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# Malaria vaccinology: from the development of a new malaria vaccine to the ethics of clinical trial participation

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# Chapter 2

# Safety and efficacy of immunization with a late-liver-stage attenuated malaria parasite

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# Abstract

Background: Immunization with live-attenuated *Plasmodium falciparum* (*Pf*) malaria parasites is an alternative vaccination strategy to existing subunit vaccines. We report the safety and efficacy of GA2, a *mei2* single knock out *Pf*NF54 parasite (sporozoite form) with extended development into the liver-stage.

Methods: We conducted a comparator-controlled double-blind clinical trial, assessing safety, tolerability and efficacy of mosquito bite (MB) immunization with GA2 in healthy malaria-naïve adults, dose-escalated from 15 to 50 MB per immunization. Following 3 immunizations with 50 MB, protective efficacy against homologous *Pf* controlled human malaria infection (CHMI) was compared to that of an early-arresting parasite (GA1) and placebo.

Results: Adverse events were comparable across dose groups. GA2 resulted in protection in 8/9 participants (89%) against subsequent CHMI, compared to 1/8 (13%) for GA1 and protection in 0/3 amongst placebo recipients. A significantly higher frequency of *Pf*-specific polyfunctional $CD4^+$ and $V\delta2^+$ $\gamma\delta$ T cells was observed in GA2 compared to GA1 recipients, whereas both GA2 and GA1 induced comparable anti-*Pf* circumsporozoite protein antibody titers.

Conclusion: In this small study, the favorable immune induction profile and protective efficacy of GA2 warrant further evaluation.
Trial registration number: NCT04577066

# Introduction

The progress in the eradication of malaria has stalled, creating a need for novel tools[1]. The currently licensed and WHO-approved malaria vaccines, Mosquirix (RTS,S/AS01) and R21, are subunit vaccines based on the major sporozoite surface antigen, circumsporozoite protein (CSP). The resulting protection is modest (50-80%), short-lived (approximately 1 year) and in high endemic areas lower in infants compared to children[2,3]. Alternate vaccine strategies based on whole attenuated *Plasmodium* parasites have the potential to improve protection[4]. Metabolically active attenuated sporozoites invade hepatocytes, the first (asymptomatic) stage of the parasite's lifecycle, 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, eliciting both humoral and cellular immune responses[5-8].

The most advanced whole sporozoite vaccine candidate, PfSPZ Vaccine, consists of *Plasmodium falciparum (Pf)* radiation-attenuated sporozoites, which arrest development early after invading hepatocytes. Albeit safe (i.e., no breakthrough blood-stage infections), radiation attenuated sporozoites require high doses to induce protection, which remains suboptimal in pre-exposed populations[9-14]. In an experimental medicine setting, repeated *Pf* inoculations attenuated through concomitant administration of chloroquine chemoprophylaxis, which kills the parasites only during the blood-stage, induced 100% protection with low numbers of sporozoites[15,16]. The late arrest of parasites in this model exposes the immune system to a broad array of antigens expressed in sporozoites, intrahepatic stage and a small number of asexual blood-stage parasites, thereby improving protective efficacy.

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), with short intrahepatic development (24hrs, similar to radiation attenuated sporozoites)[18,19] and reported low protective efficacy (12%; 3/25) against homologous controlled human malaria infection (CHMI) in malaria-naïve participants[18].

We recently generated *PfΔmei2* (GA2), a parasite showing complete growth-arrest late during the liver-stage (day 6 post-invasion) in humanized mouse models, thus resembling parasites co-administered with chemoprophylaxis but without any blood-stage exposure[20,21]. Here we describe a clinical trial designed to test the concept that extended liver-stage antigen exposure can induce better protection. To that end, we compared safety, tolerability and preliminary efficacy of late-arresting GA2, early-arresting GA1

and placebo in healthy malaria-naive participants by mosquito bite (MB) immunization followed by CHMI.

# Materials and methods

## Trial design and oversight

This multi-stage study was performed between September 2021 and December 2022 at Leiden University Medical Center (LUMC) and Radboud University Medical Center (Radboudumc). The trial consisted of two stages: stage A was an open-label, dose-escalation safety stage with participants exposed to either 15 (n=5) or 50 (n=15) GA2-infected MB; Stage B was double-blind, placebo-controlled and compared protective efficacy against homologous CHMI in GA1-MB (n=10), GA2-MB (n=10) and placebo groups (n=3) after 3 immunizations, administered at 28-day intervals, with 50 MB each. Because of COVID-19 lockdowns, the study was conducted with fewer participants than originally envisaged (refer to study protocol), with approval of the safety monitoring committee (SMC).

All participants in stage B underwent CHMI with 5 *Pf*3D7-infected MB (**Fig. 1**). *Pf*3D7 is an unattenuated clone of the parental parasite strain *Pf*NF54[22], which was used to generate GA2 and GA1.

## Primary and secondary endpoints

The primary endpoint was the number and severity of adverse events (AEs) and blood-stage parasitemia >100 Pf/ml by quantitative polymerase chain reaction (qPCR) after GA2-MB. Efficacy after CHMI was assessed by measuring blood-stage parasitemia by qPCR .

The secondary outcomes consisted of measuring plasma antibodies targeting key *Pf*-antigens using enzyme linked immunosorbent assay (ELISA) and cellular responses in peripheral blood mononuclear cells (PBMCs) stimulated with *Pf*-infected and uninfected erythrocytes *in vitro* and subsequently phenotyped and profiled for cytokine expression by spectral-flowcytometry.

Methods are described in the accompanying **Supplementary Appendix**.

## Recruitment of participants

Malaria-naïve adults, aged 18 to 35, in good health as assessed by medical history, physical examination, general laboratory evaluation, including biochemistry, hematology, a toxicological screening for illicit drugs, and electrocardiography (ECG) were included. Female participants were required to use adequate birth control throughout the study. All

participants provided written informed consent prior to inclusion. A list of inclusion and exclusion criteria is provided in the **Supplementary Appendix**.

## Ethics

The trial protocol was approved by the Dutch Central Committee for Research involving Human Subjects (CCMO) (identifier NL75555.000.21), registered in ClinicalTrials.gov (identifier NCT04577066), and EudraCT (identifier 2020-005646-41), performed under a license from the Dutch Gene Therapy Office (identifier IM-MV 20-018) and overseen by an SMC.

Recruitment and follow-up of participants were carried out at LUMC, whereas GA2, GA1 and unattenuated *Pf3D7* parasite culturing, *Anopheles stephensi* mosquito rearing and exposure of participants to MBs were performed at Radboudumc. Data analyses and drafting of the manuscript were done by both teams. All authors take responsibility for the integrity, completeness, accuracy of the data and for the fidelity of the trial to the protocol and statistical analysis plan.

## Randomization and statistical analysis

Randomization was carried out by an independent statistician. All staff dealing with participants were blinded to randomization throughout the trial.

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 CHMI in the GA2-MB group. This would provide 80% power and a margin of error of 5% (2-tailed $\alpha$) to detect 70% protective efficacy of GA2-MB compared to placebo.

Adverse events (AE) were evaluated according to intention to treat (ITT) analysis (n=43), efficacy and immunological analyses were per protocol (PP, n=40). The proportion of participants in each cohort or group reporting mild, moderate or severe AEs was calculated. The presence of parasitemia was analyzed by $\chi^2$ and Fischer's exact test.

Analyses of humoral and cellular immune responses were prespecified in the protocol. Serological data were log transformed to achieve a normal distribution. A paired t-test was used when comparing pre- and post-exposure values, and an unpaired t-test was used for comparisons between groups. For discrete variables, the $\chi^2$ test or Fisher's exact test was performed (two-tailed). Cellular responses between the groups were compared using the two-tailed Mann-Whitney test. All p-values were considered significant if $<0.05$. Graphs were produced in GraphPad Prism (version 9.3.1) and R (version 4.2.1).

