson9, Lindsey S Garver9, Niklas K Björkström10, Martha C Nason11, Barney S Graham1, Mario Roederer1, B Kim Lee Sim3,4, Stephen I Hoffman3,14, Julie E Ledgerwood1-14 & Robert A Seder1,14, for the VRC 312 and VRC 314 Study Teams

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-naive 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-naive 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 protection5,6.

Sustained sterilizing immunity against the pre-erythrocytic stages of Pf has been observed in humans immunized by whole-parasite approaches using mosquitoes for vaccination7,8. In a study

1Vaccine Research Center (VRC), National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda (NIH), Maryland, USA. 2Institute for Global Health, Center for Vaccine Development and Division of Malaria Research, University of Maryland School of Medicine, Baltimore, Maryland, USA. 3Sanaria Inc., Rockville, Maryland, USA. 4Protein Potential, LLC, Rockville, Maryland, USA. 5Pharmaceutical Development Section, Clinical Center, National Institutes of Health, Bethesda, Maryland, USA. 6Naval Medical Research Center (NMRC), Malaria Department, Silver Spring, Maryland, USA. 7Department of Laboratory Medicine, Clinical Center, National Institutes of Health, Bethesda, Maryland, USA. 8Center for Infectious Disease Research, Seattle, Washington, USA. 9Entomology Branch, Walter Reed Army Institute of Research, Silver Spring, Maryland, USA. 10Center for Infectious Medicine, Department of Medicine Huddinge, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden. 11Biostatistics Research Branch, Division of Clinical Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA. 12Present addresses: Sanofi, Swiftwater, Pennsylvania, USA (L.-J.C.), Albert Einstein College of Medicine, Montefiore Medical Center, New York, New York, USA (U.N.S.), Sanofi, Cambridge, Massachusetts, USA (S.R.). 13These authors contributed equally to this work. 14These authors jointly directed this work. Correspondence should be addressed to R.A.S. (rseder@mail.nih.gov).

Received 12 February; accepted 15 April; published online 9 May 2016; corrected online 18 May 2016 (details online); doi:10.1038/nm.4110
of malaria-naive adults, 5/6 subjects exposed to >1000 irradiated mosquitoes carrying attenuated PfSPZ were protected when CHMI occurred 23–42 weeks after immunization. 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 Vaccine (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_{H}1 cytokine–producing CD4 T cells and γδ 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 × subgroup VE). VE is calculated as ‘1 − 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 weeks^14 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
parasitemia after CHMI, as compared to parasitemia in 7/8 control subjects (Fig. 1b). In group 4 (4 doses of $2.7 \times 10^5$ 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 $1.35 \times 10^5$ PfSPZ and a fifth dose of $4.5 \times 10^5$ 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 $1.35 \times 10^5$ PfSPZ.11. Three subjects in group 1 (three doses of $2.7 \times 10^5$ 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 $2.7 \times 10^5$ 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 $2.7 \times 10^5$ 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 $2.7 \times 10^5$ 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 $2.2 \times 10^6$ 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...
Cellular responses. Cellular immunity is critical for protective efficacy by vaccination with live-attenuated SPZs in rodent and NHP models. Although interferon (IFN)-γ-producing CD8 T cells are necessary and sufficient for protection in the majority of mouse and NHP studies, NK, γδ, and CD4 T cells also influence protection. IFN-γ production was measured by 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).

Immune correlates. To identify potential immune correlates of protection, 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. PICSP- and PISPZ-specific antibody levels correlated with outcome for CHMIs that were done 3 weeks and 21–25 weeks after the final immunization. However, the absolute frequency of unstimulated Vδ2+ T cells as a percentage of total lymphocytes correlated with outcome at the 3-week or the 21- to 25-week CHMI (Supplementary Fig. 7m).
of the V82+ 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 protection14–16. 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 at 3 weeks to again be parasitemic at 21–25 weeks, the immune data from individuals who were parasitemic at 21–24 weeks (PfCSP GM = 1,860; PfSPZ GM = 1,640) were no different than those in subjects immunized with whole PfSPZ. Liver-stage burden was quantified by whole-animal imaging.

