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Live Attenuated Malaria Vaccine Designed to Protect Through Hepatic CD8$^+$ T Cell Immunity

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Our goal is to develop a vaccine that sustainably prevents *Plasmodium falciparum* (Pf) malaria in ≥80% of recipients. Pf sporozoites (PfSPZ) administered by mosquito bites are the only immunogens shown to induce such protection in humans. Such protection is thought to be mediated by CD8$^+$ T cells in the liver that secrete interferon-γ (IFN-γ). We report that purified irradiated PfSPZ administered to 80 volunteers by needle inoculation in the skin was safe, but suboptimally immunogenic and protective. Animal studies demonstrated that intravenous immunization was critical for inducing a high frequency of PfSPZ-specific CD8$^+$, IFN-γ–producing T cells in the liver (nonhuman primates, mice) and conferring protection (mice). Our results suggest that intravenous administration of this vaccine will lead to the prevention of infection with Pf malaria.

A highly effective vaccine would be the ideal tool to prevent the nearly 1 million deaths and 300 million cases caused by malaria annually and to eliminate malaria from defined locations (*1*, *2*). Since the 1970s, the gold standard for malaria vaccine developers has been immunization by bite of mosquitoes with radiation-attenuated *Plasmodium falciparum* (Pf) sporozoites (SPZ) in their salivary glands (*3*–*5*). Recently, fully infectious PfSPZ administered by mosquito bite to volunteers taking antimalarial chemoprophylaxis also induced high-level, sustained prevention of infection in volunteers (*6*, *7*). Because infected mosquitoes cannot be used for immunizing large numbers of individuals and it has been considered impossible to produce, purify, and preserve aseptic PfSPZ that met regulatory standards, no vaccine based on PfSPZ has heretofore been developed. Therefore, almost all malaria vaccine development has focused on subunit recombinant vaccines, which have shown far less efficacy (*8*–*10*).

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Clinical Trial

Because of the unmet medical need, we reassessed development of an attenuated PfSPZ vaccine (*11*), overcame production obstacles, and manufactured the PfSPZ Vaccine (*12*), and now report on results of the first clinical trial. In contrast to all licensed live attenuated vaccines, which are replication deficient or replication competent with reduced or no virulence (*13*), our vaccine consisted of ~1 × 10 μm metabolically active, nonreplicating, purified, aseptic, cryopreserved, whole parasites (*14*) (figs. S1 to S3 and table S1). Thus, there were no precedents for method, route and site of administration, or dosage.

The only human data were obtained from volunteers immunized by bite of irradiated, PfSPZ-infected mosquitoes. In those studies, volunteers were generally exposed to several hundred mosquitoes simultaneously in a surface area of ~56 cm^2 of skin, and each mosquito inoculated PfSPZ in <0.5 μl of saliva (*15*). Undoubtedly some SPZ were inoculated directly into blood vessels, but it has been shown in mice that mosquitoes deposit many SPZ in the dermis and some in subcutaneous tissues (*16*). Replicating mosquito bites is not possible with a standard needle and syringe or any licensed administration device. Recognizing this limitation, we immunized equal numbers of volunteers intradermally (id) in the forearm or subcutaneously (sc) in the upper arm by needle and syringe (120 μl at two sites). The goals were to demonstrate safety and to begin the process of optimizing immunogenicity and protective efficacy. The trial was an open-label, dose-escalation study in 80 malaria-naïve, healthy adults aged 18 to 50 years conducted at the Naval Medical Research Center (NMRC) and University of Maryland Center for Vaccine Development (UMD-CVD). Volunteers in groups 1 to 3 were to receive four doses of 7500 (N = 14), 30,000 (N = 22), or 135,000 (N = 22) PfSPZ/dose at 4-week intervals. Those in group 4 were to receive four doses of 135,000 PfSPZ/dose at 4-week intervals, and beginning 12-weeks later doses five and six at 4-week intervals (N = 22) (table S2).

Asexual erythrocytic stage (AES) parasites cause all manifestations of malaria. The PfSPZ in the vaccine are irradiated with the intention of attenuating them so that they cannot develop to the late liver stage and produce parasites that invade erythrocytes and develop to AES parasites. To assess adequacy of attenuation, we analyzed blood smears to rule out breakthrough infections 2 weeks after each dose, and for up to 6 months after last immunization in group 3. Sera acquired 2 weeks after the fourth dose were assessed for antibodies against AES parasites and PfMSP-1 (*17*)

Table 1. Adverse events. Number (%) of volunteers with adverse events (AEs) deemed possibly, probably, or definitely related to immunization in groups 1 to 3 (four immunizations) and group 4 (six immunizations) through 14 days after last dose (solicited AEs) or 28 days after last dose (unsolicited AEs). An individual volunteer was counted at most once within each type of event or class (local or general). Percent of volunteers was based on the number who received at least one dose.

