MALARIA

Infectivity of *Plasmodium falciparum* sporozoites determines emerging parasitemia in infected volunteers

Matthew B. B. McCall,[1]*† Linda J. Wammes,[1]* Marijke C. C. Langenberg,[2] Geert-Jan van Gemert,[3] Jona Walk,[3] Cornelus C. Hermsen,[3] Wouter Graumans,[3] Rob Koelewijn,[1,2] Jean-François Franetich,[4] Sandra Chishimba,[1] Max Gerdsen,[3] Audrey Lorthiois,[4] Marga van de Vegte,[3] Dominique Mazier,[4,5] Else M. Bijker,[3] Jaap J. van Hellemond,[1] Perry J. J. van Genderen,[2] Robert W. Sauerwein[3‡]

Malaria sporozoites must first undergo intrahepatic development before a pathogenic blood-stage infection is established. The success of infection depends on host and parasite factors. In healthy human volunteers undergoing controlled human malaria infection (CHMI), we directly compared three clinical *Plasmodium falciparum* isolates for their ability to infect primary human hepatocytes in vitro and to drive the production of blood-stage parasites in vivo. Our data show a correlation between the efficiency of strain-specific sporozoite invasion of human hepatocytes and the dynamics of patent parasitemia in study subjects, highlighting intrinsic differences in infectivity among *P. falciparum* isolates from distinct geographical locales. The observed heterogeneity in infectivity among strains underscores the value of assessing the protective efficacy of candidate malaria vaccines against heterologous strains in the CHMI model.

INTRODUCTION

Malaria infections are initiated by blood-feeding female *Anopheline* mosquitoes injecting *Plasmodium* sporozoites into their mammalian host. Although most of the sporozoites get trapped in the dermis or draining lymph nodes, a small but crucial fraction succeed in penetrating dermal vasculature, whence they are carried into the bloodstream, including eventually the hepatic circulation (*1*). There they invade the liver parenchyma, traversing numerous cells before a few eventually succeed in invading a permissive hepatocyte where they undergo schizogony (*2*). Each liver-stage schizont releases an estimated ≥40,000 merozoites (*3*), which invade erythrocytes, causing blood-stage disease. The respective steps in the preerythrocytic life cycle of the *Plasmodium* parasite initially form a substantial bottleneck and subsequently an enormous multiplication opportunity (*4*).

However, it is unclear how the success rate of these various preerythrocytic steps ties together to determine the total burden of parasites emerging from the liver and hence the course of pathogenic blood-stage infection. It is also unknown to what extent malaria isolates vary in infectivity with regard to preerythrocytic infection or, conversely, whether humans differ in their intrinsic permissiveness. Addressing the latter issue is central to the rational design of controlled human malaria infection (CHMI) studies for clinical evaluation of candidate malaria vaccines (*5*).

Here, we investigated whether three clinical isolates of *Plasmodium falciparum* differed in their intrinsic ability to accomplish the sequential steps of preerythrocytic development in liver hepatocytes and how this affected the course of blood-stage infection in humans. We studied parasite dynamics both in vivo in malaria-naïve volunteers undergoing CHMI and in a recently optimized in vitro model using freshly isolated human hepatocytes (*6*, *7*). In vitro experiments were performed with sporozoites harvested from exactly the same batches of infected mosquitoes used on the same day for the human infections. Alongside the long-standing laboratory strain NF54 (*8*) and the recently reported NF135.C10 clone (*9*), we described the development and use in humans of the *P. falciparum* clone NF166.C8. We showed that intrinsic differences in infectivity existed between *P. falciparum* isolates with regard to their ability to invade and multiply within human hepatocytes in vitro. The degree of liver-stage infection correlated directly with the magnitude of the first wave of blood-stage parasites to emerge from the liver in vivo and correlated inversely with the prepatent period in CHMI subjects.

RESULTS

Dynamics of parasitemia during CHMI

In total, 23 subjects across two separate CHMI studies were each exposed to sets of five mosquitoes carrying the NF54, NF135.C10, or NF166.C8 isolates of *P. falciparum* (study flowcharts, study subject demographics, and laboratory characteristics of these *P. falciparum* isolates are provided in fig. S1 and tables S1 and S2, respectively). All subjects developed patent parasitemia and were curatively treated with atovaquone-proguanil once thick smear–positive (first study, CHMI-a) or quantitative real-time fluorescence polymerase chain reaction (qPCR)–positive (second study, CHMI-b). In CHMI-a (Fig. 1A), the prepatent period as determined by thick smear was significantly shorter for subjects infected with either the NF135.C10 clone (mean, 7.2 days; range, 6.5 to 8.5 days) or the NF166.C8 clone (mean, 7.4 days; range, 6.5 to 8.5 days) than for those infected with the NF54 strain of *P. falciparum* (mean, 10.2 days; range, 9.0 to 10.5 days); mean differences were, respectively, 3.0 days [95% confidence interval (CI), 1.7 to 4.3] and 2.8 days (95% CI, 1.5 to 4.1) [$P < 0.001$ for both comparisons, one-way analysis

[1]Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, Netherlands. [2]Institute for Tropical Diseases, Harbour Hospital, Rotterdam, Netherlands. [3]Department of Medical Microbiology, Radboud University Medical Centre, Nijmegen, Netherlands. [4]Sorbonne Universités, UPMC Univ Paris 06, INSERM U1135, CNRS ERL 8255, Centre d'Immunologie et des Maladies Infectieuses (CIMI-Paris), 91 Bd de l'hôpital, F-75013 Paris, France. [5]AP-HP, Groupe hospitalier La Pitié-Salpêtrière, Service de Parasitologie Mycologie, F-75013 Paris, France.
*These authors contributed equally to this work.
†Present addresses: Centre de Recherches Médicales de Lambaréné, Lambaréné, Gabon; and Institute for Tropical Medicine, University of Tübingen, Tübingen, Germany.
‡Corresponding author. Email: robert.sauerwein@radboudumc.nl

