MALARIA

An open-label phase 1/2a trial of a genetically modified rodent malaria parasite for immunization against Plasmodium falciparum malaria

Isaie J. Reuling 1*, António M. Mendes 2*, Gerdie M. de Jong 3*, Amanda Fabra-García 1*, Helena Nunes-Cabaço 2*, Geert-Jan van Gemert 1, Wouter Graumans 1, Luc E. Coffeng 4, Sake J. de Vlas 4, Annie S. P. Yang 1, Cynthia Lee 5, Yimin Wu 5, Ashley J. Birkett 5, Christian F. Ockenhouse 5, Rob Koelewijn 3, Jaap J. van Hellemond 3, Perry J. J. van Genderen 3,6†, Robert W. Sauerwein 1†, Miguel Prudêncio 2†

For some diseases, successful vaccines have been developed using a nonpathogenic counterpart of the causative microorganism of choice. The nonpathogenicity of the rodent Plasmodium berghei (Pb) parasite in humans prompted us to evaluate its potential as a platform for vaccination against human infection by Plasmodium falciparum (Pf), a causative agent of malaria. We hypothesized that the genetic insertion of a leading protein target for clinical development of a malaria vaccine, Pf circumsporozoite protein (CSP), in its natural pre-erythrocytic environment, would enhance Pb’s capacity to induce protective immunity against Pf infection. Hence, we recently generated a transgenic Pb sporozoite immunization platform expressing Pf CSP (PbVac), and we now report the clinical evaluation of its biological activity against controlled human malaria infection (CHMI). This first-in-human trial shows that PbVac is safe and well tolerated, when administered by a total of ~300 PbVac-infected mosquitoes per volunteer. Although protective efficacy evaluated by CHMI showed no sterile protection at the tested dose, significant delays in patency (2.2 days, P = 0.03) and decreased parasite density were observed after immunization, corresponding to an estimated 95% reduction in Pf liver parasite burden (confidence interval, 56 to 99%; P = 0.010). PbVac elicits dose-dependent cross-species cellular immune responses and functional Pf CSP-dependent antibody responses that efficiently block Pf sporozoite invasion of liver cells in vitro. This study demonstrates that PbVac immunization elicits a marked biological effect, inhibiting a subsequent infection by the human Pf parasite, and establishes the clinical validation of a new paradigm in malaria vaccination.

INTRODUCTION

Malaria, a disease caused by Plasmodium parasites, remains a severe public health burden. Despite tremendous progress made over the last decade as a result of scaled-up vector control and of improved diagnosis, as well as availability of first-line treatments, progress has slowed down over recent years. Malaria still caused ~435,000 deaths in 2017 (1). Besides its direct implications on human health, malaria is a substantial contributor to ongoing poverty in affected countries.

The availability of an effective vaccine against malaria is considered among the most important tools for prevention and potential eradication of malaria. The clinical development of a malaria vaccine has been a continuous effort over the past half century (2). Most vaccine candidates so far have shown modest protection or even failed completely. This is due to the complex biology of malaria parasites, which comprises multiple stages in both the mosquito and mammalian hosts, including an asymptomatic obligatory stage of pre-erythrocytic development in the human liver, which is ideally suited for eliciting the multifaceted immune responses required for protection (3). The circumsporozoite protein (CSP) of Plasmodium falciparum (Pf) is currently the leading protein target for clinical development of pre-erythrocytic subunit malaria vaccines. Pf CSP is the predominant antigen on the surface of sporozoites, the parasite form that colonizes the mosquito’s salivary glands and is transmitted to humans, infecting their liver cells. Encouraging, but moderate, protection has been obtained with the Pf CSP-based RTS,S/AS01e (RTS,S) vaccine (4, 5). RTS,S has been associated with up to 87% protection in controlled human malaria infection (CHMI) and delays patency in nonprotected volunteers (6); further, it reduces the number of cases of malaria by 39% and severe malaria by 29% over 4 years in young African children, but the vaccine’s efficacy wanes over several years (7). A milestone in itself, as the first malaria vaccine to be given to children through routine immunization, RTS,S is potentially a valuable tool in the fight against malaria when used in combination with other malaria control interventions and is currently undergoing pilot implementation in various African countries. However, it is clear that malaria vaccines with improved and more durable efficacy are needed (8).

A promising alternative approach is whole-parasite vaccine strategies, based on attenuated Pf sporozoites, which have been shown to induce remarkably high protection rates, but are still in relatively early stages of clinical development (9). Radiation-attenuated sporozoites (RAS) were the first whole-parasite vaccine candidates, eliciting high efficacy against a homologous strain during CHMI in malaria-naïve adults (10, 11). However, a markedly lower efficacy was observed in recent
field evaluations (12–15). Similarly, a remarkable and highly efficient induction of sterile homologous (16, 17), but not heterologous (18), protection against *Pf* infection was also achieved by immunization using a combination of chemoprophylaxis and sporozoites (CPS). More recently, the first genetically attenuated parasite used as a vaccine candidate, PISPZ-GA1 Vaccine, was evaluated in the clinic. This study demonstrated the favorable profile of this candidate, but did not provide definite conclusions regarding the strength of its protective efficacy (19). Collectively, these results warrant the evaluation of novel cross-strain or cross-species approaches that might provide protection against genetically diverse human *Plasmodium* strains.

The concept of cross-species vaccination is usually attributed to Sir Edward Jenner and relies on immunization with the nonpathogenic counterpart of the causative microorganism of disease. Several human diseases, including smallpox [reviewed in (20)], tuberculosis [reviewed in (21)], and rotavirus infections [reviewed in (22)], have been the target of cross-species vaccination. Cross-species immunity between different *Plasmodium* species has been reported in multiple animal studies (23–28) and in clinical studies carried out in the last century as part of an experimental neurosyphilis treatment (29, 30). Live attenuated sporozoite-based cross-species immunity is thought to be dependent on both cellular- and antibody-based responses (28). Experimental evidence indicates that *Pf* sporozoites can protect mice from a *Plasmodium berghei* (*Pb*) infection (26), and inoculations of *Plasmodium malariae* in humans reduce parasitemia and symptoms of a subsequent *Pf* infection (29, 30).