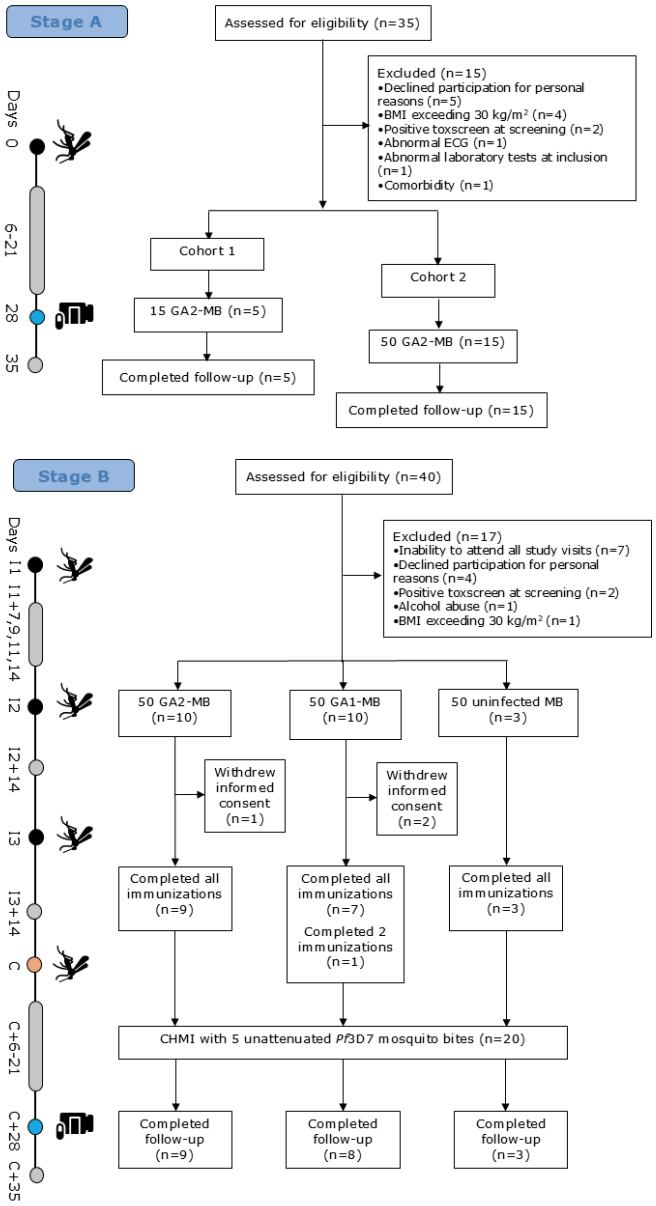


**Figure 1. CONSORT diagram and clinical trial design**
**Stage A**: Stage A was open label and eligible participants were recruited sequentially into cohort 1 and then cohort 2. In the dose-escalation stage A, participants were exposed once to GA2-MB (black circle): the first cohort received 15 MB, the second 50 MB.
**Stage B**: Allocation in stage B was randomized and double-blind. Three groups of participants were exposed thrice to either 50 GA2-MB, GA1-MB or uninfected MB (placebo) respectively, at 28-day intervals (black circles). Three weeks after the last immunizing exposure, participants underwent controlled human malaria infection (CHMI) by bites of 5 unattenuated wild-type (WT) *Pf*3D7-infected mosquitoes (orange circle). All participants were treated with a curative regimen of atovaquone/proguanil at latest day 28 after their final exposure (blue circles).
BMI: body mass index, ECG: electrocardiogram.

# Results

## Study population

Seventy-five malaria-naïve adults were screened from the 13th of September 2021 to the 28th of January 2022, of which 43 were included. No participants were lost during stage A, and 3 participants in stage B withdrew consent prior to CHMI (**Fig. 1).** In total, 22 (51%) participants were female. Median age was 23.0 years (range 19-35) and median BMI was 24.1 kg/m$^2$ (range 18.0-29.9) (**Table 1**).

**Table 1. Participant demographics**.

| | | Stage A | | Stage B | | | |
|---|---|---|---|---|---|---|---|
| | | **Cohort 1** | **Cohort 2** | **GA2** | **GA1** | **Placebo** | **Total** |
| Number of participants | | 5 | 15 | 10 | 10 | 3 | 43 |
| Age (years) | Median | 22 | 23 | 23.5 | 23.5 | 22 | 23 |
| | Min, max | 20-27 | 19-30 | 19-31 | 19-35 | 21-24 | 19-35 |
| Sex | Female | 4 | 8 | 5 | 3 | 2 | 22 |
| | Male | 1 | 7 | 5 | 7 | 1 | 21 |
| BMI (kg/m$^2$) | Median | 24.8 | 24.1 | 22.4 | 22.6 | 27.5 | 24.1 |
| | Min, max | 21.9-26.9 | 20.3-29.1 | 18.0-29.9 | 19.9 -28.6 | 24.4-28.7 | 18.0-29.9 |

BMI: body mass index.

## Safety results

No serious adverse events occurred during the trial. There were no breakthrough infections after exposure to either 15 or 50 GA2-MB, as assessed by qPCR. There was no significant difference in the incidence and severity of AEs after administration of GA2-MB, GA1-MB or placebo (ITT).

The most common related local AEs were erythema and pruritus after MB, reported by 19 (95%) and 17 (85%) participants, respectively, in stage A (**Supplementary Fig. 1 A, Supplementary Table 1**) and by 23 (100%) and 22 (96%) participants, respectively, in stage B (**Supplementary Fig. 1 B-D, Supplementary Table 3)**, some of which were treated with antihistamines or topical steroids. Myalgia was the most common related systemic AE in 4 (20%) participants in stage A (**Supplementary Fig. 1 A, Supplementary Table 1**). Headache, reported twice (9%), was the most frequently related systemic AE in the immunization phase of stage B (**Supplementary Fig. 1 B-D, Supplementary Table 3)**. Unrelated AEs are summarized in **Supplementary Table 2** and **Supplementary Table 4** in the **Supplementary Appendix**.

In two cases, troponin T levels were elevated to 18 and 19 ng/mL (reference 14 ng/ml) for reasons unrelated to the trial. Antihistamine use led to elevated liver function tests in a third participant.

The clinical course following CHMI in stage B was consistent with prior CHMI trials at our centre[23] (**Supplementary Fig. 2, Supplementary Table 5 and Supplementary Table 6**).

## Protective efficacy against CHMI

All participants who completed the immunization phase in stage B (9, 8 and 3 participants immunized with GA2-MB, GA1-MB and uninfected-MB, respectively) underwent CHMI with 5 unattenuated *Pf3D7*-infected MB, 3 weeks after the last immunization.

Immunization with GA2-MB resulted in 89% (95% CI: 31%-98%) protection, with all participants remaining qPCR negative until the end of the trial except one (1/9) who developed a positive *Pf* qPCR on day 11 post-CHMI, whereas 0/3 placebo recipients were protected with a median time to parasitemia of 12 days (IQR=3.0days) post CHMI ($\chi^2$ *P*=0.005 and Fischer's exact *P*=0.02 compared to placebo, PP). In contrast, 1/8 GA1-MB was protected, with others developing parasitemia by a median of 11 days (IQR=2.5 days). In a post hoc comparison, protection against CHMI was significantly different between GA2-MB and GA1-MB groups ($\chi^2$ *P*=0.002 and Fischer's exact *P*=0.003, log-rank *P*=0.002, PP) (**Fig. 2**).

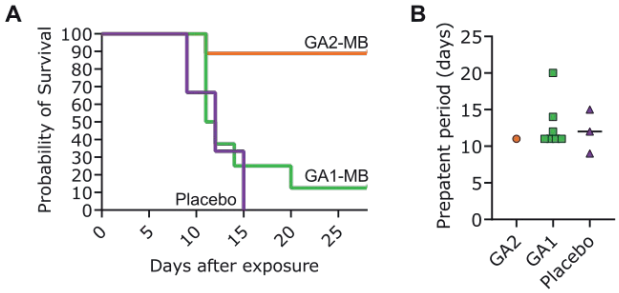


**Figure 2. Parasitemia after controlled human malaria infection (CHMI)**
**A.** Kaplan-Meier displaying the percentage of participants remaining free of blood-stage parasitemia over time after CHMI.
**B.** Prepatent period for participants who developed a positive *Pf* qPCR, measured in days post-CHMI. Lines indicate median.

## Immunogenicity

In both GA2-MB and GA1-MB participants, we detected higher anti-*Pf*CSP antibodies on the day before challenge (C-1: 1.33 ± 0.35 µg/ml) compared to baseline (baseline: 0.07 ± 0.35 µg/ml) (paired t-test, p<0.001, CI: 1.07 to 1.44) and placebo (C-1: 0.01 ± 0.16 µg/ml) (independent samples t-test, p<0.001, CI: 0.89 to 1.77) (**Fig. 3 A**). However, titers did not differ between GA2-MB (1.2 ± 0.3 µg/ml) or GA1-MB (1.5 ± 0.4 µg/ml) immunized participants or protected (1.2 ± 0.3 µg/ml) and non-protected (1.4 ± 0.4 µg/ml) participants (**Fig. 3 D**). We did not detect changes in antibodies against apical membrane antigen 1 (*Pf*AMA1; baseline:

0.63 ± 0.52 µg/ml; C-1: 0.57 ± 0.52 µg/ml) (**Fig. 3 B, E**) or a fragment of the merozoite surface protein 1 (*Pf*MSP1; baseline: -0.27 ± 0.2 µg/ml; C-1: -0.23 ± 0.3 µg/ml) (**Fig. 3 C, F**).

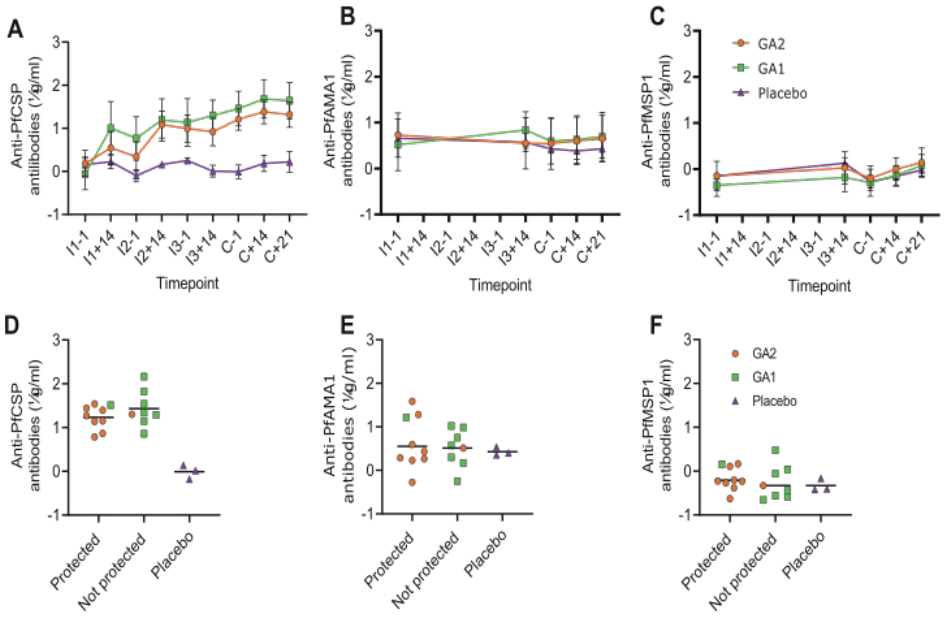


**Figure 3. Humoral immune responses**
**A-C** Plasma anti-*Pf*CSP, anti-*Pf*AMA1 and anti-*Pf*MSP1 antibodies measured at the indicated time points for the GA2-MB (orange circles), GA1-MB (green squares) and uninfected-MB placebo (purple triangles) groups. Symbols and error bars indicate mean and standard deviation. Days corresponding to first (I1), second (I2), third (I3) immunization and challenge (C) are indicated as timepoints on x-axes.
**D-F** Plasma anti-*Pf*CSP, anti-*Pf*AMA1 and anti-*Pf*MSP1 antibodies on the day before challenge for individual participants exposed to GA2-MB (orange circles), GA1-MB (green squares) and uninfected-MB placebo (purple triangles). Black lines indicate mean.
All values are log transformed.