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. 5c) clinical trials received their final immunizations, ~20–30% of PfSPZ-specific CD4 T cells were Ki-67-positive, 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
Longitudinal cellular responses in humans. In PBMCs, cytokine-producing PISPZ-specific CD4 T cell responses declined significantly over the course of 59 weeks in vaccinated subjects who remained without parasitemia \((P = 0.047; \text{Fig. } 6a)\), and cytokine-producing PRBC-specific CD8 T cell responses were induced by vaccination; however, they returned to pre-vaccine levels shortly after the final vaccination \((\text{Fig. } 6b)\). 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 PISPZ \((\text{Supplementary Fig. } 10 \text{ and Supplementary Table } 6)\). Immunogenicity studies with PISPZ Vaccine in NHPs previously provided immune data that guided successful translation to human studies.\(^{10,11}\) We had previously shown that \(1.35 \times 10^5\) PISPZ administered by the s.c. route did not induce detectable T cell responses in the blood or liver of NHPs, so we administered tenfold more PISPZ 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.
of $1.35 \times 10^5$ PfSPZ and four i.v. doses of $2.7 \times 10^5$ 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, 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. 6c). 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, γδ T cells, and CD4 T cells—were identified in the livers of NHPs, the majority (~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 $2.7 \times 10^5$ 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 $2.7 \times 10^5$ PfSPZ was 24%, as compared to 73% with four doses of $2.7 \times 10^5$ PfSPZ. Similarly, four or five doses of $1.35 \times 10^5$ PfSPZ conferred an estimated 25% efficacy against CHMI at 21 weeks, as compared to 55% with four doses of $2.7 \times 10^5$ 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 $4.5 \times 10^5$ to $2.7 \times 10^6$ 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 Vβ2+ T cells correlated with outcome of CHMI. Vβ2+ 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,
IFN-γ production (Supplementary Fig. 7m), and/or by serving as antigen-presenting cells\(^2\) at the time of immunization, leading to enhanced CD8 T cell priming\(^3\). γδ T cells have been shown to provide help to dendritic cells to facilitate protective responses to blood-stage malaria\(^3\), and IFN-γ-secreting γδ T cells are a correlate of protection against blood-stage malaria\(^4\). It is possible V\(\delta\)2+ T cells also mediate direct effector functions in the liver. A high frequency of circulating γδ T cells express CXCR6, a chemokine receptor that facilitates surveillance of the liver sinusoids\(^5\), and produce IFN-γ and perforin (Supplementary Fig. 7i–m), two potent effector molecules shown to mediate killing of intracellular Pf. γδ T cells have been shown to contribute to protection against pre-erythrocytic malaria in mouse models\(^6\), and these T cells expand in humans following whole-SPZ immunization\(^7\). The correlation between γδ T cell frequency and CHMI outcome described herein suggests that PISPZ Vaccine induces protective immunity not only through induction of conventional CD8 and CD4 T cell responses but also through induction of γδ 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\(^5\) PISPZ 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 CHMI\(^8\). The clear dose response in this prior study\(^9\) 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 PISPZ that successfully invaded hepatocytes (Fig. 4c,d)\(^10\)–\(^12\), allowing for rapid elimination of the remaining parasites in the liver by cellular responses\(^11\), thereby resulting in low Ki-67 expression by PfRBC-specific CD8 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 PISPZ-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.

**Figure 6** Circulating and liver-resident Pf-specific T cells in humans and NHPs. (a,b) Longitudinal assessment of PISPZ-specific CD4 T cell responses (a) and PIRBC-specific CD8 T cell responses (b) in subjects who received four doses of 2.7 × 10\(^5\) PISPZ/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 ± s.e.m. P values are for comparisons between immune responses at 2 and 59 weeks after the final vaccination for the subject groups outlined in c. (c) Schematic of NHP study design. (d) PISPZ-specific CD4 (top) and PIRBC-specific CD8 (bottom) T cell responses in PBMCs of NHPs 2 weeks after the final vaccination for the subject groups outlined in c. (e) PISPZ-specific CD4 (top) and PIRBC-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 PIRBC, MAIT, mucosally associated invariant T cell.
(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 PSpZV-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 PSpZV to the liver, where they presumably mediate priming of T cells that are programmed to remain as non-recirculating, liver-resident cells36. For PSpZV 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 protection10,17–20,26,37,38.

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.