Because the expression of correctly folded *Pf* proteins in heterologous systems can be challenging, *Pb* parasites have recently emerged as an alternative solution for their structurally integral and conformationally accurate expression, as exemplified by the transmission-blocking candidate antigen Pfs48/45 (31, 32). Thus, we hypothesized that PfCSP’s protective ability against *Pf* could be qualitatively enhanced compared to recombinant subunit CSP vaccine candidates, through its expression in a more native-like conformation, and in its natural pre-erythrocytic environment. This, coupled with the potential for immune responses between different *Plasmodium* species and the predicted nonpathogenic nature of *Pb* parasites in humans (33), prompted the generation of a transgenic *Pb* sporozoite expressing PfCSP (*PbVac*). Preclinical studies established the proof of principle of *PbVac* immunization through the validation of an array of core premises, including the demonstration of (i) its ability to infect and develop inside human liver cells both in vitro, in human primary hepatocytes, and in vivo, in liver-humanized mice; (ii) its inability to develop in human red blood cells (RBCs); (iii) its high cellular cross-species immunogenicity; and (iv) its ability to induce functional antibodies in various animal models, which block infection of human hepatocytes by *Pf* sporozoites (33). Additional preclinical demonstration of *PbVac’s* safety paved the way to the evaluation of this vaccine candidate in a clinical setting (34). Here, we performed a first-in-human, dose-escalation study to assess safety and tolerability of *PbVac* delivered by infectious mosquito bites and determined its protective efficacy against *Pf* CHMI.

**RESULTS**

**Study population**

Twenty-four malaria-naive volunteers were enrolled in the study from May 2017 to January 2018, of which 3 volunteers participated in group 1, 3 in group 2, and 12 in group 3, receiving 5, 25, and 75 *PbVac*-infected mosquito bites, respectively. Group 3 participants received a total of ~300 *PbVac*-infected mosquito bites (4× ~75 at 4-, 4-, and 8-week intervals) before *Pf* CHMI by 5 mosquito bites, 3 weeks after the last *PbVac* immunization. Six volunteers (group 4) served as nonimmunized controls for *Pf* CHMI (Fig. 1). All 24 study volunteers (100%) completed all immunizations and/or challenge as scheduled per group. CHMI was administered to 12 immunized participants (group 3) and 6 controls (group 4) on 30 January 2018. Baseline characteristics are shown in table S1.

![Fig. 1. CONSORT diagram.](http://stm.sciencemag.org/content/12/eaay2578/F1.large.jpg)
Safety and tolerability
Immunizations were well tolerated in all study groups without breakthrough infections, as measured by thick smear and retrospective Pb quantitative polymerase chain reaction (qPCR) analyses of samples collected daily for 10 days after parasite administration, during phase 1 of the trial, and on days 1 and 8 after immunization, during phase 2 of the trial. Only mild to moderate (grade 1 and 2) adverse events and no grade 3 or serious adverse events were recorded (table S2). There were no differences in systemic adverse events between study groups, although local reactions related to the mosquito bites were more frequent in groups 2 and 3. The most commonly reported systemic adverse events were headache (7 of 18 participants), nausea (5 of 18 participants), and malaise (3 of 18 participants). During the challenge phase, approximately 92% (11 of 12) of the participants of group 3 and 100% of group 4 reported mild to moderate adverse events per participant of groups 3 and 4 was significantly lower (P = 0.040) in the immunized subjects than in controls. A total of only eight (grade 1 or 2) laboratory abnormalities were observed, including decreases in leukocyte, lymphocyte, and platelet counts, and increases in bilirubin, aspartate aminotransferase/alanine aminotransferase, and creatinine concentrations during the immunization phase (table S3).

Protective efficacy
Sterile protection against an NF54 P. falciparum challenge was not observed, but a significant delay in time to parasitemia (prepatent period) was observed in PbVac-immunized subjects (9.9 ± 2.0 days), compared to controls (7.7 ± 1.6 days) (P = 0.026, Kolmogorov-Smirnov test), as detected by qPCR (Fig. 2A). Furthermore, there was a significantly 12.8-fold lower parasite peak density on the day of first positive PCR in immunized volunteers compared to the control group (P = 0.04, Kolmogorov-Smirnov test) (Fig. 2B), which resulted in a delayed parasitemia curve (Fig. 2C). Collectively, this corresponds to an estimated 95% average reduction in parasite liver load (confidence interval, 56 to 99%; P = 0.010, two-sample t test for logarithm of estimated first-generation parasite concentration) (Fig. 2D).

Humoral immune responses
Antibody responses to the candidate vaccine were measured in subjects by immunofluorescence assay (IFA) against PbVac sporozoites, as well as against the wild-type Pb (PbWT) and PfCSP vaccine components. Marked increases in PbVac sporozoite recognition were observed for both immunoglobulin G (IgG; 5.9-fold, P < 0.01; Fig. 3A) and IgM (3.1-fold, P < 0.01; fig. S1A) after immunization. Similar results, albeit of lower magnitude, were obtained for PbWT sporozoite-specific IgG and IgM (2.6-fold, Fig. 3B, and 2.8-fold, fig. S1B, respectively) and for PfCSP (4.2-fold, Fig. 3C, and 2.2-fold, fig. S1C).

We subsequently assessed antibody binding and functionality against Pf, by IFA, sporozoite membrane integrity, and sporozoite liver invasion inhibition assays (Fig. 3, D to F). Immunization with PbVac increased the concentration of both IgG and IgM targeting Pf sporozoites (3.5-fold, P < 0.01, Fig. 3D, and 1.71-fold, P = 0.03, fig. S1D, respectively). Of note, the capacity of plasma IgG to bind Pf sporozoites directly correlated with the titers of circulating anti-PfCSP antibodies (fig. S1E). IgG purified after immunization (fig. S1F) enhanced C3 complement protein deposition on Pf sporozoites (4.3-fold, P < 0.01; fig. S1G), resulting in a strong decrease of Pf sporozoite viability (Fig. 3E). Last, purified IgG significantly reduced Pf sporozoite invasion of HC-04 cells by a median of 61.1% (P < 0.01). Depletion of anti-PfCSP–specific IgG completely reversed the inhibitory effect on Pf sporozoite invasion (Fig. 3F), as well as the C3 complement fixation on Pf sporozoites (fig. S1G). The titers of IgG against PfCSP, the binding capacity of IgG to Pf sporozoites, and the enhancement of Pf sporozoite lysis correlated with the cumulative number of PbVac mosquito bites (Fig. 3, G to I, respectively, and table S4). The combined data suggest that induced antibodies were capable of inhibiting Pf sporozoite viability and functionality in a dose-dependent manner. However, these in vitro findings did not correlate with the delayed patency or with the reduced parasite liver load (table S4).

