

Innate Immunity Induced by *Plasmodium* Liver Infection Inhibits Malaria Reinfections

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Following transmission through a mosquito bite to the mammalian host, *Plasmodium* parasites first invade and replicate inside hepatocytes before infecting erythrocytes and causing malaria. The mechanisms limiting *Plasmodium* reinfections in humans living in regions of malaria endemicity have mainly been explored by studying the resistance induced by the blood stage of infection. However, epidemiologic studies have suggested that in high-transmission areas, preerythrocytic stages also activate host resistance to reinfection. This, along with the recent discovery that liver infections trigger a specific and effective type I interferon (IFN) response, prompted us to hypothesize that this pre-erythrocyte-stage-induced resistance is linked to liver innate immunity. Here, we combined experimental approaches and mathematical modeling to recapitulate field studies and understand the molecular basis behind such resistance. We present a newly established mouse reinfection model and demonstrate that rodent malaria liver-stage infection inhibits reinfection. This protection relies on the activation of innate immunity and involves the type I IFN response and the antimicrobial cytokine gamma IFN (IFN- γ). Importantly, mathematical simulations indicate that the predictions based on our experimental murine reinfection model fit available epidemiological data. Overall, our study revealed that liver-stage-induced innate immunity may contribute to the preerythrocytic resistance observed in humans in regions of malaria hyperendemicity.

Malaria accounts for over half a million deaths per year and is thus the most prevalent parasitic human disease worldwide (1). The disease is caused by an intracellular protozoan parasite of the genus *Plasmodium* that infects multiple hosts, such as *Anopheles* mosquitoes and humans and other mammals (2). Infection begins with a bite of a female mosquito that injects a few *Plasmodium* sporozoites, which represent the mosquito-transmitted parasite form, into the skin of the mammalian host. After migrating through skin cells (3), sporozoites enter the bloodstream and are then rapidly and specifically retained in the liver sinusoids. Sporozoites then cross the sinusoidal barrier (4) and traverse several liver cells until individual parasites invade a final hepatocyte with the formation of a parasitophorous vacuole (5). Inside this vacuolar niche, sporozoites asymptotically develop and replicate into thousands of erythrocyte-infective parasites, termed merozoites (6). Finally, merozoites are released into the bloodstream and rapidly infect erythrocytes, initiating the blood stage and the clinical phase of infection (7).

Malaria reinfections are common, especially in regions of high malaria transmission. *Plasmodium* parasites present an extraordinarily high rate of polymorphism; consequently, the host can be reinfected by different parasites that repeatedly escape the immune response (8). Therefore, efficient immunity to reinfection in one individual is obtained only after many years of facing recurrent infections with many different parasite strains (9, 10). Most of our knowledge of immune defense mechanisms acting against *Plasmodium* reinfection relies on studies focusing on the blood stage of infection. These studies have revealed that host resistance to blood-stage infection is complex and mediated by both the innate immune system, which limits the initial growth of all blood-stage parasites, irrespective of *Plasmodium* species or strain, and adaptive immunity, which is genotype specific (reviewed in references 9, 10, and 11). In addition, recent studies have suggested that high levels of blood-stage parasitemia protect the

host from secondary or superinfection, by inhibiting liver-stage reinfection (12, 13). In mice, this increased resistance seems to be mediated by the iron-regulatory hormone hepcidin, which impairs parasite growth by restricting iron availability in the liver (12). Thus, antimalarial host responses induced during the erythrocytic stage of infection are effective against reinfection of both the blood and the liver.

Interestingly, epidemiological and mathematical modeling studies have also suggested that, in addition to these blood-stage-induced defenses, resistance mechanisms must exist which are triggered by infection stages that precede blood-stage infection (14–16). However, the immune responses raised during these initial stages of infection remain poorly explored (reviewed in references 17 and 18). Recently, we have shown that *Plasmodium* liver-stage parasites are sensed by the host and specifically activate a type I interferon (IFN) response (19). This response controls par-

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asite load during this first obligatory step of infection and mediates host resistance when experimentally induced prior to infection (19). In regions of hyperendemicity, the entomological inoculation rate (EIR), which indicates the number of infective mosquito bites within a given time interval (20), can reach several hundred infective bites per person per year (21–24). Since liver-stage infection lasts 7 to 13 days in humans (25, 26), a person living in these regions of hyperendemicity is likely to be reinfected while parasites from a previous infection are still in the liver. On the basis of our recent results, we speculated that this reinfection is less efficient than the primary infection as it occurs in the context of a type I IFN response triggered by the first infection and that this effect could participate in the resistance mechanism observed in epidemiological studies.

To test this hypothesis, we have established a mouse reinfection model. We show that innate immunity induced by a primary *Plasmodium berghei* liver-stage infection contributes significantly to host resistance to reinfection, not only after intravenous injections of high sporozoite doses but, crucially, also following mosquito bite infections. Our data indicate that the mechanisms of inhibition of a secondary infection depend on the activation of type I IFN signaling and gamma IFN (IFN- γ) expression. Last, we utilized a mathematical modeling approach to understand whether the resistance observed in the murine model might explain epidemiological observations of the rates of *P. falciparum* infection. We showed that the host resistance observed in our murine model is consistent with the observed changes in infection rates seen in epidemiological studies of human infection with *P. falciparum*.

MATERIALS AND METHODS

Supplemental experimental information is available in the supplemental material.

Ethics statement. All *in vivo* protocols were approved by the internal animal care committee of the Instituto de Medicina Molecular (IMM) and performed according to national and European regulations (project license AEC_2010_034_MP_Rdt_General).

Mice. Mice were housed in the facilities of the IMM. C57BL/6J wild-type (WT) mice were purchased from Charles River Breeding Laboratories. *Ifnar1*^{-/-} mice were bred in specific-pathogen-free facilities at the Instituto Gulbenkian de Ciência. *Ifn- γ* ^{-/-} mice were purchased from The Jackson Laboratories (27). Mice used in this work were in the C57BL/6J background and had been backcrossed at least 10 times.