Adverse events	Group 1 (N = 14)		Group 2 (N = 22)		Group 3 (N = 22)		Group 4 (N = 22)	
	sc (N = 7)	id (N = 7)	sc (N = 11)	id (N = 11)	sc (N = 11)	id (N = 11)	sc (N = 11)	id (N = 11)
Any adverse event	4 (57)	3 (43)	4 (36)	6 (55)	5 (45)	7 (64)	6 (55)	7 (64)
Serious adverse event	0	0	0	0	0	0	0	0
Grade 3 adverse event	0	0	0	0	0	0	1	0
Solicited adverse event	4 (57)	3 (43)	3 (27)	5 (45)	3 (27)	7 (64)	6 (55)	6 (55)
Local	3 (43)	1 (14)	2 (18)	3 (27)	2 (18)	3 (27)	2 (18)	3 (27)
General	3 (43)	3 (43)	2 (18)	3 (27)	3 (27)	4 (36)	5 (45)	5 (45)
Unsolicited adverse event	2 (29)	2 (29)	1 (9)	3 (27)	5 (45)	3 (27)	2 (18)	4 (36)

and PfEBA-175 (*18*). PfMSP-1 and PfEBA-175 are only expressed in late liver and AES stages, and play major roles in parasite invasion of erythrocytes. There were no Pf blood stage infections and no volunteers developed antibodies against AES parasites, PfMSP-1, or PfEBA-175; therefore, the PfSPZ were adequately attenuated.

About half of volunteers in each group had a local or general adverse event (AE) considered possibly, probably, or definitely related (= related) to administration of vaccine (Table 1 and tables S3 and S4). The 42 volunteers with related AEs experienced 39, 21, 49, and 52 AEs in groups 1 to 4, respectively. Ninety-one percent of related AEs were grade 1. Eight volunteers experienced 14 related grade 2 AEs (tables S5 and S6). These included headache, malaise, myalgia, nausea, fever, chills, arthralgia, fatigue, and musculoskeletal pain. One volunteer had one grade 3 AE, fatigue, considered possibly, but unlikely, related. There were no related serious (grade 4) AEs. Increasing dose did not increase numbers of volunteers with an AE, and incidence of AEs did not increase as volunteers received additional doses of vaccine. During the immunization phase, 35 volunteers (43.8%) had a laboratory abnormality. Five volunteers experienced a grade 2 laboratory abnormality (table S7). None experienced grade 3 or 4 laboratory abnormalities. The vaccine was safe and well tolerated.

Humans protected by immunization with attenuated PfSPZ by mosquito bite develop low to modest antibody responses to whole SPZ, the Pf circumsporozoite protein (PfCSP) (*19*, *20*) and other proteins (*21*), and low-magnitude and -frequency $CD8^+$ T cell responses against several parasite proteins, such as PfCSP, PfSSP2/TRAP, and PfCelTOS (*22*–*25*). None of these individual human immune responses has been correlated with protection. Protection in multiple strains of mice immunized with radiation-attenuated *P. yoelii* (Py) and *P. berghei* (Pb) SPZ is dependent on $CD8^+$ T cells and IFN-γ (*26*–*34*). Therefore, it is thought that IFN-γ produced in response to parasite-specific stimulation of $CD8^+$ T cells is a critical effector molecule responsible for elimination of parasite-infected hepatocytes and protection, and antibodies may play a secondary role. Because no T cell responses against parasite-derived antigens have been shown to correlate with protection in humans or animals, and human T cell responses are so low, it has been suggested that protection may require additive small T cell responses against many antigens from SPZ to achieve the breadth and magnitude of protective immunity observed (*34*, *35*).

Therefore, we assessed immune responses against the antigens in whole SPZ using purified, aseptic PfSPZ (identical formulation as used in the vaccine) as well as against multiple Pf proteins. Volunteers in all groups developed low-level T cell responses to PfSPZ as measured by production of IFN-γ. The magnitude of responses and percentage of responders increased with increasing dose (groups 1 to 3) and numbers of doses (group 4) for the sc but not the id group (Fig. 1A). As the dosage of PfSPZ increased in groups 1 to 4 sc (Fig. 1A), there was a significant ($P < 0.05$) increasing trend in magnitude of IFN-γ responses when either individual values ($R^2 = 0.36$) or means ($R^2 = 0.96$) were assessed by linear regression analysis. Of the volunteers in group 4 immunized sc, 100% (9/9) developed T cell responses to PfSPZ, and the mean net spot-forming cells (SFCs) per 10^6 peripheral blood mononuclear cells (PBMCs) was greater than in any other group (Fig. 1A). We performed intracellular staining (ICS) assays on PBMCs from volunteers in groups 3 and 4. Three of 35 volunteers studied had a low frequency (net 0.05 to 0.09%) of PfSPZ-specific $CD4^+$ T cells producing IFN-γ, tumor necrosis factor–α (TNF-α), and/or interleukin-2 (IL-2). The results from the best responder are shown in Fig. 1B. PfSPZ-specific $CD8^+$ T cell responses were not detected by ICS from PBMCs in any volunteers. We also assessed immune responses against peptide pools and/or recombinant proteins based on the amino acid sequences of PfCSP, PfAMA1, PfSSP2, PfLSA1, and PfCelTOS. Responses to PfSPZ were significantly greater than to any individual protein. In group 4, 76% (13/17) of volunteers responded to PfSPZ after the sixth dose, but no more than 25% of volunteers responded to any single protein (fig. S4).