Fig. 2. Clinical efficacy of PbVac. (A) Prepatent period for nonimmunized controls (white circles; n = 6) and immunized volunteers (green circles; n = 12), presented as the number of days until parasitemia was at least 100 Pf parasites/ml of blood, as measured by qPCR. (B) Pf parasitemia at first positive qPCR. (C) Pf parasitemia curves following CHMI. (D) Estimation of the number of first-generation Pf parasites exiting the liver. Comparisons between controls and immunized volunteers in (A) and (B) were assessed by using the Kolmogorov-Smirnov nonparametric test (*P < 0.05; **P < 0.01), and bars represent mean ± SE. The shaded area below the dashed line in (B) and (C) denotes parasitemia values below the 100 Pf/ml of blood threshold. In (D), significance was calculated using a two-sample t test, and bars represent geometric mean ± SE. vxx denotes volunteer number.
Fig. 3. Humoral responses elicited by PbVac. (A and B) Plasma IgG binding to PbVac (A) and PbWT (B) sporozoites (spz) in samples collected preimmunization (I1; white circles) and before CHMI (C; green circles); median fluorescence intensity (MFI) values are for binding at a 1:1000 dilution. (C) Plasma IgG reactivity against full-length PbCSP as measured by ELISA, normalized to a pool of sera from 100 hyperimmune Tanzanians (HT), which was set at 100 arbitrary units (AU). (D) Binding of plasma IgG to Pf sporozoites. MFI values are for binding at a 1:1000 dilution. (E) Binding of Pb sporozoite–infected cells after incubation with purified plasma IgG was assessed as a surrogate for induced Pf sporozoite lysis. (F) Inhibition of Pf sporozoite invasion of HC-04 hepatic cells by purified plasma IgG from samples collected preimmunization (I1–I1) and before CHMI (C–1), depleted or not of PbCSP-specific antibodies (PbCSP-depleted, light green circles). (G to I) Correlation between the cumulative number of mosquito bites and anti-PbCSP antibody titers (G), binding capacity to Pf sporozoites (H), and induction of Pf sporozoite lysis (I). Differences between groups were assessed by using the Wilcoxon matched-pairs signed-rank test and correlations using the nonparametric Spearman correlation test (*P \leq 0.05, **P \leq 0.01, and ***P \leq 0.001). vxx denotes volunteer number (n = 12).

Cellular immune responses

Given the largely exploratory nature of the study, a wide-range analysis of cellular immune responses was performed. Peripheral blood mononuclear cells (PBMCs) sampled before immunization (I1–I1), 14 days after the first immunization (I1+14), and 1 day before CHMI (C–1) were analyzed by multiparameter flow cytometry with an initial focus on relevant cellular subsets identified in previous clinical challenge studies, including CD4\(^+\) T cells (10, 16, 35), CD8\(^+\) T cells (10, 35, 36), γδ T cells (17, 37, 38), natural killer (NK) cells (39), and monocytes (39) (fig. S2). An increased frequency of CD8\(^+\) T cells and, in particular, γδ T cells (particularly the Vγ2+ subfamily) and a decrease in circulating monocytes were observed at C–1, unrelated to trial outcome (table S5). Of note, an increase in circulating NKT cells was found before CHMI (at C–1; fig. S2), which associated with the increase in prepatent period and the decrease in parasite density (table S5). No relevant differences were observed between circulating cytokines before and 5, 7, and 10 days after the first immunization (fig. S3). However, a clear association was found between changes in serum cytokine or chemokine concentrations after the first PbVac administration and the outcome of immunization, including for interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-12 (IL-12), IL-6, and monocyte chemoattractant protein 1 (MCP1/CCL2) (table S6). Such changes may also be associated with the observed differences in the frequency of circulating immune cells (table S7). For example, serum concentrations of IFN-γ may be directly linked to changes in γδ T cell frequency before CHMI, whereas concentrations of TNF-α are more broadly associated with the frequency of not only γδ T cells but also CD4\(^+\) T and NKT cells. As expected, changes in the MCP1/CCL2 chemokine are closely linked to variations in the frequency of circulating monocytes, and are accompanied by alterations in other cytokines involved in the same pathway, such as IFN-α and IL-18 (table S7).

Next, PBMCs were incubated with PbVac or PbWT sporozoites, or with recombinant PbCSP, and the expression of IFN-γ, IL-2, and TNF-α was analyzed. Statistically significant increases were
observed in the frequency of PbVac- and PbWT-specific, but not PfCSP-specific, lymphocytes producing IFN-γ at either I1+14 (P = 0.002 and P = 0.001, respectively) or C-1 (P = 0.002 and P = 0.007, respectively) (Fig. 4, A to C). Similarly, PbVac-specific (P = 0.009 for C-1) and PbWT-specific (P = 0.034 for I1+14), but not PfCSP-specific, CD4+ (Fig. S4C) and CD8+ (Fig. 4, D to F) T cells expressing any combination of IFN-γ, IL-2, and TNF-α were significantly more frequent after immunization (P = 0.016 and P = 0.042, for C-1, respectively). The frequency of CD4+ T cells simultaneously expressing IFN-γ and TNF-α increased for both PbVac (Fig. S4, D and E) and PbWT (Fig. S4, F and G) sporozoite-specific responses.

Collectively, these data show that the genetic introduction of PfCSP bears small influence on the cellular responses induced by immunization with PbVac. In contrast, the PbWT backbone is sufficient to induce an increase in sporozoite-specific cytokine production, but no correlation was found between cytokine production by any of the cell populations analyzed and the clinical outcome. The combined data suggest the existence of a multifaceted response to PbVac that may comprise different cellular populations or a combination of cellular and humoral immune responses.

**Dose dependency of cross-species cytokine responses**

We studied cytokine responses to Pf sporozoites in PBMCs from volunteers receiving 5, 25, and 75 PbVac-infected mosquito bites (Fig. 5, A and B, and fig. S5, A to C). IFN-γ production was only statistically increased at the highest PbVac dosage in total PBMC (Fig. 5A) and CD8+ T cells (Fig. 5B), but not in CD4+ T cells producing any combination of IFN-γ, IL-2, or TNF-α (fig. S5A). Nonetheless, CD4+ T cells expressing simultaneously IFN-γ and TNF-α were significantly increased after immunization (P = 0.012) (fig. S5B). There was no statistically significant association between the frequency of cytokine-producing cells and either an increase in prepatent period or a reduction in liver load of immunized subjects. Our results show that PbVac immunization elicits dose-dependent cross-species cytokine responses against Pf, suggesting that a higher PbVac dosage might improve protective efficacy.