To study *Pf*-specific cellular immunity, participants' PBMCs were stimulated with *Pf*-infected RBC (*Pf*RBC) and uninfected RBC (unRBC) and then assessed by flow cytometry (**Supplementary Fig. 3-5**). While the overall cellular frequency of all studied T cell lineages remained comparable, we observed an increase in γδ T cells expressing the Vδ2 chain in GA2-MB but not GA1-MB participants (**Supplementary Fig. 6 A-E**). From a functional perspective, immunization induced *Pf*-specific $CD4^+$ T cells with a strong type 1 inflammatory (IFN−γ, TNF−α and IL−2) but not type 2 or regulatory (IL−10 , IL−4, IL−5 and IL−13) signature (**Fig. 4 A**). However, we observed higher frequency of *Pf*-specific $CD4^+$ T cells in GA2-MB compared to GA1-MB participants. Similarly, *Pf*-specific $V\delta2^+$ γδ T cells expressing type 1 cytokines were induced only in GA2-MB but not GA1-MB participants (**Fig. 4 B**). *Pf*-specific responses were not detected in $CD8^+$, $V\delta2^-\gamma\delta$ and NK T cell compartments (**Supplementary Fig. 6 F-H**).

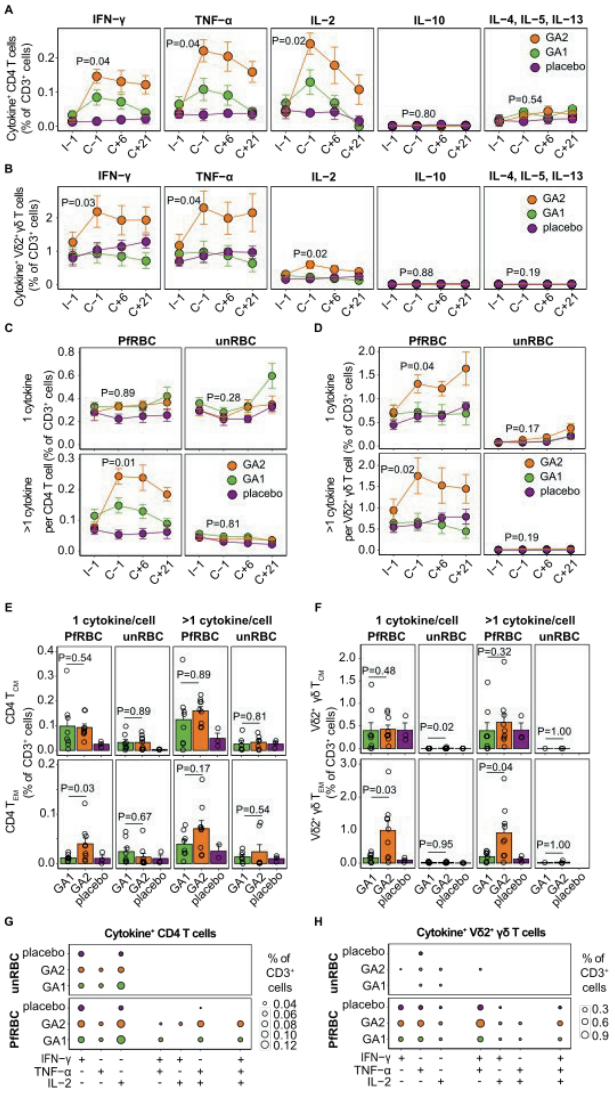


**Figure 4. GA2-MB induces stronger pro-inflammatory CD4⁺ and Vδ2⁺ γδ T cell responses compared to GA1-MB.**

**A, B**. Percentage of CD4⁺ (A) and Vδ2⁺ γδ (B) T cells secreting indicated cytokines at baseline (I-1), one day before challenge (C-1), 6 and 21 days post challenge (C+6 and C+21).

**C, D.** Percentage of CD4⁺ (C) and Vδ2⁺ γδ (D) T cells secreting either single (top) or more than one cytokine (bottom) upon stimulation with *Pf* infected (PfRBC; left) or uninfected RBC (unRBC; right) at the indicated time points.

**E, F**. Mean percentage of CD4⁺ (E) and Vδ2⁺ γδ (F) T cells secreting a single (left) or more than one cytokine (right) adopting a central ($T_{CM}$; top) or effector ($T_{EM}$; bottom) memory phenotype upon PfRBC or unRBC stimulation at C-1. Open circles indicate individual participants.

**G, H**. Mean percentage of CD4⁺ (G) and Vδ2⁺ γδ (H) T cells secreting cytokines in the indicated combination upon PfRBC (bottom) or unRBC (top) stimulation at C-1.

Data corresponding to GA1-MB, GA2-MB and uninfected-MB (placebo) immunization groups are indicated in green, orange and purple colour, respectively. A-D: Filled circle indicates arithmetic mean. A-F error bars indicate standard error mean. $p<0.05$ was considered significant, two tailed Mann-Whitney test.

Profiling the cytokine expression at a single T cell level showed that higher frequencies of polyfunctional $CD4^+$ and $V\delta2^+$ $\gamma\delta$ T cells expressing more than one cytokine acquiring an effector memory phenotype were measured in GA2-MB compared to GA1-MB participants (**Fig. 4 C-F**). These polyfunctional T cells expressed combinations of IFN−γ, TNF−α and IL−2, with the preferential expression of TNF−α and IL−2 in $CD4^+$ T cells and IFN−γ and TNF−α in $V\delta2^+$ $\gamma\delta$ T cells (**Fig.4 G, H**).

# Discussion

With this study we show evidence for higher cellular immunogenicity and protective efficacy of a late-arresting genetically attenuated parasite (GA2-MB) compared to an early-arresting one or (GA1-MB), indicating a potential role for late-liver-stage antigens in eliciting protective immunity to malaria. Protection did not associate with antibodies to *Pf*CSP, *Pf*AMA1 or *Pf*MSP1, but was related to the induction of *Pf*-specific polyfunctional $CD4^+$ T cells and $V\delta2^+$ $\gamma\delta$ T cells.

The protection induced by late-arresting GA2-MB in this model is higher than that seen in previous replication-incompetent, early-arresting attenuated sporozoites. Early-arresting genetically attenuated parasites, delivered by either intravenous injection or MB provided lower protective efficacy (12% by GA1 and 50% by PfGAP3KO[18,24]). Protection achieved by radiation attenuated sporozoites has varied between 50% to >90% when administered intravenously (dose range $1.35 \times 10^5$ to $2.7 \times 10^6$ PfSPZ Vaccine) in homologous CHMI[4,25-29]. However, administration of radiation attenuated sporozoites by MB requires higher doses (approximately 1000 MB) to reach similar levels of protection offered by GA2-MB[13]. In contrast, protection induced by GA2-MB in this study is reminiscent of parasites co-administered with chemoprophylaxis where 3 exposures to bites of 15 infected mosquitoes resulted in 100% efficacy against homologous CHMI,[15,30] thus further supporting the hypothesis that extended hepatic antigenic presentation may have a role for the induction of protective immunity.

To elucidate the correlates of protection, we measured both circulating antibodies and cellular responses. Consistent with previous reports[4], we found anti-*Pf*CSP antibodies induced in response to both GA2-MB and GA1-MB. This suggests that the differential arrest in the liver did not impact anti-sporozoite antibody responses quantitatively. Lack of plasma antibodies against late-liver-stage antigens, *Pf*AMA1 and *Pf*MSP1, indicates that their exposure is insufficient to mount a measurable antibody response.

Unlike antibody responses, *Pf*-specific cellular immune responses differed between GA2-MB and GA1-MB-immunized participants. While we observed a strong pro-inflammatory

CD4+ T cell response in both groups, induction of *Pf*-specific polyfunctional effector memory CD4+ T cells was more robust in GA2-MB compared to GA1-MB. GA2-MB also more efficiently induced mono- and poly-functional Vδ2+ γδ T cells with a strong pro-inflammatory signature. These findings are in line with previous attenuated sporozoite studies reporting Vδ2+ γδ T cells as a marker of protective immunity[16,26].