Antibodies against PfSPZ can also play a role in protection (*27*, *31*). Volunteers in all groups produced antibodies against PfSPZ by immunofluorescence assay (IFA) and PfCSP by enzyme-linked immunosorbant assay (ELISA) (Fig. 2). Geometric mean (GM) anti-PfSPZ titers and anti-PfCSP net optical density 1.0 (OD 1.0) increased with increasing dose of PfSPZ (groups 1 to 3), or number of doses (group 3 versus 4) (Fig. 2A). There was a significant ($P < 0.05$) increasing trend by linear regression analysis of anti-PfSPZ IFA titers as the total dosage increased (Fig. 2A). In all groups, volunteers immunized id had higher GM titers than did volunteers immunized sc, although differences were only statistically significant in group 1 (Fig. 2A). All volunteers in group 4 id developed antibodies to PfSPZ, albeit at quite low levels [GM anti-PfSPZ titer (297) and anti-PfCSP net OD 1.0 (203.5)]. We selected 13 sera from volunteers immunized by bite of irradiated PfSPZ-infected mosquitoes from 1990 to 2002 (*5*, *19*, *25*) and assessed them for antibodies to PfCSP by ELISA. The GM net OD 1.0 was 32 times higher than in sera from volunteers in group 4 (Fig. 2B).

Sera from the two volunteers with the highest IFA titers in group 4 after the fourth dose and the two protected volunteers in group 2 (see below) were assessed for capacity to inhibit invasion and development of PfSPZ in vitro in a human hepatocyte cell line. The inhibition at a serum dilution of 1:20 was low for the two protected volunteers (16% and 18%) and modest for the two volunteers with the highest antibody titers in group 4 (57% and 59%) (table S8).

Three weeks after the final dose, volunteers in groups 1, 2, and 4 ($N = 44$) and six nonimmunized control volunteers for each group were challenged by exposure to bites of five *Anopheles stephensi* mosquitoes infected with PfSPZ as described previously (*36*). All 18 nonimmunized

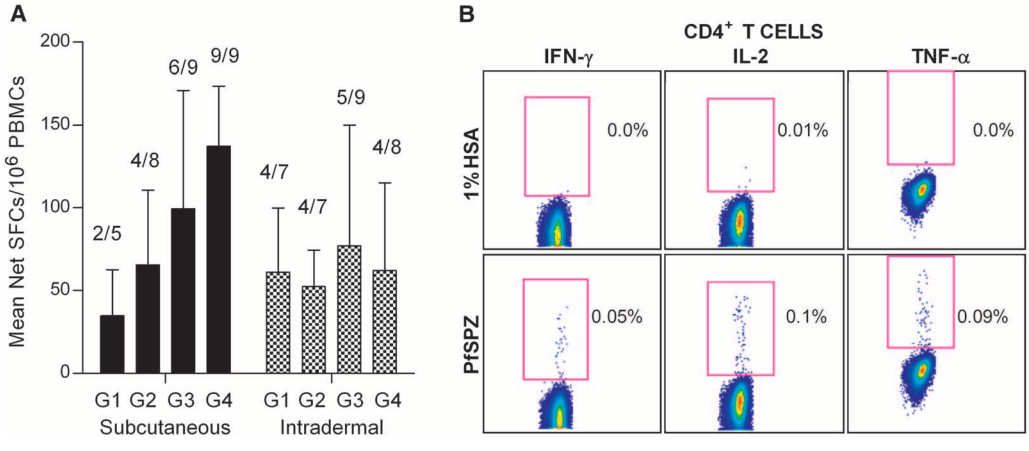

Fig. 1. T cell responses in volunteers. (**A**) IFN-γ responses by ELISpot 2 weeks after final dose of PfSPZ vaccine. To be considered positive, the net SFCs in triplicate wells (SFCs post- minus SFCs preimmunization) had to be >50 SFCs, the ratio of post-SFCs to pre-SFCs had to be >1.5, and the difference between triplicate post- and preimmunization SFCs had to be significant ($P < 0.05$). Among positives, ratios of post- to pre-SFCs ranged from 2.25 to 205.00. The number of positive responders out of total number of volunteers assessed is denoted above each bar. Data are presented as arithmetic mean ± SD net SFCs for each group. Samples from three fully immunized volunteers (table S1) were not processed, because of compliance or technical reasons. (**B**) Percentage of reactive (cytokine-producing) $CD4^+$ T cells against whole PfSPZ in PBMCs from an immunized volunteer by ICS. Nonspecific responses were measured in medium containing diluent alone (1% human serum albumin, HSA).

volunteers developed Pf parasitemia with onset 11 to 15 days after challenge (GM = 11.5 days). None of the 11 volunteers in group 1 and none of the 17 volunteers in group 4 were protected. Two of the 16 volunteers in group 2 were protected as evidenced by negative blood smears and real-time quantitative DNA polymerase chain reaction (PCR) assays. There was no delay in the time until onset of parasitemia in immunized volunteers as compared to controls (table S9). Both protected volunteers, U041 (sc) and U010 (id), had T cell responses (net SFCs of 103 and 53) and antibodies (titers of 800 and 200) to PfSPZ. Volunteer U041 had the second highest T cell response and the highest IFA titer in group 2.

In summary, we established that the PfSPZ in the PfSPZ Vaccine were adequately attenuated and the vaccine was safe and well tolerated. Low-level T cell and antibody responses against whole PfSPZ and selected parasite proteins were detected, and two individuals were protected against malaria. We postulated that the suboptimal immunogenicity and protective efficacy were due to inefficiency of administration by the sc and id routes. We therefore conducted animal studies to determine if the aseptic, purified, cryopreserved PfSPZ in the vaccine were potent and if immune responses and protection could be improved by altering route of immunization.

