ORIGINAL ARTICLE

Safety and Efficacy of Immunization with a Late-Liver-Stage Attenuated Malaria Parasite

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ABSTRACT

BACKGROUND

Currently licensed and approved malaria subunit vaccines provide modest, short-lived protection against malaria. Immunization with live-attenuated *Plasmodium falciparum* malaria parasites is an alternative vaccination strategy that has potential to improve protection.

METHODS

We conducted a double-blind, controlled clinical trial to evaluate the safety, side-effect profile, and efficacy of immunization, by means of mosquito bites, with a second-generation genetically attenuated parasite (GA2) — a *mei2* single knockout *P. falciparum* NF54 parasite (sporozoite form) with extended development into the liver stage. After an open-label dose-escalation safety phase in which participants were exposed to the bites of 15 or 50 infected mosquitoes (stage A), healthy adults who had not had malaria were randomly assigned to be exposed to 50 mosquito bites per immunization of GA2, an early-arresting parasite (GA1), or placebo (bites from uninfected mosquitoes) (stage B). After the completion of three immunization sessions with 50 mosquito bites per session, we compared the protective efficacy of GA2 against homologous *P. falciparum* controlled human malaria infection with that of GA1 and placebo. The primary end points were the number and severity of adverse events (in stages A and B) and blood-stage parasitemia greater than 100 *P. falciparum* parasites per milliliter after bites from GA2-infected mosquitoes (in stage A) and after controlled human malaria infection (in stage B).

RESULTS

Adverse events were similar across the trial groups. Protective efficacy against subsequent controlled human malaria infection was observed in 8 of 9 participants (89%) in the GA2 group, in 1 of 8 participants (13%) in the GA1 group, and in 0 of 3 participants in the placebo group. A significantly higher frequency of *P. falciparum*–specific polyfunctional CD4+ and Vδ2+ γδ T cells were observed among participants who received GA2 than among those who received GA1, whereas GA2 and GA1 induced similar antibody titers targeting the *P. falciparum* circumsporozoite protein.

CONCLUSIONS

In this small trial, GA2 was associated with a favorable immune induction profile and protective efficacy, findings that warrant further evaluation. (Funded by the Bontius Foundation; ClinicalTrials.gov number, NCT04577066.)

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The progress in the eradication of malaria has slowed, creating a need for new tools.[1] The currently licensed malaria vaccines approved by the World Health Organization, RTS,S/AS01 (Mosquirix) and R21, are subunit vaccines based on the major sporozoite surface antigen, circumsporozoite protein (CSP). The resulting protection is modest (50 to 80%), short-lived (approximately 1 year), and, in high endemic areas, lower in infants than in children.[2,3] Alternative vaccination strategies based on whole, genetically attenuated plasmodium parasites have the potential to improve protection.[4] Metabolically active attenuated sporozoites invade hepatocytes in the first (asymptomatic) stage of the parasite's life cycle but fail to develop into a patent blood-stage infection. Inoculation with attenuated whole sporozoites safely exposes the immune system to a broad array of parasite antigens, which results in both humoral and cellular immune responses.[5-8]

The most advanced whole-sporozoite vaccine candidate is the *Plasmodium falciparum* sporozoite (PfSPZ) vaccine, composed of radiation-attenuated *P. falciparum* sporozoites, in which development is arrested soon after they invade the hepatocytes. Although this vaccine is considered to be safe (i.e., no breakthrough blood-stage infections), high doses of radiation-attenuated sporozoites are needed in order to induce protection, which remains at suboptimal levels in persons previously exposed to malaria.[9-14] In an experimental medicine setting, repeated inoculations with *P. falciparum* attenuated through concomitant administration of chloroquine chemoprophylaxis, which kills the parasites only during the blood stage, induced 100% protection against malaria with low numbers of sporozoites.[15,16] In this model, the late arrest of parasites exposes the immune system to a broad array of antigens expressed in sporozoites, intrahepatic-stage parasites, and a small number of asexual blood-stage parasites, thereby improving protective efficacy.

The identification of genes essential to the parasite's intrahepatic development facilitated the creation of genetically attenuated malaria parasites, which can be produced and are amenable to vaccine applications.[17] We previously tested *PfΔb9Δslarp* (GA1), which has short intrahepatic development (24 hours), similar to that of radiation-attenuated sporozoites.[18,19] That trial showed protective efficacy against homologous *P. falciparum* controlled human malaria infection in 3 of 25 participants (12%) who had not had malaria.[18]

We then generated *PfΔmei2* (GA2), a parasite that shows complete growth arrest late during the liver stage (6 days after invasion) in humanized mouse models, similar to that in parasites that are coadministered with chemoprophylaxis but without any blood-stage exposure.[20,21] Here, we report results of a clinical trial designed to test the concept that extended liver-stage antigen exposure can induce better protection than that induced by GA1. To that end, we compared the safety, side-effect profile, and preliminary protective efficacy of late-arresting GA2 against *P. falciparum* controlled human malaria infection with that of early-arresting GA1 and placebo in healthy adults who had not had malaria.

METHODS

TRIAL DESIGN AND OVERSIGHT

We conducted this multistage trial at Leiden University Medical Center and Radboud University Medical Center in the Netherlands. The trial consisted of two stages: an open-label, dose-escalation safety phase (stage A), in which participants were exposed to the bites of either 15 or 50 mosquitoes infected with GA2, and a double-blind, placebo-controlled phase (stage B), in which we compared the protective efficacy of GA2 against homologous controlled human malaria infection with that of GA1 and placebo (bites from uninfected mosquitoes) after the completion of three immunization sessions, administered at 28-day intervals. In stage B, participants were exposed to the bites of 50 mosquitoes in each session. Owing to the coronavirus disease 2019 pandemic and lockdown restrictions, the trial was conducted with fewer participants than originally envisaged (see the trial protocol, available with the full text of this article at NEJM.org), with the approval of the data and safety monitoring committee.