Parasite strains, liver infection, and blood parasitemia. Green fluorescent protein (GFP)-expressing *P. berghei* ANKA (28) sporozoites were obtained by dissection of *Anopheles stephensi*-infected mosquitoes bred at the IMM. Mice were injected intravenously with 50,000 sporozoites. Mosquito bite infection was performed with 10 mosquitoes per mouse. Parasite liver load was quantified by quantitative real-time PCR (qRT-PCR) in extracts of total livers. The presence of erythrocyte-stage parasites was monitored by flow cytometry. Measurements were performed on a Fortessa (BD Biosciences) flow cytometer. A drop of blood in 1 \times phosphate-buffered saline (PBS) was used to measure the blood parasitemia of GFP-expressing parasites. Flow cytometry data were analyzed using FlowJo software (version 9.0.2, Tree Star Inc., OR, USA).

RNA isolation of total livers and qRT-PCR quantification. For mouse liver RNA extraction, whole livers were homogenized in 3 ml denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate [pH 7], 0.5% *N*-lauroylsarcosine, 0.7% mercaptoethanol, diethyl pyrocarbonate [DEPC]-treated water). RNA was extracted using an RNeasy minikit (Qiagen). cDNA was synthesized using a Transcriptor first-strand cDNA synthesis kit (Roche). Gene expression analysis was performed using Bio-Rad kits. For analysis, the expression levels of all target genes were normalized against the hypoxanthine guanine phosphoribosyltransferase

(*Hprt*) housekeeping gene using the delta threshold cycle (ΔC_T) method. Changes in gene expression values were calculated using the Pfaffl method (29). We used the following oligonucleotide primer pairs to detect target gene transcripts: *Hprt*-F (CATTATGCCGAGGATTTGGA) and *Hprt*-R (AATCCAGCAGGTCAGCAAAG), *Ifit1*-F (CCTTTACAGCAACCATGGAGA) and *Ifit1*-R (GCAGCTCCATGTGAAGTGAC), *Ifi44*-F (TCGATCCATGAAACCAATCAC) and *Ifi44*-R (CAAATGCAGAATGCCATGTTT), *Usp18*-F (CGTGCTTGAGAGGGTCATTTG) and *Usp18*-R (G GTCGGGAGTCCACAACCTTC), *Ifit3*-F (CTGAACTGCTCAGCCCA CAC) and *Ifit3*-R (TGGACATACTTCCTCCCTGA), *Irf7*-F (CTTCAG CACTTCTTCCGAGA) and *Irf7*-R (TGAGTGTGGTGACCCTTGC), *Ifn- γ* -F (CACACTGCATCTTGGCTTTG) and *Ifn- γ* -R (TCTGGCTCTG CAGGATTTTC), and *P. berghei* 18S rRNA-F (AAGCATTAAATAAAGC GAATACATCCTTAC) and *P. berghei* 18S rRNA-R (GGAGATTGGTTT TGACGTTATGTG).

Statistical analyses. Data are expressed as means \pm standard errors of the means (SEM). Statistically significant differences between two different groups were analyzed using the Mann-Whitney test. Statistical tests involving three groups or more were analyzed using the nonparametric Kruskal-Wallis test with posterior Dunn's multiple comparison tests. Results with a *P* value of <0.05 were considered statistically significant. Significances are represented in the figures as follows: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. All statistic tests were performed using Graph Prism 5.0 software.

Mathematical simulations. (i) Estimating the maximum "blocking" of the liver stage. In order to estimate the extent to which prior liver-stage infection is able to block subsequent liver stages, we analyzed the data from our murine experiments. There are two ways to estimate the level of blocking. First, we might consider simply the reduction in liver infection load in the second infection (Fig. 1B and C). However, this may underestimate the reduction in subsequent blood-stage infection because, although liver cells may contain *Plasmodium* DNA, this may not result in fully infectious merozoites. Therefore, the most direct way to estimate the reduction in release of infectious merozoites from the liver is to estimate the delay in infection dynamics in a second infection compared to a first infection (Fig. 2B). In order to estimate the initial number of infectious merozoites that were released from liver, we fitted the logistic growth function to the mean level of parasitemia observed in the experimental data. This function is a good approximation of the growth of the concentration of parasites over time (threshold cycle [C_T]) in blood until the concentration reaches a maximal value:

$$C(t) = \frac{MPe^{rt}}{M + P(e^{rt} - 1)} \quad (1)$$

where *P* is the initial concentration of parasites in blood, *M* is the asymptotic maximum of the concentration of parasites in blood, and *r* is the initial growth rate of parasites. We used GraphPad Prism 6.04 (GraphPad Software Inc. La Jolla, CA), to fit function 1 to the mean of experimental data. We assumed that the only parameter that is different in groups with single and repeated infections is the initial concentration of parasites (*P*); let us call the concentrations *P*₁ and *P*₂, respectively. Parameters *M* and *r* are shared between groups. The graphs of the best-fit function (equation 1) to the data are shown in Fig. S2A in the supplemental material, and the best-fit parameters are as follows: *P*₁ = 2.28% $\times 10^{-5}$ infected red blood cells (RBC), *P*₂ = 9.1% $\times 10^{-7}$ infected RBC, *M* = 3.296% infected RBC, and *r* = 2.345 parasites per day. Comparing *P*₁ and *P*₂, we find the concentration of merozoites released from the liver after reinfection is approximately 25-fold less than after the single infection.