Nonhuman Primate (NHP) and Rabbit Studies

Because NHPs are phylogenetically closest to humans, they are useful for studying vaccine-induced immune responses that may be predictive of human responses. Therefore, NHPs (rhesus macaques) were immunized sc or intravenously (iv) with four to six doses of 1.35×10^5 PfSPZ (the same dose as groups 3 and 4 of the clinical trial), produced identically to the PfSPZ Vaccine used in the clinical trial. Although mouse and human studies show that certain antigens induced by immunization with radiation-attenuated SPZ, such as CSP and SSP2/TRAP, can mediate protection through production of antibodies and/or T cells (*37*–*40*), the parasite antigens responsible for the $CD8^+$ T cell– and IFN-γ–dependent protection after immunization with attenuated SPZ are unknown.

As PfSPZ produce over 1000 proteins (*41*), rather than focusing on T cell responses against a few known proteins, we used the ICS assay that uniquely utilizes PfSPZ like those in the PfSPZ Vaccine as the antigen to stimulate cells from the immunized NHPs. First, assay conditions were optimized (fig. S5). The highest frequency of $CD8^+$ T cell cytokine responses were detected when PBMCs were cultured for 16 but not 6 hours with 1.5×10^5 PfSPZ in the assay. A marked reduction in frequency of IFN-γ–producing $CD8^+$ T cells, but not $CD4^+$ T cells, was observed when heat-inactivated PfSPZ were used for in vitro stimulation (fig. S5). Therefore, optimal detection of $CD8^+$ T cells required overnight stimulation with live, metabolically active PfSPZ.

Response in PBMCs at 2 weeks and 3 to 4 months after immunizations. Using this assay, we assessed T cell responses in PBMCs from NHPs immunized sc, as in the clinical trial, or iv, 2 weeks after doses of PfSPZ Vaccine. After administration of five or six doses of PfSPZ by the sc route, only one of five animals had a net PfSPZ-specific $CD8^+$ T cell response ≥ 0.05% (0.08%), and this response was detected after the sixth dose of PfSPZ (fig. S6). None of the NHPs immunized sc had net PfSPZ-specific IFN-γ–producing $CD4^+$ T cell frequencies ≥ 0.05% (fig. S6). In contrast, after four or five doses of PfSPZ administered by the iv route, three of the four animals had PfSPZ-specific, IFN-γ–producing $CD8^+$ T cells in PBMCs (net = 1.42, 1.28, 0.05%) (fig. S7 and Fig. 3). Among these animals, two NHPs (CL55, CV92) were first positive after the second dose, and one (DBTL) after the fifth dose (fig. S7). Because animals DBVJ and CV92 were euthanized after the fourth immunization, we could not establish whether an additional immunization would have induced (DBVJ) or boosted (CV92) the $CD8^+$ T cell responses. All four NHPs developed PfSPZ-specific $CD4^+$ T cell IFN-γ responses after two (CL55 and CV92) or three iv immunizations (DBTL and DBVJ). Such responses were boosted upon subsequent immunizations (net = 0.12, 0.54. 0.25, 0.28%) (fig. S7 and Fig. 3). Thus, based on assessment of PBMCs, PfSPZ-specific T cell immunity was elicited in all NHPs immunized iv, with some variability in the number of immunizations required to elicit $CD4^+$ or $CD8^+$ T cell responses.

To extend the analysis, we assessed the frequency of T cells producing IL-2 and TNF-α as well as IFN-γ. All three cytokines were detected in $CD4^+$ T cells from NHPs immunized by the iv route, whereas only IFN-γ and TNF-α were detected in $CD8^+$ T cells (Fig. 3A). In defining the quality of the T cell responses as determined by any combination of these cytokines produced at the single-cell level, PfSPZ-specific $CD8^+$ T cells largely comprised IFN-γ or IFN-γ– and TNF-α–producing cells, whereas $CD4^+$ T cell cytokine responses were more heterogeneous with a greater proportion of cells making IL-2 (Fig. 3, B and C). IFN-γ–producing PfSPZ-specific T cells in peripheral blood were present in both the central memory ($CCR7^{hi}CD95^{hi}$) and effector memory ($CCR7^{lo}CD95^{hi}$) T cell subsets after the final immunization with PfSPZ iv (Fig. 3D).

Assays were repeated 3 to 4 months after the last immunization. None of the animals immunized sc had positive responses. All four animals immunized iv had detectable PfSPZ-specific T cell responses in PBMCs, albeit at lower frequency than at 2 weeks, demonstrating durability of immunity (Fig. 4A).

T cell responses in liver 3 to 4 months after final immunization. On the basis of mouse studies, the primary target for protective T cells induced by immunization with attenuated SPZ is the infected hepatocyte. In this regard, previous reports using surrogate cell surface activation markers on T cells have shown that high frequencies of $CD8^+$ T cells in the livers of mice immunized with radiation-attenuated SPZ confer protection (*42*, *43*). However, there has been no analysis of SPZ-specific cytokine T cell responses in the liver of NHPs or humans