**Heterogeneity of immune responses**

Last, to directly compare the responses of each volunteer with the mean values of each parameter for the entire group before immunization, a Z score was calculated for the magnitude of the cellular and humoral immune responses measured for each volunteer (fig. S6). An analysis of the individual immune responses to immunization reveals a marked heterogeneity in the magnitude of these responses, with some volunteers developing a predominantly cellular immune response (fig. S6, A and B) and others favoring a humoral response (fig. S6C). This heterogeneity is not unexpected and has been observed not only in malaria vaccination trials of whole-sporozoite vaccines delivered by mosquito bite but also in other malaria vaccination trials (40). However, our analysis shows that all individuals display a response to PbVac in at least one of the immune response parameters assessed. For example, v70, the volunteer who showed the smallest increase in prepatent period and the lowest reduction of estimated liver load after CHMI,

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Fig. 4. Cellular immunogenicity of PbVac. (A to C) Percentage of IFN-γ-producing lymphocytes in peripheral blood samples collected preimmunization [I1 (−1), white circles], 14 days after the first immunization [I1(+14), gray circles], and before CHMI [C (−1), green circles] after stimulation with PbVac sporozoites (A), PbWT sporozoites (B), or PfCSP recombinant protein (C). (D to F) Percentage of CD8+ T cells producing any combination of IFN-γ, IL-2, and TNF-α in peripheral blood samples, after stimulation with either PbVac sporozoites (D), PbWT sporozoites (E), or PfCSP (F). Differences between groups were assessed by using the Wilcoxon matched-pairs signed-rank test (**P < 0.05, ***P < 0.01, and ****P < 0.001). In (C) and (F), bars represent the mean ± SD. vxx denotes volunteer number (n = 12). ns, not significant.
cells within lymphocytes (%)

Plasmodium based on the use of genetically modified nonhuman
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control of the UIS4 pre-erythrocytic stage promoter, belongs to a
Vac, a parasite that expresses the
Pb
Pf
Pb

Whole-sporozoite immunization constitutes a promising approach
DISCUSSION
immune response parameter.
of a direct correlation between the clinical outcome and a single
response against either PbVac (fig. S6A) or Pf sporozoites (fig. S6B),
but developed a robust functional humoral immunity, capable of
inhibiting Pf sporozoite invasion of HC-04 cells (fig. S6C). On the
contrary, v65 is among the top responders of the group in terms of
cellular immune responses against either PbVac or Pf sporozoites,
but his/her humoral immune responses against Pf sporozoites rank
as the lowest of the group. Overall, this analysis highlights the
heterogeneity of individual immune responses as well as the lack of a
direct correlation between the clinical outcome and a single
immune response parameter.

Fig. 5. Dose-dependent cross-species cytokine responses. (A) Percentage of
IFN-γ–producing lymphocytes after stimulation with Pf sporozoites in peripheral
blood samples collected preimmunization [I1 (–)] and 14 days after immunization
[I1 (+14)] with 5 and 25 mosquito bites, or collected preimmunization [I1 (–)],
14 days after the first immunization [I1 (+14d)] with 75 mosquito bites, and before
CHMI [C1 (–)]. (B) Percentage of CD8+ T cells producing any combination of IFN-γ,
IL-2, and TNF-α after stimulation with Pf sporozoites. Differences between groups
were assessed by using the Wilcoxon matched-pairs signed-rank test (*P ≤ 0.05,
**P ≤ 0.01, and ***P ≤ 0.001).

appears to be one of the volunteers with the lowest cellular immune
responses against either PbVac (fig. S6A) or Pf sporozoites (fig. S6B),
but developed a robust functional humoral immunity, capable of
inhibiting Pf sporozoite invasion of HC-04 cells (fig. S6C). On the
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immune response parameter.

DISCUSSION
Whole-sporozoite immunization constitutes a promising approach
to the much-desired goal to control and eliminate malaria (41). PbVac, a Pb parasite that expresses the PfCSP antigen under the
control of the UIS4 pre-erythrocytic stage promoter, belongs to a
distinct class of whole-sporozoite malaria vaccination approaches
based on the use of genetically modified nonhuman Plasmodium
parasites expressing heterologous target antigens of their human
counterparts (33). The present first-in-humans study shows that
administration of PbVac by mosquito bites to healthy volunteers
is safe and well tolerated, exerting a pronounced biological effect
against a subsequent Pf challenge, which appears to be associated
with components of both humoral and cellular immune responses.

The efficacy of protective humoral responses targeting the extra-
cellular, infective sporozoite depends on the concentration and
specificity of the response elicited by vaccination (42, 43). Anti-CSP
antibodies have been shown to inhibit Pf sporozoite infectivity in vitro
(44, 45) and in vivo (46), and their protective role is shown in the
context of the subunit vaccine RTS,S (47, 48). Here, we show that
the genetic insertion of PfCSP in the rodent Pb platform markedly
contributed to the antibody responses induced by PbVac, enabling
complement activation, Pf sporozoite lysis, and the inhibition of Pf
sporozoite invasion of hepatic cells in vitro. The PfCSP component of
PbVac is therefore a potential major contributor to the biological
effect observed upon vaccination.

Our results also suggest a contribution of PbVac’s PbWT backbone
to the cellular immune responses elicited and to the clinical
outcome. In RAS and CPS vaccination, an increase in the frequency
of Pf-specific CD4+ and CD8+ T cells producing any combination of
IFN-γ, IL-2, or TNF-α or the frequency of V82+ T cells associates
with sterile immunity in CHMI studies in healthy U.S./European
volunteers (10, 17, 35, 37, 49, 50). In the present study, a marked
expansion of not only CD8+ T, γδ T, and V82+ T cells but also NKT
cells was observed after immunization. Conversely, decreased fre-
cquencies of circulating monocytes were observed after vaccination,
which may be linked to early changes in MCP1/CCL2 signaling as
well as in IL-18, an inducer of MCP1 production (51, 52). These
observations suggest a possible recruitment of monocytes to the liver,
where they may contribute to the development of tissue-resident
protective immune responses, potentially affecting the outcome
of CHMI (53–56). Last, increased frequencies of IFN-γ–producing
lymphocytes and of CD8+ T cells producing any combination of
IFN-γ, TNF-α, or IL-2 were observed upon PBMC stimulation with
either PbWT or Pf sporozoites, but not with PfCSP. Collectively,
these results expose a multifactorial and complex immune signature,
without clear correlates of protection, similarly to what has been
noted for RTS,S (47, 48), as well as for whole-sporozoite immuniza-
tions (10, 50), warranting further investigation. In summary, and
in agreement with our preclinical data (33), our data suggest that PbVac
immunization elicits functional PfCSP-based antibody responses,
as well as cross-species, PbWT backbone-dependent, cellular immune
responses against Pf.