The induction of γδ T cells is observed not only after immunization with parasites co-administered with chemoprophylaxis, but also after high dose immunization with radiation attenuated sporozoites where the duration of liver-stage antigen exposure is limited[26,31]. However, a recent study reported 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 non-human primates have indicated a central role for effector memory-like liver resident CD8+ T cells[32,33]. However, detection of *Pf*-specifc CD8+ T cells in peripheral blood of participants is generally limited[18,25,34-36], although CD8+ T cells can be present at up to 100 times higher frequencies in the liver than in the blood[26,35]. Hence, it remains unclear whether there is any limitation in induction or recirculation of tissue resident *Pf*-specific CD8+ T cells in humans immunized with attenuated sporozoites. In line with these studies, we did not detect *Pf*-specific CD8+ T cells in peripheral blood in any of the immunized participants using general flow cytometric tools.

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

Nonetheless, the conclusions from this study are limited by the small sample size. More studies with greater numbers of participants are required to better understand the safety profile of GA2. In addition, the immunological assessments were exploratory and the relevance of the parameters associated with GA2-induced protection needs confirmation. To translate the promising efficacy results to the broader population affected by malaria (**Supplementary Table 7**), the immunogenicity of GA2 needs to be assessed for durability

and against heterologous *Pf* strains in malaria endemic regions[7]. Previous studies showed that co-administration of parasites with chemoprophylaxis can induce durable protection[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. If high levels of protection can be sustained in such settings, satisfying the recent WHO preferred product characteristics[39], late-arresting whole sporozoites will be considered as a next generation vaccine, complementing the two currently available subunit vaccines[2,3].

In summary, our findings suggest that parasites arresting late during the liver-stage (GA2) offer protection compared to early-arresting sporozoites (GA1) and provide a step towards a next generation malaria vaccine with potentially higher protective efficacy possibly from cellular immune mechanisms.

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# Supplementary appendix

## Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

1. Subject is aged ≥ 18 and ≤ 35 years and in good health.
2. Subject has adequate understanding of the procedures of the study and agrees to abide strictly thereby.
3. Subject is able to communicate well with the investigator, is available to attend all study visits.
4. Furthermore, the subject will remain within the Netherlands from day -1 till day +35 after each parasite exposure. After exposure to parasites, subjects have to be reachable by phone (24/7) from day -1 until day 35.
5. Subject agrees that his/her general practitioner (GP) will be informed about participation in the study.
6. Subject agrees to refrain from blood donation to Sanquin or for other purposes throughout the study period and for a defined period thereafter according to Sanquin guidelines (3 years minimum, depending on serology).
7. Non-pregnant, non-lactating, fertile (i.e., have a uterus and are neither surgically sterilized nor post-menopausal) female subjects agree to use adequate contraception and to not breastfeed for the duration of study.
8. Subject agrees to refrain from intensive physical exercise (disproportionate to the subjects' usual daily activity or exercise routine) for twenty-one days following each immunization and during the malaria challenge period.
9. Subject signs informed consent.

## Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

1. Any history, or evidence at screening, of clinically significant symptoms, physical signs or abnormal laboratory values suggestive of systemic conditions, such as cardiovascular, pulmonary, renal, hepatic, neurological, dermatological, endocrine, malignant, haematological, infectious, immune-deficient, psychiatric or other disorders, which could compromise the health of the subject during the study or interfere with the interpretation of the study results. These include, but are not limited to, any of the following:

a. Body weight <50 kg or Body Mass Index (BMI) <18.0 or >30.0 kg/m$^2$ at screening.
b. A heightened risk of cardiovascular disease, defined as:
    i. An estimated 10-year risk of fatal cardiovascular disease of ≥5% at screening, as determined by the Systematic Coronary Risk Evaluation (SCORE).
    ii. History, or evidence at screening, of clinically significant arrhythmia's, prolonged QT-interval or other clinically relevant ECG abnormalities; or
    iii. A positive family history of cardiac events in first- or second-degree relatives (according to the system used in medical genetics) <50 years old.
c. Functional asplenia, sickle cell trait/disease, thalassemia trait/disease or G6PD deficiency.
d. History of epilepsy in the period of 5 years prior to study onset, even if no longer on medication.
e. Positive HIV, HBV or HCV screening tests.
f. Chronic use of i) immunosuppressive drugs, ii) antibiotics, iii) or other drugs that might have an influence on the immune system (excluding inhaled and topical corticosteroids and incidental use of oral anti-histamines), within 3 months prior to study onset or expected use of such during the study period.
g. History of malignancy of any organ system (other than localized basal cell carcinoma of the skin), treated or untreated, within the past 5 years.
h. Any history of treatment for severe psychiatric disease by a psychiatrist in the past year.
i. History of drug or alcohol abuse interfering with normal social function in the period of one year prior to study onset, positive urine toxicology test for cocaine, amphetamines or cannabis at screening.

2. For female subjects: breastfeeding, or positive urine pregnancy test at screening or prior to immunization or prior to controlled human malaria infection (CHMI).
3. Any history of malaria, positive serology for *Pf,* or previous participation in any malaria (vaccine) study or CHMI.
4. Known hypersensitivity to or contra-indications (including co-medication) for use of atovaquone/proguanil or artemether/lumefantrine, or history of severe (allergic) reactions to mosquito bites (MB).

5. Receipt of any vaccinations in the 3 months prior to the start of the study or plans to receive any other vaccinations during the study period or up to 8 weeks thereafter. Exceptions are made for influenza vaccination and for vaccination against the coronavirus SARS-CoV2 or any other vaccination which cannot be reasonably withheld or postponed.
6. Participation in any other clinical study in the 30 days prior to the start of the study or during the study period.
7. Being an employee or student of the department of Parasitology, Medical Microbiology or Infectious Diseases of the LUMC or RUMC.
8. Any other condition or situation that would, in the opinion of the investigator, place the subject at an unacceptable risk of injury or render the subject unable to meet the requirements of the protocol or would compromise the integrity of the data.

# Materials and methods

## Trial procedures

### *Parasites and mosquito rearing procedures*

The generation and characterization of GA2 and GA1 from the *Plasmodium falciparum (Pf)* NF54 strain has been previously described[1-3]. *Pf* parasites were cultured in standard conditions using a static culture system and subsequently fed to female *Anopheles stephensi* mosquitoes by standard membrane feeding[4]. *An. stephensi* mosquitoes were reared according to standard procedures at the Radboudumc instectiary[5,6].

### *Exposure of participants to infected mosquitoes*

Exposure of participants to infected MB was carried out according to previously described procedures[7]. In short, mosquitoes were inspected after the blood meal to confirm feeding. If necessary, participants were exposed to additional mosquitoes until the predefined number of MB was reached. For practical reasons, a margin of error of 20% was accepted for the immunizations, hence 12-18 MB were accepted for the targeted 15 MB dose, whereas 40-60 MB were accepted for the targeted 50 MB dose. For CHMI, participants were exposed to exactly 5 *Pf*-infected MB. All procedures were supervised by an experienced technician and a study physician. After exposure, participants were observed for at least 15 minutes.

### *Schedule of immunization and follow-up*

#### *Stage A*

In stage A of the trial, participants visited the trial centre daily, from days 6 to day 21 after exposure. On day 28 after exposure, all participants received rescue treatment with a standard regimen of atovaquone/proguanil. Complete blood counts were done at inclusion, on days 6, 14, 21, 28, 31 and 35 after exposure to GA2-MB, whereas biochemistry tests were done on days 6, 14, 28, 31 and 35 after exposure to GA2-MB. Platelet counts, lactate dehydrogenase and highly sensitive troponin T were assessed at each ambulatory visit, except for days 100 and 188. Blood samples for immunological analysis were taken at baseline, on days 6, 14, 21, 100 and 188.

#### *Stage B*

In stage B of the trial, visits were planned on day 14 after every immunization and on the day prior to each immunization. Phone calls were scheduled on days 7 and 21 after each immunization. After the first immunization, 3 additional ambulatory visits were planned on days 7, 9 and 11. Three weeks after the last immunization, participants underwent CHMI with 5 unattenuated *Pf*3D7-MB. After CHMI, participants visited the trial centre daily from days 6 to 21 post CHMI. Participants received rescue treatment with atovaquone/proguanil as soon as they developed parasitemia or, at the latest, at day 28 post CHMI.

During the immunization period, complete blood counts and biochemistry tests were done at inclusion, on day 14 after each immunization and on the day prior to each subsequent immunization and prior to CHMI. After CHMI, complete blood counts were performed on days 6, 14, 21, 28 and 31 if the participant had not yet received rescue treatment and always on day 35, whereas biochemistry tests were performed on days 6, 14, 28 and 31 if the participant had not yet received rescue treatment and always on day 35.

Platelet counts, lactate dehydrogenase and highly sensitive troponin T were assessed at every ambulatory visit, except for days 100 and 188 post CHMI. Blood samples for immunological analysis were taken at baseline, on day 14 after each immunization, on the day prior to each new immunization, prior to CHMI and after CHMI on days 6, 14, 21, 35, 100 and 188 (**Fig. 1**).

### *Blood stage parasitemia*

Blood stage parasitemia was assessed by qPCR, which was carried out according to previously described methods[8]. Samples were considered positive if >100 *Pf*/ml.

In stage A qPCR analysis was performed at inclusion, daily on days 6 to 21, 28, 31 and on day 35. whereas in stage B qPCR was performed at inclusion, prior to each new immunization and prior to challenge and on days 7, 9, 11, 14 after the first immunization and on day 14 after the second and third immunization. After CHMI, qPCR was carried out daily from days 6 to 21 and on days 28, 31 and 35. If participants developed parasitaemia before rescue treatment, qPCR was also repeated on the afternoon of qPCR positivity and on the third day after treatment initiation (**Fig.1**).