Three weeks after completion of the immunization phase in stage B, all participants underwent controlled human malaria infection by means of 5 bites from mosquitoes infected with unattenuated *P. falciparum* parasite strain 3D7 (Pf3D7) (Fig. 1). Pf3D7 is an unattenuated clone of the parental parasite strain PfNF54,[22] which was used to generate GA2 and GA1.

The trial protocol, available with the full text of

this article at NEJM.org, was approved by the Dutch Central Committee for Research Involving Human Subjects. The trial was performed with a license from the Dutch Gene Therapy Office and overseen by a data and safety monitoring committee.

Recruitment and follow-up of participants occurred at Leiden University Medical Center. Culturing of GA2, GA1, and unattenuated Pf3D7 parasites, the rearing of *Anopheles stephensi* mosquitoes, and the exposure of participants to mosquito bites were performed at Radboud University Medical Center. Both teams at Radboud University Medical Center and Leiden University Medical Center analyzed the data and prepared the first draft of the manuscript. The authors vouch for the integrity, completeness, and accuracy of the data and for the fidelity of the trial to the protocol and statistical analysis plan.

PRIMARY AND SECONDARY END POINTS

The primary end points in stage A were the number and severity of adverse events after exposure to GA2 and the number of participants with blood-stage parasitemia (defined as >100 *P. falciparum* parasites per milliliter), as assessed by means of a quantitative polymerase-chain-reaction (qPCR) assay, performed after immunization with bites from GA2-infected mosquitoes. In stage B, the primary end points were the number and severity of adverse events after exposure to GA2 and the number of participants with blood-stage parasitemia after controlled human malaria infection, assessed by means of a qPCR assay, to measure protective efficacy.

The secondary end points in both stages were humoral immune responses, as assessed by the measurement of plasma antibodies targeting key *P. falciparum* antigens with the use of an enzyme-linked immunosorbent assay (ELISA), and cellular immune responses in peripheral blood mononuclear cells that were stimulated with *P. falciparum*–infected erythrocytes and uninfected erythrocytes in vitro and subsequently phenotyped and profiled for cytokine expression by means of spectral-flow cytometry. Details on these methods are provided in the Supplementary Appendix.

RECRUITMENT AND RANDOMIZATION

Adults who were 18 to 35 years of age and in good health, as assessed by medical history, physical examination, general laboratory evaluation (including biochemical and hematologic testing, toxicologic screening for illicit drugs, and electrocardiography) were included. Women who had reproductive potential were required to use adequate birth control throughout the trial. All participants provided written informed consent before inclusion in the trial. A list of inclusion and exclusion criteria is provided in the protocol and Supplementary Appendix.

Randomization was conducted by an independent statistician. All staff who had contact with participants were unaware of the group assignments throughout the trial.

STATISTICAL ANALYSIS

No formal sample size calculations were performed for stage A of the trial. For stage B, we aimed for a total of 10 participants to undergo controlled human malaria infection by means of mosquito bites in the GA2 group; we calculated that this sample size would provide the trial with 80% power, at a two-sided alpha level of 5%, to detect 70% protective efficacy of GA2 against *P. falciparum* infection, as compared with placebo.

Adverse events were assessed in the intention-to-treat population, which consisted of all participants who were exposed to the GA2 parasite in stage A and all participants who underwent at least one immunization in stage B. Analyses of efficacy and immunologic response were conducted in the per-protocol population, which consisted of all participants who completed follow-up. We calculated the percentage of participants in each cohort or group who reported mild, moderate, or severe adverse events. The presence of parasitemia was analyzed by means of a chi-square test and Fisher's exact test.

Some analyses of humoral and cellular immune responses were prespecified in the protocol and statistical analysis plan. Serologic data were log-transformed to follow a normal distribution. Values obtained before and after exposure were compared with the use of a paired t-test, and comparisons between groups were performed with the use of an unpaired t-test. Discrete variables were analyzed with the use of the two-sided chi-square or Fisher's exact test. Cellular responses between the groups were compared with the use of the two-sided Mann-Whitney test. P values of less than 0.05 were used to indicate statistical significance, and no adjustment was made for multiplicity. Graphs were generated with the use of GraphPad Prism, version 9.3.1, and R software, version 4.2.1.