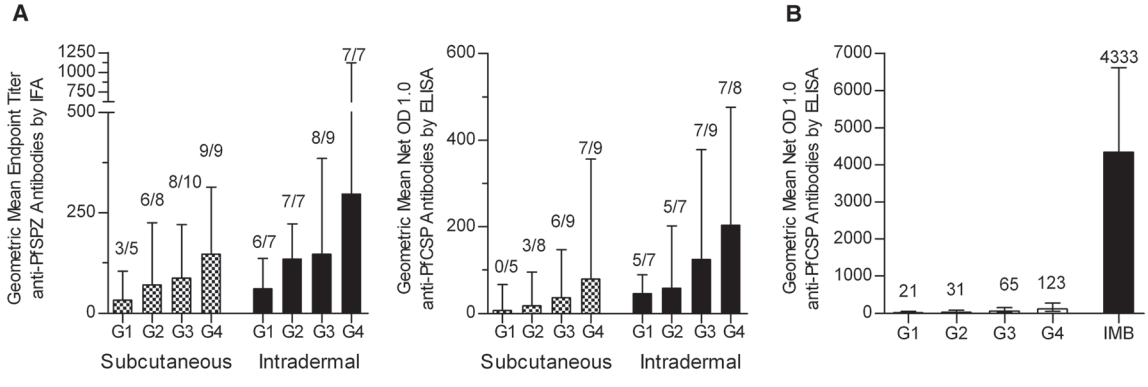

Fig. 2. Antibody responses in volunteers. (**A**) Endpoint antibody titers to PfSPZ by IFA and net OD 1.0 to PfCSP by ELISA in volunteers in groups 1 to 4 2 weeks after last dose of PfSPZ vaccine. An IFA titer ≥50 was considered positive. OD 1.0 was the serum dilution at which the optical density was 1.0, and net OD 1.0 was the difference between post- and preimmunization OD 1.0. Anti-PfCSP responses were considered positive if net OD 1.0 was ≥50, and the ratio of post- to pre-OD 1.0 was ≥2. The number of positive responders out of total number of volunteers assessed is denoted above each bar. (**B**) Comparative net OD 1.0 titers to PfCSP by ELISA in volunteers in groups 1 to 4 after last dose of PfSPZ vaccine or after 6th to 10th immunization by irradiated mosquito bite (IMB) in 13 volunteers. Geometric mean net OD 1.0 for each group is denoted above each bar. Data in all three graphs are presented as geometric mean titer or net OD 1.0 for each group with 95% confidence intervals. Samples from four fully immunized volunteers in groups 2 to 4 (table S2) were not processed, because of compliance or technical reasons.

after immunization with radiation-attenuated SPZ. Here, PfSPZ-specific, T cell responses were assessed in livers of NHPs immunized sc or iv 3 to 4 months after the final immunization.

As in the PBMCs, there were low to undetectable T cell responses in livers of sc-immunized NHPs (Fig. 4A). The most notable finding of our studies was the prevalence and magnitude of PfSPZ-specific T cell responses in the livers of iv-immunized animals. Despite the low numbers of animals per group, there were consistently high levels of PfSPZ-specific, IFN-γ–producing, $CD8^+$ T cells (net = 3.1, 2.8, and 3.6%) and $CD4^+$ T cells (net = 1.7, 4.6, and 1.9%) detected in the livers of all three of the NHPs (CL55, CV92 and DBVJ, respectively) immunized iv (Fig. 4A). It is notable that whereas $CD8^+$ T cell responses were not detected in the PBMCs of animal DBVJ at