(ii) Deterministic model of the impact of the innate liver-stage immunity on the force of infection. We developed a mathematical model in order to investigate whether the effects of innate immunity blocking subsequent liver-stage infections could contribute to the relationship between the entomological inoculation rate (EIR) and the force of infection (FOI) that was observed in field studies. In the absence of induced resistance, we would expect that the proportion of infected bites reaching the

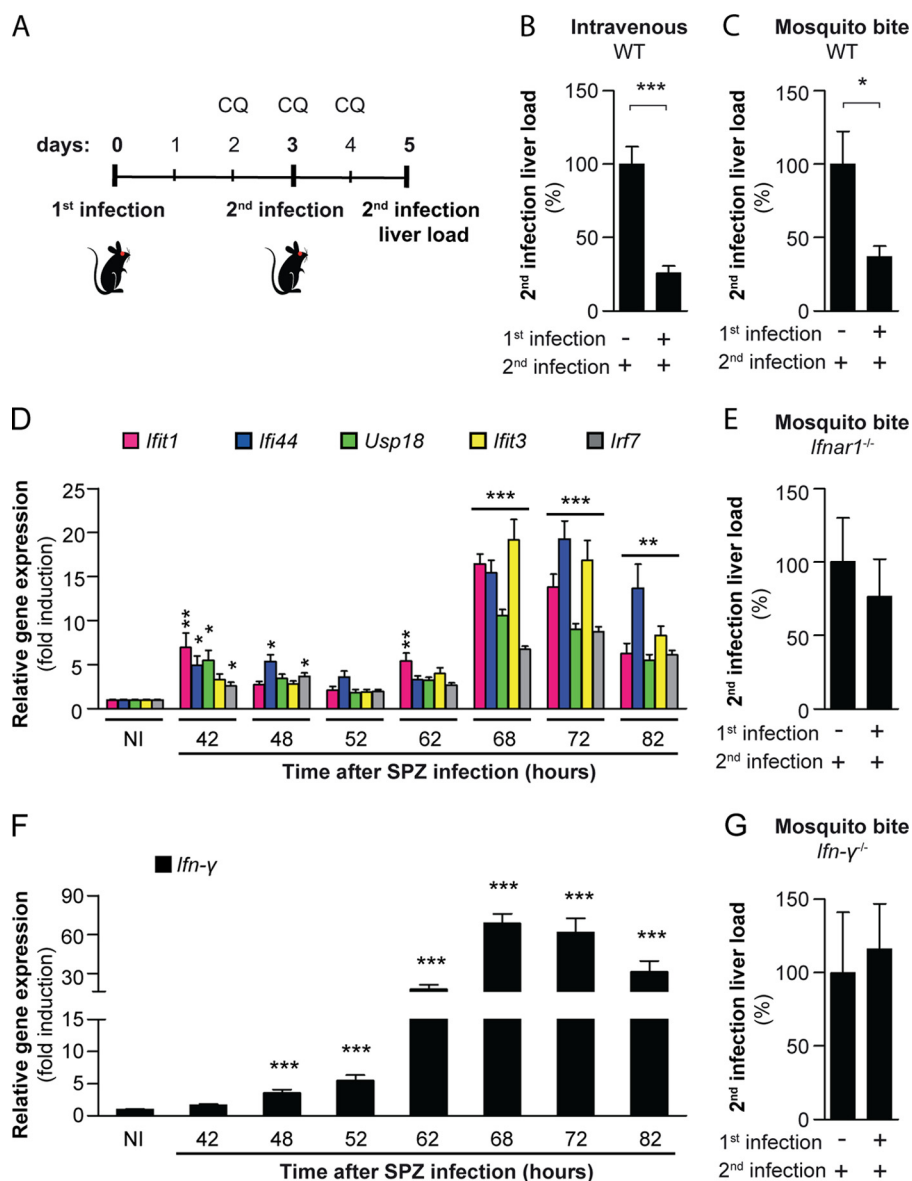


FIG 1 Role of type I IFN and IFN- γ in a *P. berghei* liver-stage reinfection model. (A) Schematic representation of the reinfection protocol. Mice were injected intravenously (i.v.) with 50,000 *P. berghei* sporozoites or an equivalent amount of noninfected (NI) salivary glands on day 0. Both groups of animals were treated with 700 μ g of chloroquine (CQ) on days 2, 3, and 4 after injection. On day 3, mice were reinfected by the i.v. injection of 50,000 *P. berghei* sporozoites. Alternatively, mice were subjected to bites of infected *Anopheles* mosquitoes or an equivalent number of noninfected mosquitoes, treated with CQ on days 2, 3, and 4 after inoculation, and infected or reinfected on day 3 by mosquito bite. The amount of parasite 18S ribosomal transcripts (18S rRNA), corresponding to the parasite liver load of the second infection, was measured by qRT-PCR 44 h after reinfection. (B) Parasite liver loads in reinfected WT mice, measured by qRT-PCR of *P. berghei* 18S rRNA 44 h after the second infection with *P. berghei* sporozoites and plotted as the percentages of the *P. berghei* 18S rRNA levels in control WT mice. The results shown represent the means and SEM of the results of two independent experiments (single infection, $n = 14$; reinfection, $n = 14$). The Mann-Whitney test demonstrated a statistically significant difference between control and reinfected mice ($P < 0.001$). (C) Parasite liver loads in reinfected WT mice, measured by qRT-PCR of *P. berghei* 18S rRNA, 44 h after two consecutive mosquito bite infections (10 mosquitoes/mouse) plotted as percentages of the levels in control WT mice. The results shown represent the means and SEM of the results of three independent experiments (single infection, $n = 16$; reinfection, $n = 18$). The Mann-Whitney test demonstrated a statistically significant difference between control and reinfected mice ($P < 0.05$). (D) Expression of genes *lfit1*, *lfi44*, *Usp18*, *lfit3*, and *lrf7* in total liver extracts of WT mice collected at multiple time points after infection with 50,000 *P. berghei* sporozoites (SPZ). For each time point, the values shown are the means and SEM of the results of two independent experiments (NI, $n = 10$; 42 h, $n = 8$; 48 h, $n = 8$; 52 h, $n = 16$; 62 h, $n = 11$; 68 h, $n = 10$; 72 h, $n = 10$; 82 h, $n = 10$). The Kruskal-Wallis test followed by Dunn's multiple-comparison test revealed statistically significant differences between uninfected control samples and liver samples 42 h, 68 h, 72 h, and 82 h after infection ($P < 0.05$). (E) Parasite liver loads in reinfected *Ifnar1*^{-/-} mice, measured by qRT-PCR