## Adverse events

Solicited and unsolicited adverse events (AEs) were recorded at every visit until day 35 after exposure to GA2-MB in stage A and until day 35 after CHMI in stage B of the trial. Solicited local AEs were: bruising, erythema, induration, pain, pruritus, swelling and tenderness. Solicited systemic AEs were: arthralgia, chills, oedema, fatigue, fever, headache, malaise, pruritus, rash and urticaria. Participants were instructed to fill in a diary with daily temperature and AEs. AEs were graded according to the following scale:

- Mild (grade 1): awareness of symptoms that are easily tolerated and do not interfere with usual daily activity;
- Moderate (grade 2): discomfort that interferes with or limits usual daily activity;
- Severe (grade 3): disabling, with subsequent inability to perform usual daily activity, resulting in absence or required bed rest.

For additional information regarding AE toxicity please refer to the study protocol. Causality of the AEs was assessed by the investigator and classified as definitively, probably, possibly, unlikely or not related. In a dichotomous analysis, the first 3 categories were classified as related, the latter 2 as unrelated. AEs were considered unrelated if there was a more plausible explanation or if timing made the relatedness unlikely.

## ELISA to measure antibody responses

Plasma samples taken at the indicated time points were assessed for IgG antibodies targeting the circumposporozoite protein (*Pf*CSP), apical membrane antigen 1 (*Pf*AMA1) and merozoite surface protein 1 (*Pf*MSP1) by enzyme-linked immunosorbent assay (ELISA). Full length CSP was recombinantly expressed in *Escherichia coli* at the European Molecular Biology Laboratory (EMBL), Heidelberg, Germany. Full length recombinant AMA1 and a fragment of MSP-1, both expressed in *Pichia pastoris* were kindly offered by Dr. B. Faber and Dr. C. Kocken (Biomedical Primate Research Center,Rijswijk, The Netherlands). 96-well Microplates (Corning® Half Area Clear Flat Bottom Polystyrene

High Bind) were coated overnight at 4 °C with 1 µg/ml of antigen at 25 µl/well in 0.1 M sodium carbonate buffer (pH 9.6). Plates were washed 3 times with washing buffer (0.05% Tween in phosphate buffered saline (PBS)) and blocked with 50 µl 5% skim milk in PBS for 2 h at 37 °C. Plasma samples were serially diluted in 0.5% skim milk with the initial dilution of 1:500 for 8 steps each by 1:2.5. After washing the plates, diluted plasma samples were added at 25 µl/well and incubated at room temperature for 2h. Plates were washed 5 times and incubated with 25 µl/well of goat-anti-human immunoglobulin G (IgG) conjugated with horseradish peroxidase at a concentration of 0.2 µg/ml (in 0,5% skim milk in PBS and 0.05% EDTA) for 1 hour at room temperature. After 6 washes, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added. Colour development was blocked with 10% sulfuric acid after 8 minutes. Plates were read with the Multiskan EX reader at 450 nm. Measurements were normalized to a standard curve, consisting of polyclonal IgGs with a known concentration, purified from human plasma (Sigma-Aldrich/ Merck). Measurements were repeated in at least 2 independent experiments with overall coefficient of variance below 30%.

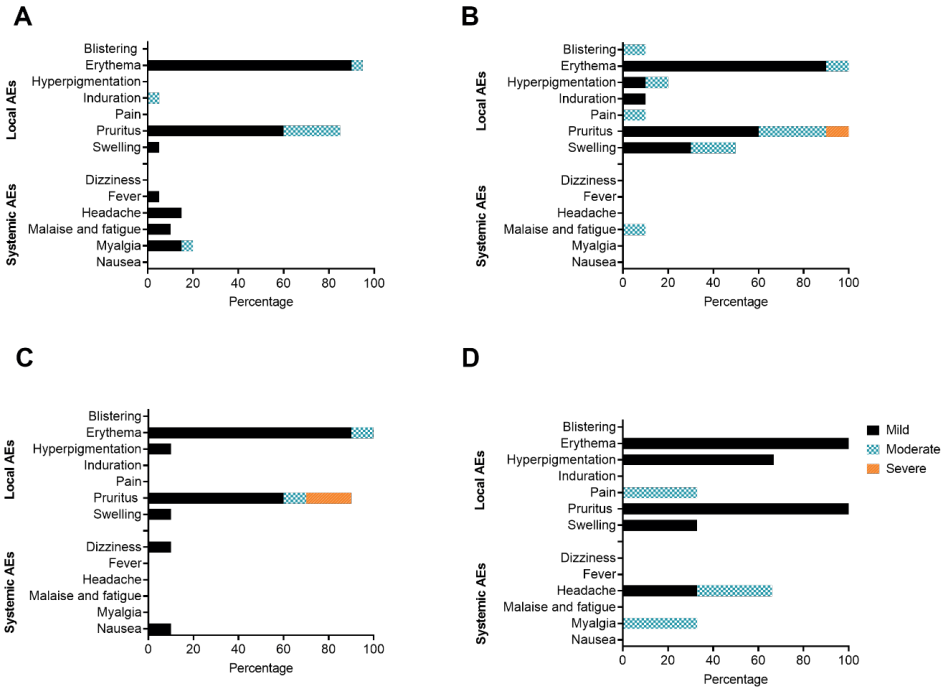
## Peripheral blood mononuclear cells (PBMCs) restimulation and flow cytometry to measure T cell responses

*Pf* stimulation and flow cytometry analyses were performed as described before[2]. In brief, frozen PBMCs were thawed incubated overnight at 37°C and 5% $CO_2$ in complete Roswell Park Memorial Institute (RPM) medium (cRMPI consisting of RPMI 1640 (Invitrogen), 100 U/ml Penicillin (Euroco-Pharma), 100 µg/ml Streptomycin (Sigma), 1 mM Pyruvate (Sigma) and 2 mM L-Glutamate (Sigma), supplemented with 20% heat-inactivated fetal calf serum (FCS) (Bodinco). After incubation, cells were resuspended in cRPMI for 4 h, 10 µg/ml Brefeldin A (Sigma) was added to the culture. PBMCs from 1 malaria-naïve donor (Sanquin, the Netherlands) and a donor previously infected with malaria were used as negative and positive controls, respectively.

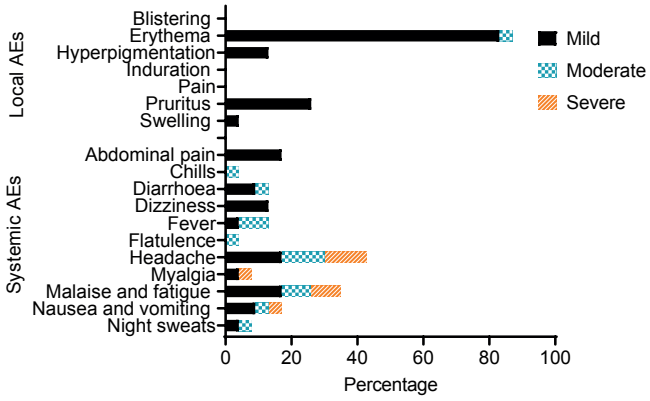
After stimulation for 24 h, the cells were transferred into a V bottom plate and all the flow cytometry staining steps were performed on ice using the panel described in **Supplementary Table 8**. Cells were treated with Fc block and surface-stained for the pan-γδ T cell receptor for 20 min in the dark and followed by a 20-min surface-staining for the markers CD56, γδ-Vδ2 TCR, CCR7, CD3, CD4, CD25 and CD11c. Aqua Live/ Dead dye (Invitrogen) was used as per the manufacturer's instructions to stain the dead cells. Upon washing, fixing, and permeabilizing using the Intracellular Fixation & Permeabilization Buffer Set (Invitrogen), cells were stained intracellularly for the markers CD45RA, IFN-γ, TNF-α, IL-10, IL-2, IL-4, IL-5, IL-13, FoxP3, and CD8 for 30

min. The cells were resuspended in fluorescence activated cell sorting (FACS) buffer (PBS (Invitrogen), 0.5% BSA, 2mM EDTA) and acquired on the 3-laser spectral analyzer Aurora (configuration 16V-14B-8R) and the data were analyzed using FlowJo 10.8.2 as described in **Supplementary Fig. 3**. Fluorescence Minus Many were used to define gates for cytokine positive cells. *Pf*-specific cytokine positive frequencies in *Pf*-infected red blood cells (PfRBC) stimulated samples are reported upon subtraction of the frequencies from the same gate on the same sample stimulated with uninfected red blood cells (unRBC).