ACKNOWLEDGMENTS

The authors thank the vaccine trials participants for their contribution and commitment to vaccine research. We acknowledge the contributions of our US National Institutes of Health (NIH) Clinical Center (CC) and National Institute of Allergy and Infectious Diseases (NIAID) colleagues, especially J. Stein, J. Prierson, R. Eches, P. Driossal, L. Ediger, and the nursing staff of the SNW, SCNU, SSE, and SSE-N units; our VRC colleagues, especially A. Mittelman, H. Harvey, M. Young, C. Arts, R. Hicks, P. Darrah, and T. Abram; our Sanaria and Protein Potential colleagues, especially Y. Abebe, J. Jackson, A. Awe, M. King, H. Huang, A. Patil, S. Matheny, T. Overby, Y. Wen, K. Nelson, V. Pich, M. Orozco, D. Padilla, E. Saverino, B. Jiang, L. Gao, R. Xu, E. Fomumbod, M. Laskowski, T.T. Wai, F. Beams, R. Thompson, and A. Hoffman for administrative, operations, and legal support, and T. Luke for insight and inspiration; the EMMES Corporation; the NIAID Institutional Review Board; the NIAID Office of Communications and Government Relations; the NIH CC Department of Laboratory Medicine; the NIH CC Patient Recruitment and Public Liaison Office. We appreciate the expert reviews of the Safety Monitoring Committee (A. Durbin, K. Kester, and A. Cross) and the assistance from the US Military Malaria Vaccine Program, the Walter Reed Army Institute of Research Entomology Branch, and the Naval Medical Research Center (NMRC), especially A. Reyes, Y. Alicorta, G. Banania, C. Fedders, M. Dowler, T. Savransky, D. Patel, C. Brando, K. Kobylinski, and K. Walker. This work was supported by the intramural research program of the VRC, NIAID, NIH. Production and characterization of the PSpZV Vaccine were supported in part by NIAID Small Business Innovation Research grants SR44AI05229-11 (S.L.H.), SR44AI058499-08 (S.L.H.), and SR44AI058375-08 (S.L.H.). The humanized mouse experiments reported here were funded by the Bill and Melinda Gates Foundation (Investment ID: 24922; to S.H.I.K.).

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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, designed to assess the safety, immunogenicity, and protective efficacy of PfSPZ Vaccine that was administered by intravenous (i.v.) injection. The VRC 312 clinical trial protocol, funding, study oversight, and data reporting have been previously described [40]. 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 (Malaron) 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-naive, 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%) [40]. 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 \textit{P. falciparum} sporozoites, was manufactured as described [40] 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 \textit{Anopheles stephensi} mosquitoes, which met standard infectivity criteria [8,11]. 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 described [11]. Treatment with atovaquone and proguanil (Malaron) 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, 

\begin{equation}
\text{VE} = 1 - \frac{P \text{value}}{0.05}
\end{equation}

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 – 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 parasitic following CHMI and who were treated remained parasitic following a second CHMI. Subgroup VE was VE among the subgroup of subjects who were not parasitic at the prior CHMI. Cumulative VE is first VE multiplied by subgroup VE.

 PICSP ELISA. For ELISA measurement of IgG against PICSP (rPICSPV2) was used. The rPICSP (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 rPICSP are S-L-G-E-N-D. The central repeat region of this rPICSP 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 rPICSP/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′-azonio-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-PICSP 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 PICSP antibody levels 2 weeks after final vaccination.

PfSPZ aIFA. Purified PfSPZ (NF54 strain) from aseptic \textit{A. stephensi} mosquitoes, which were produced by Sanaria, was sonicated in PBS (pH 7.4). 0.5 × 10^7 PfSPZ in 40 μl were added to each well of Greiner CELLSTAR Clear-bottom black 96-well plate (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 PISPZ Vaccine in the VRC 312 clinical trial.11

The data was plotted to fit a four-parameter sigmoidal curve. Sera from malaria-naive 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 PISPZ, 2.0 × 10^5 AFU falls in the exponential portion of the sigmoidal curve. Therefore 2.0 × 10^5 was chosen as the threshold in the aIFA assay, and the level of anti-PISPZ 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 PISPZ 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 Benzonase (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) PISPZ Vaccine diluent (1% human serum albumin; CSL Behring); (ii) 1.5 × 10^5 viable, irradiated, aseptic, purified cryopreserved PISPZ from a single production lot; (iii) 2 × 10^5 lysed, infected RBCs consisting of >90% parasitemic late-stage schizonts (PiRBC) from a single production lot; or (iv) a single lot of donor-matched uninfected erythrocytes (uRBCs). PISPZ and PiRBC 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 × 10^6 PISPZ by i.v. injection and a negative malaria-naive control were included for each day the subjects were analyzed to determine the reproducibility of antigen stimulation.

After stimulation, cells were stained as previously described.14 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 cell-surface-stained for the chemokine receptor CCRC2 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 Vδ, TCR γδ, perforin, or Ki-67. 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 PISPZ 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 protocol.14

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 hepatic donor-matched FRR-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 Animals.46 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 described.37 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/midgit. 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.44–48 Briefly, mice were intraperitoneally injected with 100 µl of RediJect δ-Tifericin (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 PICSP 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 Animals.46 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). PISPZ Vaccine was formulated and administered as for human injection with the following exceptions. For i.v. groups, PISPZ in 500 µl was administered by direct venous inoculation into the saphenous vein. For the i.m. group, PISPZ was administered to the left and right deltoid, and each injection was 500 µl in volume. For the i.d. group, PISPZ 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 described.10 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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**Corrigendum: Retinal lipid and glucose metabolism dictates angiogenesis through the lipid sensor Ffar1**