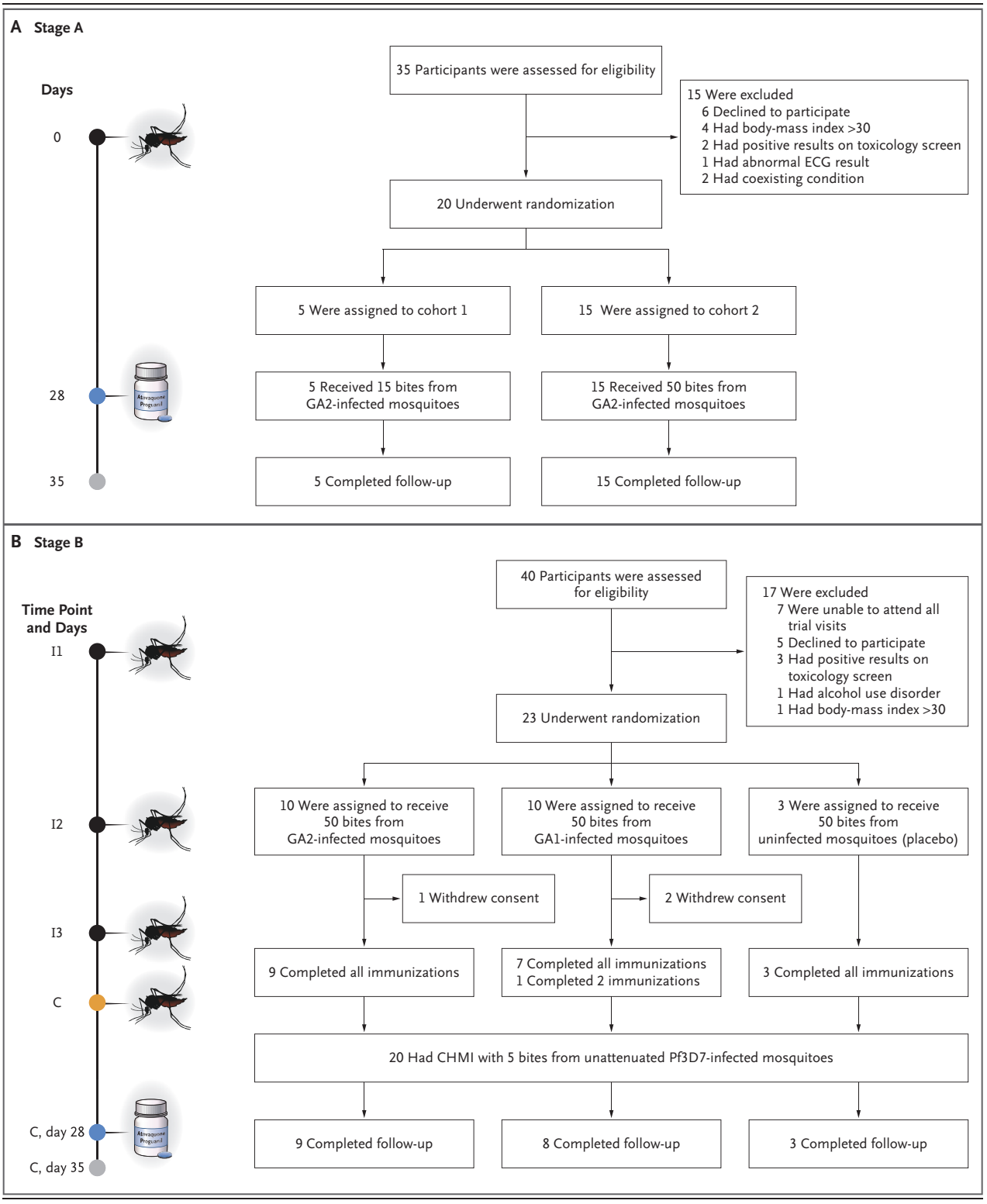

Figure 1 (facing page). Clinical Trial Design.
Stage A, shown in Panel A, was an open-label phase in which eligible participants were assigned sequentially into cohort 1 and then cohort 2. In the dose-escalation phase of stage A, participants underwent one exposure to GA2 by means of mosquito bites (black circle); cohort 1 received 15 mosquito bites, and cohort 2 received 50 mosquito bites. Stage B, shown in Panel B, was a double-blind stage, in which eligible participants were randomly assigned to one of three groups; participants were exposed three times to 50 bites from GA2-infected mosquitoes, 50 bites from GA1-infected mosquitoes, or 50 bites from uninfected mosquitoes (placebo) at 28-day intervals (black circles). Three weeks after the last immunizing exposure, participants underwent controlled human malaria infection (CHMI) by means of 5 bites from mosquitoes infected with unattenuated wild-type *P. falciparum* strain 3D7 (Pf3D7) (orange circle). All participants were treated with a curative regimen of atovaquone–proguanil, 28 days, at the latest, after their final exposure (blue circles). Throughout the trial, participants were monitored strictly and had to attend multiple ambulatory visits. The final ambulatory visit occurred 35 days after exposure to GA2 in stage A and 35 days after CHMI in stage B (gray circles). Body-mass index is the weight in kilograms divided by the square of the height in meters. C denotes challenge, ECG electrocardiogram, and I immunization.

RESULTS

TRIAL POPULATION

From September 13, 2021, to January 28, 2022, a total of 75 adults who had not had malaria underwent screening, of whom 43 were enrolled in the trial. No participants withdrew from the trial during stage A. In stage B, 3 participants withdrew consent before they could undergo controlled human malaria infection (Fig. 1). A total of 22 participants (51%) were women. The median age was 23 years (range, 19 to 35) and the median body-mass index (BMI, the weight in kilograms divided by the square of the height in meters) was 24.1 (range 18.0 to 29.9) (Table 1).

SAFETY

No serious adverse events occurred during the trial. No breakthrough infections occurred after exposure to either 15 or 50 bites from GA2-infected mosquitoes, as assessed by qPCR. Among participants in the intention-to-treat population, no significant difference in the incidence and severity of adverse events was observed after bites from GA2-infected mosquitoes, GA1-infected mosquitoes, or uninfected mosquitoes (placebo).

The most common local adverse events that were considered by the investigators to be related to the trial intervention were erythema and pruritus after mosquito bites, some of which were treated with antihistamines or topical corticosteroids. In stage A, erythema was reported by 19 participants (95%) and pruritus by 17 participants (85%) (Fig. S1A and Table S1 in the Supplementary Appendix); in stage B, erythema and pruritus were reported by 23 (100%) and 22 participants (96%), respectively (Fig. S1B through S1D and Table S3). The most common systemic adverse event considered by the investigators to be related to the trial intervention was myalgia (in 4 participants [20%]) in stage A and headache (in 2 participants [9%]) during the immunization phase of stage B. Adverse events that were not considered by the investigators to be related to the trial intervention are summarized in Table S2 and Table S4.

Two participants had elevated levels of troponin T (18 and 19 ng per milliliter; upper limit of the normal range, 14 ng per milliliter); the investigator assessed these elevations to be unrelated to the trial intervention. Another patient had elevated results of liver-function tests that were considered by investigator to be related to antihistamine use.