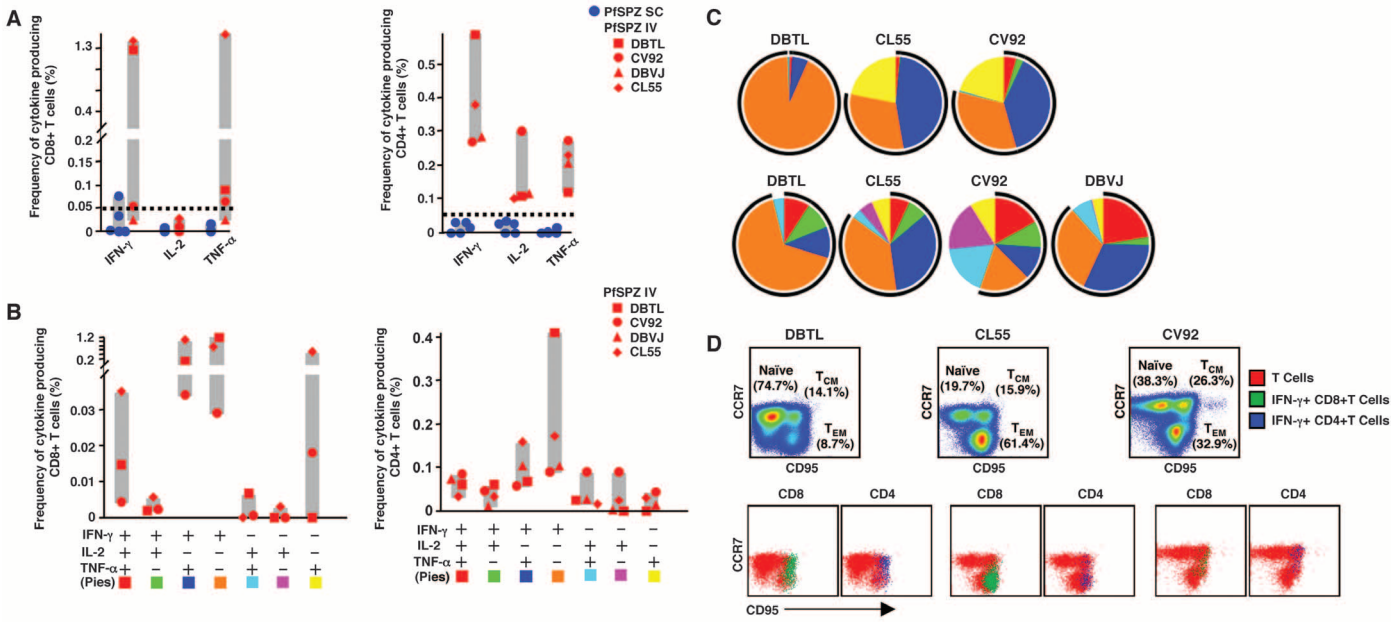

Fig. 3. PfSPZ-specific T cell cytokine responses in PBMCs from NHPs immunized iv and sc. (**A**) PBMCs were analyzed for PfSPZ-specific cytokine (IFN-γ, IL-2, TNF-α)–producing T cells from sc- or iv-immunized NHPs, as shown in fig. S5A. The frequencies of net PfSPZ-specific cytokine-producing $CD8^+$ and $CD4^+$ T cells were calculated by subtracting preexisting response (response to PfSPZ minus response to 1% HSA in preimmune PBMCs) from peak responses generated postimmunization (response to PfSPZ minus response to 1% HSA). A net response ≥0.05% was considered positive and is demarcated by a dotted line. (**B** to **C**) Using SPICE analysis (*52*), we divided cytokine-producing $CD8^+$ and $CD4^+$ T cell responses into seven distinct subpopulations producing any combination of IFN-γ, IL-2, and TNF-α. The total frequency (B) and pie chart representation (C) of combination cytokine-producing $CD8^+$ (top) and $CD4^+$ (bottom) T cells are shown for individual iv-immunized NHPs. The black arcs highlight the IFN-γ–producing T cells among the total responses (C). (**D**) The distribution of PfSPZ-specific IFN-γ–producing $CD8^+$ (green) and $CD4^+$ (blue) T cells at the peak of the response within central memory ($CCR7^{hi}CD95^{hi}$) and effector memory ($CCR7^{lo}CD95^{hi}$) phenotype T cells is shown for three NHPs.

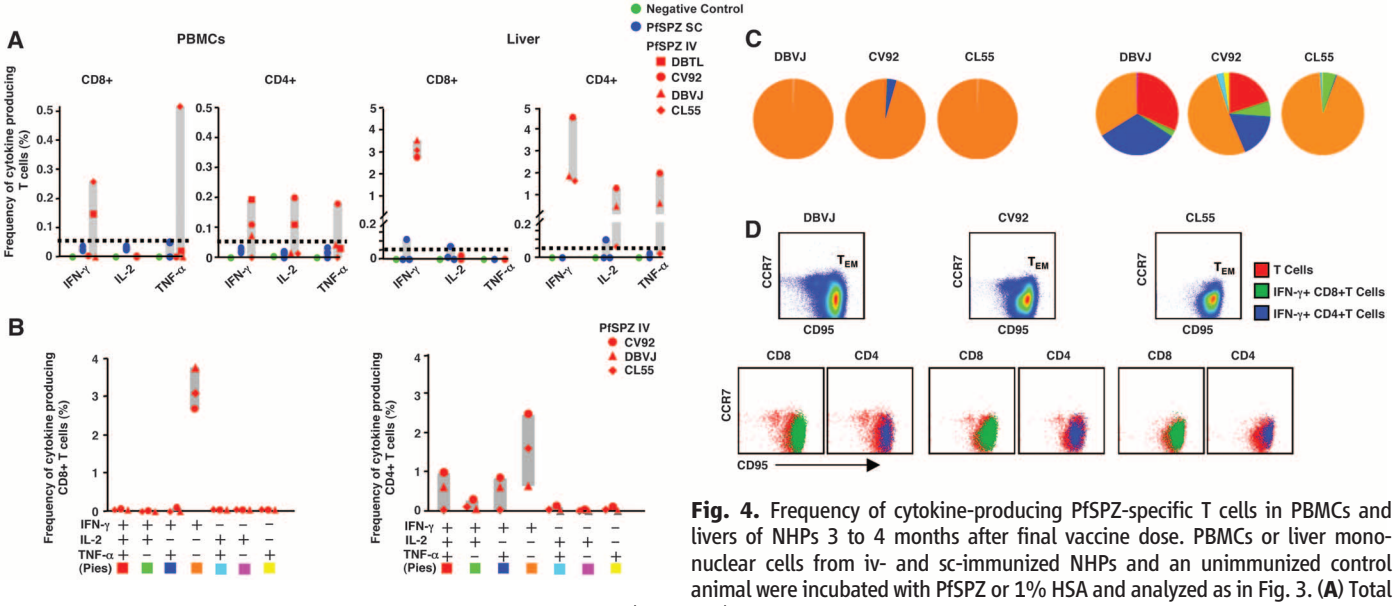

Fig. 4. Frequency of cytokine-producing PfSPZ-specific T cells in PBMCs and livers of NHPs 3 to 4 months after final vaccine dose. PBMCs or liver mononuclear cells from iv- and sc-immunized NHPs and an unimmunized control animal were incubated with PfSPZ or 1% HSA and analyzed as in Fig. 3. (**A**) Total frequency of PfSPZ-specific cytokine (IFN-γ, IL-2, TNF-α)–producing $CD8^+$ and $CD4^+$ T cells present in PBMCs and liver. (**B** to **D**) Analyses as in Fig. 3, but only for T cells isolated from livers of NHPs immunized iv. Pie chart representation (C) of combination cytokine-producing $CD8^+$ (left) and $CD4^+$ (right) T cells are shown for individual iv-immunized NHPs. For technical reasons, liver-derived T cells from NHP DBTL could not be assessed.

any time point, robust responses were observed in the liver. The liver is apparently a more sensitive site than the peripheral blood for detecting PfSPZ-specific T cell immunity after iv immunization with the PfSPZ Vaccine. Consistent with this interpretation, at 3 to 4 months, responses in PBMCs underestimated the T cell response in the liver by more than 10-fold (Fig. 4A).