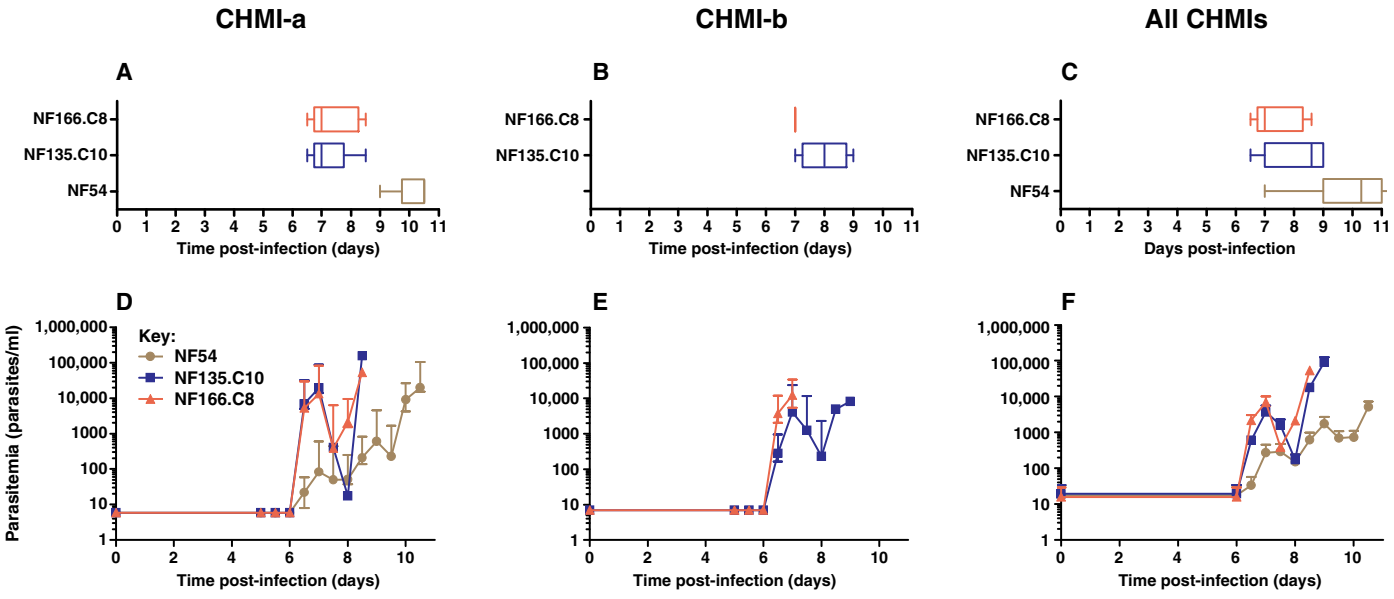

Fig. 1. Prepatent period and kinetics of parasitemia in CHMI with three *P. falciparum* strains. (**A**) Prepatent periods in the CHMI-a study ($n = 5$ per group). (**B**) Prepatent periods in the CHMI-b study ($n = 4$ per group). All subjects infected with the NF166.C8 strain in the CHMI-b study had prepatent periods of exactly 7.0 days, which is shown as a single line in (B). (**C**) Combined data from all 10 comparable CHMI studies performed at our center using thick smear microscopy as an end point ($n = 56$ NF54, $n = 13$ NF135.C10, and $n = 5$ NF166.C8). Box-and-whisker plots represent median/interquartile range and range per strain. In the CHMI-a study (A) and all studies in (C), prepatent periods were determined by time to positive thick blood smear; in the CHMI-b study (B) and subsequent studies at our center, prepatent periods were determined by time to positive qPCR. (**D** to **F**) Development of submicroscopic parasitemia after infection, as determined retrospectively by qPCR in the CHMI-a study (D) ($n = 5$ per group), the CHMI-b study (E) ($n = 4$ per group), and combined data from all 18 CHMI studies at our center using either thick smear microscopy or qPCR as an end point (F) ($n = 90$ NF54, $n = 22$ NF135.C10, and $n = 14$ NF166.C8). Data represent geometric mean ± 95% CI of all pretreatment samples per group per time point. All subjects in the CHMI-a and CHMI-b studies subsequently received a standard course of atovaquone-proguanil treatment and had their parasitemia cleared completely. Brown circles, NF54 strain; blue squares, NF135.C10 strain; red triangles, NF166.C8 strain.

of variance (ANOVA)/Tukey's post hoc test, $n = 5$ per group]. No significant difference existed between NF135.C10 and NF166.C8 (mean difference, 0.2 day; 95% CI, −1.1 to 1.5; $P > 0.05$). In CHMI-b (Fig. 1B), the prepatent period as determined by qPCR criteria did not differ significantly between NF135.C10 (mean, 8.0 days; range, 7.0 to 9.0 days) and NF166.C8 (7.0 days in all four subjects) *P. falciparum* strains (mean difference, 1.0 day; 95% CI, −0.3 to 2.3) ($P = 0.09$, one-sample t test, $n = 4$ per group). The prepatent period observed for NF54 in the CHMI-a study was similar to that of $n = 56$ malaria-naïve subjects in all 10 of our CHMI studies (mean, 10.3 days; range, 7.0 to 16.0 days), in which treatment was initiated upon positive standardized thick blood smear analysis (table S3). Likewise, the prepatent period for NF135.C10 in CHMI-a was similar to that of $n = 13$ subjects across all 3 of those 10 studies that also included infections with NF135.C10 strain parasites (mean, 8.0 days; range, 6.5 to 9.0 days; table S3). In a meta-analysis of all 10 studies, the prepatent period of NF54 remained significantly ($P < 0.001$) longer than that of either NF135.C10 or NF166.C8 (Fig. 1C).

As determined retrospectively by qPCR in CHMI-a subjects, submicroscopic parasitemia became detectable before thick smear positivity in all subjects. Parasitemia was evident 6.5 days after infection in all NF135.C10-infected and NF166.C8-infected subjects and by day 7.5 (6.5 to 8.5) in the NF54-infected group (mean difference, 1.0 day; 95% CI, 0.1 to 1.9; Fig. 1D and Table 1). The magnitude of the initial wave of parasitemia emerging from the livers of both NF135.C10-infected and NF166.C8-infected subjects was greater compared to that of NF54-infected subjects, although this magnitude did not itself differ significantly between NF135.C10-infected and NF166.C8-infected subjects (Fig. 1D and Table 1). Overall, peak parasitemia per subject before initiation of antimalarial treatment with atovaquone-proguanil did not differ between groups (Table 1).

In CHMI-b, submicroscopic parasitemia was first detected by qPCR on day 6.5 after infection in all four subjects in both the NF135.C10 group and the NF166.C8 group. The magnitude of the initial wave of parasitemia emerging from the liver did not differ between NF135.C10 and NF166.C8 (Fig. 1E and Table 1). Although the initial wave of parasitemia in CHMI-a was slightly lower for the NF54 strain ($P = 0.046$) and somewhat higher for the NF135.C10 strain ($P = 0.0045$), as compared to our other CHMI studies (table S3), the magnitude of the first wave of NF54 parasitemia was around 10-fold lower compared to either NF135.C10 or NF166.C8 parasitemias in a meta-analysis of all 18 of our CHMI studies (Fig. 1F and Table 1).

Clinical course and adverse events in subjects undergoing CHMI

Mild-moderate adverse events were experienced by all CHMI-a subjects after infection (table S4A). Grade 3 adverse events occurred in five subjects, including general malaise/fatigue prohibiting daily activities in four subjects (across all three groups) and fever >39.0°C in two NF166.C8-infected subjects (table S4A). The duration of general fatigue/malaise was greater in NF54-infected subjects [5.0 (3.0 to 7.8) days] compared to NF135.C10-infected [2.7 (1.3 to 4.6) days] or NF166.C8-infected subjects [2.7 (1.6 to 4.0) days]; the mean difference for both comparisons was 2.6 days (95% CI, 0.0 to 5.1) ($P < 0.05$, one-way ANOVA/Tukey's post hoc test, $n = 5$ per group; table S4A). Overall, no other statistically significant difference in frequency, duration, or gradation of adverse events was observed between the three groups (table S4A).

Table 1. Characteristics of *P. falciparum* parasitemia in CHMI subjects in vivo.

	NF54†	NF135.C10†	NF166.C8†	NF54 versus NF135.C10‡	NF54 versus NF166.C8‡	NF135.C10 versus NF166.C8‡
CHMI-a						
No. of subjects per group	5	5	5			
First detection of parasitemia by qPCR (days after infection)	7.5 (6.5 to 8.5)	6.5 (6.5 to 6.5)	6.5 (6.5 to 6.5)	**1.0 (0.1 to 1.9)***	**1.0 (0.1 to 1.9)***	—
Peak parasitemia (log_{10} parasites/ml)						
First wave¤	1.96 (1.23 to 3.52)	4.28 (3.41 to 4.79)	3.89 (2.83 to 4.79)	**−2.32 (−3.69 to −0.96)****	**−1.93 (−3.30 to −0.56)****	0.39 (−0.97 to 1.76)
Overall◊	4.43 (3.84 to 5.17)	4.64 (4.24 to 5.19)	4.22 (2.83 to 4.79)	−0.20 (−1.19 to 0.78)	0.21 (−0.77 to 1.20)	0.42 (−0.56 to 1.40)
CHMI-b						
No. of subjects per group	—	4	4			
First detection of parasitemia by qPCR (days after infection)	—	6.5 (6.5 to 6.5)	6.5 (6.5 to 6.5)	—	—	—
Peak parasitemia (log_{10} parasites/ml)						
First wave¤	—	3.61 (2.59 to 4.19)	4.09 (3.77 to 4.34)	—	—	−0.48 (−1.63 to 0.66)
Overall◊	—	3.94 (3.82 to 4.19)	4.09 (3.77 to 4.34)			−0.15 (−0.62 to 0.32)
All CHMI studies						
No. of subjects per group§	90	22	14			
First detection of parasitemia by qPCR (days after infection)§	7.2 (6.3 to 10.6)	6.7 (6.5 to 7.0)	6.5 (6.5 to 6.5)	0.52 (−0.004 to 1.0)	**0.70 (0.070 to 1.3)***	0.18 (−0.57 to 0.93)
Peak parasitemia (log_{10} parasites/ml)						
First wave¤§	2.72 (1.00 to 4.67)	3.67 (2.59 to 4.79)	3.78 (2.78 to 4.79)	**−0.95 (−1.41 to −0.49)*****	**−1.06 (−1.61 to −0.51)*****	−0.11 (−0.79 to 0.58)
Overall◊‖	4.28 (3.13 to 5.17)	4.72 (3.35 to 5.84)	4.22 (2.83 to 4.97)	**−0.44 (−0.86 to −0.009)***	0.065 (−0.58 to 0.71)	0.50 (−0.23 to 1.23)