The clinical course after controlled human malaria infection in stage B was consistent with that in previous trials involving controlled human malaria infection at our center (Fig. S2 and Tables S5 and S6).[23]

PROTECTIVE EFFICACY AGAINST CONTROLLED HUMAN MALARIA INFECTION

All participants who completed the immunization phase in stage B (9 participants in the GA2 group, 8 in the GA1 group, and 3 in the placebo group) underwent controlled human malaria infection. Participants received 5 bites from unattenuated Pf3D7-infected mosquitoes, administered 3 weeks after the last immunization.

Immunization with GA2 resulted in 89% protection (95% confidence interval [CI], 31 to 98): 8 of the 9 participants in the GA2 group had

negative qPCR tests until the end of the trial; 1 participant had a positive qPCR test for *P. falciparum* 11 days after controlled human malaria infection. Protective efficacy was observed in no participants in the placebo group (0 of 3); the median time to parasitemia was 12 days (interquartile range, 10.5 to 13.5) after controlled human malaria infection (P=0.005 by a chi-square test and P=0.02 by Fisher's exact test for the difference between the GA2 group and the placebo group in the per-protocol population). In contrast, protection was observed in 1 of 8 participants who underwent immunization with bites from GA1-infected mosquitoes, with parasitemia that developed in the other 7 participants by a median of 11 days (interquartile range, 9.8 to 12.2). In a post hoc comparison, protection against controlled human malaria infection differed significantly between the GA2 group and the GA1 group in the per-protocol population (P=0.002 by chi-square test; P=0.003 by Fisher's exact test; and P=0.002 by log-rank test) (Fig. 2).

IMMUNOGENICITY

In the GA2 and GA1 groups combined, we detected higher titers of antibodies against *P. falciparum* CSP (PfCSP) in participants on the day before challenge (1.33±0.35 μg per milliliter) than at baseline (0.07±0.35 μg per milliliter), a difference of 1.26 μg per milliliter (95% CI, 1.07 to 1.44; P<0.001 by paired t-test). On the day before challenge, titers of antibodies in the GA2 and GA1 groups combined were also higher than those observed in the placebo group (1.33±0.35 vs. 0.01±0.16), a between-group difference of 1.32 μg per milliliter (95% CI, 0.89 to 1.77; P<0.001 by independent samples t-test) (Fig. 3). However, titers did not differ between participants immunized with bites from GA2-infected mosquitoes (1.2±0.3 μg per milliliter) and participants immunized with bites from GA1-infected mosquitoes (1.5±0.4 μg per milliliter), or between protected (1.2±0.3 μg per milliliter) and unprotected (1.4±0.4 μg per milliliter) participants. We did not detect notable changes in antibodies against *P. falciparum* apical membrane antigen 1 (PfAMA1) from baseline to one day before challenge (0.63±0.52 μg per milliliter vs. 0.57±0.52 μg per milliliter) or against a fragment of the *P. falciparum* merozoite surface protein 1 (PfMSP1) from baseline to one day before challenge (−0.27±0.2 μg per milliliter vs. −0.23±0.3 μg per milliliter).

To study cellular immunity specific to *P. falciparum*, the peripheral-blood mononuclear cells of participants were stimulated with *P. falciparum*–infected red cells and uninfected red cells and then assessed by means of flow cytometry (Figs. S3 through S5). Although the overall cellular frequency of all studied T-cell lineages remained similar, we observed an increase in γδ T cells expressing the Vδ2 chain in participants who received bites from GA2-infected mosquitoes but not in those who received bites from GA1-infected mosquitoes (Fig. S6A through E). From a functional perspective, immunization induced *P. falciparum*–specific CD4+ T cells with a strong type 1 inflammatory signature (interferon-γ, tumor necrosis factor α [TNF-α], and interleukin-2) but not a type 2 or regulatory signature (interleukin-10, interleukin-4, interleukin-5, and interleukin-13) (Fig. 4A). However, a higher frequency of *P. falciparum*–specific CD4+ T cells was observed in the GA2 group than in the GA1 group. Similarly, *P. falciparum*–specific Vδ2+ γδ T cells expressing type 1 cytokines were induced only in participants who received GA2 (Fig. 4B). *P. falciparum*–specific

Table 1. Demographics of the Participants at Baseline.

Characteristic	Stage A		Stage B			Total
	Cohort 1 (N=5)	Cohort 2 (N=15)	GA2 (N=10)	GA1 (N=10)	Placebo (N=3)	(N=43)
Median age (range) — yr	22 (20–27)	23 (19–30)	23.5 (19–31)	23.5 (19–35)	22 (21–24)	23 (19–35)
Sex						
Female	4	8	5	3	2	22
Male	1	7	5	7	1	21
Median BMI (range)	24.8 (21.9–26.9)	24.1 (20.3–29.1)	22.4 (18.0–29.9)	22.6 (19.9–28.6)	27.5 (24.4–28.7)	24.1 (18.0–29.9)

responses were not detected in CD8+, Vδ2– γδ, and natural-killer T-cell compartments (Fig. S6F through H).

Profiling the cytokine expression at a single T-cell level showed that higher frequencies of polyfunctional CD4+ and Vδ2+ γδ T cells expressing more than one cytokine acquiring an effector memory phenotype were observed in the GA2-group than in the GA1 group. These polyfunctional T cells expressed combinations of interferon-γ, TNF-α, and interleukin-2, with the preferential expression of TNF-α and interleukin-2 in CD4+ T cells and interferon-γ and TNF-α in Vδ2+ γδ T cells.