of *P. berghei* 18S rRNA, 44 h after two consecutive mosquito bite infections (10 mosquitoes/mouse) plotted as percentages of the levels in control *Ifnar1*^{-/-} mice. The results shown represent the means and SEM of the results of two independent experiments (single infection, $n = 14$; reinfection, $n = 13$). The Mann-Whitney test demonstrated no statistically significant difference between control and reinfected *Ifnar1*^{-/-} mice. (F) *Ifn-γ* gene expression in whole livers of WT mice collected at multiple time points after infection with 50,000 *P. berghei* sporozoites. For each time point, the values represented are the means and SEM of the results of four (42 h, 48 h, and 52 h) and two (62 h, 72 h, and 82 h) independent experiments (NI, $n = 38$; 42 h, $n = 20$; 48 h, $n = 37$; 52 h, $n = 20$; 62 h, $n = 10$; 68 h, $n = 10$; 72 h, $n = 10$; 82 h, $n = 10$). The Kruskal-Wallis test followed by Dunn's multiple-comparison test revealed statistically significant differences between uninfected control samples and liver samples 48 h, 52 h, 62 h, 72 h, and 82 h after infection ($P < 0.001$). (G) Parasite liver loads in reinfected *Ifn-γ*^{-/-} mice, measured by qRT-PCR of *P. berghei* 18S rRNA, 44 h after two consecutive mosquito bite infections (10 mosquitoes/mouse), plotted as percentages of the levels in control *Ifn-γ*^{-/-} mice. The results shown represent the means and SEM of the results of two independent experiments (single infection, $n = 8$; reinfection, $n = 8$). The Mann-Whitney test demonstrated no statistically significant difference between control and reinfected *Ifn-γ*^{-/-} mice.

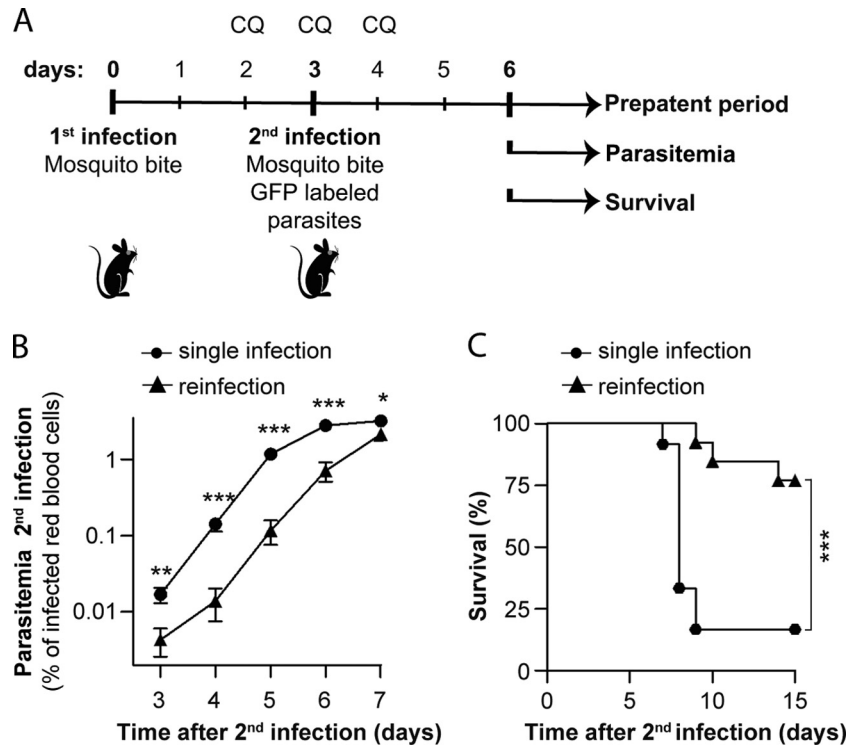


FIG 2 Effect of a primary *P. berghei* liver infection on the blood stage of a subsequent sporozoite-initiated reinfection. (A) Schematic representation of the reinfection protocol. Mice were injected with 50,000 *P. berghei* sporozoites or noninfected salivary glands on day 0. Both groups of animals were treated with 700 µg of chloroquine (CQ) on days 2, 3, and 4 after injection. On day 3, mice were reinfected with 50,000 *P. berghei* GFP-expressing sporozoites. Parasitemia (percentage of red blood cells infected with GFP-expressing parasites) was measured by flow cytometry starting at day 3 after the second infection. Survival was monitored daily. (B and C) Parasitemia and survival in reinfected WT mice after mosquito bite infection (10 mosquitoes/mouse). The results shown represent the means and SEM of the results of two independent experiments (single infection, $n = 11$; reinfection, $n = 12$). The Mann-Whitney test demonstrated a statistically significant difference in parasitemia levels between control and reinfected mice ($P < 0.01$). The log-rank test demonstrated a statistically significant difference in survival rates between control and reinfected mice ($P < 0.001$).

liver would be constant at different biting rates and that the slope of this would be determined by the baseline success rate of an infected bite reaching the blood stage (r). In this case, the relation between EIR and FOI would be a straight line:

$$y = rx \tag{2}$$

where x represents EIR and y represents FOI.