We observed proportionality between humoral/cellular immune
responses and the cumulative number of immunizing bites, as
previously determined after RAS and CPS immunizations (10, 35). In
early clinical studies of PfRAS vaccination by mosquito bite, sterile
protection was only achieved when volunteers underwent a mean of
9 immunizations and >1000 immunizing bites before CHMI (57).
In the present study, the total number of immunizing bites was sub-
stantially lower (four immunizations with ~75 immunizing bites
each, for a total of ~300 bites). Therefore, PbVac immunization may
induce sterilizing protection against Pf challenge with an increase in
the number of sporozoites administered. Because markedly increas-
ing the dose of parasites used for immunization through increasing
the number of mosquito bites appears impractical, achieving this
goal will potentially require the inoculation of sporozoites through
the injection of sporozoites by intravenous route. This method
is amply documented as an efficacious method for delivery of
whole-sporozoite RAS and chemoattenuated vaccine candidates
(10, 11, 17, 37). Such an endeavor poses several challenges, chief
among which is the requirement for a good manufacturing practice
(GMP)–compliant sterile Pb/PbVac production system, an absolute
require for the intravenous administration of such parasites to humans.

Whole-sporozoite vaccines against malaria still require relatively
high doses of sporozoites to elicit sterile immunity, demanding the
production of large numbers of infected mosquitoes reared in highly
specialized facilities, with a consequent impact on the cost of goods.
This is particularly relevant in the case of Pf-based vaccines, whose
production commands compliance with very stringent safety require-
mements. The biological effect observed upon PbVac administration

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in the present study warrants the exploration of Pb as one possible alternative platform for whole-sporozoite vaccination. Besides its relative safety, Pb-based vaccines may offer an alternative to Pf-based vaccination, with potential advantages over the latter in terms of cross-species immunization. We have previously shown not only that Pb infectivity of human primary hepatocytes appears to be higher than that of Pf but also that >24,000 in silico–predicted CD8+ T cell epitopes, encoded in >60% of both the Pf and Pb proteins, are shared between the two parasite species (33). One may further speculate that, given the co-evolution of humans and Pf parasites, immune evasion mechanisms may be at play during immunization with the latter that may be absent in the case of Pb-based immunization. On the other hand, whereas the expression of Plasmodium proteins in heterologous systems for subunit vaccination is notoriously difficult (31, 58), the presence of several genetic loci permissive for gene-insertion modifications on Pb (59–61) offers opportunities for the introduction of multiple correctly folded antigens in their native conformation (31). Thus, next-generation Pb-based vaccine candidates may include combinations of not only antigens from different human-infective Plasmodium spp. but also blood-stage or transmission-blocking immunogens, as well as multiple alleles of an antigen (62).

In addition, the hepatotropic nature of Pb sporozoites promotes the exposure of antigens in the liver, where tissue-resident immunity is most likely to develop (53–56). Nevertheless, whether sufficiently protective immune responses can be elicited by Pb-based immunization, as well as the durability of such responses, need to be addressed in further clinical studies. Such studies will potentially include both PbWT- and PbVac-immunized volunteers and appropriate PfRAS or PfCPS arms for a comparative analysis of protective efficacy and immune responses elicited by vaccination. These features, along with the presently reported strong biological effect elicited by immunization with PbVac, even at suboptimal doses, make the Pb-based platform a promising tool for the development of a next-generation malaria vaccine.

**MATERIALS AND METHODS**

**Study design**

This study was a multicenter phase 1/2a, open-label, dose-escalation trial in malaria-naïve healthy participants to assess safety and tolerability of PbVac (phase 1) and to determine the protective efficacy of immunization with PbVac against CHMI (phase 2). The primary endpoint of phase 1 was determined by frequency and magnitude of adverse events, as well as by the presence of parasitemia after exposure to PbVac, as detected by thick blood smear. The primary endpoint of phase 2 was determined by the time to parasitemia after CHMI, as detected by 18S qPCR analysis. Secondary endpoints were to determine the immunogenicity of PbVac as assessed by enzyme-linked immunosorbent assay (ELISA) and IFA, as well as the composition and function of immune responses after exposure to PbVac, which were analyzed using various assays.

The study protocol was approved by the Dutch Central Committee on Research Involving Human Subjects (NL60019.091.16) and the Western Institutional Review Board (20170356), and PbVac was released for study use by the Gene Therapy Office in the Netherlands, licensed to the Radboud University Medical Centre (IM-MV 15-010), Erasmus Medical Centre (IM-MV 16-008), and Harbour Hospital (IM-MV 16-010). The trial was conducted according to the principles outlined in the Declaration of Helsinki and Good Clinical Practice standards, and registered at ClinicalTrials.gov, identifier NCT03138096.

In phase 1, 18 participants were divided in three groups and exposed to bites of 5 (n = 3), 25 (n = 3), or 75 (n = 12) PbVac-infected mosquitoes. Study subjects of all groups were followed once daily on an outpatient basis from day 1 to day 10 and every other day on days 12 to 20 after exposure. During the visits, blood was drawn for routine hematological and biochemical analysis, peripheral Giemsa-stained thick blood smears, and retrospective real-time qPCR (Pb18S qPCR) analysis. Signs and symptoms were recorded by a physician and graded as follows: grade 1 (no interference with daily activities), grade 2 (interferes with normal activity), or grade 3 (prevents normal activity). Participants of groups 1 and 2 were treated with a curative regimen of atovaquone/proguanil (Malarone, GlaxoSmithKline) on day 28 and received an end of study visit on day 100 after immunization. Participants of group 3 received three additional immunizations with 75 PbVac-infected mosquitoes after phase 1 at 4-, 4-, and 8-week intervals (phase 2). Three weeks after the last immunization, all 12 immunized participants, including 6 unimmunized infectivity controls, were subjected to a standard CHMI with five P. falciparum (strain NF54)–infected mosquito bites (63). All participants were monitored once daily on an outpatient basis from day 6 to day 21 for symptoms and signs of malaria, and blood was drawn for routine laboratory tests and for Pf18S qPCR analysis, as previously described (64). Participants were treated with a curative regimen of atovaquone/proguanil (Malarone, GlaxoSmithKline) after a single positive qPCR (≥100 parasites/ml) after challenge. Additional information can be found in the Clinical Trial Protocol, data file S1. Primary data are reported in data file S2.