DISCUSSION

This trial showed evidence of higher cellular immunogenicity and protective efficacy with a late-arresting genetically attenuated parasite (GA2) than with an early-arresting parasite (GA1), which indicates a potential role for late-liver-stage antigens in eliciting protective immunity to malaria. Protection was not associated with antibodies to PfCSP, AMA1, or PfMSP1 but was related to the induction of *P. falciparum*–specific polyfunctional CD4+ T cells and Vδ2+ γδ T cells.

The protection induced by late-arresting GA2 in this trial (89%) is higher than that seen in previous trials involving replication-incompetent, early-arresting attenuated sporozoites. Early-arresting genetically attenuated parasites, delivered by means of either intravenous injection or mosquito bites, provided lower protective efficacy (12% by GA1 and 50% by PfGAP3KO[18,24]). Protection induced by radiation-attenuated sporozoites has varied from 50% to greater than 90% when administered intravenously (dose range, 1.35×10^5 to 2.7×10^6 for the PfSPZ vaccine) in homologous controlled human malaria infection.[4,25-29] However, administration of radiation-attenuated sporozoites by means of mosquito bites requires higher doses (approximately 1000 mosquito bites) to reach levels of protection similar to the level offered by GA2.[13] In contrast, the protection induced by bites from GA2-infected mosquitoes in this trial is reminiscent of that from parasites coadministered with chemoprophylaxis, in which three exposures to bites of 15 infected mosquitoes resulted in 100% efficacy against homologous controlled human malaria infection,[15,30] which supports the hypothesis that extended hepatic antigenic presentation may have a role in the induction of protective immunity.

To explore potential correlates of protection, we measured both circulating antibodies and cellular responses. We found that anti-PfCSP antibodies were induced in response to bites from GA2-infected and GA1-infected mosquitoes, a finding consistent with previous reports.[4] This finding suggests that the differential arrest in the liver did not affect antisporozoite antibody responses quantitatively. A lack of plasma antibodies against late-liver-stage antigens (PfAMA1 and PfMSP1) indicates that exposure to those antigens is insufficient to mount a measurable antibody response.

In contrast to the antibody responses, differences between the GA2 and GA1 groups were observed in the *P. falciparum*–specific cellular

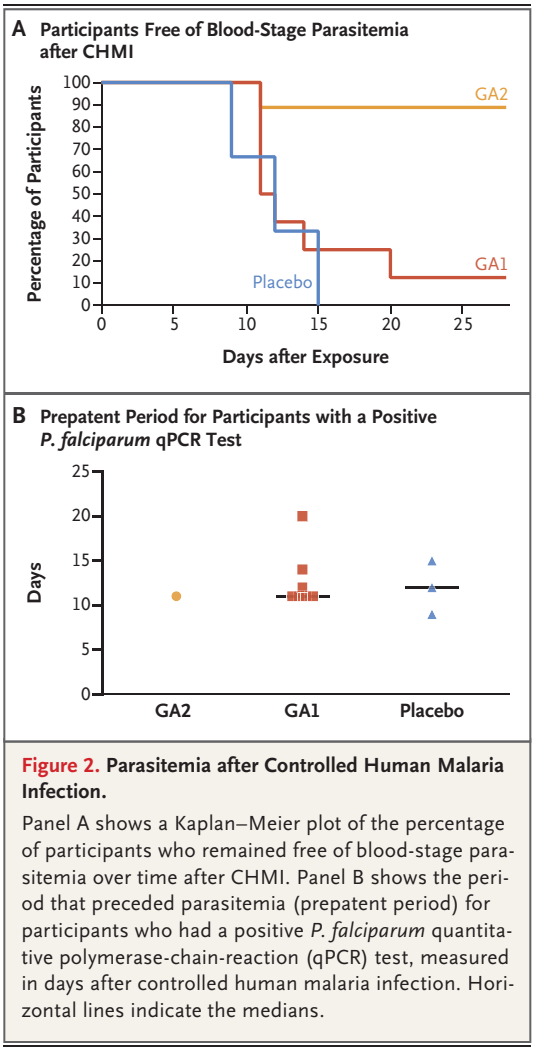

Figure 2. Parasitemia after Controlled Human Malaria Infection.

Panel A shows a Kaplan–Meier plot of the percentage of participants who remained free of blood-stage parasitemia over time after CHMI. Panel B shows the period that preceded parasitemia (prepatent period) for participants who had a positive *P. falciparum* quantitative polymerase-chain-reaction (qPCR) test, measured in days after controlled human malaria infection. Horizontal lines indicate the medians.