†Data represent mean per group (range). ‡Data represent mean differences between groups (95% CI of difference). Boldface indicates statistical significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by one-way ANOVA/Tukey's post hoc test). §Data included from all 18 comparable CHMI studies performed at our center using either thick smear microscopy or qPCR as an end point (table S3). ‖Data included from only those 10 CHMI studies performed at our center in which subjects were treated upon positive standardized thick blood smear ($n = 56$ NF54-infected, $n = 13$ NF135.C10-infected, and $n = 5$ NF166-infected subjects, respectively). ¤Peak height of parasitemia during the first wave to emerge from the liver, defined as the highest parasite density measured by qPCR per subject between day 6.5 and 8.0 after infection; data represent geometric mean (range) per group. ◊Peak height of parasitemia per subject at any time after infection (generally at start of treatment); data represent geometric mean (range) per group.

The clinical characteristics of the CHMI-b study did not differ markedly from those of the CHMI-a study, and no statistically significant differences were observed between the NF135.C10-infected and NF166.C8-infected groups (table S4B). No serious adverse events (SAEs) occurred in either study.

Genetic characterization of P. falciparum isolates

The genetic identity of the three *P. falciparum* isolates used in the CHMI-a and CHMI-b studies was assessed by PCR for the polymorphic repetitive RII region of Glutamate-Rich Protein (GLURP); the K1, MAD20, and R033 allelic variants of block 2 of Merozoite Surface

Protein 1 (MSP1); and the IC1 and FC27 variants of block 3 of MSP2 (fig. S2). GLURP bands were observed corresponding to amplified DNA fragment lengths of about 950, 980, and 680 base pairs (bp) for NF54, NF166.C8, and NF135.C10 strains, respectively (fig. S2). MSP1 K1 bands corresponding to fragment lengths of 220 and 210 bp were observed for NF54 and NF166.C8 strains, but these isolates failed to produce an MSP1 MAD20 band (fig. S2). NF135.C10, in contrast, did produce an MSP1 MAD20 band with a fragment length of 180 bp (fig. S2). MSP2 IC1 bands corresponded to fragment lengths of 480, 580, and 560 bp for NF54, NF166.C8, and NF135.C10 strains, respectively (fig. S2). 18*S* bands were detectable as amplified DNA fragments of around 130 bp in all isolates (fig. S2).

Hepatocyte invasion and schizogony by sporozoites in vitro

In parallel, we assessed the infectivity of the three clinical *P. falciparum* isolates NF54, NF135.C10, and NF166.C8 for fresh human hepatocytes in vitro, using sporozoites dissected from the same batches of mosquitoes used to infect subjects in the CHMI-a study (experiment A) and derived from independent gametocyte cultures used in the CHMI-b study up to 2 years later (experiments B1 and B2) (Fig. 2). A higher proportion of NF135.C10 and NF166.C8 sporozoites established infection in human hepatocytes compared to the NF54 strain in these three experiments ($P < 0.001$ overall by repeated-measures ANOVA and $P < 0.01$ for NF54 versus NF135 and NF54 versus NF166 individually by Tukey's post hoc test; Fig. 2). In addition, mature NF166.C8 and NF135.C10 schizonts tended to be larger than NF54 schizonts, and concomitantly, the increase in the number of nuclei per schizont from day 2 to day 6 appeared to be greater for NF135.C10 and NF166.C8 compared to NF54 (Table 2). These combined data indicated that both sporozoite invasion and schizont development were more efficient in the NF135.C10 and NF166.C8 strains than in the NF54 strain.

Correlations between the in vitro human hepatocyte invasion assay and in vivo parasite dynamics

Given that identical sporozoite batches were used for both the human hepatocyte invasion assays and human infections in the CHMI-a study, we queried whether sporozoite infectivity in vitro might be predictive of blood-stage infection in vivo. The magnitude of the first wave of parasitemia emerging from the liver in study subjects inoculated with each strain (Table 1) appeared to correlate with the infectivity of sporozoites of that strain in the human hepatocyte invasion assay in vitro (Table 2) (Pearson's $R = 0.994$, $P = 0.068$, $n = 3$ strains). Furthermore, a strong negative association existed between sporozoite infectivity in vitro and the prepatent period of that strain in vivo ($R = -1.0$, $P = 0.005$).

DISCUSSION

Here, we systematically assessed the dynamics of the obligate liver stage of human malaria infection, from mosquito bite to the emergence of blood-stage parasites, by using three different clinical isolates of *P. falciparum* and directly linking data on sporozoite infectivity in an in vitro human hepatocyte invasion model with in vivo measurements of parasitemia in subjects undergoing CHMI.

Both in vitro and in vivo findings support the notion that a substantially greater initial burden of blood-stage parasites is released from the livers of subjects infected with the NF135.C10 or NF166.C8 *P. falciparum* strains than from those infected with the NF54 strain. This in turn has significant effects on the subsequent course of blood-stage infection, with total parasite numbers more quickly reaching levels detectable by thick smear microscopy, shortening the prepatent period by several days. The difference in parasite burden between strains in vivo was even greater than the differences in invasion and development observed in vitro, a phenomenon that may be partially explained by suboptimal conditions for sporozoite invasion and development in vitro as compared to in vivo. In support of this explanation, the diameter of the NF54 strain liver-stage schizonts in our human hepatocyte cultures in this study (Table 2), as well as in a previous study (*10*), was 1.5- to 5-fold smaller than those measured in vivo in two humanized mouse models [the fumarylacetoacetate hydrolase–deficient mouse (*3*) and the severe combined immunodeficient mouse homozygous for the urokinase-type plasminogen activator transgene under the albumin promoter (*11*)]. Measuring the size of liver-stage schizonts in these mouse models might reveal even bigger differences between *P. falciparum* isolates than those found here in vitro. Whereas more than a decade separates the original isolation date of each of these three strains of *P. falciparum* (table S2), the use of specific master cell banks (MCBs) for CHMI studies restricts the cumulative culture period for the generation of gametocytes of each strain. In our opinion, this is unlikely to explain the marked differences in phenotype of the strains in vivo with regard to liver-stage burden.