In the case of induced immunity that blocks infections from subsequent bites, we can also find the expected relationship between EIR and FOI. For a given EIR, the waiting times between infected bites have an exponential distribution. If we again assume a baseline success of an infection from a bite reaching the blood stage of r , then the waiting time between instances of “successful” liver-stage infection is rx . Therefore, the cumulative distribution function (CDF) of an exponential distribution with parameter rx at point T gives us the probability that next bite would occur during the period T from a previous infection—and would be blocked by the innate immune response. Thus, the relation between EIR and FOI can be expressed by the following formula:

$$y = rx[1 - p \text{CDF}_{\text{exp}}(rx, T)] \tag{3}$$

where p is the probability that a liver-stage infection will be blocked by the innate immune response induced by a previous bite, given that it occurred during the period T of the active immunity response. We then fitted model 2 and model 3 to the experimental data and compared the fits using the Akaike information criterion (AIC).

(iii) Stochastic model of the impact of the innate liver-stage immunity on the force of infection. We also developed a stochastic model that is able to take into account a more complex infection kinetics than the

deterministic model, such as multiple consecutive bites and gradual acquisition and loss of immunity. This allows us to investigate whether the effects of innate immunity blocking subsequent liver-stage infections would produce the same relationship between entomological inoculation rate (EIR) and force of infection (FOI) as that predicted by the deterministic model and observed in field studies (30–33). This model simulated biting at different rates and the proportion of infectious bites that successfully initiated blood-stage infections.

(iv) Modeling infection. The simulation takes into account 3 possible types of infection: (i) infections that survive the liver stage and pass on to the blood stage, which induce strong innate liver stage resistance to subsequent liver-stage infections (with the maximal level of $R_s = 1$); (ii) infections that are cleared at the liver stage by the innate liver-stage immunity induced by previous infections—we assume that they would induce a lower level of resistance than completed liver-stage infections (at a level of R_p , which we assume to be 25% of the maximal inhibition [i.e., $R_p = 0.25 \times R_s$]); and (iii) bite infections that do not reach the liver stage. We assume that there is a baseline probability (R_b) that infectious bites would fail to result in liver-stage infection. They do not induce liver-stage immunity. According to model 2, R_b is equal to 0.93 (rate of progression to blood-stage infection $r = 0.07$).

(v) Simulation of biting rates. The model assumes that for a given EIR, infective bites arrive randomly with exponentially distributed waiting times (13, 34, 35). Each bite infection then has a probability of reaching the blood stage, which is determined by the baseline probability of success (R_b) and the level of induced resistance at a given time [$R(t)$]. After a successful bite infection, the level of induced resistance rises to its max-

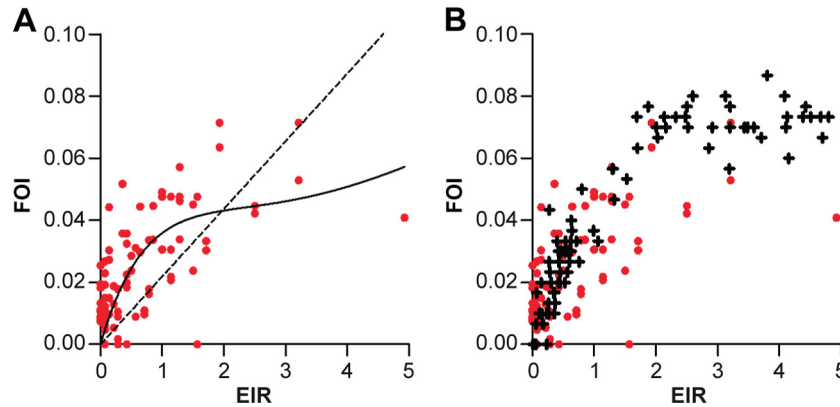


FIG 3 Linking murine reinfection data to epidemiological observations. (A) Deterministic modeling of the experimental relationship between the EIR (entomological inoculation rate; average number of infectious bites per person per unit time) and the FOI (force of infection; number of infections per person per unit of time). Using published data on EIR and FOI from a field study in western Kenya (extracted from Table 2 of reference 30; red dots), we fitted either a baseline model (fixed proportion of infectious bites progressing to blood stage) (dashed line) or a model with induced liver-stage immunity (solid line). The model of induced liver-stage immunity showed a better fit to the experimental data compared by AIC. The best-fit parameters for the models are as follows: for model 2, $r = 0.022$; for model 3, $r = 0.069$, $p = 0.85$, $t = 12.2$ days. (B) Results of the stochastic simulation of the relation between EIR and FOI in the presence of induced resistance. Red dots represent the field study data (extracted from Table 2 of reference 30); crosses represent the estimates from the simulation.

imum and then falls over time (see formula 4 below). Thus, for each simulated bite, a uniformly distributed random number α is assigned to it. The value of this number and the current level of induced resistance determine which of categories 1 to 3 this infection belongs to as follows. (i) If $R(t)$ plus R_b is $< \alpha$, where $R(t)$ is the current level of the liver stage resistance, then the infection is considered of type 1 and induces subsequent resistance to infection. (ii) If R_b is less than α and α is less than $R(t)$ plus R_b , where $R(t)$ is the current level of liver stage resistance, then the infection is considered of type 2. (iii) If α is less than R_b , then the infection does not reach the liver stage and the infection is considered of type 3 and does not contribute to the innate liver-stage immunity.