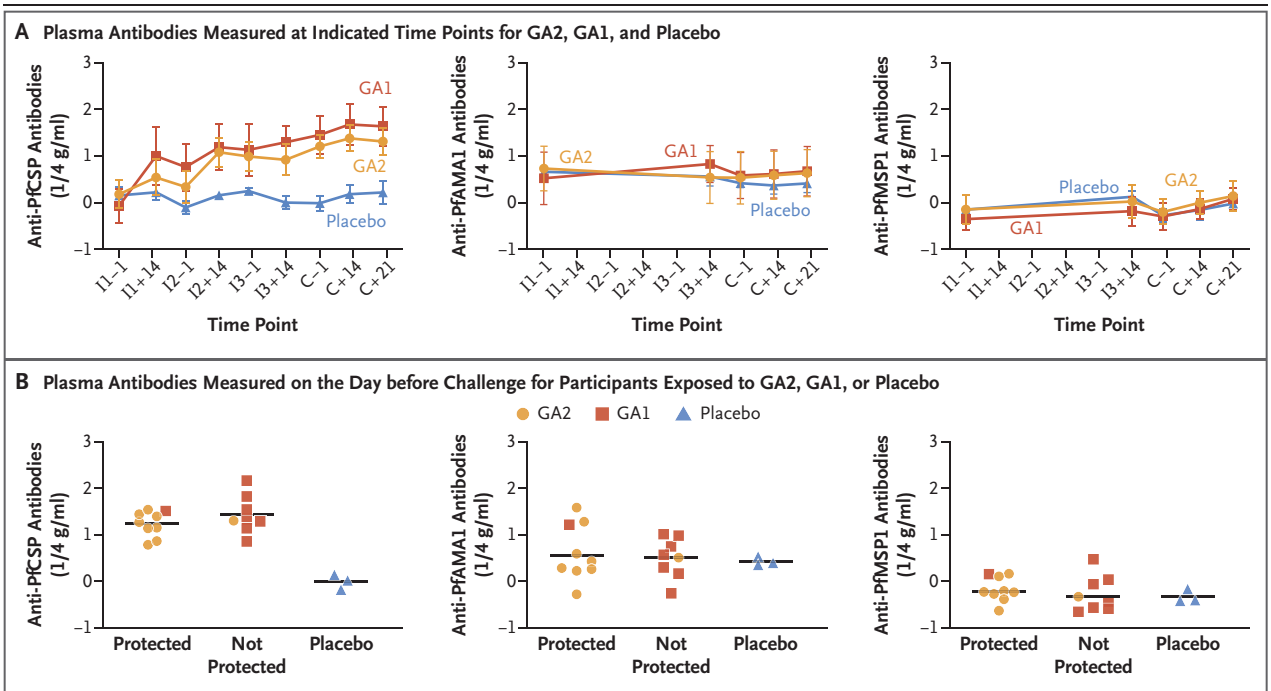

Figure 3. Humoral Immune Responses.

Panel A shows the concentrations of plasma antibodies against *P. falciparum* circumsporozoite protein (PfCSP), *P. falciparum* apical membrane antigen 1 (PfAMA1), and *P. falciparum* merozoite surface protein 1 (PfMSP1), measured at the indicated time points for the participants who received bites from GA2-infected mosquitoes (orange circles), bites from GA1-infected mosquitoes (red squares) and placebo (bites from uninfected mosquitoes) (blue triangles). Symbols indicate the means, and I bars standard deviations. Days relative to the first immunization (I1), second immunization (I2), third immunization (I3), and challenge (C) are indicated as time points on x axes. Panel B shows frequencies of plasma anti-PfCSP, anti-PfAMA1, and anti-PfMSP1 antibodies on the day before challenge for individual participants exposed to bites from GA1-infected mosquitoes (orange circles), bites from GA1-infected mosquitoes (red squares), and placebo (bites from uninfected mosquitoes) (blue triangles). Horizontal lines indicate means. All values are log transformed.

immune responses. Although we observed a strong proinflammatory CD4+ T-cell response in both groups, induction of *P. falciparum*–specific polyfunctional effector memory CD4+ T cells was more robust in participants who received bites from GA2-infected mosquitoes than in those who received bites from GA1-infected mosquitoes. GA2 also more efficiently induced monofunctional and polyfunctional Vδ2+ γδ T cells with a strong proinflammatory signature. These findings are in line with previous studies of attenuated sporozoites that have shown Vδ2+ γδ T cells to be a marker of protective immunity.[16,26]

The induction of γδ T cells was observed not only after immunization with parasites coadministered with chemoprophylaxis, but also after high-dose immunization with radiation-attenuated sporozoites, in which the duration of liver-stage antigen exposure is limited.[26,31] However, a recent study showed that Vδ2+ γδ T-cell induction was dose-dependent in an early-arresting chemo-attenuation strategy.[9] In contrast, late chemo-attenuation induced Vδ2+ γδ T cells irrespective of the dose. This suggests an important independent role of late-liver-stage antigens in inducing γδ T-cell responses.[9]

Studies with attenuated sporozoites in mice and nonhuman primates have indicated a central role for effector memory-like liver-resident CD8+ T cells.[32,33] However, detection of *P. falciparum*–specific CD8+ T cells in peripheral blood of participants is generally limited,[18,25,34-36] although CD8+ T cells can be present at frequencies up to 100 times higher in the liver than in the blood.[26,35] Hence, whether there is any limitation in induction or recirculation of tissue-resident *P. falciparum*–specific CD8+ T cells in humans immunized with attenuated sporozoites remains unclear. In line with these studies, we did not detect *P. falciparum*–specific CD8+ T cells in peripheral