Both sporozoite infectivity (Fig. 2) and the size of mature liver-stage schizonts of strain NF135.C10 in particular (Table 2) appeared to be greater than those for the NF54 strain, suggesting that two distinct mechanisms may be contributing to the greater number of merozoites released in vivo. In contrast, the duration of liver-stage development was markedly similar between isolates (Fig. 1, D to F). Although

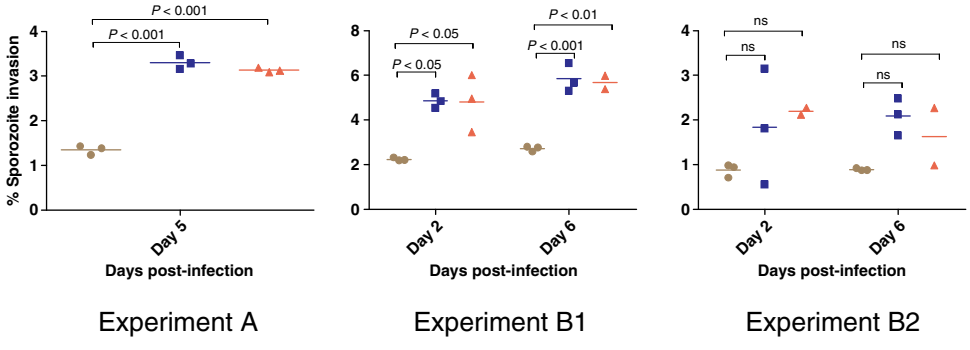

Fig. 2. Invasion of human hepatocytes in vitro by three *P. falciparum* strains. Freshly isolated human hepatocytes were co-incubated with freshly isolated NF54 sporozoites (brown circles), NF135.C10 sporozoites (blue squares), or NF166.C8 sporozoites (red triangles) in vitro. The number of infected hepatocytes per well was counted by immunofluorescence microscopy at the given time points after infection. Shown are the results of three independent experiments. Experiment A was performed with 40,000 sporozoites per well harvested from mosquito batches used to infect subjects in the CHMI-a study and assessed at 5 days after infection. Experiments B1 and B2 were performed with 50,000 sporozoites per well harvested from mosquitoes fed on gametocyte cultures used in the CHMI-b study and assessed at 2 and 6 days after infection. Experiment A was performed at a different institute to experiments B1 and B2, and each of the three experiments was performed with human hepatocytes from a different donor. Data represent the percentage of sporozoites achieving successful invasion in each of three triplicate wells per condition (some NF166.C8 strain wells only in duplicate); horizontal lines represent group means. *P* values by one-way ANOVA with Tukey's posttest. ns, not significant.

Table 2. Characteristics of *P. falciparum* infectivity of human hepatocytes in vitro. ND, not determined.

			NF54	NF135.C10	NF166.C8
Experiment B1	Day 2	#Schizonts per well*	1,100 (1,091 to 1,158)	2,420 (2,273 to 2,595)	2,479 (1,727 to 3,005)
		Schizont Ø (μm)†	3.0 (2.3 to 4.1)	3.4 (0.9 to 4.5)	2.8 (1.8 to 3.6)
		#Nuclei per well‡	18,533 (15,187 to 20,077)	20,090 (17,823 to 26,446)	17,980 (15,731 to 25,785)
		#Nuclei/schizont§	16.8	8.3	7.3
	Day 6	#Schizonts per well*	1,383 (1,289 to 1,398)	2,840 (2,656 to 3,276)	2,843 (2,692 to 2,993)
		Schizont Ø (μm)†	11.2 (9.0 to 13.5)	18.3 (12.5 to 25.0)	16.1 (8.9 to 26.8)
		#Nuclei per well‡	56,181 (52,810 to 69,893)	216,449 (181,107 to 313,381)	248,961 (201,453 to 394,328)
		#Nuclei per schizont§	40.6	76.2	87.6
		Multiplication rate‖	2.4	9.2	12.1
Experiment B2	Day 2	#Schizonts per well*	373 (320 to 458)	902 (275 to 1,575)	1,098 (1,059 to 1,137)
		Schizont Ø (μm)†	1.7 (0.9 to 3.6)	2.8 (1.8 to 5.4)	3.4 (1.8 to 7.1)
		#Nuclei per well‡	8,858 (7,226 to 9,585)	16,726 (10,228 to 16,726)	21,968 (20,742 to 23,195)
		#Nuclei per schizont§	23.8	18.5	20.0
	Day 6	#Schizonts per well*	1,061 (826 to 124)	813 (492 to 1,134)	
		Schizont Ø (μm)†	11.4 (6.3 to 16.1)	18.9 (12.5 to 28.6)	12.5 (8.9 to 17.0)
		#Nuclei per well‡	95,295 (94,347 to 11,184)	382,508 (364,489 to 451,483)	ND
		#Nuclei per schizont§	218	361	ND
		Multiplication rate‖	11.5	19.4	ND

*Number of liver-stage schizonts per well, counted by immunofluorescence microscopy after staining with anti-HSP70 MAbs, as in Fig. 2. Data represent median (range) across triplicate wells. †Schizont diameters, as measured by immunofluorescence microscopy and digital image analysis. Data represent mean (range) of $n = 19$ to 49 schizonts per sample. NF135.C10 schizonts were larger than NF54 schizonts at day 2 ($P < 0.05$) and day 6 ($P < 0.001$) in both experiments and were also larger than NF166.C8 schizonts ($P < 0.05$ to $P < 0.001$ in all comparisons except experiment B2, day 2); NF166.68 schizonts were larger than NF54 schizonts at day 2 in experiment B2 ($P < 0.001$) and at day 6 in experiment B1 ($P < 0.001$). *P* values by one-way ANOVA with Tukey's posttest. ‡Number of parasite nuclei per well, as determined by qPCR; data represent median (range) of triplicate wells. §Number of nuclei per schizont, as calculated by dividing the median #nuclei per well by the median #schizonts per well. ‖Fold increase in number of nuclei per infected human hepatocyte from day 2 to day 6 after infection, as calculated by dividing the median #nuclei per schizont at day 6 after infection by that at day 2.

the mean time to qPCR positivity was slightly longer for the NF54 strain, we regularly detected NF54 parasites in blood samples by day 6.5 after infection (Table 1) (*12*). The small magnitude of the first wave of NF54 parasitemia suggested that the earliest parasites to emerge from the liver may fall below the detection limit of the qPCR. In any case, this short delay in qPCR positivity (0.52 and 0.70 day relative to the NF135.C10 and NF166.C8 strains, respectively, across all studies, Table 1) would appear to form only a minor determinant of the overall delay in prepatent period (2.3 and 2.9 days, respectively; Fig. 1C).

Finally, the variability in both the timing and the magnitude of the first wave of parasitemia among subjects within each study group was relatively limited in comparison to the difference in that magnitude between groups, suggesting that innate host factors may have little influence on either the duration or efficiency of liver-stage development in primary *P. falciparum* infections in our study population.

Although no clear relationship has been found between salivary gland sporozoite load and the number of sporozoites inoculated by probing/feeding mosquitoes (*13*), higher salivary gland loads have been suggested to enhance the infectivity of sporozoites (*14*). However, NF54-infected mosquitoes used in the CHMI-a study actually contained higher salivary gland sporozoite loads than did NF166.C8-infected or NF135.C10-infected mosquitoes from the same colony (table S2), a finding that is consistent with batches of mosquitoes used for other CHMIs at our center (table S3). The advent of CHMIs using parenteral sporozoite inoculation allowing better control over the number of administered sporozoites may further help to disentangle this point.

As a direct consequence of their higher liver burden, the time frame between release of the first NF135.C10 and NF166.C8 parasites from the liver and the initiation of antimalarial treatment with atovaquone-proguanil (upon parasitemia attaining the threshold by thick smear microscopy) incorporated only a single erythrocytic cycle. This is insufficient to reliably determine their blood-stage multiplication rate (*5*).

A possible limitation of this study is that sporozoite infectivity and the development of parasitemia were only assessed in malaria-naïve volunteers from malaria nonendemic countries. The observed infectivity between the parasite strains may be different in endemic populations with variable degrees of semi-immunity. Therefore, it will be of interest to compare these clones in CHMI in endemic settings. A further limitation is that only three *P. falciparum* isolates from different geographical backgrounds were tested for comparison in human hepatocyte

cultures and by CHMI. It remains to be seen to what extent the three strains are representative of the overall diversity among the global *P. falciparum* population (*15*). Increasing the portfolio of *P. falciparum* clones for CHMI studies may provide further support for our findings. Both the NF135.C10 (*9*) and NF166.C8 strains described here were developed and characterized with the specific intent to increase the currently limited portfolio of *P. falciparum* strains available for heterologous challenge CHMI studies. For NF54 or its derivative clone 3D7, the minimum infecting dose to guarantee a 100% infection rate in malaria-naïve subjects in such CHMI studies is generally held to be five mosquito bites (*16*), 3200 cryopreserved sporozoites inoculated intravenously (*17*), or 75,000 cryopreserved sporozoites inoculated intramuscularly (*18*). Given the higher observed infectivity of NF135.C10 and NF166.C8 sporozoites, the minimum dose to reliably achieve 100% infection must be explored in further studies. Whereas interventions that target hepatocyte invasion aim at one of the two most constrained bottlenecks in the parasite's life cycle (*4*), our data indicate that relatively limited but relevant heterogeneity does exist among strains with regard to sporozoite infectivity (Fig. 2), which should be taken into account when designing and evaluating such interventions.

