Supporting Information

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SI Materials and Methods

Screening of Study Subjects. Forty-two subjects without a history of malaria or residence in a malaria-endemic area in the 6 mo before study entry were screened for eligibility based on medical and family history, physical examination, and general hematological and biochemical tests. Serologic analyses for HIV, hepatitis B, hepatitis C, and *Plasmodium falciparum* were negative in all subjects. Urine toxicology screening was negative, and none of the volunteers was pregnant or lactating. All subjects had an estimated 10-y risk of developing a cardiac event lower than 5%, as estimated by the systematic coronary evaluation system (1).

Preparation of *P. falciparum*-Infected Mosquitoes for Chemoprophylaxis and Sporozoites Immunization. *Anopheles stephensi* mosquitoes were reared at our insectary and infected by allowing them to feed on cultured gametocytes of *P. falciparum* parasites according to standard procedures as described previously (2). We intended to immunize the subjects with 3D7 parasites only, because this *P. falciparum* strain was to be used for the challenge infections. Initial stocks of 3D7 parasites for immunization and challenge were kindly provided by Adrian Hill, Oxford. However, because of difficulties in culturing 3D7 parasites during the immunization period, we immunized each volunteer with seven 3D7-infected plus eight NF54-infected mosquito bites per immunization instead. The 3D7 strain is a clone of NF54, and both are sensitive to chloroquine. The percentage of infected mosquitoes in the batches used for immunization 1, 2, and 3 was 75, 85, and 90 for 3D7 and 95, 90, and 100 for NF54, respectively. Mosquitoes were infected with an average of 5.042 and 109.000 sporozoites for 3D7 and NF54, respectively.

Chemoprophylaxis and Sporozoites Immunization Procedure. Chloroquine was administered to all subjects according to a standard prophylactic regimen for a period of 14 wk (98 d) as described previously by Roestenberg et al. (3). While receiving chloroquine, groups 1 and 2 (15 subjects) were immunized three times at monthly intervals, starting 8 d after the first chloroquine dose. Immunization was performed by exposure to the bites of exactly 15 *P. falciparum*-infected mosquitoes for 15 min, briefly interrupted twice. Following each feeding session, the salivary glands of all blood-engorged mosquitoes were dissected to confirm the presence of sporozoites. All 10 control subjects (groups 3 and 4) received chloroquine prophylaxis only, but no mosquito bites.

On days 6–10 after each immunization, we checked all 15 immunized subjects once daily at our outpatient clinical research department. Blood was drawn for thick blood smears, standard hematological markers, and retrospective assessment of blood-stage parasitemia by real-time quantitative PCR (qPCR) using *P. falciparum* standard curves prepared by DNA extraction from titrated samples of ring-infected cells [qPCR (4)]. All signs and symptoms were recorded by the attending physician as mild (grade 1, easily tolerated), moderate (grade 2, interferes with normal activity), or severe (grade 3, prevents normal activity), or in the case of fever, grade 1 (>37.5–38.0 °C), grade 2 (>38.0–39.0 °C), or grade 3 (>39.0 °C). For safety reasons related to a previously reported cardiac event (5), we measured cardiovascular markers throughout the trial (highly sensitive troponin, platelets, D-dimer, and lactate dehydrogenase). Whenever abnormal, blood samples were checked for the presence of fragmentocytes and von Willebrand-cleaving protease activity, according to previous protocols (6).

Challenge Infections. Seventeen weeks after discontinuation of chloroquine prophylaxis, corresponding to 21 wk after the last immunization, all subjects received a challenge infection. Group 1 ($n = 9$; 1 lost to follow-up) and control group 3 ($n = 5$) subjects were challenged by i.v. administration of 3D7 *P. falciparum*-infected erythrocytes (blood-stage challenge), whereas group 2 ($n = 5$) and group 4 ($n = 5$) were exposed to the bites of five 3D7 *P. falciparum*-infected *A. stephensi* mosquitoes (sporozoite challenge).

Subjects were checked daily on an outpatient basis for symptoms and signs of malaria, thick blood smears, hematologic tests, and cardiovascular markers. Subjects who received a blood-stage challenge were checked from the first day after challenge onward, sporozoite-challenged subjects from day 5 onward. All signs and symptoms (solicited and unsolicited) were recorded as described for the chemoprophylaxis and sporozoites (CPS) immunization. At the time of thick smear positivity, each subject was treated with a curative regimen of 1,000 mg atovaquone and 400 mg proguanil once daily for 3 d according to Dutch national guidelines. Subjects who remained thick smear negative until day 21 after challenge were treated presumptively with the same curative regimen. Complete cure was confirmed by two consecutive parasite-negative blood smears after treatment.

Blood-stage inoculum. Inocula for blood-stage challenge were derived from a stock of 3D7 *P. falciparum*-infected erythrocytes (blood group O, rhesus negative) produced at the Queensland Institute of Medical Research, as described previously (7). The donor was seronegative or PCR negative, or both, for a panel of parenterally transmissible viruses at the time of donation and 1 y later, but was IgG positive for EBV and CMV (7). The blood-stage inoculum was cryopreserved in Glycerolyte 57 and stored in liquid nitrogen under temperature-monitored conditions.