In assessing the quality of the T cell responses, we found that PfSPZ-specific $CD8^+$ T cells in the liver were monofunctional, producing only IFN-γ in the three individual animals examined (Fig. 4, B and C). PfSPZ-specific $CD4^+$ T cells in the liver were multifunctional in two of three (DBVJ and CV92) animals examined (Fig. 4, B and C). Phenotypically, all PfSPZ-specific T cells in the liver were $CD95^{hi}CCR7^{lo}$ effector memory cells (Fig. 4D). Taken together, these data show that a high frequency of $CD8^+$ effector T cells producing IFN-γ were induced in the liver of NHPs by iv, but not sc immunization with PfSPZ, consistent with the central premise for how this vaccine platform mediates protection.

To further substantiate the differences in adaptive immunity following sc or iv PfSPZ immunization in NHPs, we assessed humoral responses. The GM antibody titers against PfSPZ by IFA or PfCSP by ELISA were 38 and 153 times higher, respectively, in the iv group compared to the sc group after immunization with four doses (iv) or 5 doses (sc) of PfSPZ (tables S10 and S11). Similarly, rabbits immunized iv had 81 and 131 times higher antibody titers by IFA and ELISA than rabbits immunized sc (tables S12 and S13). Collectively, iv immunization with PfSPZ induced substantially higher T cell and antibody responses in the blood than did sc immunization. The data regarding T cell and antibody responses against the whole parasite, when combined with analyses of T cell (*25*) and antibody (*35*) responses in volunteers immunized by the bite of mosquitoes carrying radiation-attenuated PfSPZ, further highlight the probable role of immune responses against many antigens in achieving protection.

PfSPZ invade and develop in NHP hepatocytes. The specific in vivo mechanisms by which iv immunization induced such potent T cell immunity in the livers of NHPs are unknown. The observation that incubation of PBMCs in vitro with live attenuated, as compared to heat-killed PfSPZ, induced far better PfSPZ-specific $CD8^+$ T cell responses (fig. S5) suggests that the PfSPZ invade antigen-presenting cells and are processed in the major histocompatibility complex class I pathway to recall vaccine-elicited $CD8^+$ T cell responses. Another possibility is that PfSPZ invade hepatocytes, leading to direct antigen presentation by the hepatocytes or indirect cross presentation by dendritic cells (*34*, *44*). Rhesus monkeys cannot develop a blood-stage infection with Pf because their erythrocytes lack the appropriate receptors for merozoite invasion of the cells, which explains their limited utility as a Pf challenge model (*45*). However, SPZ can invade many different cell types without fully developing (*46*), and rhesus hepatocytes have been infected with "human-like" malaria parasites (*47*). Therefore, we compared invasion rates in vitro of PfSPZ in rhesus and human hepatocytes. Irradiated PfSPZ invaded rhesus hepatocytes, albeit less efficiently than human hepatocytes (fig. S8), and expressed a protein, PfEXP-1, not expressed in sporozoites (fig. S9). These data indicate that antigen presentation in the liver could have been responsible for induction and maintenance (*48*) of PfSPZ-specific T cells in the NHP livers.

Mouse Studies

Because rhesus macaques cannot be challenged with Pf to assess protection, we used the Py rodent malaria model to determine if iv administration of radiation-attenuated, purified, cryopreserved PySPZ could induce high-level protective immunity and was superior to sc or id immunization. In three experiments, we achieved 71 to 100% sterile protective immunity after iv immunization with a total of 6×10^3 (three doses of 2×10^3) purified, cryopreserved PySPZ, a total dosage far lower than generally used (*28*, *30*, *33*) (Table 2). Protection was achieved if the mice were challenged by iv or mosquito bite inoculation of infectious PySPZ (Table 2), and was abrogated by depletion of $CD8^+$ T cells before challenge (table S14). In comparing the efficacy of vaccine route, 7 to 10 times more PySPZ were required after sc or id as compared to iv immunization to achieve ≥80% protection in the mouse model system (table S15), and the percentage of experiments in which >80% protection was achieved was higher overall in iv-immunized as compared to sc- or id-immunized mice. Last, 2.7 to 4 times as many cryopreserved as compared to freshly isolated PySPZ were required to achieve ≥80% protection (table S15). These data substantiate that the radiation-attenuated purified, cryopreserved PySPZ are potent, even at low doses, and that route is critical for optimizing protection.