A major advantage of CHMI studies is the relatively small number of subjects required for the evaluation of vaccine efficacy, due to the homogeneity of the profile of parasitemia within each group and between homologous groups, as illustrated here by our CHMI-a and CHMI-b studies. Given the clear-cut difference in parasite dynamics between *P. falciparum* isolates, however, homologous challenge studies of malaria vaccines will remain insufficiently representative, and CHMIs with heterologous strains are required to more accurately predict the efficacy of promising vaccine candidates.

MATERIALS AND METHODS

Study design

Two CHMIs were performed using the well-established *P. falciparum* research strain NF54 and the new clones NF135.C10 and NF166.C8. The primary objective of the first study (CHMI-a) was to compare parasite dynamics of these isolates during infection, and that of the second (CHMI-b) study was to determine the proportion of subjects in each group who developed patent parasitemia, as described below. Secondary objectives of both studies included clinical parameters and further measures of parasite kinetics.

Healthy malaria-naïve adult (age 18 to 35 years) Dutch volunteers were recruited at the Harbour Hospital, Rotterdam, after signing informed consent. Our screening procedures have been described previously (*19*), and a complete list of inclusion/exclusion criteria is provided in table S5. In the first CHMI study described here (CHMI-a), 15 subjects (9 men and 6 women; table S1) were randomly allocated (ratio 1:1:1) to three parallel groups of $n = 5$, to be infected by bites of five mosquitoes per subject carrying, respectively, the NF54 strain of *P. falciparum*, the NF135.C10 clone, or the new NF166.C8 clone. In the second study (CHMI-b), 24 subjects were randomly allocated (ratio 1:1:1:1:1:1) to six parallel groups of $n = 4$, to bites by one, two, or five mosquitoes carrying, respectively, either NF135.C10 or NF166.C8 strains. For comparison with CHMI-a, only the two CHMI-b groups exposed to the bites of five infectious mosquitoes are reported here (that is, eight subjects in total, of whom three were men, and five were women; table S1). Flow charts for both studies are shown in fig. S1. Sample sizes were chosen pragmatically, based on longstanding experience with NF54 and (limited) NF135.C10 data. The CHMI-a study was powered to detect a 0.9 log (7.9-fold) difference in parasite burden between strains.

At inclusion, subjects received a unique pseudonomized study code. Once the predetermined number of subjects per study was included, two departmental employees not otherwise involved in the study linked a random number generated in Excel to each study code and allocated the required number of study codes to each group, using simple ranking of the associated random numbers. This allocation list was provided only to insectary technicians, who prepared feeding cages containing the required strain and number of *P. falciparum*–infected mosquitoes and labeled these otherwise identical cages with only the respective study code. Thus, study subjects, clinical investigators, and laboratory personnel assessing study end points (thick smear microscopy and qPCR) remained blinded to group allocation throughout each study.

All subjects within each study underwent infection on the same day, and considering the extremely close monitoring of CHMI study subjects for safety reasons, all subjects were expected to complete follow-up. Data from all subjects were included in the study, and there were no specific rules for data exclusion, outliers, or premature cessation of data collection (other than if required for safety reasons after consultation with the sponsor, safety monitor, and Data Safety and Monitoring Board).

Culture and characterization of clinical parasite isolates

P. falciparum blood-stage parasites were introduced into semi-automated culture from peripheral whole-blood samples of patients with clinical malaria and used to generate infective *Anopheles stephensi* mosquitoes, as described before (*20*). The origins of NF54 and NF135 strains of *P. falciparum* have been described previously (*8*, *9*). The NF166 strain of *P. falciparum* originated in 2010 as a clinical isolate (parasitemia 0.5 to 1%) from a child who had recently visited Guinea (West Africa). The clone NF166.C8 was obtained by limiting dilution culturing and established as a stable producer of fertile gametocytes and subsequently oocysts in infected mosquitoes (table S2). To minimize a potential confounding effect, gametocytes used to infect mosquitoes for our CHMI studies are generated following a strict culture procedure. When a cryopreserved aliquot of any isolate from our MCB is thawed and brought into culture, new aliquots of the culture are frozen down again for future use after the first four rounds of subculture. The remaining culture is then maintained for the generation of gametocytes, generally between the 15th and 25th rounds of subculture. Because in vitro parasites lose their ability to generate gametocytes beyond ±25 rounds of subculture, such cultures are discontinued. The MCB of NF54 was generated from the estimated 10th to 15th subculture since its original isolation in the 1970s. For NF135, the MCB was generated from the sixth subculture since isolation, and for NF166 from the fourth subculture.

The identities of NF54, NF135.C10, and NF166.C8 were defined by assessing the polymorphic regions of three *P. falciparum* antigenic genes by PCR in a method adapted from Snounou *et al.* (*21*). Briefly, parasite DNA was isolated using QIAamp DNA Blood Mini Kit (Qiagen) and amplified with GoTaq G2 Flexi Polymerase (Promega) using specific primers for the polymorphic repetitive RII region of *P. falciparum* GLURP (*22*); the K1, MAD20, and R033 allelic variants of block 2 of MSP1 (*23*); and the IC1 and FC27 variants of block 3 of MSP2 (*24*) (all primers from Invitrogen); 18*S* ribosomal DNA was used as a loading control. The sensitivity of NF166.C8, NF135.C10, and NF54 to dihydroartemisinin (Sigma-Tau), chloroquine diphosphate salt (Sigma-Aldrich), mefloquine, proguanil (British Pharmacopoeia), atovaquone (GlaxoSmithKline), and lumefantrine (Novartis) was tested

by the Malaria SYBR Green I-Based Fluorescence Assay in triplicate experiments (*25*). See also table S2 and fig. S2.

Controlled human malaria infections

Mosquitoes in small cages were allowed to feed for 10 min on the forearms of study subjects. All blood-engorged mosquitoes were dissected to confirm sporozoite carriage. Where necessary, study subjects were exposed to additional mosquitoes until precisely the predetermined number of infective bites was achieved. From day 5 after infection, subjects were seen twice daily as outpatients. Solicited and unsolicited adverse events and vital parameters were recorded at each visit and venous whole blood was drawn for thick blood smear, qPCR (see below), safety parameters, and exploratory assays. Adverse events were graded as mild (not interfering with), moderate (interfering with), or severe (prohibiting daily activities). Respective grading of fever was 37.5° to 38.0°C, 38.0° to 39.0°C, and >39.0C°. Adverse events were recorded by clinical investigators as being probably, possibly, or unlikely related to study procedures.

In CHMI-a, subjects were treated with a standard regimen of atovaquone-proguanil (Malarone, 1000/400 mg daily for 3 days) upon their first positive thick blood smear (defined as ≥2 parasites per 225 high-powered fields, equating to 0.5 μl of whole blood examined). In CHMI-b, subjects were treated with the same regimen as soon as two consecutive whole-blood samples were positive by qPCR (defined as >500 parasites/ml, calibrated against a daily standard curve of known parasite concentrations). The change in methodology between studies was proposed (*12*) and implemented to minimize potential safety risks upon a request by Netherlands' Central Committee on Human Research (CCMO), following concerns over a cardiac SAE possibly related to CHMI in an earlier study (*26*). Sequential daily thick smears/qPCRs were continued after treatment in each subject until complete clearance of blood-stage parasites. CHMI-a whole-blood samples were also retrospectively analyzed by qPCR. These studies were approved by Netherlands' CCMO (NL41004.078.12 and NL48704.000.14).