(vi) Modeling changes in induced resistance. The dynamics of induced resistance to liver-stage infection in humans is unknown. However, we can estimate the likely duration of resistance using the data from the studies in *P. berghei* and scaling for the longer duration of the intrahepatic stage of infection in *P. falciparum*. We assume that resistance in *P. falciparum* infection has a duration of 12 days and is induced over time during liver-stage infection (peaking at d_1 days after the initial infection; we assume $d_1 = 6$ days) and then decays over some period (for d_2 days after the peak; we assume $d_2 = 6$ days). We chose the logistic growth function to describe the growth and decay of liver stage resistance over time. For this function, we need an initial level of resistance immediately after initiation of the liver stage ($R_0 = 0.05$) and a peak level of resistance (M , the asymptotic maximum resistance [which will never be attained], which we assume to be slightly larger than the maximum resistance $M = R_{\max}/0.98$). The function that describes both the growth and decay is defined by formula 4.

$$f(t) = \begin{cases} \frac{MR_0e^{r_1(t-t_0)}}{M + R_0(e^{r_1(t-t_0)} - 1)}, & t_0 \leq t < t_0 + d_1, \\ \frac{MR_{\max}e^{-r_2(t-t_0-d_1)}}{M + R_{\max}(e^{-r_2(t-t_0-d_1)} - 1)}, & t_0 + d_1 \leq t < t_0 + d_1 + d_2, \\ 0, & t \geq t_0 + d_1 + d_2 \end{cases} \quad (4)$$

where $r_i = \ln[(R_0R_{\max}) + MR_{\max}]/R_0(M - R_{\max})/d_i$, $i = 1, 2$, and R_{\max} is the maximal level of induced liver-stage immunity, which can take a value of R_s or R_p , depending on conditions i to iii as described above. Fig. S2B in the supplemental material shows the trajectory of resistance over time after a successful bite.

The total level of induced resistance $R(t)$ induced by all previous infections is calculated using formula 5.

$$R(t) = \text{Min} \left[\sum_{i=1}^n f_i(t), R_{\max} \right] \quad (5)$$

where function $f_i(t)$ is the immunity induced by the i -th infection ($i = 1, \dots, n$).

Using this stochastic model, we can then simulate different biting rates and analyze the observed FOI that we obtain in the simulation. We compared the relationship of EIR to FOI predicted by the simulations with that observed in a published field study presented in Table 2 in a study by Beier et al. (30). We ran simulations with EIR values ranging from 0.01 to 5 infective bites/day and observed the rate of infection expected. The stochastic simulation showed the same relationship between EIR and FOI as in model 3, namely, as the EIR increases, infections from an increasing proportion of bites are blocked by induced resistance, and we see a decreasing proportion of infections from bites making it to the blood. This is shown in Fig. 3B. A sample of the simulation calculated with an EIR of 1 is shown in Fig. S2C in the supplemental material.

RESULTS

Induction of type I IFN and IFN- γ by a first *P. berghei* liver infection protects against reinfection. To test the hypothesis that a primary *Plasmodium* liver infection induces protection from reinfection, we set up an assay in which mice were first injected with *P. berghei* parasites or, for control animals, with an equivalent amount of salivary gland material from noninfected mosquitoes. Three days later, mice were reinfected and the liver parasite burden 42 h after the rechallenge was monitored by quantitative real-time PCR, targeting the parasite 18S ribosomal small-subunit transcripts (Fig. 1A). Since a primary liver-stage infection lasts 2 to 3 days in our rodent model (see Fig. S1 in the supplemental material), we were able to attribute the measured liver parasite burden to the second infection. To minimize the influence of blood-stage-induced immune responses, mice were treated with chloroquine from day 2 to day 5 after the primary infection, effectively killing developing erythrocyte-stage parasites without affecting the liver stages (36–38) (Fig. 1A). This was also confirmed by the absence of blood-stage parasitemia on day 2 after the second sporozoite infection, when any blood-stage parasites would likely have resulted from the first infection (observation of Giemsa-stained blood smears; data not shown). When mice were

infected by intravenous injection of 50,000 sporozoites, we found that primed mice were significantly more resistant to a second challenge with 50,000 sporozoites than control animals (Fig. 1B). Thus, our data indicate that, within 3 days, a primary exposure to a high number of sporozoites activates a significant host resistance to a malaria liver-stage reinfection. We next carried out a similar experiment in which mice were infected by mosquito bites. A significant 2-fold decrease of liver parasite load in reinfected mice was also observed when infections were performed via this natural route (Fig. 1C). Overall, our data show that, 3 days after an initial liver-stage infection, the host benefits from increased defenses against *Plasmodium* sporozoite reinfection. Notably, this protection is observed not only after a primary infection by intravenous injection with (nonphysiologically) high numbers of sporozoites but also when sporozoites are delivered by mosquito bites.

We next sought to understand the molecular mechanisms underlying this phenomenon. Recently, we have shown that a primary liver-stage infection induces a hepatic type I IFN signature (19). The recognition mechanism relies on the detection of *Plasmodium* RNA by the cytosolic RNA sensor MDA5 and likely other as-yet-unidentified receptors. The signaling cascade involves the mitochondrial antiviral signaling protein (MAVS) adapter molecule and transcription factors interferon regulatory factor 3 (IRF3) and IRF7. Our data further suggested a model in which released type I IFN binds, in both an autocrine manner and a paracrine manner, to the heterodimeric alpha interferon receptor (IFNAR, composed of IFNAR1 and IFNAR2), activating transcription of IFN-inducible genes, with a peak of expression at 42 h after infection (19, 39). Here we have extended our analysis of the liver type I IFN response to later time points. We challenged mice with 50,000 *P. berghei* sporozoites and treated them with chloroquine on the second, third, and fourth days after infection. We first confirmed a significant expression of several type I IFN-inducible genes (*Ifit1*, *Ifi44*, *Usp18*, *Ifit3*, and *Irf7*) at 42 h after infection, followed by a progressive decline of the response (Fig. 1D). Strikingly, we found another significant and strong induction at 68 and 72 h after infection, which suggests that the liver reinitiates *de novo* type I IFN signaling at those later time points (Fig. 1D). To analyze the *in vivo* contribution of type I IFN-dependent signaling to host resistance to sporozoite reinfection, we monitored the liver parasite load in reinfected IFNAR1-deficient (*Ifnar1*^{-/-}) mice following infections by mosquito bites. In contrast to wild-type (WT) mice, preinfected *Ifnar1*^{-/-} mice did not display a decreased liver parasite load after a secondary challenge by *P. berghei* sporozoites (Fig. 1E). This result demonstrates that the type I IFN response in the liver is physiologically relevant in host defense against sporozoite reinfection in mice.