Because $CD8^+$ T cells were required for protection in mice, we directly assessed how route of immunization influenced frequency of such responses. To perform T cell analyses as in the NHP studies would have required aseptic PySPZ, which are not yet available. Therefore, we adapted a recently developed assay based on changes in the phenotypic expression of cell surface molecules on $CD8^+$ T cells after Py or Pb attenuated SPZ immunization (*43*). Such immunization increased expression of integrin CD11a ($CD11a^{hi}$) and reduced expression of CD8 alpha chain ($CD8\alpha^{lo}$), causing an increased frequency of $CD11a^{hi}CD8\alpha^{lo}$ T cells. Moreover, because $CD8^+$ T cells against a single epitope on the Py and Pb CSP eliminate infected hepatocytes in vitro and are fully protective in vivo (*30*, *49*), we used PyCSP280-288 peptide stimulation to enumerate the frequency of IFN-γ–producing $CD11a^{hi}CD8\alpha^{lo}$ T cells to demonstrate antigen specificity and effector function. Mice repetitively immunized iv with 2×10^4 irradiated PySPZ had ~10-fold higher frequency of PyCSP-specific, $CD11a^{hi}CD8\alpha^{lo}$ T cells secreting IFN-γ in the liver and spleen than mice receiving the same dosage id (fig. S10). These data further demonstrate the advantage of iv as compared to id immunization with irradiated SPZ in inducing the IFN-γ–producing $CD8^+$ T cells in the liver required for protection. This is most likely due to more SPZ reaching the liver. These data suggest that protective immune responses following iv immunization may be generated in the liver, which is an uncommon organ for priming T cell immunity.

Summary and Conclusions

We report that a radiation-attenuated, aseptic, purified, cryopreserved whole-parasite malaria vaccine, the PfSPZ Vaccine, can be manufactured and administered safely to humans and protected two individuals in one dosage group. Because the immunogenicity and protective efficacy of the vaccine were suboptimal, we conducted animal studies to determine if this was due to poor potency of the vaccine or inefficient administration. The animal studies demonstrated that purified, cryopreserved, attenuated SPZ were potent for inducing a high frequency of effector memory IFN-γ–producing $CD8^+$ T cell responses in the livers of NHPs and conferred protection in mice. They also showed that altering route of administration improved immunity and protection.

The low antibody and T cell responses found in the NHPs after sc administration were comparable in prevalence and magnitude to the responses in the human volunteers immunized sc or id. Thus,

Table 2. Consistency of protection in mice immunized with PySPZ. Purified, radiation-attenuated PySPZ were produced and cryopreserved. More than a year later, the PySPZ were thawed and BALB/c mice were immunized iv with three doses of 2000 PySPZ at 2-week intervals. Two weeks after the last dose, mice were challenged by iv administration of freshly isolated, infectious PySPZ or by bite of PySPZ-infected mosquitoes. Four weeks after first challenge, protected mice were rechallenged by mosquito bite. ND, not determined.

Group (date of first immunization)	Challenge (no. PySPZ or infected mosquitoes)	Percent protected (no. protected/ no. immunized)	Rechallenge (no. infected mosquitoes)	Rechallenge percent protected (no. protected/ no. immunized)
1 (3/11/10)	200 SPZ	100% (7/7)	5–9	100% (7/7)
2 (3/26/10)	200 SPZ	71% (5/7)	5–9	100% (5/5)
3 (4/09/10)	5 to 9 mosquitoes	90% (9/10)	ND	ND

these animal studies provide a possible explanation for the suboptimal immunogenicity and protective efficacy of the PfSPZ Vaccine in this first clinical trial and provide a clear direction and rationale for design of the next clinical trial, in which this whole-parasite vaccine will be administered iv. This trial is intended to prove that administration of the PfSPZ Vaccine to humans can induce high-level protective immunity and provide the foundation for its development, regardless of method of attenuation (*50*, *51*), for prevention and elimination (*2*) of Pf malaria.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1211548/DC1
Materials and Methods
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Clonal Production and Organization of Inhibitory Interneurons in the Neocortex

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The neocortex contains excitatory neurons and inhibitory interneurons. Clones of neocortical excitatory neurons originating from the same progenitor cell are spatially organized and contribute to the formation of functional microcircuits. In contrast, relatively little is known about the production and organization of neocortical inhibitory interneurons. We found that neocortical inhibitory interneurons were produced as spatially organized clonal units in the developing ventral telencephalon. Furthermore, clonally related interneurons did not randomly disperse but formed spatially isolated clusters in the neocortex. Individual clonal clusters consisting of interneurons expressing the same or distinct neurochemical markers exhibited clear vertical or horizontal organization. These results suggest that the lineage relationship plays a pivotal role in the organization of inhibitory interneurons in the neocortex.

The neocortex contains two major classes of neurons: glutamatergic excitatory neurons and γ-aminobutyric acid (GABA)–ergic inhibitory interneurons. Proper functioning of the neocortex critically depends on the production of a correct number of excitatory and inhibitory neurons, which largely occurs during the embryonic stages. Extensive studies over the past decade have provided a comprehensive view of excitatory neuron neurogenesis in the developing neocortex. In contrast, our knowledge of neocortical interneuron neurogenesis remains incomplete.

Most, if not all, neocortical interneurons are generated in the developing ventral (i.e., subcortical) telencephalon, including the ganglionic eminences (GEs) and the preoptic area (PoA), and migrate tangentially over long distances to reach their destination in the neocortex (*1*–*3*). Genetic and transplantation studies have demonstrated that different regions of the GEs generate distinct interneuron subgroups that differ in morphology, expression of neurochemical markers, biophysical properties, and synaptic connectivity (*4*–*9*). Distinct temporal origins of physiologically defined interneuron subgroups have also been reported

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