Quantitative real-time fluorescence polymerase chain reaction

P. falciparum parasitemia was quantified by qPCR as described before (*27*) with some modifications. Briefly, isolated DNA was resuspended in 100 μl of H_2O, using 5 μl as template and the TaqMan MGB probe AAC AAT TGG AGG GCA AG-FAM. Samples for post hoc analysis were stored at −80°C. For each sample (including standard curve dilutions), two qPCR replicates were performed; if these values differed by >5%, then the sample was remeasured a third time. In addition, all samples were spiked with heterologous DNA as an extraction control; if the qPCR value of the extraction control differed by >5% from the running average of all other samples, then the extraction process for that sample was repeated. Quantification of *P. falciparum* nuclei in wells of the human hepatocyte infectivity assay was performed using the same qPCR.

Primary human hepatocyte infectivity assay

In vitro sporozoite infectivity assays in human hepatocytes were performed, as described previously (*6*), at two different laboratories. Briefly, fresh primary hepatocytes derived from patients undergoing elective (partial) hepatectomy were plated in 96-well plates (50,000 cells per well). Two to 5 days after plating the human hepatocytes, sporozoites were dissected from the salivary glands of 80 to 110 infected mosquitoes per *P. falciparum* strain and added to the human hepatocyte wells in triplicate. In the first in vitro experiment (experiment A), sporozoites were harvested from the exact same batches of mosquitoes used on the same day to infect human subjects in CHMI-a; 40,000 sporozoites were added per human hepatocyte well. In subsequent in vitro experiments (B1 and B2), sporozoites were harvested from mosquitoes fed on independent gametocyte cultures used to subsequently infect subjects in CHMI-b, and 50,000 sporozoites were added per well. After 2 to 6 days, the number of human hepatocytes in each well harboring viable parasites was assessed by intracellular staining with anti–*P. falciparum* HSP-70 rabbit polyclonal antibody (SPC-186C/D, StressMarq Biosciences) followed by fluorescence microscopy; no instances of >1 invaded sporozoite per human hepatocyte were observed. Discrimination between sporozoite invasion of/adherence to human hepatocytes was achieved by staining with fluorescent monoclonal antibodies against circumsporozoite protein (CSP) before cell permeabilization (*28*); the proportion of adherents (that is, HSP-70$^+$CSP$^+$-double positive, noninvaded parasites) was generally <5% at day 2 after infection and consistently <0.5% at day 6 after infection. The diameter of n = 19 to 49 liver-stage schizonts per strain was measured at day 2 and day 6 after infection by fluorescence microscopy and digital image analysis (Leica DMI6000B). Total numbers of parasite nuclei per well were determined by qPCR in separate triplicate wells after discarding the culture supernatant. It was technically infeasible to perform both microscopy and qPCR on the same wells. The median number of merozoites per liver-stage schizont was calculated for each strain as follows: ([median #parasite nuclei in triplicate wells] − [median #adhered (noninvaded) parasites in triplicate wells])/([median #infected human hepatocytes in triplicate wells]). Investigators carrying out the in vitro hepatocyte infectivity assay were blinded as to the strain of the sporozoites.

Statistical analysis

Differences in continuous variables between groups of study subjects were compared by one-way ANOVA/Tukey's post hoc test (CHMI-a, three groups), unpaired t test (CHMI-b, two groups), or one-sample t test (CHMI-b, two groups when all values for one group were identical). Differences in categorical variables between groups were compared by χ^2/Fisher's exact test. Differences in in vitro invasive capacity between isolates were compared by repeated-measures ANOVA/Tukey's post hoc test (three groups). Correlations between in vitro and in vivo variables were assessed by Pearson's test on the mean value of each variable per strain; where appropriate, variables were first log-transformed. Two-sided $P < 0.05$ was considered statistically significant in all tests.

Parasite densities below the lower quantification limit of the qPCR (35 parasites/ml in CHMI-a and 50 parasites/ml in CHMI-b) were plotted at 0.5× the log value of that limit. The magnitude of the initial wave of parasitemia emerging from the liver was defined as the highest parasite density measured by qPCR per subject between day 6.5 and 8.0 after infection.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/9/395/eaag2490/DC1

Fig. S1. Study flow charts.

Fig. S2. Genetic characterization of *P. falciparum* isolates.

Table S1. Study demographics.

Table S2. Characteristics of *P. falciparum* isolates used in CHMI studies.

Table S3. Overview of all comparable CHMI studies performed at our center.

Table S4. Adverse events by severity grade during CHMI-a and CHMI-b studies.

Table S5. Inclusion and exclusion criteria for CHMI studies.

References (*31*–*36*)