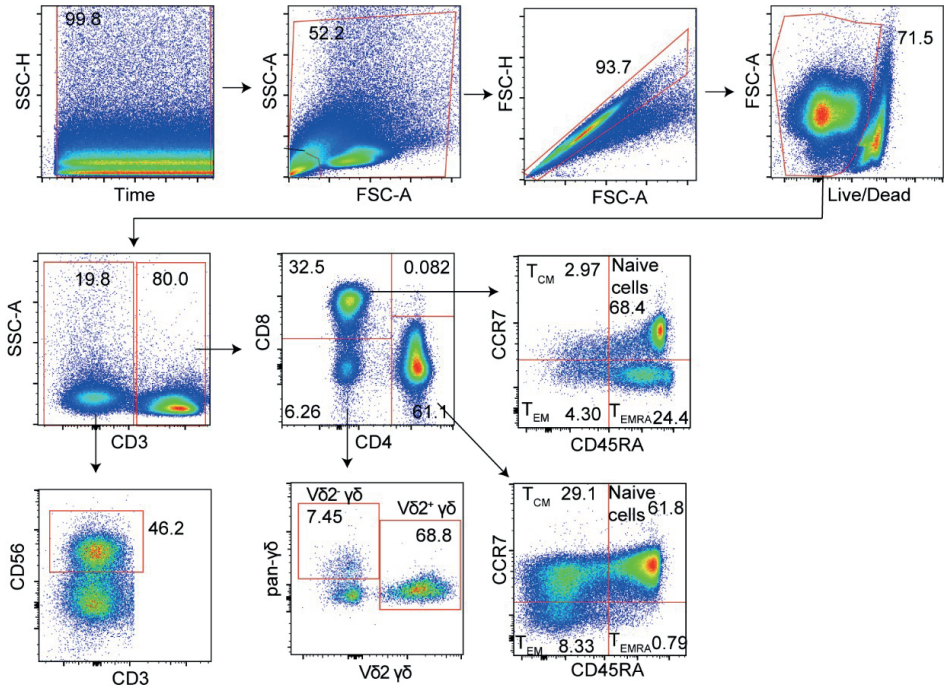
## Supplementary figures



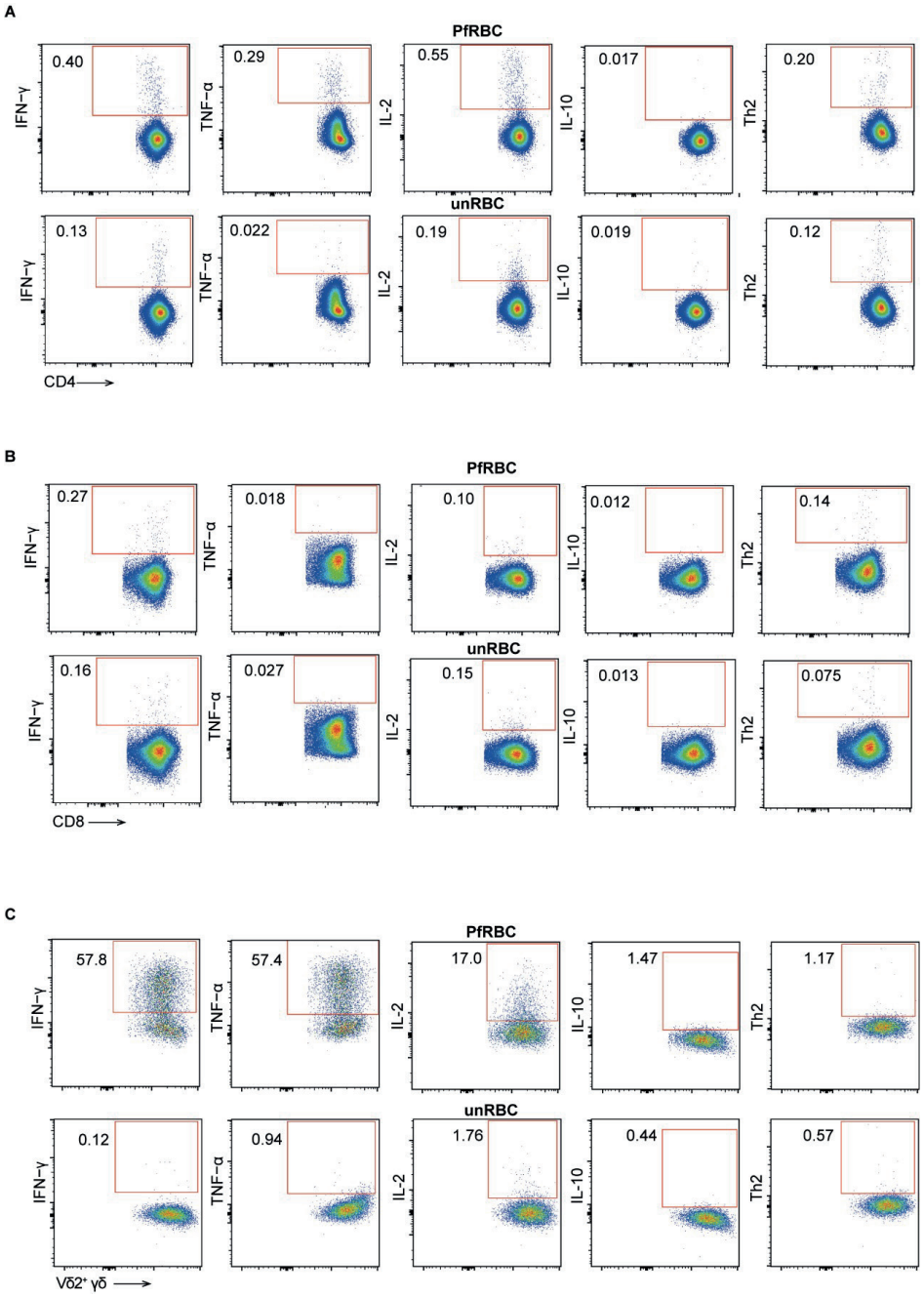

**Supplementary Figure 1. Adverse events (AEs) reported during stage A and the immunization phase of stage B of the clinical trial**
Stacked bars showing percentage of participants reporting any AEs related to GA2-MB in stage A (**A**) and GA2-MB, GA1-MB and uninfected-MB (placebo) groups in stage B (**B-D**), of mild (black), moderate (blue) and severe (orange) intensity.



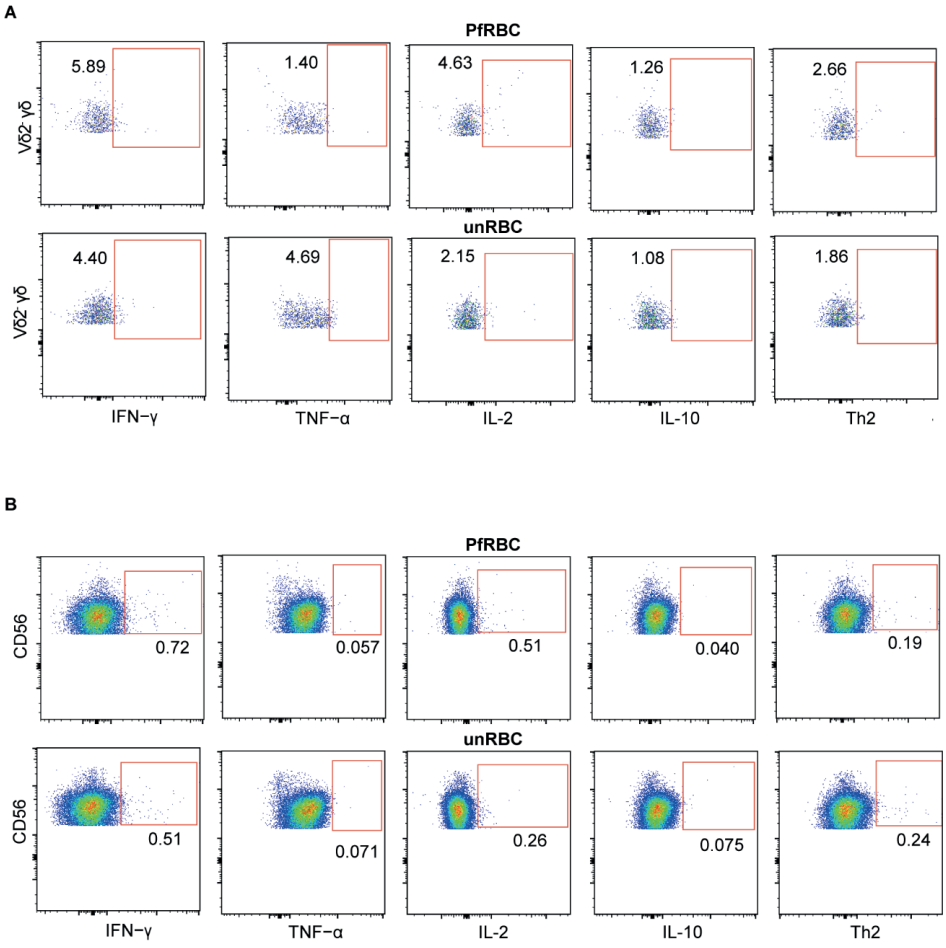

**Supplementary Figure 2. Adverse events (AEs) reported after controlled human malaria infection (CHMI) in stage B of the clinical trial**
Stacked bars showing percentage of participants reporting any AEs related to CHMI in stage B, of mild (black), moderate (blue) and severe (orange) intensity.