Next, we sought to approach the effector mechanisms that are involved in the observed phenotype downstream of the type I IFN response. Previously, we have provided evidence that the hepatocyte-mediated type I IFN response *per se* is not able to eliminate the parasite during a primary infection. However, this response is critical for the recruitment of immune cells to the infected hepatocyte, which leads to *Plasmodium* elimination (19). As IFN- γ has been identified as a cytokine that is able to mediate the killing of intrahepatic *Plasmodium* parasites (40–42), we hypothesized that the mechanism of parasite elimination in the liver after reinfection is IFN- γ dependent. We monitored transcript expression of the gene encoding this cytokine in whole-liver extracts at different time points after infection, using the previously established infec-

tion protocol and chloroquine treatment. *Ifn- γ* expression started to be induced by 48 h after *P. berghei* liver infection, followed by a slow but steady increase in transcript levels until 52 h postchallenge. Interestingly, and similarly to the observation of the type I IFN response, we observed a peak of *Ifn- γ* gene induction at 68 h after infection (Fig. 1F). To assess the functional role of IFN- γ in innate immunity to *Plasmodium* reinfection, IFN- γ -deficient (*Ifn- γ* ^{-/-}) mice were reinfected by mosquito bites after a primary mosquito bite infection. We found that, in contrast to WT animals but similarly to *Ifnar1*^{-/-} mice, *Ifn- γ* ^{-/-} mutants lost their protection against sporozoite reinfection (Fig. 1G). Taken together, our data suggest that liver-stage infection by *P. berghei* first stimulates type I IFN pathway activation and later stimulates IFN- γ expression, both of which play a critical role in host defense against reinfection.

A primary liver infection affects the blood stage of a sporozoite reinfection. In contrast to the liver stage, which is asymptomatic, the blood stage of infection is responsible for all the symptoms associated with malaria. Thus, we next investigated whether *P. berghei*-induced host protection also impacts the development of the subsequent blood-stage infection and the associated pathogenesis. We performed both primary and secondary infections by mosquito bites, following the same protocol as previously described, and used *P. berghei* parasites expressing green fluorescence protein (GFP) for reinfection (Fig. 2A). First, we measured the length of the prepatent period, i.e., the time between sporozoite injection and the appearance of blood-stage parasites, by observing Giemsa-stained blood smears under a light-field microscope. Strikingly, we observed that 50% of the reinfected mice demonstrated a 1-day delay in the appearance of blood-stage parasites from the secondary infection compared to primary-infected controls (data not shown). As the prepatent period is a good indicator of the number of infective merozoites produced in the liver (43), this result suggests that fewer merozoites are released from reinfected mice than from control mice. Next, we measured the percentage of GFP-expressing infected red blood cells (blood-stage parasitemia of the second infection) by flow cytometry. Our results indicate that growth of blood-stage parasites from a secondary infection in primed mice was significantly delayed compared to that seen in nonprimed, control animals (Fig. 2B). Moreover, reinfected mice presented a delay in malaria-associated mortality compared to control animals (Fig. 2C). These data indicate that the host resistance activated by a primary sporozoite liver infection is physiologically relevant as it not only inhibits liver infection but also delays blood-stage patency, resulting in a decrease in the associated mortality.

Mathematical modeling of murine reinfection correlates with epidemiological data. Several studies carried out in low-, medium-, and high-transmission areas have measured the time required for *Plasmodium* parasites to reappear in the blood of individuals after parasitemia has been cleared with blood-stage-specific antimalarial compounds (14, 15, 21, 30, 44–47). Interestingly, these and other modeling studies have revealed a discrepancy between the estimated number of infective bites per human per time unit (i.e., the EIR) and the resulting force of infection (FOI; i.e., the rate of new blood-stage infections) (16, 33). These data indicate that, first, even at a low EIR, not all infective bites translate into subsequent infections, and second, as the transmission rate increases (i.e., when EIR increases), the proportion of infective bites that result in blood-stage infection progressively

decreases (33). Thus, the observed FOI is lower than what would be expected if it remained proportional to the rate of infective bites. These data suggest that a high proportion of sporozoite re-infections are blocked under high-transmission conditions and that the preerythrocytic stage and/or early blood stage of infection may activate effective host defenses. Therefore, we decided to test whether our experimental data could explain the increased preerythrocytic resistance with increasing FOI observed in humans (33).

We created two models linking FOI to EIR, one with a constant proportion of infections from bites reaching the blood stage and another in which infections from bites could be blocked by innate liver-stage immunity. The reduction in the infection that would be induced by a prior liver-stage infection was estimated based on the analysis of the reduction in the numbers of blood-stage parasites observed after primary and secondary murine infections (for more details, see Materials and Methods; also see Fig. S2A in the supplemental material). In order to determine which model better explains the field study data, we compared the results of the fitting of the two models to experimental data on *P. falciparum* EIR and FOI from a field trial in western Kenya published in reference 30. The results of fitting are shown in Fig. 3A. Using the Akaike information criterion (AIC) to compare the two models, we found that the model taking into account induced liver-stage immunity provided a significantly better fit to the field data (the AICs for constant-proportion model 2 and for innate-immunity model 3 are -660 and -700 , respectively).