**Study participants and eligibility criteria**

All 57 volunteers that underwent screening signed an informed consent form after the nature and possible consequences of the studies have been explained. Four of the volunteers included withdrew their informed consent because of change of job and relocation from the area of the study center and, therefore, four backup volunteers were enrolled in the study. A total of 33 participants were excluded after screening. Twenty-four healthy malaria-naïve males and non-pregnant females (aged 18 to 35 years) were recruited at the Harbour Hospital, Rotterdam, after signing informed consent. Extensive screening was performed before inclusion in the Erasmus Medical Centre, Rotterdam, as previously described (65). The complete screening procedures, including the list of inclusion and exclusion criteria, can be found in the clinical trial protocol document (data file S1) and on ClinicalTrials.gov, identifier NCT03138096.

**Sample collections**

Blood collections for separation and cryopreservation of plasma and PBMCs were performed at the beginning of the trial and the day before each immunization or CHMI using sodium citrate BD Vacutainer Mononuclear Cell Preparation Tubes. Further details are provided in the Clinical Trial Protocol (data file S1).

**Production and administration of PbVac**

The generation and characterization of PbVac, which consists of genetically modified sporozoites of rodent Pb parasites expressing PfCSP, has been previously described in detail (33, 34). Briefly, a transgenic Pb parasite line containing a Pfs2 expression cassette was generated using the “gene insertion/marker out” (GIMO) technology. Correct integration of the construct into the genome of transgenic parasites was analyzed by diagnostic PCR analysis of
genomic DNA and Southern analysis of pulsed-field gel-separated chromosomes.

**PbVac parasite detection by qPCR**
Retrospective PbVac parasite detection on whole-blood samples was performed by the use of a qPCR with Pb 18S ribosomal RNA (rRNA)–specific primers as previously described (34). A standard curve consisting of 10-fold serial dilutions from 10⁶ to 1 PbVac sporozoite was included in all qPCR assays.

**Estimation of *Pf* liver burden reduction**
Liver burden reduction calculation was estimated by back-calculating the initial number of first-generation parasites released from the liver and then comparing the means of the logarithms of estimated concentration in controls and immunized individuals with a two-sample t test. Back-calculations were performed on the basis of a published mathematical model (66, 67) based on the following assumptions: (i) the first generation of parasites is released at 6.87 days after inoculation, (ii) the time between parasite generation is 1.84 days, and (iii) asexual parasite numbers increase with a factor of 11.8 per parasite generation. Back-calculation was performed using the highest measured parasite concentration for each subject, which yielded the most conservative estimate of the difference between controls and immunized.

**IgG purification and *PfCSP*-specific IgG depletion**
Pre- and post-immunization (before challenge) IgGs from citrated plasma samples were precipitated with a saturated ammonium sulfate solution (Pierce, Thermo Fisher), following the manufacturer’s instructions, and resuspended in phosphate-buffered saline (PBS) (Gibco, Thermo Fisher). IgGs were purified by affinity chromatography using 1 ml of HiTrap protein G HP columns (GE Healthcare Life Science) and eluted with an amine-based buffer at pH 2.8 (Pierce, Thermo Fisher). To deplete the IgGs with specificity for *PfCSP*, post-immunization IgGs were run several times through a 1 ml HiTrap N-hydroxysuccinimide (NHS)–activated HP column coupled to a full-length *PfCSP* (Genova Biotechniques Pvt. Ltd.), as previously described (68). Anti-*PfCSP* IgGs were eluted from the column with the previously used buffer. After purification and depletion, IgGs (anti-*PfCSP* fraction and non-anti-*PfCSP* fraction) were buffer-exchanged with PBS and concentrated using Vivaspin 30 concentrator columns (Sartorius). The final concentration of IgGs was measured by the bicinchoninic acid assay (Pierce, Thermo Fisher) following the manufacturer’s indications.

**Anti-*PfCSP* ELISA**
The reactivity of the IgGs from citrated plasma samples at indicated time points and the depletion efficiency were tested against the full-length *PfCSP* (Genova Biotechniques Pvt. Ltd.) or the repeat region of *PfCSP* with the amino acid sequence (NANP)₄NDPC using a standardized ELISA, as previously described (68). Briefly, Immunolun polystyrene flat-bottom 96-well plates (Thermo Fisher) were coated overnight at 4°C with full-length *PfCSP* (2 μg/ml) or the repeat region of *PfCSP* at 5 μg/ml. The plates were blocked [0.05% Tween 20/1× PBS (PBST) with 5% milk] for 1 hour at room temperature (RT). Plasma samples were diluted 1:50 in 1% milk PBST, and a four-point 1:3 dilution was carried out for each sample. To determine the depletion efficiency, pre- and post-anti-*PfCSP*–depleted samples were tested at a final concentration of 1 mg/ml in duplicates.

After 3-hour incubation at RT, plates were washed three times with washing solution (PBST) and incubated for 1 hour at RT with the secondary antibody, goat anti-human IgG coupled to horseradish peroxidase (1:3000, Jackson Immunoresearch). After six washes, the reaction was developed with tetra-methyl-benzidine followed by the addition of 0.2 M sulfuric acid to stop the reaction. Absorbance was immediately measured at 450 nm using an iMark microplate absorbance reader (Bio-Rad) or a Tecan Infinite M200 plate reader. Data were analyzed in relation to a plasma pool from 100 hyperimmune Tanzanian (HIT) adults living in a highly malaria-endemic area. The plasma pool was diluted 1:200 in 1% milk PBST, and a seven-point 1:2 dilution curve was generated. Analysis was performed using Audit-able Data Analysis and Management System for ELISA (ADAMSEL FPL v1.1), and the data were expressed as arbitrary units (AU), as described elsewhere (18, 68).