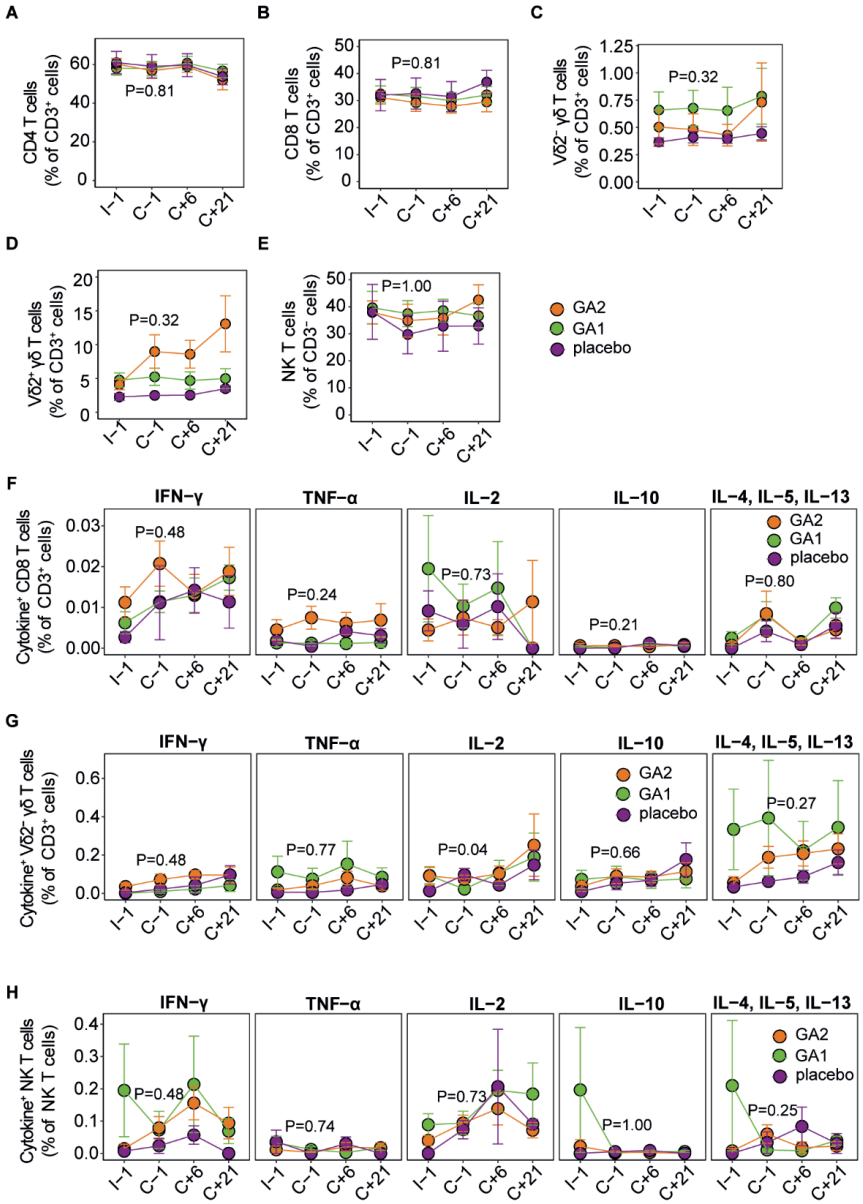

**Supplementary Figure 3. Representative gating panels indicating identification of target T cell populations**




**Supplementary Figure 4. Cytokine expression in CD4⁺, CD8⁺ and Vδ2⁺ γδ T cell populations A-C**. PBMCs stimulated either with PfRBC (top) or uninfected RBC (bottom) measured for the indicated cytokine expression in CD4⁺ (A), CD8⁺ (B) and Vδ2⁺ γδ (C) T cell populations.




**Supplementary Figure 5. Cytokine expression in Vδ2$^-$ γδ and NK T cell populations**
**A,B**. PBMCs stimulated either with PfRBC (top) or uninfected RBC (bottom) measured for the indicated cytokine expression in Vδ2$^-$ γδ (A) and NK (B) T cell populations.




**Supplementary Figure 6. T cell analysis**

**A-E.** Percentage of CD4+ (A), CD8+ (B), Vδ2– γδ (C), Vδ2+ γδ (D) and NK T cells at baseline (I-1), one day before challenge (C-1), 6 and 21 days post challenge (C+6 and C+21) upon stimulation with medium control.

**F-H.** Percentage of CD8+ (F) and Vδ2– γδ (G) and NK (H) T cells secreting indicated cytokines at baseline (I-1), one day before challenge (C-1), 6 and 21 days post challenge (C+6 and C+21).

Data corresponding to GA1-MB, GA2-MB and uninfected-MB (placebo) immunization groups are indicated in green, orange and purple colour, respectively.

A-H: Filled circle indicates arithmetic mean and error bars indicate standard error mean. $P < 0.05$ is considered significant, two tailed Mann-Whitney test.

## Supplementary tables

**Supplementary Table 1. Number of participants with related adverse events (AEs) reported during stage A of the clinical trial (percentage in brackets, N=20)**

| Adverse events | Mild | Moderate | Severe | Total |
|---|---|---|---|---|
| **Local** | | | | |
| **Erythema** | 18 (90%) | 1 (5%) | | 19 (95%) |
| **Induration** | | 1 (5%) | | 1 (5%) |
| **Pruritus** | 12 (60%) | 5 (25%) | | 17 (85%) |
| **Swelling** | 1 (5%) | | | 1 (5%) |
| **Systemic** | | | | |
| **Fever** | 1 (5%) | | | 1 (5%) |
| **Headache** | 3 (15%) | | | 3 (15%) |
| **Malaise and fatigue** | 2 (10%) | | | 2 (10%) |
| **Myalgia** | 3 (15%) | 1 (5%) | | 4 (20%) |

**Supplementary Table 2. Number of participants with unrelated adverse events (AEs) reported during stage A of the clinical trial (percentage in brackets, N=20)**

| Adverse events | Mild | Moderate | Severe | Total |
|---|---|---|---|---|
| **Abdominal pain** | 6 (30%) | 2 (10%) | | 8 (40%) |
| **Acute kidney abnormality** | 1 (5%) | | | 1 (5%) |
| **Acute pharyngitis** | 7 (35%) | | | 7 (35%) |
| **Gastroenteritis** | 2 (10%) | | | 2 (10%) |
| **Change in bowel habit** | 1 (5%) | | | 1 (5%) |
| **Chills** | 1 (5%) | | 1 (5%) | 2 (10%) |
| **Cough** | 1 (5%) | | | 1 (5%) |
| **Diarrhea** | 3 (15%) | 1 (5%) | | 4 (20%) |
| **Dizziness** | 1 (5%) | | | 1 (5%) |
| **Epistaxis** | 1 (5%) | | | 1 (5%) |
| **Fever** | 1 (5%) | | | 1 (5%) |
| **Headache** | 3 (15%) | 5 (25%) | 1 (5%) | 9 (45%) |
| **Hematoma** | 3 (15%) | | | 3 (15%) |
| **Limb pain** | 1 (5%) | | | 1 (5%) |
| **Low back pain** | 1 (5%) | | | 1 (5%) |
| **Lymph node swelling** | 1 (5%) | | | 1 (5%) |
| **Malaise and fatigue** | 8 (40%) | 2 (10%) | | 10 (50%) |
| **Myalgia** | 2 (10%) | 1 (5%) | | 3 (15%) |
| **Nausea and vomiting** | 9 (45%) | 2 (10%) | 1 (5%) | 12 (60%) |
| **Night sweats** | 1 (5%) | | | 1 (5%) |
| **Otitis media** | 1 (5%) | | | 1 (5%) |
| **Restlessness** | 1 (5%) | | | 1 (5%) |

**Supplementary Table 3. Number of participants with related adverse events (AEs) reported during the immunization phase of stage B of the clinical trial (percentage in brackets)**

| **GA2 (immunization) (N=10)** | | | | |
|---|---|---|---|---|
| **Adverse event** | **Mild** | **Moderate** | **Severe** | **Total** |
| **Local** | | | | |
| **Blistering** | | 1 (10%) | | 1 (10%) |
| **Erythema** | 9 (90%) | 1 (10%) | | 10 (100%) |
| **Hyperpigmentation** | 1 (10%) | 1 (10%) | | 2 (20%) |
| **Induration** | 1 (10%) | | | 1 (10%) |
| **Pain** | | 1 (10%) | | 1 (10%) |
| **Pruritus** | 6 (60%) | 3 (30%) | 1 (10%) | 10 (100%) |
| **Swelling** | 3 (30%) | 2 (20%) | | 5 (50%) |
| **Systemic** | | | | |
| **Malaise and fatigue** | | 1 (10%) | | 1 (10%) |
| **GA1 (immunization) (N=10)** | | | | |
| **Adverse event** | **Mild** | **Moderate** | **Severe** | **Total** |
| **Local** | | | | |
| **Erythema** | 9 (90%) | 1 (10%) | | 10 (100%) |
| **Hyperpigmentation** | 1 (10%) | | | 1 (10%) |
| **Pruritus** | 6 (60%) | 1 (10%) | 2 (20%) | 9 (90%) |
| **Swelling** | 1 (10%) | | | 1 (10%) |
| **Systemic** | | | | |
| **Dizziness and giddiness** | 1 (10%) | | | 1 (10%) |
| **Nausea and vomiting** | 1 (10%) | | | 1 (10%) |
| **Placebo (immunization) (N=3)** | | | | |
| **Adverse event** | **Mild** | **Moderate** | **Severe** | **Total** |
| **Local** | | | | |
| **Erythema** | 3 (100%) | | | 3 (100%) |
| **Hyperpigmentation** | 2 (66%) | | | 2 (66%) |
| **Swelling** | 1 (33%) | | | 1 (33%) |
| **Pain** | | 1 (33%) | | 1 (33%) |
| **Pruritus** | 3 (100%) | | | 3 (100%) |
| **Systemic** | | | | |
| **Headache** | 1 (33%) | 1 (33%) | | 2 (66%) |
| **Myalgia** | | 1 (33%) | | |