REFERENCES AND NOTES

1. R. Amino, S. Thiberge, B. Martin, S. Celli, S. Shorte, F. Frischknecht, R. Ménard, Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. *Nat. Med.* **12**, 220–224 (2006).
2. M. M. Mota, G. Pradel, J. P. Vanderberg, J. C. R. Hafalla, U. Frevert, R. S. Nussenzweig, V. Nussenzweig, A. Rodríguez, Migration of *Plasmodium* sporozoites through cells before infection. *Science* **291**, 141–144 (2001).
3. A. M. Vaughan, S. A. Mikolajczak, E. M. Wilson, M. Grompe, A. Kaushansky, N. Camargo, J. Bial, A. Ploss, S. H. I. Kappe, Complete *Plasmodium falciparum* liver-stage development in liver-chimeric mice. *J. Clin. Invest.* **122**, 3618–3628 (2012).
4. R. E. Sinden, A biologist's perspective on malaria vaccine development. *Hum. Vaccines* **6**, 3–11 (2010).
5. M. Roestenberg, S. J. de Vlas, A.-E. Nieman, R. W. Sauerwein, C. C. Hermsen, Efficacy of preerythrocytic and blood-stage malaria vaccines can be assessed in small sporozoite challenge trials in human volunteers. *J. Infect. Dis.* **206**, 319–323 (2012).
6. A. Siau, O. Silvie, J.-F. Franetich, S. Yalaoui, C. Marinach, L. Hannoun, G.-J. van Gemert, A. J. F. Luty, E. Bischoff, P. H. David, G. Snounou, C. Vaquero, P. Froissard, D. Mazier, Temperature shift and host cell contact up-regulate sporozoite expression of *Plasmodium falciparum* genes involved in hepatocyte infection. *PLOS Pathog.* **4**, e1000121 (2008).
7. L. Dembélé, J.-F. Franetich, A. Lorthiois, A. Gego, A.-M. Zeeman, C. H. M. Kocken, R. Le Grand, N. Dereuddre-Bosquet, G.-J. van Gemert, R. Sauerwein, J.-C. Vaillant, L. Hannoun, M. J. Fuchter, T. T. Diagana, N. A. Malmquist, A. Scherf, G. Snounou, D. Mazier, Persistence and activation of malaria hypnozoites in long-term primary hepatocyte cultures. *Nat. Med.* **20**, 307–312 (2014).
8. H. A. Delemarre-van de Waal, F. C. de Waal, A 2d patient with tropical malaria contracted in a natural way in the Netherlands. *Ned. Tijdschr. Geneeskd.* **125**, 375–377 (1981).
9. A. C. Teirlinck, M. Roestenberg, M. van de Vegte-Bolmer, A. Scholzen, M. J. L. Heinrichs, R. Siebelink-Stoter, W. Graumans, G.-J. van Gemert, K. Teelen, M. W. Vos, K. Nganou-Makamdop, S. Borrmann, Y. P. A. Rozier, M. A. A. Erkens, A. J. F. Luty, C. C. Hermsen, B. K. L. Sim, L. van Lieshout, S. L. Hoffman, L. G. Visser, R. W. Sauerwein, NF135.C10: A new *Plasmodium falciparum* clone for controlled human malaria infections. *J. Infect. Dis.* **207**, 656–660 (2013).
10. D. Mazier, R. L. Beaudoin, S. Mellouk, P. Druilhe, B. Texier, J. Trosper, F. Miltgen, I. Landau, C. Paul, O. Brandicourt, M. Gentilini, Complete development of hepatic stages of Plasmodium falciparum in vitro. *Science* **227**, 440–442 (1985).
11. S. Morosan, S. Hez-Deroubaix, F. Lunel, L. Renia, C. Giannini, N. Van Rooijen, S. Battaglia, C. Blanc, W. Eling, R. Sauerwein, L. Hannoun, J. Belghiti, C. Brechot, D. Kremsdorf, P. Druilhe, Liver-stage development of *Plasmodium falciparum* in a humanized mouse model. *J. Infect. Dis.* **193**, 996–1004 (2006).
12. J. Walk, R. Schats, M. C. C. Langenberg, I. J. Reuling, K. Teelen, M. Roestenberg, C. C. Hermsen, L. G. Visser, R. W. Sauerwein, Diagnosis and treatment based on quantitative PCR after controlled human malaria infection. *Malar. J.* **15**, 398 (2016).
13. J. C. Beier, J. R. Davis, J. A. Vaughan, B. H. Noden, M. S. Beier, Quantitation of *Plasmodium falciparum* sporozoites transmitted in vitro by experimentally infected *Anopheles gambiae* and *Anopheles stephensi*. *Am. J. Trop. Med. Hyg.* **44**, 564–570 (1991).
14. C. B. Pumpuni, C. Mendis, J. C. Beier, Plasmodium yoelii sporozoite infectivity varies as a function of sporozoite loads in Anopheles stephensi mosquitoes. *J. Parasitol.* **83**, 652–655 (1997).
15. T. Mita, T. Jombart, Patterns and dynamics of genetic diversity in *Plasmodium falciparum*: What past human migrations tell us about malaria. *Parasitol. Int.* **64**, 238–243 (2015).
16. M. Roestenberg, G. A. O'Hara, C. J. A. Duncan, J. E. Epstein, N. J. Edwards, A. Scholzen, A. J. A. M. van der Ven, C. C. Hermsen, A. V. S. Hill, R. W. Sauerwein, Comparison of clinical and parasitological data from controlled human malaria infection trials. *PLOS ONE* **7**, e38434 (2012).
17. B. Mordmuller, C. Supan, K. L. Sim, G. P. Gómez-Pérez, C. L. Ospina Salazar, J. Held, S. Bolte, M. Esen, S. Tschan, F. Joanny, C. Lamsfus Calle, S. J. Z. Lohr, A. Lalremruata, A. Gunasekera, E. R. James, P. F. Billingsley, A. Richman, S. Chakravarty, A. Legarda, J. Muñoz, R. M. Antonijoan, M. R. Ballester, S. L. Hoffman, P. L. Alonso, P. G. Kremsner, Direct venous inoculation of *Plasmodium falciparum* sporozoites for controlled human malaria infection: A dose-finding trial in two centres. *Malar. J.* **14**, 117 (2015).
18. G. P. Gómez-Pérez, A. Legarda, J. Muñoz, B. K. L. Sim, M. R. Ballester, C. Dobaño, G. Moncunill, J. J. Campo, P. Cisteró, A. Jimenez, D. Barrios, B. Mordmüller, J. Pardos, M. Navarro, C. J. Zita, C. A. Nhamuave, A. L. García-Basteiro, A. Sanz, M. Aldea, A. Manoj, A. Gunasekera, P. F. Billingsley, J. J. Aponte, E. R. James, C. Guinovart, R. M. Antonijoan, P. G. Kremsner, S. L. Hoffman, P. L. Alonso, Controlled human malaria infection by intramuscular and direct venous inoculation of cryopreserved *Plasmodium falciparum* sporozoites in malaria-naïve volunteers: Effect of injection volume and dose on infectivity rates. *Malar. J.* **14**, 306 (2015).
19. E. M. Bijker, G. J. H. Bastiaens, A. C. Teirlinck, G.-J. van Gemert, W. Graumans, M. van de Vegte-Bolmer, R. Siebelink-Stoter, T. Arens, K. Teelen, W. Nahrendorf, E. J. Remarque, W. Roeffen, A. Jansens, D. Zimmerman, M. Vos, B. C. L. van Schaijk, J. Wiersma, A. J. A. M. van der Ven, Q. de Mast, L. van Lieshout, J. J. Verweij, C. C. Hermsen, A. Scholzen, R. W. Sauerwein, Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 7862–7867 (2013).
20. T. Ponnudurai, A. H. Lensen, G. J. A. van Gemert, M. P. E. Bensink, M. Bolmer, J. H. Meuwissen, Infectivity of cultured Plasmodium falciparum gametocytes to mosquitoes. *Parasitology* **98** (Pt. 2), 165–173 (1989).
21. G. Snounou, X. Zhu, N. Siripoon, W. Jarra, S. Thaithong, K. N. Brown, S. Viriyakosol, Biased distribution of msp1 and msp2 allelic variants in *Plasmodium falciparum* populations in Thailand. *Trans. R. Soc. Trop. Med. Hyg.* **93**, 369–374 (1999).
22. M. B. Borre, M. Dziegiel, B. Høgh, E. Petersen, K. Rieneck, E. Riley, J. F. Meis, M. Aikawa, K. Nakamura, M. Harada, A. Wind, P. H. Jakobsen, J. Crowland, S. Jepsen, N. H. Axelsen, J. Vuust, Primary structure and localization of a conserved immunogenic *Plasmodium falciparum* glutamate rich protein (GLURP) expressed in both the preerythrocytic and erythrocytic stages of the vertebrate life cycle. *Mol. Biochem. Parasitol.* **49**, 119–131 (1991).
23. L. H. Miller, R. J. Howard, R. Carter, M. F. Good, V. Nussenzweig, R. S. Nussenzweig, Research toward malaria vaccines. *Science* **234**, 1349–1356 (1986).
24. J. A. Smythe, R. L. Coppel, K. P. Day, R. K. Martin, A. M. Oduola, D. J. Kemp, R. F. Anders, Structural diversity in the Plasmodium falciparum merozoite surface antigen 2. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1751–1755 (1991).
25. J. D. Johnson, R. A. Dennull, L. Gerena, M. Lopez-Sanchez, N. E. Roncal, N. C. Waters, Assessment and continued validation of the malaria SYBR green I-based fluorescence assay for use in malaria drug screening. *Antimicrob. Agents Chemother.* **51**, 1926–1933 (2007).
26. M. P. A. van Meer, G. J. H. Bastiaens, M. Boulaksil, Q. de Mast, A. Gunasekera, S. L. Hoffman, G. Pop, A. J. A. M. van der Ven, R. W. Sauerwein, Idiopathic acute myocarditis during treatment for controlled human malaria infection: A case report. *Malar. J.* **13**, 38 (2014).
27. E. M. Bijker, R. Schats, J. M. Obiero, M. C. Behet, G.-J. van Gemert, M. van de Vegte-Bolmer, W. Graumans, L. van Lieshout, G. J. H. Bastiaens, K. Teelen, C. C. Hermsen, A. Scholzen, L. G. Visser, R. W. Sauerwein, Sporozoite immunization of human volunteers under mefloquine prophylaxis is safe, immunogenic and protective: A double-blind randomized controlled clinical trial. *PLOS ONE* **9**, e112910 (2014).
28. B. C. L. van Schaijk, C. J. Janse, G.-J. van Gemert, M. R. van Dijk, A. Gego, J.-F. Franetich, M. van de Vegte-Bolmer, S. Yalaoui, O. Silvie, S. L. Hoffman, A. P. Waters, D. Mazier, R. W. Sauerwein, S. M. Khan, Gene disruption of *Plasmodium falciparum p52* results in attenuation of malaria liver stage development in cultured primary human hepatocytes. *PLOS ONE* **3**, e3549 (2008).
29. https://clinicaltrials.gov/ct2/show/NCT01627951.
30. https://clinicaltrials.gov/ct2/show/NCT02149550.
31. D. F. Verhage, D. S. C. Telgt, J. T. Bousema, C. C. Hermsen, G. J. A. van Gemert, J. W. M. van der Meer, R. W. Sauerwein, Clinical outcome of experimental human malaria induced by Plasmodium falciparum-infected mosquitoes. *Neth. J. Med.* **63**, 52–58 (2005).
32. M. Roestenberg, M. McCall, J. Hopman, J. Wiersma, A. J. F. Luty, G. J. van Gemert, M. van de Vegte-Bolmer, K. Teelen, T. Arens, L. Spaarman, Q. de Mast, W. Roeffen, G. Snounou, L. Rénia, Andre van de Ven, C. C. Hermsen, R. Sauerwein, Protection against a malaria challenge by sporozoite inoculation. *N. Engl. J. Med.* **361**, 468–477 (2009).
33. M. Roestenberg, A. C. Teirlinck, M. B. McCall, K. Teelen, K. N. Makamdop, J. Wiersma, T. Arens, P. Beckers, G. van Gemert, M. van de Vegte-Bolmer, A. J. van der Ven, A. J. F. Luty, C. C. Hermsen, R. W. Sauerwein, Long-term protection against malaria after experimental sporozoite inoculation: An open-label follow-up study. *Lancet* **377**, 1770–1776 (2011).
34. E. M. Bijker, A. C. Teirlinck, R. Schats, G.-J. van Gemert, M. van de Vegte-Bolmer, L. van Lieshout, J. IntHout, C. C. Hermsen, A. Scholzen, L. G. Visser, R. W. Sauerwein, Cytotoxic markers associate with protection against malaria in human volunteers immunized with *Plasmodium falciparum* sporozoites. *J. Infect. Dis.* **210**, 1605–1615 (2014).
35. E. M. Bijker, R. Schats, L. G. Visser, R. W. Sauerwein, A. Scholzen, Ex vivo lymphocyte phenotyping during *Plasmodium falciparum* sporozoite immunization in humans. *Parasite Immunol.* **37**, 590–598 (2015).
36. G. J. H. Bastiaens, M. P. A. van Meer, A. Scholzen, J. M. Obiero, M. Vatanshenassan, T. van Grinsven, B. K. L. Sim, P. F. Billingsley, E. R. James, A. Gunasekera, E. M. Bijker, G.-J. van Gemert, M. van de Vegte-Bolmer, W. Graumans, C. C. Hermsen, Q. de Mast, A. J. van der Ven, S. L. Hoffman, R. W. Sauerwein, Safety, immunogenicity, and protective efficacy of intradermal immunization with aseptic, purified, cryopreserved *Plasmodium falciparum* sporozoites in volunteers under chloroquine prophylaxis: A randomized controlled trial. *Am. J. Trop. Med. Hyg.* **94**, 663–673 (2016).