**In vitro sporozoite invasion inhibition assay**
The ability of the induced antibodies to inhibit sporozoite invasion was assessed in vitro using the human hepatoma cell line HC-04 (MRA-965, deposited by J. Sattabongkot). Briefly, HC-04 cells (5 × 10⁴ cells per well) were seeded onto rat tail collagen (BD Bioscience) pretreated flat-bottom 96-well plates (Corning, Merck) for 16 to 24 hours. Pre- or post-immunization or anti-*PfCSP*–depleted IgGs were preincubated at 6 mg/ml with *PfCSP* (Genova Biotechniques Pvt. Ltd.) or the repeat region of *PfCSP* with 10% heat-inactivated human serum (HIHS). Sporozoites were also incubated with PBS to determine the percentage of invasion and with the mouse anti-CSP antibody, 2A10 (100 μg/ml; MR4, MRA-183A), as invasion inhibition control. In addition, to neutralize possible anti-*PfCSP* IgGs remaining after the depletion, soluble *PfCSP* protein (500 μg/ml) was added to the depleted samples and to the 2A10 control. After 30 min on ice in each of these conditions, 5 × 10⁴ sporozoites were directly seeded in triplicates onto HC-04 cells. The plates were centrifuged for 10 min at 3000 rpm with low brake and incubated for 3 hours at 37°C in 5% CO₂. Cells were then washed with PBS (Gibco) to eliminate non-invasing sporozoites and trypsinized (0.005% EDTA; Gibco) for 5 min at 37°C to obtain a single-cell suspension. HIHS (10%)/PBS was added at 1:1 ratio to neutralize the trypsins. Cells were transferred to a V-bottom 96-well plate, washed with PBS (Gibco), and centrifuged at 1700 rpm for 4 min at 4°C. Subsequently, a cell viability dye [1:2000; fixable viability dye (FVD) eFluor 780, eBioscience, Thermo Fisher Scientific] was added for 20 min at 4°C. After washing with PBS, the cells were fixed and permeabilized (eBioscience) for 30 min at 4°C and stained to detect intracellular and invading sporozoites with a fluorescently labeled mouse anti-CSP antibody (69) [1:200; 3SP2–fluorescein isothiocyanate (FITC)] for 30 min at 4°C. Cells were then washed with 2% fetal bovine serum (FBS)/PBS and fixed with 1% paraformaldehyde (PFA). The readout was done by flow cytometry using Gallios (Beckman Coulter) and the analysis with FlowJo v10.0.8 (FlowJo, BD).

**In vitro complement deposition and membrane-compromised sporozoite assay**
The capacity of antibodies to fix complement (C3b) and therefore compromise the sporozoite membrane integrity was assessed by flow cytometry, as previously described (68). Briefly, mosquito salivary gland–dissected *P. falciparum* (NF54) sporozoites were seeded onto V-bottom 96-well plates (5 × 10⁴ sporozoites per well; Costar). Infected salivary glands were washed with PBS and centrifuged at
3200g for 5 min at RT. The supernatant was carefully removed, and the sporozoites were then incubated in duplicate with pre- or post-immunization IgGs (10 mg/ml) and 10% fresh human serum (NHS; active complement); all components were diluted in 1× veronal buffer (Lonza Bioscience). After incubating for 30 min at 37°C, complement was inactivated with 10 mM EDTA/PBS for 5 min at 4°C. The sporozoites were then washed with 2% bovine serum albumin (BSA)/PBS and stained with a fixable cell viability dye (1:2000; eFluor 780 eBioscience), a fluorescently labeled mouse anti-PCSP antibody (1:10,000; 3SP2-dylight 650), and the anti-C3b complement protein deposition (1:500; C3-FITC Cappel, MP Biomedicals) for 30 min at 4°C. The samples were washed and fixed with 1% PFA. Positive cells were quantified by flow cytometry. Data were acquired with Gallios (Beckman Coulter) and analyzed with FlowJo v10.0.8 (FlowJo, BD).

**Quantification of plasma IgG and IgM by a flow cytometry–based IFA**

Quantification of sporozoite-specific antibody titers in the plasma of immunized volunteers was performed by a flow cytometry–based immunofluorescence assay. Cryopreserved PfNF54, PbVac, or PbWT sporozoites were thawed and washed with PBS by centrifuging at 16,900g for 15 min at 4°C. Sporozoites were labeled by incubating with 20 μM SYTO 61 red fluorescent nucleic acid stain (Molecular Probes) for 30 min at 4°C, centrifuged, and resuspended in PBS. Total plasma or purified IgGs (at 5000 ng/ml) were diluted 1:500 in PBS, and 20 μl of each dilution was mixed with 20 μl of sporozoites in PBS (containing 25 × 10^3 to 50 × 10^3 parasites) in a U-bottom 96-well plate. After a 30-min incubation at 4°C, the plate was centrifuged (10 min at 3900g and 4°C) and the supernatant was removed. Anti-human IgG CF488A (Sigma) and anti-human IgM Pacific Blue (BioLegend) antibodies were added to the wells for 30 min at 4°C. Sporozoites were fixed in 1% formaldehyde (Sigma) before acquisition on a BD LSFRFortessa X-20 (BD Biosciences). Data were analyzed using FlowJo v10.5.3 (FlowJo, BD).

**Ex vivo phenotyping of PBMCs**

The impact of PbVac immunization on circulating immune populations was assessed by flow cytometry. PBMCs were thawed and rested overnight at 37°C in complete RPMI [RPMI 1640 containing 10% heat-inactivated FBS, 2 mM l-glutamine, penicillin (100 U/ml), 100 μg/ml streptomycin, 10 mM Hepes, and 1× minimum essential medium (MEM) nonessential amino acids, all from Gibco, Thermo Fisher Scientific]. PBMCs (1 × 10^6) were incubated with FVD for 15 min at RT, washed with PBS containing 2% FBS, and stained with the following anti-human antibodies: CD4 (RPA-T4), CD8 (RPA-T8), CD3ε (UCHT1 or HIT3a), T cell receptor γδ (TCRγδ) (IMMU510), TCR Vδ2 (B6), CD56 (MEM-188), and CD14 (HCD14), all from BioLegend, eBioscience, or Beckman Coulter. Cells were washed with PBS containing 2% FBS, fixed in 1% formaldehyde for 10 min, and finally washed and resuspended in PBS with 2% fetal calf serum (FCS) before acquisition of approximately 5 × 10^6 events on a BD LSFRFortessa X-20 (BD Biosciences). After exclusion of dead cells (based on positive FVD staining) and doublets (based on FSC-A and FSC-H parameters), the following populations were assessed: CD4^+ T cells (CD3^+CD4^+CD8^-), CD8^+ T cells (CD3^+CD4^-CD8^+), γδ T cells (CD3^+TCRγδ^-), TCRγδ Vδ2 T cells (CD3^+TCRγδ^-Vδ2^+), NKT cells (CD3^+TCRγδ^-CD56^+), NK cells (CD3^+CD56^-), and monocytes (FSC^highSSC^highCD3^+CD14^-). Data were analyzed using FlowJo v10.5.3 (FlowJo, BD).