To prepare the inocula for i.v. administration, an aliquot of the seed stock was thawed and washed under sterile conditions as described previously (8) using solutions licensed for clinical use and sterile, single-use consumables. Bacterial culture plates of the hood and both aerobic and anaerobic blood culture of the inoculum did not show any bacterial growth. The inoculum was prepared by diluting to the appropriate dose and dispensed aseptically into 5-mL syringes for administration. Based on microscopic estimates of the donor's parasite density before freezing of blood samples, each inoculum contained 4,289 infected erythrocytes. The inocula were kept on ice during preparation. Alternating between CPS-immunized and control subjects, all volunteers were inoculated i.v. between 103 and 111 min after thawing of the inocula. The number of viable parasites in the inoculum was verified in retrospect by limiting dilution culture and qPCR detection as previously described (4, 8), and the inoculum was calculated to contain 1,962/4,289 (46%) viable/total parasites per subject. This recovery of viable parasites was in the range of what has been reported for the inoculum elsewhere (8–10).

Sporozoite challenge. *A. stephensi* mosquitoes fed on cultured gametocytes of *P. falciparum* strain 3D7 were 100% infected with an average of 100,000 sporozoites per mosquito. A total of 10 volunteers from group 2 ($n = 5$) and group 4 ($n = 5$) were exposed to bites of five infected *A. stephensi* mosquitoes for 10 min, as described previously (3). One feeding session was sufficient for five volunteers, whereas a second session was required in the remaining five volunteers to obtain an infectious challenge by exactly five infected mosquitoes in all 10 subjects.

Study Outcomes. The primary study outcome was time to parasitemia after challenge, as assessed by microscopy. Sampling

started on a daily basis for groups 1 and 3 (blood-stage challenge) on days 1–4 and continued for all groups post challenge twice daily on days 5 and 6, thrice daily on days 7–11, again twice daily on days 12–15, and finally once daily on days 16–21. Thick blood smears were made from 15 μL of EDTA-anticoagulated blood spread over the standardized surface of one well of a three-well glass slide according to harmonized standard protocol for Controlled Human Malaria Infections (Laurens MB, Roestenberg M, and Moorthy VS; manuscript in preparation). After drying, wells were stained with Giemsa for 30 min. Slides were read at 1,000× magnification by assessing 200 high-power fields, equal to about 0.5 μL of blood. The smear was deemed positive if two unambiguously identifiable parasites were found. Lymphocyte and platelet counts were determined in EDTA-anticoagulated blood with the Sysmex XE-5000. D-dimer concentrations were assessed in citrate plasma by STA-R (Roche).

Immunological Measurements. Concentrations of malaria antigen-specific antibodies were determined in serially diluted citrate plasma by standardized ELISA in Nunc MaxiSorp plates (Thermo Scientific) coated with 1 μg/mL full-length circumsporozoite protein (CSP), apical membrane antigen 1 (AMA-1; FVO-allele) (11), or glutamate-rich protein (GLURP) (12) diluted in PBS. ELISAs were developed using biotinylated polyclonal goat anti-human IgG (1/2,500), streptavidin-conjugated HRP (1/2,000), and tetramethylbenzidine (all from Mabtech). Spectrophotometrical absorbance was measured at 450 nm. Levels of antibody reactivity were analyzed in relation to a pool of sera from adults living in a highly endemic area in Tanzania (3), which was defined to contain 100 arbitrary units (AU) of IgG directed against an antigen. Antibody responses were considered positive when they had increased at least twofold after immunization, compared with baseline.

Antibodies for growth inhibition assay (GIA) were isolated from citrate plasma collected the day before the start of chloroquine prophylaxis and the day before challenge infection. IgG was purified using protein G columns (HiTrapTM Protein G HP, GE Healthcare). Eluted samples were exchanged into RPMI-1640 on a Vivaspin 20 30-kDa molecular mass cutoff ultrafiltration unit (Sartorius), concentrated to 20 mg/mL, filter sterilized, and stored at −20 °C until used. IgG protein concentrations were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The GIA was performed as previously described (11). Briefly, the effect of IgG on in vitro parasite growth was evaluated at an IgG concentration of 10 mg/mL. Samples were run in triplicate using alanine-synchronized cultures of *P. falciparum* 3D7 schizonts at an initial parasitemia of 0.7%. After 40–42 h, parasite growth was assessed by measuring parasite lactate dehydrogenase levels in culture supernatants with the lactate diaphorase 3-acetylpyridine adenine dinucleotide substrate system. EDTA (4 mM) was included in every test plate as a positive control. Induction of growth inhibitory activity by CPS immunization was assessed by subtracting for each volunteer the preimmunization percentage of growth inhibition from the postimmunization value.

Monokine-induced-by-IFN-γ (MIG) and IFN-γ concentrations in frozen and stored EDTA anticoagulated plasma samples were determined retrospectively. MIG was measured using a DuoSet ELISA (R&D Systems) and IFN-γ was measured using a Ready-SET-Go! ELISA kit (eBioscience) according to the manufacturer's recommendations.