Acknowledgments: We thank the subjects who participated in the two CHMI studies described here. We are indebted to N. Molhoek, M. IJsselstijn, S. Goeijenbier, and K. Teelen for logistical support and to R. Siebelink-Stoter for parasite culture. We thank J. Klaassen, A. Pouwelsen, L. Pelser-Posthumus, and J. Kuhnen for assistance in generating infectious mosquitoes and the technicians of the laboratory of the Harbour Hospital and the Department

of Medical Microbiology of the Erasmus MC for reading thick smears and performing qPCRs. We are grateful to B. Krenning for assistance in the cardiac screening of study subjects and to E. van Gorp for acting as safety monitor. H. de Wilt supplied fresh liver tissue from patients undergoing elective (partial) hepatectomy. These studies are registered with ClinicalTrials.gov with registration numbers NCT01627951 (*29*) and NCT02149550 (*30*). **Funding:** The CHMI studies described here were supported by Top Institute Pharma (grant T4-102) and the Bill and Melinda Gates Foundation (grant opp1080385). Neither financial sponsor was involved in the respective study design, collection, analysis or interpretation of data, or writing of the report or decision to submit for publication. **Author contributions:** M.B.B.M., L.J.W., G.-J.v.G., C.C.H., D.M., E.M.B., J.J.v.H., P.J.J.v.G., and R.W.S. conceived and initiated the studies. M.B.B.M., L.J.W., M.C.C.L., and J.W. were responsible for the daily medical care of study subjects, the recording of adverse events, and the collection of blood samples, under the supervision of P.J.J.v.G. and R.W.S. R.K. and J.J.v.H. were responsible for measurements of parasitemia in study subjects (thick blood smears and qPCR). G.-J.v.G., W.G., and M.v.d.V. were responsible for generating gametocyte cultures and infected mosquitoes. W.G. was responsible for parasite genotyping. S.C. was responsible for processing blood samples from study subjects. C.C.H., J.-F.F., M.G., A.L., and D.M. were involved in performing human hepatocyte invasion assays. M.B.B.M., L.J.W., M.C.C.L., J.W., C.C.H., W.G., J.-F.F., M.G., J.J.v.H., P.J.J.v.G., and R.W.S. analyzed the data. M.B.B.M., L.J.W., C.C.H., J.J.v.H., P.J.J.v.G., and R.W.S. wrote the manuscript. All authors critically read and approved the manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** R.W.S. should be contacted with requests to obtain the parasite strains, which are available under a material transfer agreement.

Submitted 14 June 2016
Resubmitted 12 October 2016
Accepted 1 March 2017
Published 21 June 2017
10.1126/scitranslmed.aag2490

Citation: M. B. B. McCall, L. J. Wammes, M. C. C. Langenberg, G.-J. van Gemert, J. Walk, C. C. Hermsen, W. Graumans, R. Koelewijn, J.-F. Franetich, S. Chishimba, M. Gerdsen, A. Lorthiois, M. van de Vegte, D. Mazier, E. M. Bijker, J. J. van Hellemond, P. J. J. van Genderen, R. W. Sauerwein, Infectivity of *Plasmodium falciparum* sporozoites determines emerging parasitemia in infected volunteers. *Sci. Transl. Med.* **9**, eaag2490 (2017).

Science Translational Medicine

Infectivity of *Plasmodium falciparum* sporozoites determines emerging parasitemia in infected volunteers

Matthew B. B. McCall, Linda J. Wammes, Marijke C. C. Langenberg, Geert-Jan van Gemert, Jona Walk, Cornelus C. Hermsen, Wouter Graumans, Rob Koelewijn, Jean-François Franetich, Sandra Chishimba, Max Gerdsen, Audrey Lorthiois, Marga van de Vegte, Dominique Mazier, Else M. Bijker, Jaap J. van Hellemond, Perry J. J. van Genderen and Robert W. Sauerwein

Sci Transl Med **9**, eaag2490.
DOI: 10.1126/scitranslmed.aag2490

New insights into malaria parasite infectivity

A malaria infection starts by inoculation of *Plasmodium falciparum* parasites into the skin by blood-feeding mosquitoes. These sporozoites rapidly invade liver cells and mature and multiply over 6 to 7 days. Infected liver cells then release new parasites into the bloodstream, which then multiply repeatedly inside red blood cells, causing clinical symptoms. McCall and colleagues now show that sporozoites of diverse geographic and genetic origin differ in their capacity to infect and develop within human liver cells in culture. This matched their ability to form blood-stage parasites in human volunteers undergoing a controlled malaria infection. Thus, the fitness of sporozoites apparently determines their virulence.

Science Translational Medicine (ISSN 1946-6242) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. The title *Science Translational Medicine* is a registered trademark of AAAS.

Science Translational Medicine (ISSN 1946-6242) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. The title *Science Translational Medicine* is a registered trademark of AAAS.