**Serum cytokine and chemokine analysis**

Serum quantities of nine different cytokines and chemokines were determined using the bead-based LEGENDplex (BioLegend) assay, following the manufacturer’s instructions. Samples were analyzed at baseline and after the first immunization (days 1, 2, 3, 5, 7, and 10). Flow cytometry data were obtained on a Gallios (Beckman Coulter) apparatus and analyzed with FlowJo v10.0.8 (FlowJo, BD).

**Measurement of in vitro T cell responses by intracellular cytokine staining assay**

The cellular responses of immunized volunteers to PfNF54, PbVac, or PbWT sporozoites or to PfCSP were assessed using an intracellular cytokine staining assay. Thawed PBMCs (1 × 10^6) were stimulated in a total of 200 μl per well in a U-bottom 96-well plate with 1.5 × 10^5 purified, cryopreserved, thawed, and washed PfNF54, PbVac, or PbWT sporozoites, or with media alone as a negative control, for 22 hours at 37°C. For PfCSP stimulation, PBMCs were rested overnight at 37°C and stimulated for 6 hours with PfCSP (1 μg/ml; Genova Biotechniques Pvt. Ltd.) plus BD FastImmune CD28/CD49d (1 μg/ml) costimulation, or costimulation alone as a negative control. Stimulation with phorbol 12-myristate 13-acetate (10 ng/ml; Sigma) and ionomycin (500 ng/ml; Sigma) for 5 hours was used as positive control. Under all stimulation conditions, brefeldin (10 μg/ml; Sigma) was added to the cultures for the last 5 hours. Then, culture cells were incubated with FVD, washed with PBS containing 2% FCS, and surface-stained with TCRγδ and CD56 antibodies for 30 min at RT. After fixation and permeabilization using the Transcription Factor Staining Buffer Set, according to the manufacturer’s instructions (eBioscience, Thermo Fisher Scientific), cells were stained intracellularly with anti-human CD4, CD8, CD3, IFN-γ (4B3, TFN-α (Mab11), and IL-2 (MQ1-17H12) antibodies for 30 min at 4°C. Last, cells were washed and resuspended in PBS with 2% FCS, and about 5 × 10^5 events were acquired on a BD LSFRFortessa X-20 (BD Biosciences). Data were analyzed using FlowJo v10.5.3 (FlowJo, BD). Results are presented as the frequency of cytokine-producing cells in the stimulated condition minus in the respective negative control.

**Analysis of heterogeneity of individual immune responses**

To analyze the heterogeneity of individual responses to immunization, a Z score was calculated for the magnitude of the cellular and humoral immune responses measured for each volunteer in each assay, by subtracting the population mean before immunization from an individual raw score and then dividing the difference by the population’s SD before immunization. This score reflects the direct comparison of the responses of each volunteer before CHMI with the mean values of each parameter before immunization for the entire group and enables ranking the volunteers according to their responses, for each immune response parameter assessed.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism software (version 5, GraphPad Software Inc.) and Microsoft Excel (version 16.16.8). All volunteers exposed to PbVac were included in the intention-to-treat analysis. All safety data were descriptive to characterize safety and appearance of parasitemia (“breakthrough” infection). After challenge infection, the prepatent period to *P. falciparum* positivity (time for first positive qPCR, >100 parasites/ml) and difference in parasitemia at first positive qPCR were compared between
groups by applying the Kolmogorov-Smirnov nonparametric test. Liver burden reduction calculation was estimated as described above. Differences in antibody titers, as well as in cytokine production, upon stimulation across the immunization groups were analyzed by applying the Wilcoxon matched-pairs signed-rank test. The nonparametric Spearman correlation test was applied for all analysis of correlation. P value ≤0.05 was considered statistically significant.

SUPPLEMENTARY MATERIALS
stm.sciencemag.org/cgi/content/full/12/544/eaay2578/DC1

Fig. S1. Humoral responses elicited by immunization with PfPVac.

Table S2. Adverse events.

Fig. S1. Humoral responses elicited by immunization with PfPVac, total number of mosquito bites after four immunizations, and CHMI outcome.

Differences in antibody titers, as well as in cytokine production, were analyzed by applying the Kolmogorov-Smirnov nonparametric test. The nonparametric Spearman correlation test was applied for all analysis of correlation. P value ≤0.05 was considered statistically significant.

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Competing interests: A.M.M. and M.P. are inventors on a patent or patent application issued, allowed, or filed internationally “Genetically modified rodent Plasmodium parasites as platforms for a whole-organism malaria vaccine” (WO 2013156949 A1, also published as CN104302313A, EP2838554A1, US20150071966), covering parts of this work. All other authors declare no competing interests. Data and materials availability: All data associated with this study are present in the paper or the Supplementary Materials. PbVac (also known as Pb(PfCS@UIS4)), Pb ANKA (line 2266 c1), and PbPks@Pbis4 (in GIMO motherline), or Pb ANKA (line 2266 c1, PbPks@Pbis4 in GIMO motherline) is covered by a material transfer agreement (MTA) between Academich Ziekenhuis Leiden, also acting under the name Leiden University Medical Center (“provider”), and Instituto de Medicina Molecular (IMM) (“recipient”) dated 28 November 2012 and amended on 1 August 2016.

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An open-label phase 1/2a trial of a genetically modified rodent malaria parasite for immunization against *Plasmodium falciparum* malaria


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**GMOs for good**

Despite decades of progress and a multitude of approaches, a durable malaria vaccine remains elusive. Two new clinical studies in this issue report initial testing of genetically engineered malaria vaccines in malaria-naïve adults. Roestenberg *et al.* studied PISPZ-GA1, a *Plasmodium falciparum* sporozoite vaccine attenuated by deletion of b9 and slarp. Reuling *et al.* examined PbVac, sporozoites of the rodent-specific parasite *P. berghei* modified to express the circumsporozoite protein from *P. falciparum*. Both vaccines were well tolerated and immunogenic. Controlled malaria challenge also indicated some evidence of protection. These genetically engineered vaccines are part of the new wave of malaria vaccines and warrant further clinical testing.