This deterministic model assumed a constant level of blocking of new infections for a fixed period after the first infection. However, it is likely that innate immunity is induced and subsequently decays over time. To take these dynamics into consideration, we developed a stochastic model of malaria infection that takes into account more-complex factors such as multiple consecutive bites and gradual acquisition and loss of immunity. Our model is based on four assumptions: (i) infective bites arrive randomly at a given biting rate (13, 34, 35); (ii) only a proportion of bites result in infection (e.g., in a number of cases, mosquito injection of sporozoites is unsuccessful or sporozoites remain in the skin); (iii) a successful liver-stage infection (i.e., an infection that progresses to the blood stage) induces strong host resistance and thus inhibits the establishment of a subsequent liver-stage infection; and (iv) a liver-stage infection that is blocked by innate immunity and does not progress to a blood-stage infection nonetheless induces a small amount of resistance to subsequent infections.

To parameterize the model, we estimated the baseline proportion of infectious bites that result in blood-stage infection (baseline FOI/EIR ratio) at 7%, based on the best-fit parameters of deterministic model 2. Finally, we estimated how the reduction in infectivity (or increase in host resistance) changes over time after infection. Fig. S2B in the supplemental material illustrates how the probability of success of a reinfection changes with time after a preceding successful infective bite, based on our assumptions. Full details of the parameters and mathematical formalism of the model are provided in Materials and Methods.

Based on this model, we were able to simulate different rates of infectious biting (the EIR) and observed the expected number of blood-stage infections arising from this (the FOI). We then overlaid our simulated data onto the EIR and FOI relationship observed in field studies. We found that the stochastic simulation predicts that, similarly to the deterministic model of the inhibitory

effect of prior liver-stage infection, the number of infections per bite does not remain proportional to EIR but instead reduces with increasing biting rates, as was observed in regions of hyperendemicity (Fig. 3B). Overall, our combined modeling and experimental data suggest for the first time that inhibition of reinfection in high-transmission areas can be at least partly explained by the activation of innate immunity in the liver.

DISCUSSION

We have established a mouse reinfection model and shown that the innate immunity induced by a primary *P. berghei* liver-stage infection impairs a second sporozoite reinfection. Importantly, we also observed a significant inhibition of reinfection when both infections were initiated by mosquito bites, as well as a delay in blood-stage parasitemia and in malaria-associated mortality. Using an interdisciplinary approach, we incorporated our experimental data into a mathematical model and were able to show that the predictions based on our experimental results are sufficient to explain the available epidemiological data.

The issue treated in this study deals with a situation experienced daily by the populations living in areas of high malaria transmission, which face regular and repeated reinfections. Interestingly, in regions of malaria hyperendemicity, individuals in whom blood parasitemia has been cleared with blood stage-specific antimalarial compounds resist a large number of consecutive mosquito bite infections before parasites become detectable in the blood smears compared to individuals living in areas of medium or low transmission (14–16, 21, 33). Several attempts have been made to explain this phenomenon, including genetic factors and heterogeneous mosquito biting preferences that would result in a few people receiving most of the infective bites (33). However, the observed resistance could also be the consequence of human immune defenses triggered by the preerythrocyte stage and/or early blood stage of infection. As this resistance was also associated with the age of study participants, a role for acquired adaptive immunity has been suggested (18), although an explanation of how it would participate in this protection remains elusive (9, 18). Our murine reinfection model, combined with mathematical simulations, suggests for the first time that liver innate immunity induced by hepatic *Plasmodium* infection can also account for these observed protective effects. Our data show that this kind of interdisciplinary approach can improve the mechanistic understanding of the antimalarial resistance operating in humans.

Importantly, our findings seem to extend to other *Plasmodium* species, as another recent study with another rodent parasite strain, *P. yoelii*, showed that a first liver infection inhibits a second reinfection (48). In regions of malaria endemicity, the prevalence of multiple distinct genetic *Plasmodium* variants and species is the norm (49) and humans can be successively infected with several different genotypes (50, 51) or even with different parasite species (52, 53) at one time. A major problem in fighting malaria is the poor cross-reactivity of adaptive immune responses to different *Plasmodium* species (54). Dissecting the level of cross-protective effects of innate immune responses between different parasite strains and species would contribute to a better understanding of the relevance of innate immunity during liver-stage reinfections in the field and could be of medical interest as this knowledge could be exploited to improve the efficiency of currently available antimalarial therapies.

Our report provides genetic evidence of an important role for

the type I IFN response and IFN- γ in the host defense against malaria liver-stage reinfection. IFN- γ treatment is known to efficiently kill intrahepatic parasites (40–42), but we provide the first evidence of a biologically relevant IFN- γ response *in vivo* after mosquito bite-transmitted malaria infection (*P. berghei*). Our data are in accordance with the recent report of an IFN- γ response in mice injected intravenously with high numbers of *P. yoelii* sporozoites (48). As hepatocytes are not substantial producers of IFN- γ (55), we hypothesize that the type I IFN response likely recruits myeloid cells, in particular, NK and NK-T cells, which are known to secrete large amounts of IFN- γ , to the infected hepatocytes (56). Interestingly, Miller et al. have shown that NK-T cells constitute the only effector cell population that is crucial for host resistance to sporozoite reinfection (48). However, more data will be critical to gain further insight into the effector molecules mediating the IFN- γ -dependent host resistance to reinfections and to pinpoint when exactly these defense mechanisms are most effective in eliminating parasites during liver reinfection. Indeed, the decrease in parasite load we observed in comparisons of reinfected mice to nonprimed animals was of considerably higher magnitude at the early blood stage (~10-fold decrease) than at the liver stage (2- to 3-fold decrease at 44 h after infection). This suggests that the observed resistance could be linked to a more efficient elimination of parasites in their last maturation steps in the liver, i.e., when they start to egress from hepatocytes and transition to blood-stage infection. Taken together, our data strongly suggest that the liver-stage-induced type I IFN and IFN- γ response is a major host defense mechanism against sporozoite reinfection in our mouse model. A detailed characterization of the mechanisms by which the host detects and kills intrahepatic parasites will be helpful to pave the way for innovative strategies in the development of effective preerythrocyte-based vaccines and prophylactic immunotherapies.

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