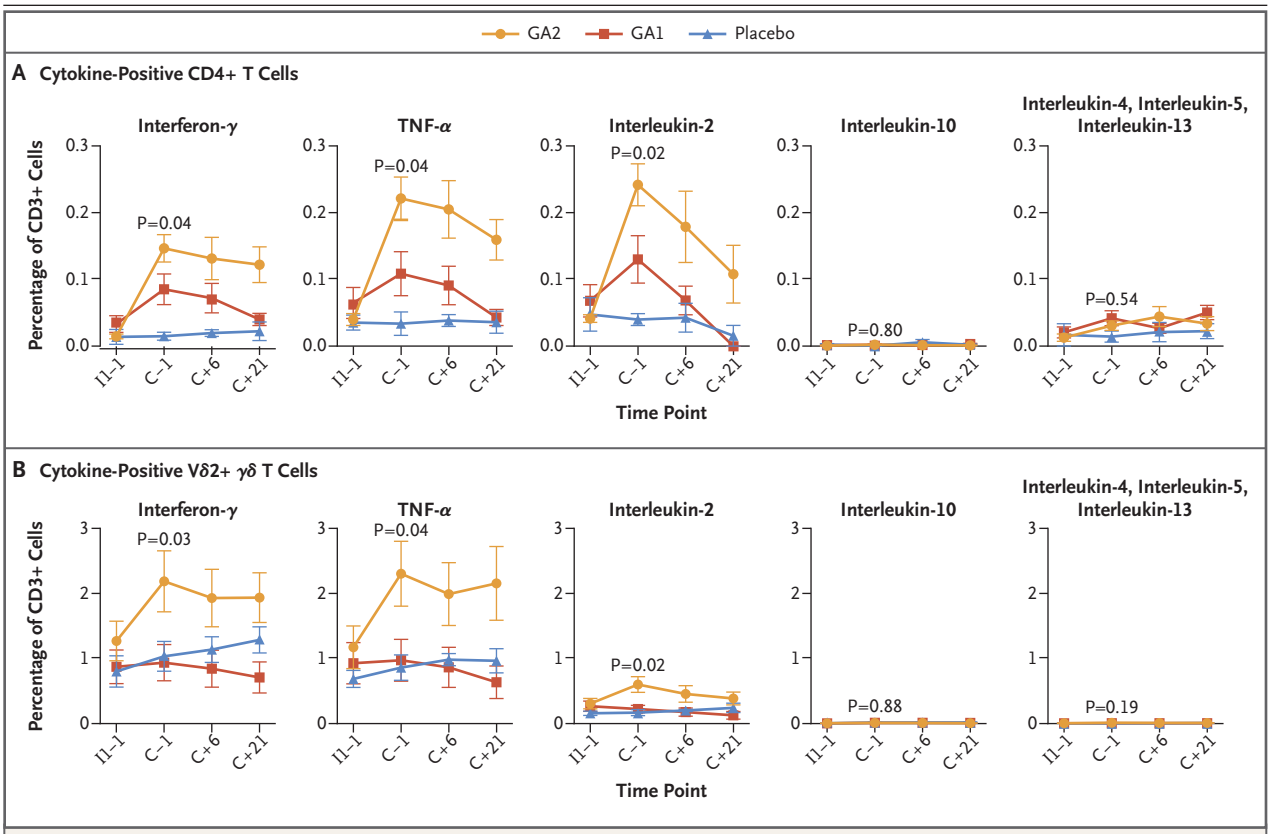

Figure 4. Proinflammatory CD4+ and Vδ2+ γδ T-cell Responses with GA2 as Compared with GA1 and Placebo.

Shown are the percentages of CD4+ T cells (Panel A) and Vδ2+ γδ T cells (Panel B) secreting indicated cytokines at baseline (I1–1), one day before challenge (C–1), and 6 and 21 days after challenge (C+6 and C+21). TNF-α denotes tumor necrosis factor α.

blood in any of the immunized participants when assessed with the use of general flow cytometric tools.

Safety and a lack of blood-stage breakthrough infections are important considerations for the further development of genetically attenuated vaccines for malaria. Because a *mei2* knockout resulted in blood-stage breakthrough infections in rodent models (*P. yoellii* and *P. berghei*), we used a dose-escalation approach in case of functional redundancy of the MEI2 protein.[21,37] However, preclinical studies showed that the *mei2* knockout in *P. falciparum* did not result in blood-stage breakthrough infections, as tested in the humanized mouse model.[21] The results of this trial support this attenuation phenotype in *P. falciparum*, with no evidence of blood-stage parasites after multiple doses (50 mosquito bites) in 25 participants.

Nonetheless, the conclusions from this trial are limited by the small sample size and the large number of immunologic analyses. More studies with greater numbers of participants are required to better understand the safety profile of GA2. In addition, the immunologic assessments were exploratory, and the relevance of the variables associated with GA2-induced protection needs confirmation. To translate the promising efficacy results to the broader population affected by malaria (Table S7), the immunogenicity of GA2 needs to be assessed for durability and against heterologous *P. falciparum* strains in regions in which malaria is endemic.[7] Previous studies showed that coadministration of parasites with chemoprophylaxis can induce protection for up to 28 months after immunization[38] and protection against heterologous challenge.[9] Replicating such findings in malaria-endemic areas is critical to determine the potential for late-arresting attenuated sporozoites. Our findings suggest that parasites arresting late during the liver stage (GA2) offer improved protection, as compared with early-arresting sporozoites

(GA1), and provide a step toward a next-generation malaria vaccine.

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A data sharing statement provided by the authors is available with the full text of this article at NEJM.org.