**Supplementary Table 4. Number of participants with unrelated adverse events (AEs) reported during the immunization phase of stage B of the clinical trial (percentage in brackets)**

| **GA2 (N=10)** | | | | |
|---|---|---|---|---|
| **Adverse events** | **Mild** | **Moderate** | **Severe** | **Total** |
| **Acute pharyngitis** | 5 (50%) | | | 5 (50%) |
| **COVID-19** | 3 (30%) | 1 (10%) | | 4 (40%) |
| **Diarrhea** | 1 (10%) | | 1 (10%) | 2 (20%) |
| **Dysmenorrhea** | 1 (10%) | | | 1 (10%) |
| **Elevated troponin** | 2 (20%) | | | 2 (20%) |
| **Epistaxis** | 1 (10%) | | | 1 (10%) |
| **Headache** | 1 (10%) | 1 (10%) | | 2 (20%) |
| **Influenza** | | 1 (10%) | | 1 (10%) |
| **Insomnia** | 1 (10%) | | | 1 (10%) |
| **Irritability and anger** | 1 (10%) | | | 1 (10%) |
| **Malaise and fatigue** | 1 (10%) | 1 (10%) | | 2 (20%) |
| **Myalgia** | 1 (10%) | | | 1 (10%) |
| **Nausea and vomiting** | 1 (10%) | | 1 (10%) | 2 (20%) |
| **Palpitations** | 1 (10%) | | | 1 (10%) |
| **Scabies** | | 1 (10%) | | 1 (10%) |
| **Unspecified feeling of malaise** | | 1 (10%) | | 1 (10%) |
| **Urinary tract infection** | 1 (10%) | | | 1 (10%) |
| **Urticaria** | | | 1 (10%) | 1 (10%) |
| **GA1 (immunization) (N=10)** | | | | |
| **Adverse events** | **Mild** | **Moderate** | **Severe** | **Total** |
| **Abdominal pain** | | 1 (10%) | | 1 (10%) |
| **Acute pharyngitis** | 2 (20%) | 2 (20%) | | 4 (40%) |
| **Allergic rhinitis** | 1 (10%) | 1 (10%) | | 2 (20%) |
| **Chest pain** | 1 (10%) | | | 1 (10%) |
| **COVID19** | severity not reported | | | 1 (10%) |
| **COVID19** | 2 (20%) | | | 2 (20%) |
| **Diarrhea** | | | 2 (20%) | 2 (20%) |
| **Enuresis** | 1 (10%) | | | 1 (10%) |
| **Epistaxis** | 1 (10%) | | | 1 (10%) |
| **Fecal abnormality** | severity not reported | | | 1 (10%) |
| **Foot fracture** | | 1 (10%) | | 1 (10%) |
| **Headache** | 3 (30%) | 2 (20%) | 1 (10%) | 6 (60%) |
| **Malaise and fatigue** | severity not reported | | | 1 (10%) |
| **Placebo (immunization) N=3** | | | | |
| **Adverse events** | **Mild** | **Moderate** | **Severe** | **Total** |
| **Acute pharyngitis** | 2 (66%) | | | 2 (66%) |
| **Chills** | 1 (33%) | | | 1 (33%) |
| **COVID19** | 2 (66%) | | | 2 (66%) |
| **Dysmenorrhea** | 1 (33%) | | | 1 (33%) |
| **Headache** | 1 (33%) | | | 1 (33%) |
| **Headache** | 2 (66%) | | | 2 (66%) |
| **Influenza** | 1 (33%) | | | 1 (33%) |
| **Nausea and vomiting** | 1 (33%) | | | 1 (33%) |

**Supplementary Table 5. Number of participants with related adverse events (AEs) reported after controlled human malaria infection (CHMI) in stage B of the clinical trial (percentage in brackets, N=23)**

| Adverse events | Mild | Moderate | Severe | Total |
|---|---|---|---|---|
| **Local** | | | | |
| **Erythema** | 19 (83%) | 1 (4%) | | 20 (87%) |
| **Hyperpigmentation** | 3 (13%) | | | 3 (13%) |
| **Pruritus** | 6 (26%) | | | 6 (26%) |
| **Swelling** | 1 (4%) | | | 1 (4%) |
| **Systemic** | | | | |
| **Abdominal pain** | 4 (17%) | | | 4 (17%) |
| **Chills** | | 1 (4%) | | 1 (4%) |
| **Diarrhoea** | 2 (9%) | 1 (4%) | | 3 (13%) |
| **Dizziness** | 3 (13%) | | | 3 (13%) |
| **Fever** | 1 (4%) | 2 (9%) | | 3 (13%) |
| **Flatulence** | | 1 (4%) | | 1 (4%) |
| **Headache** | 4 (17%) | 3 (13%) | 3 (13%) | 10 (43%) |
| **Malaise and fatigue** | 4 (17%) | 2 (9%) | 2 (9%) | 8 (35%) |
| **Myalgia** | 1 (4%) | | 1 (4%) | 2 (9%) |
| **Nausea and vomiting** | 2 (9%) | 1 (4%) | 1 (4%) | 4 (17%) |
| **Night sweats** | 1 (4%) | 1 (4%) | | 2 (9%) |

**Supplementary Table 6. Number of participants with unrelated adverse events (AEs) reported after controlled human malaria infection (CHMI) in stage B of the clinical trial (percentage in brackets, N=23)**

| Adverse events | Mild | Moderate | Severe | Total |
|---|---|---|---|---|
| **Abdominal pain** | | | 1 (4%) | 1 (4%) |
| **Abnormal findings of blood chemistry** | 1 (4%) | | | 1 (4%) |
| **Acute pharyngitis** | 7 (30%) | 3 (15%) | | 10 (43%) |
| **Allergic rhinitis** | 4 (17%) | 1 (4%) | | 5 (22%) |
| **Anorexia** | 1 (4%) | | | 1 (4%) |
| **Diarrhea** | 1 (4%) | | 1 (4%) | 2 (9%) |
| **Dysmenorrhea** | 2 (9%) | | | 2 (9%) |
| **Dyspnea** | 1 (4%) | | | 1 (4%) |
| **Headache** | 3 (13%) | 1 (4%) | | 4 (4%) |
| **Malaise and fatigue** | | 1 (4%) | | 1 (4%) |
| **Migraine** | | | 1 (4%) | 1 (4%) |
| **Myalgia** | 2 (9%) | | | 2 (9%) |
| **Nausea and vomiting** | 2 (9%) | 1 (4%) | 1 (4%) | 4 (17%) |
| **Night sweats** | | 1 (4%) | | 1 (4%) |
| **Oral aphthae** | | 1 (4%) | | 1 (4%) |
| **Otalgia** | | 1 (4%) | | 1 (4%) |

**Supplementary Table 7. Representativeness of study participants**

| Category | Details |
|---|---|
| Disease under investigation | *Plasmodium falciparum*, agent of malaria. |
| Special considerations related to: | |
| Sex and gender | Malaria affects both men and women. However, women may be more vulnerable to the effects of the disease, especially during pregnancy[9,10]. |
| Age | Approximately 78% of malaria-related casualties globally are children under the age of 5[11]. |
| Race or ethnic group | While malaria vulnerability does not depend on race or ethnicity, the disease is more prevalent in Africa and therefore Africans are disproportionally affected[11]. |
| Geography | Africa has the highest incidence of *Plasmodium falciparum* malaria: in 2022 Africa accounted for 94% of morbidity and 95% of mortality worldwide. The 4 most affected African countries are Nigeria, the Democratic Republic of Congo, Uganda and Mozambique[11]. |
| Other considerations | About 10.000 travelers acquire malaria each year, although this number may in reality be higher[12]. |
| Overall representativeness of this trial | Participants of this clinical trial were malaria-naive healthy individuals residing in The Netherlands, aged 18 to 35. Gender and age were self-reported by the participants during the screening process. The male to female ratio was 49% to 51%. While this population differs from that most affected by malaria, this study was meant as a proof of concept to assess the safety and preliminary protective efficacy of a new late-arresting GAP, relative to that of an early-arresting GAP. Further studies will be required to confirm the efficacy of GA2 against naturally-acquired malaria in younger, pre-exposed populations. |

**Supplementary Table 8. Staining antibody panel used in multi-parameter spectro-flow cytometry.**

| Marker | Fluorophore | Dilution | Manufacturer | Cat. Number | Clone |
|---|---|---|---|---|---|
| TCR γδ | PerCP-Cy5.5 | 1:50 | Biolegend | 331224 | B1 |
| CD56 | BV786 | 1:200 | BD | 744222 | R19-760 |
| TCR γδ Vδ2 | BV605 | 1:200 | BD | 743751 | B6 |
| CCR7 | AF700 | 1:100 | Biolegend | 353244 | G043H7 |
| CD3 | APC-ef780 | 1:800 | Invitrogen | 47-0038-42 | UCHT1 |
| CD4 | PE-Cy7 | 1:800 | BD | 557852 | SK3 |
| CD25 | PE-fire 640 | 1:200 | Biolegend | 356148 | M-A251 |
| CD11c | AF532 | 1:100 | Invitrogen | 58011641 | ITGAX |
| CD45RA | BV480 | 1:100 | BD | 746799 | 5H9 |
| INF-γ | BV421 | 1:500 | Biolegend | 502532 | 4S.B3 |
| TNF-α | BV650 | 1:500 | Biolegend | 502938 | MAB11 |
| IL-10 | BV711 | 1:125 | BD | 564050 | JES-9D7 |
| IL-2 | APC | 1:100 | Biolegend | 500310 | MQ1-17H12 |
| IL-4 | PE | 1:20 | BD | 340451 | 3.010.211 |
| IL-5 | PE | 1:250 | Biolegend | 504304 | TRFK5 |
| IL-13 | PE | 1:100 | Biolegend | 501903 | JES10-5A2 |
| FoxP3 | PE-CF594 | 1:50 | BD | 562421 | 259D/C7 |
| CD8 | FITC | 1:400 | BD | 555366 | RPA-T8 |

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