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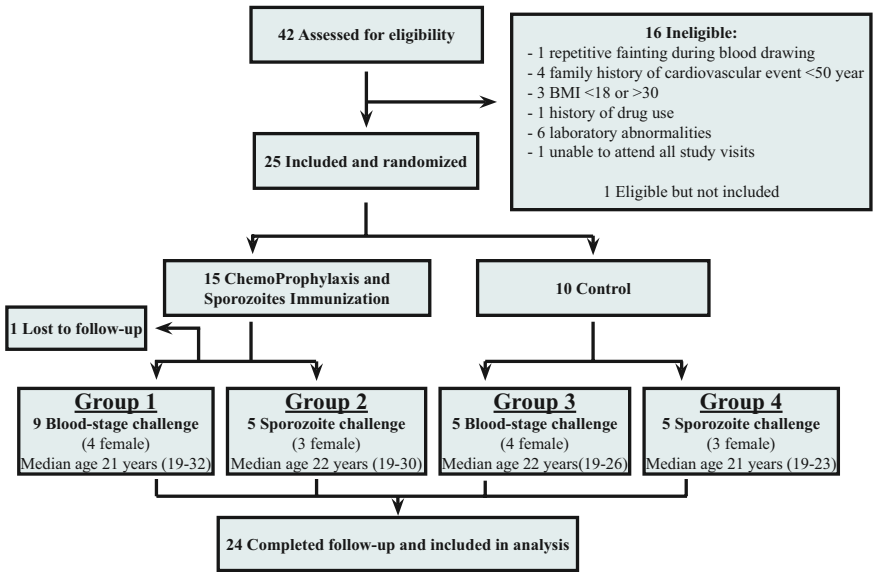

Fig. S1. Study flow diagram with relevant baseline data. Fifteen subjects were CPS immunized by the bites of 3 × 15 *P. falciparum*-infected mosquitoes while taking chloroquine prophylaxis (groups 1 and 2). Ten control subjects received chloroquine prophylaxis but no infected mosquito bites (groups 3 and 4). One subject withdrew informed consent after the immunization for reasons unrelated to the trial. Seventeen weeks after discontinuation of chloroquine prophylaxis, nine immunized (group 1) and five control volunteers (group 3) received a blood-stage challenge, whereas five immunized (group 2) and five control volunteers (group 4) received a sporozoite challenge by mosquito bites.

Table S1. Possibly or probably related solicited adverse events after challenge infection

	Blood-stage challenge				Sporozoite challenge			
	CPS-immunized (n = 9)		Control (n = 5)		CPS-immunized (n = 5)		Control (n = 5)	
Adverse event	No. of volunteers	Mean duration ± SD, d	No. of volunteers	Mean duration ± SD, d	No. of volunteers	Mean duration ± SD, d	No. of volunteers	Mean duration ± SD, d
Any								
Abdominal pain	3	1.5 ± 0.4	2	0.6 ± 0.3	0	N/A	1	0.0
Arthralgia	1	1.6	0	N/A	0	N/A	0	N/A
Chills	2	0.8 ± 0.0	0	N/A	0	N/A	1	0.0
Fatigue	3	2.2 ± 4.3	2	2.8 ± 0.3	0	N/A	2	1.9 ± 2.5
Fever	8	1.4 ± 0.6	5	1.6 ± 1.0	1	0.3	4	0.8 ± 0.3
Headache	8	1.6 ± 0.9	5	1.4 ± 1.2	2	1.4 ± 2.2	5	1.4 ± 1.1
Malaise	3	2.3 ± 1.4	0	N/A	0	N/A	2	1.5 ± 0.7
Myalgia	6	1.6 ± 0.9	3	2.9 ± 2.7	0	N/A	1	1.5
Nausea	8	0.9 ± 0.6	4	1.8 ± 1.5	2	0.4 ± 0.6	4	1.1 ± 1.5
Vomiting	4	0.9 ± 0.8	1	1.4	0	N/A	1	0.0
Any	9	1.6 ± 1.5	5	1.7 ± 0.8	3	0.8 ± 1.4	5	1.0 ± 0.8
Grade 3								
Abdominal pain	1	1.8	0	N/A	0	N/A	0	N/A
Arthralgia	0	N/A	0	N/A	0	N/A	0	N/A
Chills	0	N/A	0	N/A	0	N/A	0	N/A
Fatigue	0	N/A	1	3.0	0	N/A	1	3.7
Fever	4	1.3 ± 0.7	0	N/A	0	N/A	2	0.9 ± 0.4
Headache	1	1.0	2	1.2 ± 1.0	0	N/A	1	1.8
Malaise	3	2.3 ± 1.7	0	N/A	0	N/A	1	2.0
Myalgia	0	N/A	0	N/A	0	N/A	0	N/A
Nausea	6	0.9 ± 0.5	1	0.8	0	N/A	1	0.0
Vomiting	4	0.9 ± 0.8	1	1.4	0	N/A	1	0.0
Any	8	1.2 ± 0.9	3	1.5 ± 1.0	0	N/A	3	1.3 ± 1.3

N/A, not applicable.