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APPENDIX

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REFERENCES

1. World Health Organization. World malaria report 2021 (https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2021).
2. RTS,S Clinical Trials Partnership. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. Lancet 2015;386:31-45.
3. Datoo MS, Natama MH, Somé A, et al. Efficacy of a low-dose candidate malaria vaccine, R21 in adjuvant Matrix-M, with seasonal administration to children in Burkina Faso: a randomised controlled trial. Lancet 2021;397:1809-18.
4. Richie TL, Church LWP, Murshedkar T, et al. Sporozoite immunization: innovative translational science to support the fight against malaria. Expert Rev Vaccines 2023;22:964-1007.
5. Richie TL, Billingsley PF, Sim BKL, et al. Progress with *Plasmodium falciparum* sporozoite (PfSPZ)-based malaria vaccines. Vaccine 2015;33:7452-61.
6. Hollingdale MR, Sedegah M. Development of whole sporozoite malaria vaccines. Expert Rev Vaccines 2017;16:45-54.
7. Duffy PE, Patrick Gorres J. Malaria vaccines since 2000: progress, priorities, products. NPJ Vaccines 2020;5:48.
8. Goh YS, McGuire D, Rénia L. Vaccination with sporozoites: models and correlates of protection. Front Immunol 2019;10:1227.
9. Mwakingwe-Omari A, Healy SA, Lane J, et al. Two chemoattenuated PfSPZ malaria vaccines induce sterile hepatic immunity. Nature 2021;595:289-94.
10. Jongo SA, Church LWP, Mtoro AT, et al. Increase of dose associated with decrease in protection against controlled human malaria infection by PfSPZ vaccine in Tanzanian adults. Clin Infect Dis 2020;71:2849-57.
11. Jongo SA, Church LWP, Nchama VUNN, et al. Multi-dose priming regimens of PfSPZ vaccine: safety and efficacy against controlled human malaria infection in equatoguinean adults. Am J Trop Med Hyg 2022;106:1215-26.
12. Jongo SA, Shekalaghe SA, Church LWP, et al. Safety, immunogenicity, and protective efficacy against controlled human malaria infection of *Plasmodium falciparum* sporozoite vaccine in Tanzanian adults. Am J Trop Med Hyg 2018;99:338-49.
13. Hoffman SL, Goh LML, Luke TC, et al. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. J Infect Dis 2002;185:1155-64.
14. Sissoko MS, Healy SA, Katile A, et al. Safety and efficacy of a three-dose regimen of *Plasmodium falciparum* sporozoite vaccine in adults during an intense malaria transmission season in Mali: a randomised, controlled phase 1 trial. Lancet Infect Dis 2022;22:377-89.
15. Roestenberg M, McCall M, Hopman J, et al. Protection against a malaria challenge by sporozoite inoculation. N Engl J Med 2009;361:468-77.
16. Mordmüller B, Surat G, Lagler H, et al. Sterile protection against human malaria by chemoattenuated PfSPZ vaccine. Nature 2017;542:445-9.
17. Vaughan AM, Kappe SHI. Genetically attenuated malaria parasites as vaccines. Expert Rev Vaccines 2017;16:765-7.
18. Roestenberg M, Walk J, van der Boor SC, et al. A double-blind, placebo-controlled phase 1/2a trial of the genetically attenuated malaria vaccine PfSPZ-GA1. Sci Transl Med 2020;12(544):eaaz5629.
19. van Schaijk BCL, Ploemen IHJ, Annoura T, et al. A genetically attenuated malaria vaccine candidate based on *P. falciparum b9/slarp* gene-deficient sporozoites. Elife 2014;3:e03582.
20. Goswami D, Betz W, Locham NK, et al. A replication-competent late liver stage-attenuated human malaria parasite. JCI Insight 2020;5(13):e135589.
21. Franke-Fayard B, Marin-Mogollon C, Geurten FJA, et al. Creation and preclinical evaluation of genetically attenuated malaria parasites arresting growth late in the liver. NPJ Vaccines 2022;7:139.
22. Walliker D, Quakyi IA, Wellems TE, et al. Genetic analysis of the human malaria parasite *Plasmodium falciparum*. Science 1987;236:1661-6.
23. Roestenberg M, Mordmüller B, Ockenhouse C, Mo A, Yazdanbakhsh M, Kremsner PG. The frontline of controlled human malaria infections: a report from the controlled human infection models workshop in Leiden University Medical Centre 5 May 2016. Vaccine 2017;35:7065-9.
24. Murphy SC, Vaughan AM, Kublin JG,

et al. A genetically engineered *Plasmodium falciparum* parasite vaccine provides protection from controlled human malaria infection. Sci Transl Med 2022;14(659): eabn9709.
25. Seder RA, Chang L-J, Enama ME, et al. Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. Science 2013;341: 1359-65.
26. Ishizuka AS, Lyke KE, DeZure A, et al. Protection against malaria at 1 year and immune correlates following PfSPZ vaccination. Nat Med 2016;22:614-23.
27. Epstein JE, Paolino KM, Richie TL, et al. Protection against *Plasmodium falciparum* malaria by PfSPZ vaccine. JCI Insight 2017;2(1):e89154.
28. Lyke KE, Ishizuka AS, Berry AA, et al. Attenuated PfSPZ vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection. Proc Natl Acad Sci U S A 2017;114:2711-6.
29. Mordmüller B, Sulyok Z, Sulyok M, et al. A PfSPZ vaccine immunization regimen equally protective against homologous and heterologous controlled human malaria infection. NPJ Vaccines 2022;7:100.
30. Bijker EM, Bastiaens GJH, Teirlinck AC, et al. Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by pre-erythrocytic immunity. Proc Natl Acad Sci U S A 2013;110:7862-7.
31. Sissoko MS, Healy SA, Katile A, et al. Safety and efficacy of PfSPZ vaccine against *Plasmodium falciparum* via direct venous inoculation in healthy malaria-exposed adults in Mali: a randomised, double-blind phase 1 trial. Lancet Infect Dis 2017;17: 498-509.
32. Nunes-Cabaço H, Moita D, Prudêncio M. Five decades of clinical assessment of whole-sporozoite malaria vaccines. Front Immunol 2022;13:977472.
33. Butler NS, Schmidt NW, Vaughan AM, Aly AS, Kappe SHI, Harty JT. Superior antimalarial immunity after vaccination with late liver stage-arresting genetically attenuated parasites. Cell Host Microbe 2011;9:451-62.
34. Hoffman SL, Doolan DL. Malaria vaccines-targeting infected hepatocytes. Nat Med 2000;6:1218-9.
35. Epstein JE, Tewari K, Lyke KE, et al. Live attenuated malaria vaccine designed to protect through hepatic $CD8^+$ T cell immunity. Science 2011;334:475-80.
36. Spring M, Murphy J, Nielsen R, et al. First-in-human evaluation of genetically attenuated *Plasmodium falciparum* sporozoites administered by bite of Anopheles mosquitoes to adult volunteers. Vaccine 2013;31:4975-83.
37. Vaughan AM, Sack BK, Dankwa D, et al. A *Plasmodium* parasite with complete late liver stage arrest protects against pre-erythrocytic and erythrocytic stage infection in mice. Infect Immun 2018;86(5): e00088-18.
38. Roestenberg M, Teirlinck AC, McCall MBB, et al. Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study. Lancet 2011;377:1770-6.