Jean-Sébastien Joyal, Ye Sun, Marin I. Gantner, Zhuo Shao, Lucy P Evans, Nicholas Saba, Thomas Fredrick, Samuel Burnim, Jin Sung Kim, Gauri Patel, Aimee M Juan, Christian G Hurst, Colman J Hatton, Zhenghao Cui, Kerry A Pierce, Patrick Bherer, Edith Aguilar, Michael B Powner, Kristis Vevis, Michel Boisvert, Zhongjie Fu, Emile Levy, Marcus Fruttiger, Alan Packard, Flavio A Rezende, Bruno Maranda, Przemyslaw Sapieha, Jing Chen, Martin Friedlander, Cbry C Lish & Lois E H Smith

*Nat. Med.*; doi:10.1038/nm.4059; corrected 24 March 2016

In the version of this article initially published online, there were two errors. There was a typographical error in the text, which should have stated that the ‘dark current’ is an electrochemical gradient required for photon-induced polarization (rather than depolarization, as incorrectly stated). In addition, some funding sources were inadvertently omitted from the Acknowledgments. The errors have been corrected for the print, PDF and HTML versions of this article.

**Corrigendum: ROR-γ drives androgen receptor expression and represents a therapeutic target in castration-resistant prostate cancer**

Junjian Wang, June X Zou, Xiaqian Xue, Demin Cai, Yan Zhang, Zhijian Duan, Qiuping Xiang, Joy C Yang, Maggie C Louie, Alexander D Borowsky, Allen C Gao, Christopher P Evans, Kit S Lam, Jianzhen Xu, Hsing-Jien Kung, Ronald M Evans, Yong Xu & Hong-Wu Chen

*Nat. Med.*; doi:10.1038/nm.4070; corrected online 22 April 2016

In Figure 2a of the version of this article initially published online, one phenyl ring was inadvertently deleted from the chemical structure of compound SR2211. One affiliation of H.-W. C. (Veterans Affairs Northern California Health Care System–Mather, Mather, California, USA) was also inadvertently omitted. These errors have been corrected for the print, PDF and HTML versions of this article.

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

Andrew S Ishizuka, Kirsten E Lyke, Adam DeZure, Andrea A Berry, Thomas L. Richie, Floreliz H Mendoza, Mary E Enama, Ingelise J Gordon, Lee-Jah Chang, Uzma N Sarwar, Kathryn I. Zephir, LaSonji A Holman, Eric R James, Peter F Billingsley, Anusha Gunasekara, Sumana Chakravarty, Anita Manoj, MingLin Li, Adam J Ruben, Tao Li, Abraham G Eappen, Richard E Stafford, Natasha K C, Tooba Mursheedkar, Hope DeCederfelt, Sarah H Plummer, Cynthia S Hendel, Laura Novik, Pamela J M Costner, Jamie G Saunders, Matthew B Laurens, Christopher V Plowe, Barbara Flynn, William R Whalen, J P Todd, Jay Noor, Srinivas Rao, Kailan Sierra-Davidson, Geoffrey M Lynn, Judith E Epstein, Margaret A Kemp, Gary A Fahle, Sebastian A Mikolajczak, Matthew Fishbaugh, Brandon K Sack, Stefan H I Kappe, Silas A Davidson, Lindsey S Garver, Niklas K Björkström, Martha C Nason, Barney S Graham, Mario Roederer, B Kim Lee Sim, Stephen L Hoffman, Julie E Ledgerwood & Robert A Seder, for the VRC 312 and VRC 314 Study Teams

*Nat. Med.*; doi:10.1038/nm.4110; corrected online 18 May 2016

In the version of this article initially published online, the authors omitted a funding source, The Bill and Melinda Gates Foundation (Investment ID: 24922). The error has been corrected for the print, PDF and HTML versions of this article.

**Erratum: Modulation of splicing catalysis for therapeutic targeting of leukemia with mutations in genes encoding spliceosomal proteins**

Stanley Chun-Wei Lee, Heidi Dvinge, Eunhee Kim, Hana Cho, Jean-Baptiste Micol, Young Rock Chung, Benjamin H Durham, Akihide Yoshimi, Young Joon Kim, Michael Thomas, Camille Lobry, Chun-Wei Chen, Alessandro Pastore, Justin Taylor, Xujun Wang, Andrei Krivtsov, Scott A Armstrong, James Palacino, Silvia Buonamici, Peter G Smith, Robert K Bradley & Omar Abdel-Wahab

*Nat. Med.*; doi:10.1038/nm.4097; corrected 11 May 2016

In the version of this article initially published online, the graphs in Figure 3b–d were laid out incorrectly and were not consistent with the figure legend and text. The error has been corrected for the print, PDF and HTML versions of this article.